Universidad del País Vasco
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DOCTORAL THESIS

Characterization of edible oils main and minor components and study of their evolution during oxidation, nixtamalization and *in vitro* digestion processes by non-conventional techniques DI-SPME-GC/MS and ^{1}H NMR spectroscopy

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CHARACTERIZATION OF EDIBLE OILS MAIN AND MINOR COMPONENTS AND STUDY OF THEIR EVOLUTION DURING OXIDATION, NIXTAMALIZATION AND *IN VITRO* DIGESTION PROCESSES BY NON-CONVENTIONAL TECHNIQUES

DI-SPME-GC/MS AND ^{1}H NMR SPECTROSCOPY

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ABSTRACT

This work, carried out to qualify for the Doctor's Degree whose title is: **"Characterization of edible oils main and minor components and study of their evolution during oxidation, nixtamalization and *in vitro* digestion processes by non-conventional techniques DI-SPME-GC/MS and ^{1}H NMR spectroscopy"**, is divided into two main blocks. **Block 1** focuses on the advancement of knowledge of the edible oil composition and on its compositional changes that can take place during oxidative and/or processing conditions. **Block 2** of this work focuses on advances in the knowledge of the *in vitro* digestion process of edible oils.

In recent years, increasing attention has been paid to edible vegetable oils due to their importance in the food industry, nutrition, and human health. Vegetable oils are widely used and their quality is a function of their composition, which depends, among other factors, on its vegetable origin, on what processing took place, on its oxidation status, as well as on its level of those compounds which are able to exhibit antioxidant activity and other healthy properties. The evaluation of oil quality, safety and authenticity requires as deep a knowledge of its composition as possible. However, this is a very difficult challenge due to the great number of compounds that may be present. Besides the triglycerides, which are a well known main component of oils, edible oils have lower content in other minor components, such as, tocols, sterols, phenolic compounds and hydrocarbons among others, to which bioactive activities are attributed. The study of these minor oil components is much more difficult than that of the main ones not only because they are very numerous, but also because they have very different functional groups. In this context, the **objectives 1.1.1.** and **1.1.2.** of the **aim 1.1.** corresponding to **Block 1** of this doctoral thesis are presented, in which the development of new

methodology and its application for the characterization of the main and minor oil components was tackled.

In order to fulfil the **objective 1.1.1.** five commercial edible oils of different vegetable origins, one sunflower oil, one corn oil, two soybean oils (one refined and the other virgin) and one virgin linseed oil were used. The analysis of their minor components was performed with a new methodology based on the Direct Immersion Solid-Phase Microextraction (DI-SPME) fiber of PDMS/DVD in the oil matrix, followed by Gas Chromatography/Mass Spectrometry (GC/MS). This method enables a deep and comprehensive insight into oils composition, as well as a differentiation of edible oils in a simple way, in only one run and without either solvents or sample modification, which is unachievable with other methodologies. For the first time, the identification and quantification of sterols, tocols, hydrocarbons of different natures, fatty acids, esters, monoglycerides, fatty amides, aldehydes, ketones, alcohols, epoxides, furans, pyrans and terpenic oxygenated derivatives is simultaneously carried out. This data allows one to obtain a great deal of information on diverse aspects of the oils such as, nutritional value, oxidative stability, technological performance, quality, processing, safety and even fraudulent practices.

The **objective 1.1.2.** focuses on the qualitative and semi-quantitative characterization of minor nitrogenated compounds in eleven edible oils, namely extra virgin olive, virgin olive, olive, sunflower, virgin soybean, refined soybean, virgin flaxseed and four corn oils, by using the methodology (DI-SPME-GC/MS) developed in the objective 1.1.1. The obtained results showed for the first time the presence of an important number of cyclic dipeptides or 2,5-diketopiperazines (DKPs) and other nitrogenated compound such as, oxazolines, pyrrolidines and other pyrrol derivatives, as well as indol, pyrazol, pyridine and β-phenylethylamine derivatives, only in the four corn

oils studied. The occurrence of these compounds in corn oil is of great importance since all of them are bioactive and for this reason can give corn oil interesting properties from technological, nutritional and health points of view. Moreover, DKPs could be considered corn oil markers in authentication and fraud studies, and their concentration can be used for differentiation and classification corn oils.

Likewise, it is well known that once the oils are obtained, they are subjected to several technological processes in both industrial and culinary treatment in which either their main or minor oils components can be degraded and/or transformed. These involve a complex process which provokes not only the reduction of their nutritional and sensorial value, as well as the reduction of the shelf-life of the products, but also the generation of new compounds, some of them still unknown, coming mainly from the oxidation reactions. In this sense, in order to try to monitor simultaneously the evolution of the original main and minor oil components as well as of other compounds coming from the oxidation of both, during technological processing, the development of new techniques and/or the improvement of already existed analytical tools is of great importance. In this context, the **objective 1.1.3.** (**aim 1.1.; Block 1**) and **objectives 1.2.1.** and **1.2.2.** (**aim 1.2.; Block 1**) were addressed.

The **objective 1.1.3.** and **1.2.1.** of this Doctoral Thesis address the characterization of the oxidative status of corn oil, monitoring of the degradation of its main and minor components, as well as the formation of other compounds coming from the oxidation of both components during accelerate storage process (70 ºC). **Objective 1.1.3.** tackles the monitoring of the original minor corn oil components and of other compounds coming from the oxidation of main and minor oil components formed under the same aforementioned conditions by means of a new methodology, developed in the objective 1.1.1., (DI-SPME-GC/MS). This methodology provided information in a single

run about the degradation rate of the corn oil antioxidant minor components, such as, tocols, sterols, squalene and cyclic dipeptides and also about the identity and formation rate of compounds coming from both main and minor oil components oxidation. In this study, for the first time, some of the compounds formed from the degradation of oil's minor components, such as tocopherol´s oxidation products, are proposed as new oxidation markers of corn oil, and therefore of any edible oils containing tocopherols. The usefulness of this technique for the study of edible oil oxidation processes is shown for the first time, providing new information which would be difficult, if not impossible, to obtain by other methods.

Regarding **objective 1.2.1.,** the goal of this study is to shed light on the degradation rate of original corn oil components named main and minor components under accelerate storage process (70 ºC), as well as the identification and quantification of new compounds coming from their degradation, paying particular attention to the compounds to which scarce attention is paid or even to those never described before in edible oils, through the information provided by proton nuclear magnetic resonance (^{1}H NMR). For the first time, a global oxidation study has been made in great detail in which the degradation rate of original oil components throughout the oxidation process, and also the formation rate of new compounds, is studied. The results obtained reveal that during the oxidation of corn oil, a great number of oxidation compounds, both primary and secondary, are generated, such as dihydroperoxides, epoxy-keto-hydroxy derivatives, structures having poly-hydroxy, poly-formates, poly-ester and poly-ether groups, as well as structures having furane groups not described before in oxidized corn oil samples or even in other edible oil. This work has evidenced and reinforced again the usefulness and versatility of ^{1}H NMR to study in a global way the changes that take place during edible oil processing.

Likewise, lipid components may suffer changes when they are submitted to different processing techniques. In this sense, **objective 1.2.2.** was outlined. The purpose was to investigate by means of proton nuclear magnetic resonance (^{1}H NMR) the changes caused by nixtamalization and tortilla-making in the lipid composition of two corn varieties (blue and white). This processing technique involves an alkaline thermal treatment of the corn grains with a calcium hydroxide solution, keeping the grains in this solution for a certain period of time, after which they are washed with water and ground to obtain the nixtamalized masa or dough with which tortillas are made. The results of this study show that ^{1}H NMR is a very efficient and useful technique to study not only the lipids of two corn varieties, but also the of tortillas derived, and consequently the changes proved by nixtamalization and tortilla-making in the corn lipids. Ferulates have been found for the first time among the minor components of these lipids. Moreover, it has been proved that nixtamalization and tortilla-making provoke a total or a partial loss of minor components, a small reduction in the unsaturation degree of the main lipid components and a slight oxidation undergone by the unsaturated acyl groups in both corn varieties in a similar way.

In addition to storage and processing, it has been shown that food lipid oxidation can also occur during digestion. In this context, in the last few years, in order to delay the oxidative degradation of food lipids and to try to reduce the presence of compounds coming from its degradation (some of them with well known adverse health effects), the enrichment of oils and foods with compounds to which antioxidant activity is attributed is becoming popular. Nevertheless, it has been shown that some compounds' attributed antioxidant ability act as prooxidants in some foods when subjected to certain oxidative and digestion conditions. Besides oxidation reactions during gastrointestinal digestion, hydrolysis of triglycerides take place, giving rise to smaller building blocks, which are

able to be absorbed. However, regarding the factors that affect the lipolysis yield and its pattern, the information is still rather unclear. Indeed, a better understanding in that issue is needed. Taking all above-commented into account, **Block 2** (**aims 2.1., 2.2.** and **2.3.**) of this Doctoral Thesis was outlined. In this, **Block 2** tackles the analysis of the *in vitro* digestion of different vegetable oils differing in both main and minor components (corn, virgin flaxseed and olive oils) and of the effect of their enrichment with different levels of phenolic compounds, including *gamma*- and *alpha*-tocopherol, hydroxytyrosol acetate and dodecyl gallate by using ^{1}H NMR and SPME-GC/MS. Attention is focused on the hydrolysis degree and the possible difference on its extent and pattern, taking into account the oils' acyl group composition, the degradation of the oil main components, the occurrence of oxidation reactions and main compounds formed, as well as on the bioaccessibility of the oil main components, of compounds formed in the oxidation, and of the minor components and added phenolic compounds.

The information obtained by accomplishing **Block 2** showed that the extent and pattern of lipolysis is very different in each one of these oils. The obtained data showed that the lipolysis extent reached in the *in vitro* digestion of these oils is higher as lower the unsaturation degree. Hence, higher bioaccessibility of the oil main components is reached for olive oil than for corn oil and in both to a higher degree than in flaxseed oil. Furthermore, it should be pointed out that the effect of the enrichment with phenolic compounds does not prove significant changes in this process, which means that none inhibition of the lipase activity occurs due to the presence of these phenolic compounds.

Apart from hydrolysis, it is again demonstrated that the *in vitro* digestion provokes lipid oxidation in the oils, which is confirmed by the diminution in the molar percentage of the main fatty acids plus acyl groups in some cases or by the generation of different oxidation compounds. The oxidation extent reached during *in vitro* digestion of olive oils

is much smaller than that reached during the digestion of other edible oil such as corn and virgin flaxseed oil, with the consequent repercussions on health due to the toxicity of oxidation compounds. Furthermore, the behaviour and the efficiency of the added phenolic compounds is proved. *Gamma*-tocopherol, hydroyxytyrosol acetate and dodecyl gallate act as antioxidants in line with the number of phenolic groups in its molecule in a dose-dependent manner. However, *alpha*-tocopherol behaves as a prooxidant in line with its higher enrichment degree. Moreover, in the more *alpha*-tocopherol enriched samples, hydroperoxy-, hydroxy-, and keto-dienes as well as keto-epoxy-monoenes and aldehydes are generated, all of them well known oxidation markers and some of them associated to degenerative diseases.

In addition, an important result derived from this second block is the evidence of the bioaccessibility during *in vitro* digestion of both minor components naturally present in these oils and of the added phenolic compounds to which several biological activities are attributed. In general terms, the bioaccessibility of naturally present minor components cannot be considered low, but rather high. Bioaccessiblity of these compounds in the oil samples enriched in phenolic compounds is higher than in unenriched samples, in line with its enrichment level.

RESUMEN

Este trabajo, realizado para optar al Grado de Doctor cuyo título es: **"Characterization of edible oils main and minor components and study of their evolution during oxidation, nixtamalization and *in vitro* digestion processes by non-conventional techniques DI-SPME-GC/MS and ^{1}H NMR spectroscopy"**, está dividido en dos grandes bloques. El **Bloque 1** tiene como objetivo principal el contribuir al avance del conocimiento de la composición de aceites comestibles y sobre los cambios que éstos pueden sufrir en su composición durante condiciones oxidativas y/o de procesado. El **Bloque 2** de este trabajo se centra en la profundización del conocimiento de la digestión *in vitro* de aceites comestibles.

En los últimos años, se está prestando cada vez más atención a los aceites vegetales comestibles debido a la importancia que tienen tanto dentro de la industria alimentaria, como en la salud y en la nutrición. Su calidad está relacionada con su composición, la cual, depende entre otros factores de su origen vegetal, del tipo de procesado que han sufrido, de su estado oxidativo, así como, de la concentración de aquellos compuestos capaces de exhibir actividad antioxidante y otras propiedades saludables. La evaluación de la calidad, seguridad y autenticidad de los aceites requiere un conocimiento tan profundo de su composición como sea posible. Sin embargo, éste es un desafío muy complicado debido que su composición es muy compleja ya que existen una gran variedad de compuestos que pueden estar presentes. Además de los triglicéridos, que son los componentes principales de los aceites, éstos contienen una serie de componentes minoritarios como tocoles, esteroles, compuestos fenólicos e hidrocarburos que, aunque minoritarios en cuanto a su concentración, en muchos casos han mostrado tener propiedades bioactivas. El estudio de estos componentes minoritarios es más complicado que el de los componentes principales, no solo por ser muy numerosos sino

porque contienen grupos funcionales muy diferentes. En este contexto, y dentro del **Bloque 1** y del **objetivo 1.1.,** se contemplaron dos **sub-objetivos** el **1.1.1.** y el **1.1.2.** de la presente tesis doctoral, en los que se aborda el desarrollo de una nueva metodología y su aplicación para la caracterización de los componentes minoritarios principales del aceite.

Para alcanzar el **sub-objetivo 1.1.1.** se analizaron cinco aceites comestibles de diferente origen vegetal; un aceite de girasol, un aceite de maíz, dos aceites de soja (uno refinado y otro virgen) y un aceite de lino virgen. El estudio de los componentes minoritarios de estos aceites se llevó a cabo mediante la nueva metodología basada en la Inmersión Directa de una fibra de Microextracción en Fase Sólida (ID-MEFS) de PDMS/DVB en el aceite, seguida del análisis de los componentes extraídos mediante Cromatografía de Gases con detector de Espectrometría de Masas (ID MEFS-CG/EM). Los resultados de este estudio demuestran que, esta técnica permite obtener una visión completa y en profundidad de la composición en componentes minoritarios de los aceites, así como una diferenciación entre éstos, de una manera simple, y de forma simultánea, sin la utilización de disolventes o reactivos adicionales y sin la necesidad de modificar la muestra, lo cual es imposible de obtener mediante otras metodologías. Por primera vez se ha llevado a cabo, la identificación y semicuantificación de compuestos tales como esteroles, tocoles, hidrocarburos de diferente naturaleza, ácidos grasos, ésteres, monoglicéridos, amidas lipídicas, aldehídos, cetonas, alcoholes, epóxidos, furanos, piranos y derivados oxigenados presentes en aceites.

El **sub-objetivo 1.1.2.** se centra en la caracterización cualitativa y semicuantitativa de compuestos nitrogenados minoritarios en once aceites comestibles, entre los que se encuentran, aceite de oliva virgen extra, de oliva virgen, de oliva, de girasol, de soja virgen y refinado, de lino virgen y cuatro aceites de maíz, utilizando la

metodología (ID-MEFS-CG/EM) desarrollada en el sub-objetivo 1.1.1. Los resultados obtenidos con esta metodología muestran por primera vez, que únicamente los cuatro aceites de maíz presentan un importante número de dipéptidos cíclicos o 2,5-diketopiperacinas (DKPs) y otros compuestos nitrogenados como, oxazolinas, pirrolidinas y otros derivados de pirrol, así como derivados de indol, pirazol, piridina y *β*-feniletilamina. La presencia de estos compuestos en el aceite de maíz es de gran interés, debido a que todos ellos se consideran como compuestos bioactivos y por consiguiente pueden contribuir a que este aceite tenga propiedades interesantes, desde un punto de vista tecnológico, nutricional o de salud. Además, los DKPs podrían considerarse marcadores de aceite de maíz en los estudios de autenticación y fraude, y su concentración se puede utilizar para diferenciar y clasificar estos aceites.

Una vez obtenidos los aceites, éstos se pueden someter a varios procesos tecnológicos, tanto durante el tratamiento industrial como en el culinario, en los que los componentes mayoritarios y minoritarios pueden degradarse y/o transformarse. Estos tratamientos pueden implicar la reducción de su valor nutricional y sensorial, así como la reducción de la vida útil de los aceites y también la generación de nuevos compuestos, algunos de ellos aún no identificados, derivados fundamentalmente de reacciones de oxidación que pueden sufrir. En este sentido, con el objetivo de estudiar la evolución que experimentan tanto los componentes mayoritarios como minoritarios de los aceites durante el procesado tecnológico del mismo, además de identificar compuestos que se pueden generar de la oxidación y/o degradación de éstos, es de gran importancia desarrollar nuevas técnicas y/o mejorar las herramientas analíticas existentes. En este contexto fueron abordados el **sub-objetivo 1.1.3.** (**objetivo 1.1.; Bloque 1**) y los **sub-objetivos 1.2.1.** y **1.2.2.** (**objetivo 1.2; Bloque 1**).

Los **sub-objetivos 1.1.3.** y **1.2.1.** de esta tesis doctoral se establecieron con la finalidad profundizar en el conocimiento del estado oxidativo del aceite de maíz cuando se somete acondiciones de almacenamiento acelerado a 70ºC, llevando a cabo el seguimiento de la degradación de sus componentes mayoritarios y minoritarios, así como la formación y posterior evolución de otros compuestos que pueden proceder de la oxidación de los mismos. El **sub-objetivo 1.1.3.** se centra en el seguimiento de la evolución de los componentes minoritarios presente en el aceite de maíz, así como de los compuestos provenientes de la oxidación de los componentes mayoritarios y minoritarios, bajo las condiciones de almacenaje anteriormente mencionadas, mediante la metodología (ID-MEFS-CG/EM), desarrollada en el sub-objetivo 1.1.1. Esta metodología es capaz de proporcionar información sobre la degradación y sobre la cinética/velocidad de degradación de componentes minoritarios del aceite de maíz, tales como tocoles, esteroles, escualeno y dipéptidos cíclicos, muchos de ellos con propiedades antioxidantes; asimismo la metodología proporciona información sobre la identidad y la cinética de formación de los compuestos que se generan durante la oxidación tanto de los compuestos mayoritarios como minoritarios del aceite. En este estudio se proponen por primera vez algunos compuestos derivados de degradación de tocoferoles, como nuevos marcadores de oxidación del aceite de maíz y de cualquier aceite comestible que los contenga. Asimismo, se muestra la utilidad de esta técnica para el estudio de los procesos de oxidación de aceites comestibles debido a que la información que proporciona sería difícil, si no imposible de obtener mediante otros métodos.

Respecto al **sub-objetivo 1.2.1.** la finalidad de éste es arrojar luz a través de la información proporcionada por la resonancia magnética nuclear de protón (RMN de ^{1}H), sobre la tasa de degradación de los componentes tanto mayoritarios como minoritarios del aceite de maíz, durante un proceso de almacenamiento acelerado (70 ºC), así como la

identificación y cuantificación de nuevos compuestos procedentes de su degradación, poniendo especial atención en alguno que hasta el momento no se le ha prestado especial interés y/o no se han descrito en aceites comestibles. Por primera vez se ha realizado un estudio de oxidación global y detallado, en el que se analiza, a lo largo del proceso de oxidación, tanto la velocidad de degradación de los componentes originales del aceite como la de formación de nuevos compuestos. Los resultados obtenidos revelan que, durante la oxidación del aceite de maíz, se generan un gran número de compuestos de oxidación, tanto primarios como secundarios, alguno de ellos no descritos en aceite de maíz oxidado u otros aceites comestibles anteriormente, como, dihidroperóxidos, además de compuestos con estructuras que contienen grupos epóxido ceto, hidróxido, poli-hidróxidos, poli-ester and poli-eter y grupos furano. Este trabajo evidencia y refuerza de nuevo la utilidad y versatilidad de la RMN de ^{1}H para estudiar de manera global los cambios que tienen lugar durante el procesado/oxidación de aceites comestibles.

Igualmente, los componentes lipídicos pueden sufrir cambios cuando se someten a otras diferentes técnicas de procesado. En este sentido, se planteó el **sub-objetivo 1.2.2.** cuyo propósito fue estudiar mediante RMN de ^{1}H, los cambios producidos por la procesos como la nixtamalización y la elaboración de tortillas en la composición lipídica de dos variedades de maíz (una azul y otra blanca). La nixtamalización implica un tratamiento térmico alcalino de los granos de maíz con una solución de hidróxido de calcio, manteniendo los granos en esta solución durante cierto tiempo, posteriormente se lavan con agua y se muelen con el fin obtener la masa nixtamalizada con la cual se elaboran las tortillas. Los resultados de este estudio muestran que la RMN de ^{1}H es una técnica útil y muy eficiente para estudiar no solo la composición lipídica de las dos variedades de maíz, sino también la de las tortillas elaboradas con su masa resultante, y como consecuencia para evaluar los cambios derivados de la nixtamalización y durante la elaboración de

tortillas en los lípidos de las dos variedades de maíz. Entre los componentes minoritarios de estos lípidos, se han encontrado por primera vez ferulatos. Asimismo, se ha observado que la nixtamalización y la elaboración de tortillas provocan una pérdida total o parcial de componentes minoritarios, una pequeña reducción en el grado de insaturación de los componentes lipídicos mayoritarios y una ligera oxidación de los grupos acilo insaturados en ambas variedades de maíz de manera similar.

Además del almacenamiento y procesado, se ha demostrado que durante la digestión "*in vitro*" pueden producirse oxidación de los lípidos de los alimentos. En este contexto, en los últimos años, con el fin de retrasar la degradación oxidativa de los lípidos alimentarios y tratar de reducir la presencia de compuestos provenientes de su degradación, algunos de ellos con efectos adversos para la salud, se ha convertido en algo muy popular el enriquecimiento de aceites y alimentos con compuestos a los que se atribuye actividad antioxidante. Sin embargo, se ha demostrado que algunos de estos compuestos, cuando se someten a ciertas condiciones oxidativas y de digestión *in vitro* actúan como prooxidantes en algunos alimentos. Además de las reacciones de oxidación, durante la digestión gastrointestinal se produce la hidrólisis de los triglicéridos, dando lugar a moléculas más pequeños, que pueden ser absorbidas. Sin embargo, con respecto a los factores que afectan al rendimiento de la lipólisis y su extensión, la información aún no está clara, por lo que, se necesitaría una mejor comprensión de este tema. Teniendo en cuenta todo lo mencionado anteriormente, se planteó el **Bloque 2** (**objetivos 2.1., 2.2.** y **2.3.**) de esta Tesis Doctoral. En este **Bloque 2** se aborda el análisis mediante RMN de ^{1}H y MEFS-CG/EM de la digestión *in vitro* en 3 diferentes aceites vegetales, de muy diferente composición, aceites de maíz, lino virgen y de oliva, y del efecto de su enriquecimiento con compuestos fenólicos a diferentes concentraciones como, *gamma*- y *alfa*-tocoferol, acetato de hidroxitirosol y galato de dodecilo. La atención del estudio se

centra en el estudio del grado de hidrólisis y de la posible diferencia en su extensión y patrón teniendo en cuenta la composición en grupos acilo de los aceites, la degradación de los componentes mayoritarios del aceite, el desarrollo de reacciones de oxidación y estudio de los principales compuestos formados, además del análisis de la bioaccesibilidad de componentes mayoritarios del aceite, de compuestos formados durante la oxidación y, de los componentes minoritarios, así como de los compuestos fenólicos añadidos. La información obtenida en el **Bloque 2** mostró que la extensión y el patrón la lipolisis es muy diferente en cada uno de los aceites. Los datos obtenidos mostraron que la extensión de la lipolisis alcanzada durante la digestión *in vitro* de estos aceites es mayor cuanto menor es su grado de insaturación. Por lo tanto, se alcanza una mayor bioaccesibilidad de los componentes mayoritarios del aceite para el aceite de oliva que para el aceite de maíz y de lino. Así mismo, cabe señalar que el enriquecimiento con compuestos fenólicos no afecta de forma significativa a este proceso, lo que pone de manifiesto que no se produce inhibición de la actividad de la lipasa debido a la presencia de estos compuestos fenólicos.

Además de la hidrólisis, se ha demuestra nuevamente que durante la digestión *in vitro* se produce la oxidación de los aceites, lo que se pone de manifiesto por la disminución en el porcentaje molar de los principales ácidos grasos más grupos acilo en algunos casos o por la generación de diferentes compuestos de oxidación. El grado de oxidación alcanzado durante la digestión *in vitro* de los aceites de oliva es mucho menor que el alcanzado durante la digestión de otros aceites comestibles como el de maíz y el de aceite de lino virgen, con lo que conllevará consecuencias sobre la salud de quien los consuman debido a la toxicidad que presentan los compuestos que se generan durante la oxidación. Además, se describe el comportamiento y la eficiencia de los compuestos fenólicos añadidos durante la digestión. El *gamma*-tocoferol, el acetato de hidroxitirosol

y el galato de dodecilo actúan como antioxidantes en línea con el número de grupos fenólicos en su molécula, de una manera dependiente de la dosis. Sin embargo, el *alfa*-tocoferol se comporta como prooxidante siendo mayor cuanto mayor grado de enriquecimiento. Además, en las muestras enriquecidas con más cantidad/concentración *alfa*-tocoferol, se generan hidroperoxi, hidroxi y ceto-dienos, así como ceto-epoxi-monoenos y aldehídos, todos ellos conocidos marcadores de oxidación y en algún caso asociados a enfermedades degenerativos.

Finalmente, otro resultado importante derivado de este **Bloque 2**, es la evidencia de la bioaccesibilidad durante la digestión *in vitro* de tanto los componentes minoritarios presentes naturalmente en estos aceites y de los compuestos fenólicos añadidos, a los que se atribuyen diferentes actividades biológicas. En términos generales, se observa una elevada bioaccesibilidad de los componentes minoritarios presentes de forma natural en los aceites. La bioaccesibilidad de estos compuestos en los aceites enriquecidos en compuestos fenólicos es mayor que en los no enriquecidos, en línea con su nivel de enriquecimiento.

ABBREVIATIONS

A	Area
AG	Acyl group
Ala	Alanine
ANOVA	Analysis of variance
B	Blue corn
BHT	2,6-di-*tert*-butyl-hydroxytoluene
B_{OMC}	Bioaccessibility of oil main components
Bp	Base peak
C	Campesterol
CTN	Campestanol
CD	Conjugated dienes
$CDCl_3$	Deuterated cloroform
DKPs	Cyclic dipeptides / 2,5-diketopiperazines
DJ	Juices submitted to digestion
DG	Dodecyl gallate
DI	Direct Immersion
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
FA	Fatty acid
FE	Ferulates
GC/MS	Gas Chromatography/Mass Spectrometry
Gly	Glycine
Gol	Glycerol
HTA	Hydroxytyrosol acetate
HO-c-dEs	Hydroxy-conjugated-dienes
HPO-c-dEs	Hydroperoxy-conjugaged-dienes
HPLC	High Performance Liquid Chromatography
Ile	Isoleucine
KO-c-dEs	Keto-conjugated-dienes
KO-EPO-mEs	Keto-epoxy-monoenes

L	Linoleic
Leu	Leucine
Ln	Linolenic
ME	Methyl Ester
MG	Monoglycerides
MW	Molecular weight
O	Oleic
PV	Peroxide Value
Phe	Phenilalanine
Pro	Proline
^{1}H NMR	Proton nuclear magnetic resonance
P	Pristene
PC	Phosphatidylcholine
PDMS/DVB	Polydimethylsiloxane/divinylbenzene
PyroGlu	Pyroglutamic
S	Saturated
SC	Specific compounds
SPME	Solid Phase Microextraccion
SQ	Squalene
ST	Stigmasterol
STN	Sitostanol
TBARS	Thiobarbituric acid reactive substances
TG	Triglycerides
TMS	Tetramethylsilane
TrMPD	6,10,14-trimethylpentadecan-2-one
TrMD	3,7,11-trimethyl-3-dodecanol
TeMHD	4,8,12,16-tetramethylheptadecan-4-olide
TQ23E	α-tocopherylquinone-2,3-epoxide
TQ56E	α-tocopherylquinone-5,6-epoxide
Val	Valine

W	White corn
αT	*alpha*-tocopherol
γT	*gamma*-tocopherol
Δ7A	Δ7-avenasterol
Δ5A	Δ5-avenasterol
β-S	β-sitosterol
1,2-DG	1,2-diglyceride
1,3-DG	1,3-diglyceride

LIST OF ARTICLES/MANUSCRIPTS

The present doctoral thesis relies on the 8 articles/manuscripts listed below:

1- Alberdi-Cedeño, J., Ibargoitia, M. L., Cristillo, G., Sopelana, P., & Guillén, M. D. (**2017**). A new methodology capable of characterizing most volatile and less volatile minor edible oils components in a single chromatographic run without solvents or reagents. Detection of new components. ***Food Chemistry***, *221*, 1135-1144. **(Article 1)**.

2- Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D. (**2017**). Bioactive compounds detected for the first time in corn oil: Cyclic dipeptides and other nitrogenated compounds. ***Journal of Food Composition and Analysis***, *62*, 197-204. **(Article 2)**.

3- Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D. (**2019**). Monitoring of minor compounds in corn oil oxidation by direct immersion-solid phase microextraction-gas chromatography/mass spectrometry. New oil oxidation markers. ***Food Chemistry***, *290*, 286-294. **(Article 3)**.

4- Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D. Toxic oxylipins associated to current diseases detected for the first time in an edible oil rich in linoleic acyl groups submitted to oxidative conditions. A global, broad and in-depth study by ^{1}H NMR spectroscopy. (***Under revision, Antioxidants (2020))*** **(Manuscript 4).**

5- Alberdi-Cedeño, J., Molina, M., Yahuaca-Júarez, B., Ibargoitia, M. L., & Guillén, M. D. (**2020**). Changes provoked by nixtamalization and tortilla making in the lipids of two corn varieties. A study by ^{1}H NMR. ***Food Chemistry***, *313*, 126079. **(Article 5)**.

6- Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D. (**2020**). Effect of the Enrichment of Corn Oil With *alpha*- or *gamma*-Tocopherol on Its *In Vitro* Digestion Studied by ^{1}H NMR and SPME-GC/MS; Formation of Hydroperoxy-, Hydroxy-, Keto-Dienes and Keto-*E*-epoxy-*E*-Monoenes in the More *alpha*-Tocopherol Enriched Samples. ***Antioxidants***, *9*(3), 246. **(Article 6)**.

7- Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D. (**2020**). A Global Study by ^{1}H NMR Spectroscopy and SPME-GC/MS of the *in Vitro* Digestion of Virgin Flaxseed Oil Enriched or not with Mono-, Di- or Tri-Phenolic Derivatives. Antioxidant Efficiency of These Compounds. ***Antioxidants***, *9*(4), 312. **(Article 7)**.

8- Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D. Study of the *in vitro* digestion of olive oil enriched or not with antioxidant phenolic compounds. Relationships between bioaccessibility of main components of different oils and their composition. (***Accepted, Antioxidants (2020)***) **(Manuscript 8).**

INTRODUCTION

1. An overall idea about edible oils. Their composition in main and minor components.

In recent years, increasing attention has been paid to edible vegetable oils due to their importance in the food industry, nutrition and human health. Edible oil quality is a function of its composition, which depends, among other factors, on its vegetable origin, on what processing took place, on its oxidation status, and especially on its level of those compounds which are able to exhibit antioxidant activity and other healthy properties. The evaluation of oil quality, safety and authenticity requires as deep a knowledge of its composition as possible. However, this is a very difficult challenge due to the great number of compounds that may be present.

Edible oils are complex mixtures represented mainly by triglycerides. Determination of the triglyceride composition of edible oils has been the subject of many studies. The classical methodology involves the hydrolysis of triglycerides and the transformation of the resulting fatty acids into methyl esters which are then identified and quantified by Gas Chromatography (GC) coupled with different detectors (Andrikopoulos, 2002; Seppänen-Laakso et al., 2002). High-performance Liquid Chromatography (HPLC) performed in different modes and coupled to different detectors is also a widely used approach for the analysis of TG species of oils and fats. Among the different variants, reverse phase (RP-HPLC) is the most widely applied mode (Andrikopoulos, 2002). On the other hand, other non-chromatographic alternative methods have been developed for the determination of unsaturation degree and acyl groups composition, such as, fourier-transform infrared spectroscopy (FTIR) and proton Nuclear Magnetic Resonance (^{1}H NMR), which allows this determination to be made more quickly and simply and not requiring any chemical modification of the sample (Guillén & Cabo, 1997a,b; Guillén & Ruiz, 2003 a,b; Guillén et al., 2003; Guillén & Uriarte, 2012a; Martínez-Yusta et al., 2014).

Besides the triglycerides, however, edible oils have lower content in other minor components, such as tocols, sterols and hydrocarbons among others, to which bioactive activities are attributed (Kamal-Eldin & Appelqvist, 1996; Singh 2013). It is noteworthy that not only oil's saturation degree but also the content and nature of those minor components play a very important role in food industry because together they are responsible for oil's oxidative stability (Guillén & Ruiz, 2005a,b,c) and for oil's behaviour in processing at industrial and domestic level.

The study of minor oil components is much more difficult than that of the main ones, not only because they are very numerous, but also because they have very different functional groups. The study of these components has been traditionally carried out by extraction of the compounds of interest by a specific extraction technique for each kind of compound and their subsequent separation, identification and quantification by chromatography using diverse detectors (Cert et al., 2000). In this way, phenols, tocols, sterols and other minor oxygenated components have been quantified in several oils. However, taking into account that the composition of edible oils is very complex, it might be thought that some of their minor components still remain to be discovered. A group of compounds that until now has received very scarce attention in oils is that of nitrogenated compounds, such as cyclic dipeptides. These have been found in certain foods such as bread, beer, coffee and cocoa among others (Gautschi et al., 1997; Ginz & Engelhardt, 2000, 2001; Stark & Hofman, 2005; Ryan et al., 2009), and some of them have been attributed bioactive abilities (Kumar et al., 2013; Ser et al., 2015; Vázquez-Rivera et al., 2015). Although the occurrence of compounds of this nature has not been described in edible oils to date, it cannot be discounted. In this sense, it is of great importance to have the option of a simple method which can provide at the same time, in only one run, information about most of the minor edible oil components. It should be noted that the

determination of all the compounds mentioned above is important, because they have been shown to provide useful information about botanical origin, previous processing, the oxidation status of the oil, and in addition because some of them are responsible for its odour and its taste (Uriarte et al., 2011).

In this context, the **objectives 1.1.1.** and **1.1.2.** of the **aim 1.1.** corresponding to **Block 1** of this doctoral thesis are presented, in which the development of new methodology and its application for the characterisation of the main and minor oil components was tackled.

2. Oil lipidic components oxidation during technological processing and/or thermo-oxidative conditions: mechanisms and methodologies for its study.

It is well known that once the oils are obtained, they are subjected to several technological processes in both industrial and culinary treatment where either main or minor oils components can be degraded and/or transformed. These involve a complex process which provokes not only the reduction of their nutritional and sensorial value, as well as the reduction of the shelf-life of the products, but also the generation of new compounds, some of them still unknown, deriving mainly from the oxidation reactions. The rate of lipidic components oxidation might be influenced by many factors, including the degree of lipid unsaturation, the type and the concentration of oxygen, the temperature and light irradiation and the presence of minority compounds, among others (Choe & Min, 2006; Martínez-Yusta et al., 2014). It has been described for years that lipid oxidation reactions take place via a free radical chain reaction which is divided into three stages: initiation, propagation and termination (Frankel, 2014). Although, over the years, the knowledge and the analytical technologies have advanced, some studies have observed the presence of alternate pathways in lipid oxidation that take place simultaneously and compete with the traditional ones, integrating alternated reactions of internal rearrangement, addition, scission and disproportion of peroxyl and alkoxyl

radicals with a core of traditional hydrogen abstraction (Schaich, 2005). For all these reasons, it is of great importance to know the evolution of the original oil's components, as well as the identity, quantity and characteristics of the new formed compounds when the oils are submitted to oxidative conditions or any other technological processes.

The most common methods used to evaluate the oxidation degree of edible oils require chemical modification of the sample and provide limited information about the identity of compounds whose functional groups are measured. Among those widely used, peroxide value (PV) and conjugated dienes (CD) evaluate the occurrence of primary oxidation compounds, and thiobarbituric acid reactive substances (TBARS) evaluate the occurrence of secondary oxidation products. These techniques have the advantage of being fast and simple, although they only provide information about certain compounds or functional groups and do not provide information about the specific nature of the compounds involved in each determination, which could sometimes give rise to inaccurate conclusions (Guillén & Cabo, 2002; Devasagayam et al., 2003). Chromatographic techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) coupled to different detectors has been used in the study of the volatile compounds, as well as non-volatile compounds formed during thermo-oxidative processes (Thompson et al., 1978; Frankel, 1991; Steenhorst-Slikkerveer et al., 2000; Guillén et al., 2005; Velasco et al., 2018).

Another approach with the same aim is based on the use of ^{1}H NMR spectroscopy. This technique permits the qualitative and quantitative characterization of main edible oil components (Guillén & Ruiz, 2003a,b; Guillén & Uriarte, 2012b; Martínez-Yusta et al., 2014). It is a very useful technique in monitoring their oxidation because it allows for the evaluation of the rate of degradation of the oil's main and minor components as well as the formation rate of compounds derived from them, such as primary and many secondary

oxidation compounds (Guillén & Ruiz, 2004; Guillén & Ruiz, 2005a,b,c; Guillén & Uriarte, 2012a; Martínez-Yusta et al., 2014). Among the primary oxidation compounds, hydroperoxides, either supported in a conjugated diene systems with (*Z,E*)- or (*E,E*)-isomers, or in an unconjugated diene system or monoene system, are included (Gardner & Weisleder, 1972; Zhang et al., 2006; Guerreo et al., 2007; Martínez-Yusta et al., 2014; Ito et al., 2019). These primary oxidation compounds are unstable and are involved in different chemical reactions that give rise to the formation of secondary oxidation compounds with different functional groups in their structure, such as aldehydes, ketones, alcohols and epoxides, among others (Frankel, 1991; Martínez-Yusta et al., 2014). In fact, these functional groups can be presented either alone or combined with other functional groups, for example, hydroperoxy-epoxy, hydroxy-epoxy, hydroxy-keto and epoxy-keto, among others (Gardner et al., 1978; Gardner & Kleiman, 1981; Gardner & Crawford, 1981; Lin et al., 2007; Ramsden et al., 2017). While the study of these last compounds in edible oils has received scare attention, some authors have recently described the presence of some of them in oxidized oil lipids (Xia & Budge, 2018; Martin-Rubio et al., 2018a,b).

However, either during technological processing or oxidative conditions, not only the main components but also the minor ones, such as tocopherols, squalene and sterols among others originally present in the oils, may suffer changes in their concentration and give rise to the formation of other new compounds. Regarding this issue, some researchers have highlighted the degradation of some minor compounds present in edible oils throughout different temperature storage or thermal conditions (Player et al., 2006; Wang et al., 2010; Gawrysiak-Witulska et al., 2015; Naziri et al., 2014). In most of these studies different techniques are employed to study the diverse nature and family of compounds and, in most of them, previous extraction and/or purification steps are performed which modify the structure of the molecules. Therefore, there are very few examples of joint

studies of the evolution of these minor compounds in a sample of oils using a unique technique and, as far as is known, there has never been carried out the a study of the oxidation compounds in which these latter are transformed.

For all the reasons mentioned above and in order to try to simultaneously monitor the evolution of the original main and minor oil components and other compounds arising from their oxidation during technological processing or oxidation process, the development of new techniques and/or the improvement of already existing analytical tools is of great importance. In this context, the **objective 1.1.3.** (**aim 1.1.; Block 1**) and **objectives 1.2.1.** and **1.2.2.** (**aim 1.2.; Block 1**) were addressed.

3. The extent of lipid hydrolysis and the oxidation reactions during *in vitro* digestion of edible oils.

The digestion process is an important and determinant step in which the nutritional quality and safety of dietary lipids undergo modifications due to several reactions that take place in the gastrointestinal tract that have important repercussions in food component bioaccessibility. Among those, hydrolysis of proteins, carbohaydrates and triglycerides are the main ones, giving rise to smaller blocks which are able to be absorbed. In fact, the hydrolysis extent and pattern of triglycerides predetermines the bioaccessibility and health effect of dietary lipids. This last reaction depends mainly on the degree of unsaturation thereof, as well as on other factors, such as type of food, pH of the medium, presence of oxygen and presence of anti- or pro-oxidant compounds, among others (Nieva-Echevarría et al., 2018). As is well known, lipids are mainly formed by triglycerides (TG), which need to be hydrolyzed to be absorbed by human organism. Lipolysis or lipid hydrolysis reactions are catalyzed in the gastrointestinal track, mainly in the duodenum, due to the presence of digestive lipases such as pancreatic lipase. This is a regiospecific enzyme, which gives rise first to the formation of one molecule of 1,2-

diglycerides (1,2-DG) and one fatty acid (FA), and due to the subsequent hydrolysis of 1,2-DG, one molecule of 2-monoglyceride (2-MG) and another of FA are released, resulting, at the end of the process, in one molecule of 2-MG and two molecules of FA. Furthermore, the complete hydrolysis of TG into three molecules of FA and glycerol (Gol) have been described, after isomerization of 2-MG, into 1-MG (Desnuelle & Savary, 1963; Mattson & Volpenhein, 1964; Nieva-Echevarria et al., 2015).

Furthermore, some evidence suggests that apart from the above-mentioned reactions, others might be possible, such as Maillard reaction, esterification and oxidation (Halliwell et al., 2000; Gorelik et al., 2005; Goicoechea et al., 2011). Of these, oxidation could be cause for concern because of the subsequent loss of nutritional value of foods due to the degradation of lipids (Goicoechea et al., 2008; Nieva-Echevarría et al., 2017a,b) and the formation of compounds resulting from this degradation; some of which have been linked to several degenerative diseases, such as Alzheimer, Parkinson, respiratory distress and diminution of cardiac functions, among others (Fukushima et al., 1988; Ozawa et al., 1990; Esterbauer et al., 1991; Guillén & Goicoechea, 2008; Ramsden et al., 2017). This reaction depends mainly on the degree of unsaturation thereof, as well as on other factors, such as, type of food, pH of the medium, presence of oxygen and presence of anti- or pro-oxidant compounds, among others (Nieva-Echevarría et al., 2020). Likewise, several authors describe that during the digestion process some micronutrients present in foods, to which healthy properties haven been attributed, also undergo degradation, and therefore their bioaccessibility is also reduced (Granado-Lorencio et al., 2007; Kenmogne-Domguia et al., 2014; Martin-Rubio et al., 2019; Nieva-Echevarría et al., 2019). Accordingly, with the objective to delay and/or avoid lipid oxidation reactions, the use of compounds, to which antioxidant capacity is attributed, is becoming a common strategy.

3.1. Strategies to delay and/or to reduce lipid oxidation reactions during in vitro digestion conditions. The potential influence of the used of phenolic compounds.

As commented on above, lipid oxidation is, therefore, a very complex process which provokes not only the reduction of nutritional and sensorial quality, but also the generation of new compounds, some of which have well-known toxic effects on human health. Antioxidants, meanwhile, are molecules stable enough to donate an electron to free radicals and neutralize them, thereby inhibiting and/or delaying their oxidation and consequently the formation of oxidation products derived from them. Nowadays, it seems to be well established that diets high in vegetable and fruit content are healthy mainly due to these commodities being rich in compounds with antioxidant ability. For this reason, the enrichment of foods with compounds of natural origin with potential antioxidant ability is becoming common in the industry, for both technological and health reasons. In spite of this, knowledge of the effect that this enrichment provokes in foods when they are subjected to different processes is scarce. Recently, it has been shown that some compounds' attributed antioxidant ability act as prooxidants in some foods when submitted to certain oxidative conditions (Martin-Rubio et al., 2018a, b; Martínez-Yusta et al., 2019). Considering all the above, the study into the effect of the enrichment of lipid foods with compounds with potential antioxidant ability on their behaviour during digestion is important for both industry and consumers.

Among the compounds that exhibit antioxidant activity are phenolic compounds. These are usually secondary metabolites found mainly in vegetables and fruits in very low concentrations, and to them the healthy properties of these foods have been attributed. These compounds are considered free radical scavengers. They are characterized by having in their structure at least one hydroxyl group (see Figure 1).

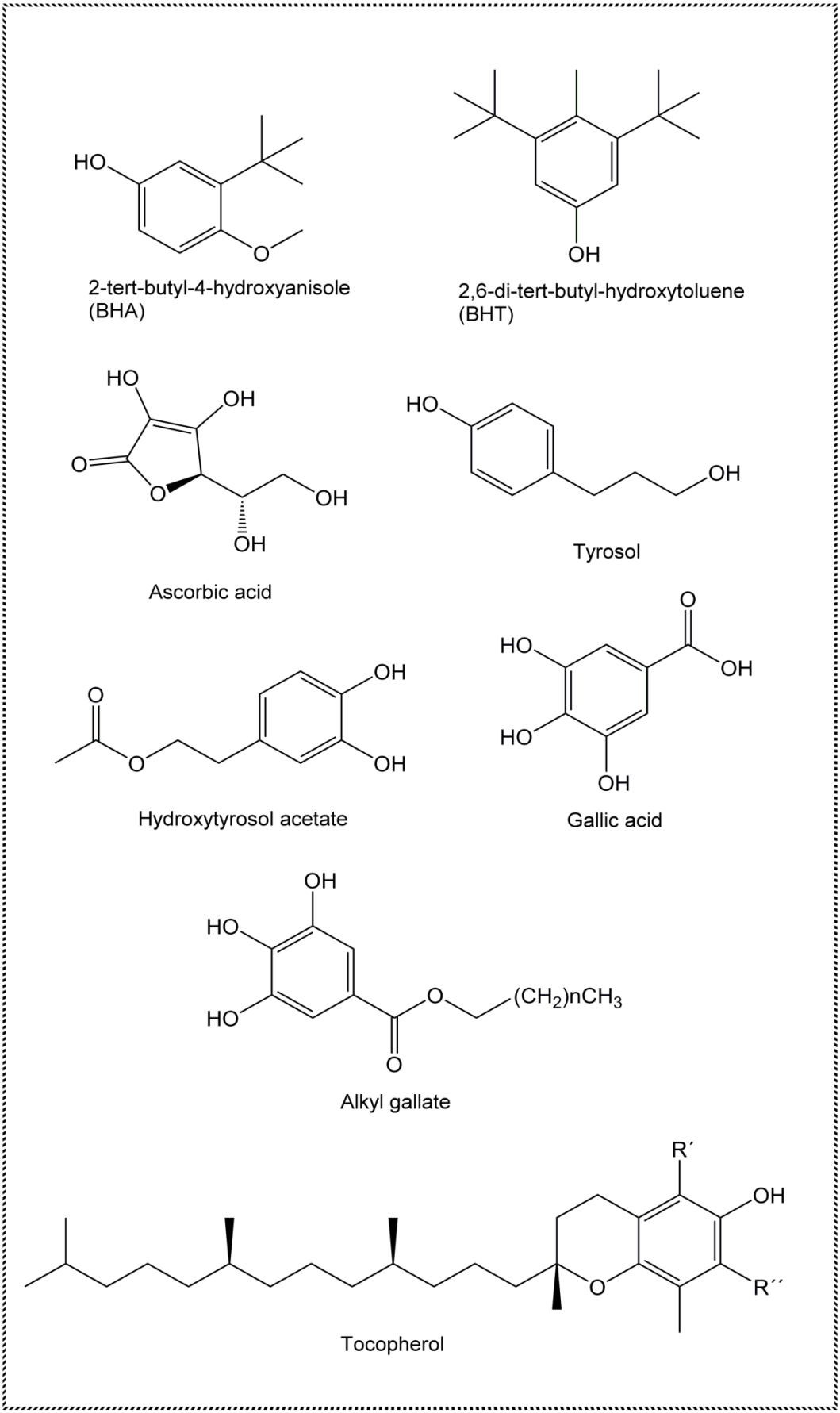

Figure 1. Chemical structures of some phenolic compounds.

Some authors have related their antioxidant capacity to the number and the arrangement of their hydroxyl groups (Shahidi & Ambigaipalan, 2015). Due to their potential antioxidant ability and to their attributed beneficial health effects (Shahidi & Ambigaipalan, 2015; Bhuyan & Basu, 2017), some studies have been focused in the enrichment of lipid food with different natural or synthetic phenolic compounds, to prevent oxidation when they are submitted to gastrointestinal digestion.

Regarding the behaviour of these compounds during the digestion process of food and oil, the efficiency of 2,6-di-*tert*-butyl-hydroxytoluene (BHT), tocopherols, resveratrol, epicatechin, caffeic and gallic acid among others, has been investigated. For this, Nieva-Echevarría et al., (2017a) carried out an *in vitro* gastrointestinal digestion of cod liver oil in the presence 2,6-di-*tert*-butyl-hydroxytoluene (BHT), in concentrations of 0.002% and 0.08%, which concluded that BHT greatly limits the extent of oxidation reactions, almost inhibiting them at 0.08%. With respect to other polyphenols, Keren et al., (2006) studied the effect of different polyphenols on the inhibition of the oxidation of linoleic acid in authentic fluid from rat small intestine, paying attention to the production of hexanal. These authors observed that epicatechin and resveratrol were the most potent antioxidants inhibiting hexanal production, while caffeic acid proved to be the less effective compound, and the effect of gallic acid was negligible.

Conversely, during the digestion of high-fat and low-fat beef meat in the presence of different amounts (0.05% to 0.5%) of some hydrophilic (gallic and caffeic acid among others) and lipophilic (quercetin and silibinin, among others) phenolic compounds, different behaviour was observed depending on the amount of fat present in the matrix and the type of phenolic compound and its concentration (Van Hecke et al., 2016). Likewise, Martini et al., (2018) reported a significant inhibition of turkey meat lipid oxidation during co-digestion with low concentration (2.5%) of extra virgin olive oil,

whereas lipid peroxidation was greatly enhanced when the amount of extra virgin olive oil in the gastrointestinal system increased to 5% and 10%. This pro-oxidant effect was associated with hydroxytyrosol-derivates, the main polyphenols in extra virgin olive oil.

Controversial results were also obtained when tocopherols were used. For example, Larsson et al., (2012), reported no effect of α-tocopherol when adding 1 mg/ml to cod liver oil. Nevertheless, these results differ from those obtained by other authors (Van Hecke et al., 2016; Tullberg et al., 2019; Nieva-Echevarría et al., 2019). A clear antioxidant effect was observed after *in vitro* gastrointestinal digestion of high-fat beef meat and *ex vivo* gastrointestinal digestion of cod liver oil in the presence of different amounts of α-T, ranging from 0.05% to 0.45% (Van Hecke et al., 2016; Tullberg et al,. 2019)., Recently, however, other authors reported that addition of α-T at 0.2%, 2% and 5% showed a pro-oxidant activity *in vitro* during digestion of sunflower and flaxseed oils (Nieva-Echevarría et al., 2019).

However, phenolic compounds, in addition to exhibiting antioxidant or prooxidant ability, could also take part in other reactions during digestion because they come in contact with all food components and also with the digestive juices containing enzymes. It should be remembered that phenolic compounds can react with proteins, and for this reason with enzymes (Kroll et al., 2003, Ozdal et al., 2013), thereby reducing its activity and provoking negative effects on the digestion by diminishing the extent of the hydrolytic reactions. In fact, it has been described that tea polyphenols are able to inhibit the pancreatic lipase activity reducing the gastrointestinal lipolysis (Uchiyama et al., 2011) and consequently the absorption of lipids. Likewise, it has been described that alkyl gallates are able to inhibit the activity of amylase, reducing in this way the absorption of carbohydrates (Chi et al., 2018; Gutierrez et al., 2020).

All these studies suggest that phenolic compounds could not only influence decreasing or increasing oxidative reactions that can occur during lipid digestion, but also, in some cases, could affect the hydrolytic reactions that constitute the essence of the digestion process to release absorbable building blocks.

Taking all the above-commented into account, **Block 2** (**aims 2.1., 2.2.** and **2.3**) of this doctoral thesis aims to evaluate the efficiency of mono- and poly-phenolic compounds on the *in vitro* digestion of olive, corn and flaxseed oils, by means of ^{1}H NMR and SPME-GC/MS, to obtain a global view of the changes occurring during this complex process, as well as whether the composition in acyl groups could have any effect in the lipolysis extent and pattern.

AIMS

The present doctoral thesis is divided in two main blocks, which are focussed on the achievement of several aims.

BLOCK 1: Methodological development and its application on the advancement of the knowledge about edible oils composition and the processes they are involved in, such as nixtamalization and oxidation. The Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography coupled to Mass Spectrometry (DI-SPME-GC/MS) and the Proton Nuclear Magnetic Resonance (^{1}H NMR). (Articles 1, 2, 3, 5 and Manuscript 4)

To carry out this block, the following **specific aims** were formulated:

1.1. Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography coupled to Mass Spectrometry (DI-SPME-GC/MS) as a very useful new tool.

1.1.1. Development and application of a **new methodology** based on DI-SPME-GC/MS, for the **characterization** of **volatile and less volatile** edible oils **components**. (Article 1)

1.1.2. New corn oil components detected for the first time by means of DI-SPME-GC/MS. (Article 2)

1.1.3. To **address** by means of DI-SPME-GC/MS the **effect** of **accelerate storage process** (70 ºC) **on corn oil,** throughout the **evolution** of some of its **minor components**. Detection of **new** oil **oxidation markers**. (Article 3)

1.2. Proton Nuclear Magnetic Resonance (^{1}H NMR) as a powerful tool in the study of the edible oil oxidation processes. Advancement in the knowledge of the oxidation process.

1.2.1. Detection and **quantification** of **compounds previously not described during edible oil oxidation processes** by means of **^{1}H NMR. A global analysis of the oxidation process.** (Manuscript 4)

1.2.2. Study by means of ^{1}H NMR the **changes caused** by **Nixtamalization and Tortilla-making** processes in the **lipid composition** of two corn varieties. (Article 5)

Accomplishing this **first block** will lead to an increase of knowledge and to shed light on the analytical methodologies able to provide simultaneous (without either solvents or sample modification), qualitative and quantitative information concerning most edible oils' components, either naturally present in the oils or generated during oxidation reactions (**aim 1.1.**). Moreover, with the accomplishment of **aim 1.2**., the usefulness of the ^{1}H NMR technique to monitor the changes that take place during edible oil oxidation process will be established, with attention given to the detection and quantification of oxidation compounds previously not described, as well as to the changes caused during nixtamalization and tortilla-making processes (a processing technique used for thousands of years by American people) in the lipid composition of two corn varieties.

BLOCK 2: Advances in the knowledge of the edible oil *in vitro* digestion process. Antioxidant effectiveness of mono- or poly-phenolic compounds. Bioaccessibility of main and minor oil components. (Articles 6-7 and Manuscript 8)

For this purpose, the following **specific aims** were addressed:

2.1. To **investigate** by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the **effect** of the addition of ***alpha*-tocopherol (α-T) and *gamma*-tocopherol (γ-T)** on the

lipolysis extent, advance of the **oxidation** reactions and **bioaccessibility** of main and minor compounds, **during *in vitro* digestion** of **corn oil.** (Article 6)

2.2. To **address** by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the **antioxidant effectiveness** of mono-, di- and tri-phenolic compounds (***gamma*-tocopherol (γ-T), hydroxytyrosol acetate (HTA)** and **dodecyl gallate (DG))** during the ***in vitro* digestion** of **virgin flaxseed oil**. (Article 7)

2.3. To **study** by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the **behaviour** of **olive oil** during ***in vitro* digestion**. Study of the **lipolysis** extent and pattern and **oxidation** reactions, as well as of the **bioaccessibility** of main and minor compounds. **Comparison** with **corn oil** and **virgin flaxseed oil** submitted to the same digestive conditions (Manuscript 8)

The consecution of the **second block** will provide important information about the lipolysis and oxidation reactions taking place on corn oil, virgin flaxseed oils and olive oil as a model of omega-6, omega-3 and omega-9 lipids, as well as the bioaccessibility of their main and minor compounds during *in vitro* digestion. Additionally, it also will shed light on the behaviour of different phenolic compounds differing in the number of phenol groups in their structure added at different concentrations to these oils, the chemical reactions affecting their lipids, as well as the bioaccessibility of the naturally present minor compounds (*gamma*-tocopherol, cycloartenol and methylencycloartenol, terpenes and squalene) during *in vitro* digestion. Moreover, it will also provide information about the bioaccessibility of some of these added phenolic compounds.

EXPERIMENTAL DESIGN

BLOCK 1

Methodological development and its application on the advancement of the knowledge about edible oils composition and the processes they are involved in, such as nixtamalization and oxidation. The Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography coupled to Mass Spectrometry (DI-SPME-GC/MS) and the Proton Nuclear Magnetic Resonance (^{1}H NMR).

(Articles 1, 2, 3, 5 and **Manuscript 4)**

Aim 1.1. Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography coupled to Mass Spectrometry (DI-SPME-GC/MS) as a very useful new tool. **(Articles 1-3)**

Aim 1.2. Proton Nuclear Magnetic Resonance (^{1}H NMR) as a powerful tool in the study of the edible oil oxidation processes. Advancement in the knowledge of the oxidation process. **(Manuscript 4 and Article 5)**

Aim 1.1. Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography coupled to Mass Spectrometry (DI-SPME-GC/MS) as a very useful new tool. **(Articles 1-3)**

Objective 1.1.1. Development and application of a new methodology based on DI-SPME-GC/MS, for the characterization of volatile and less volatile edible oils components.

(Article 1)

Objective 1.1.2. New corn oil components detected for the first time by means of DI-SPME-GC/MS.

(Article 2)

Objective 1.1.3. To address by means of DI-SPME-GC/MS the effect of accelerate storage process (70 ºC) on corn oil, throughout the evolution of some of its minor components. Detection of new oil oxidation markers.

(Article 3)

Objective 1.1.1. Development and application of a new methodology based on DI-SPME-GC/MS, for the characterization of volatile and less volatile edible oils components. (**Article 1**)

1. Samples subject of studies

The samples subject of study were: one sunflower oil, SF, one corn oil, C, two soybean oils (one refined, RSB and the other virgin, VSB) and one virgin linseed oil, VL. All were acquired in local supermarkets. It is well-known that the composition of these oils in main component is closely related to their botanical origin. This composition, expressed as molar percentages of the different kinds of acyl groups, was determined from ^{1}H NMR spectral data as in previous studies (Guillén & Uriarte, 2009) and it is given in Table S1 of **article 1**.

2. Extraction of the oil minor components

The oils were submitted to extraction directly without any previous sample preparation. The method used is based on that described by Mikuma and Kaneko (2010). The extraction of the oil components was carried out by solid-phase microextraction. To this aim a fiber of 65 μm StableFlex polydimethylsiloxane/divinylbenzene (PDMS/DVB) acquired from Supelco (Bellefonte, PA, U.S.) was immersed directly into 6 ml of edible oil at room temperature for 45 minutes. This fiber was selected on the basis of its ability to extract the widest range of minor oil components with the highest possible yield. Its selection was made after analyzing the results obtained by Mikuma and Kaneno and after testing some fibers such as 100 μm PDMS, 65 μm PDMS/DVB and 50/30 μm DVB/CAR/PDMS. The extraction time selected provided the best results in terms of extraction yield after testing extraction times of 15, 30, 45 and 90 minutes.

No influence of the concentration of a compound in the oil on the adsorption and extraction yield pf the other ones was observed in studied of enrichment of the edible oils

with several proportion either of sterol, or of different tocopherols, or of other compounds.

3. Gas Chromatography-Mass Spectrometry

The oil components picked up by the fiber were directly injected into the port of a gas chromatograph-mass spectrometer (GC/MS). For their thermal desorption, the plunger was pushed down to expose the fiber to the GC carrier gas stream and held for 10 minutes. During the first 5 min, which constitute the effective desorption time, the split valve was closed (splitless mode) to ensure the complete introduction of the desorbed compounds into the chromatographic column, and afterwards it was opened.

After the injection of the extracted compounds, the fiber was submitted to two additional desorption processes in the gas chromatograph in order to ensure the complete removal of minor oil components traces and prevent cross-sample contamination. It was confirmed that these two fiber cleanings were sufficient to avoid carry-over effects.

The desorbed compounds were separated, identified and semi-quantified by gas chromatography-mass spectrometry. The equipment used was an Agilent gas chromatograph model 6890N equipped with a mass selective detector 5973 Network and a Hewlett-Packard Compaq Pentium 4 computer. A fused silica capillary column was used (60 m length x 0.25 mm inside diameter x 0.25 μm film thickness; from Agilent Technologies Inc., Palo Alto, CA), coated with a nonpolar stationary phase (HP-5MS, 5% phenyl methyl siloxane). The operation conditions were the following: the injector and interface temperatures were held at 250°C and 305ºC respectively, and helium at a constant pressure of 117 kPa (16.9 psi) was used as the carrier gas. The oven temperature was initially held at 50ºC for 5 min, increased from 50 to 300ºC at a rate of 4ºC/min, and then held at 300ºC for 30 min. Mass spectra were recorded at an ionisation energy of 70 eV, with data acquisition in Scan mode. The temperatures of the ion source and the

quadrupole mass analyzer were kept at 230 and 150ºC, respectively. A reference sample of known composition was periodically analyzed in order to verify the sensitivity of the GC/MS equipment.

Many components were identified by comparison of their retention times and mass spectra with those of standards acquired from Sigma-Aldrich (St. Louis, MO, USA), Larodan Fine Chemicals AB (Malmo, Sweden), Chem Faces (Wuhan, China) and Cayman Chemical (Ann Arbor, MI, USA); these are asterisked in the different tables of **article 1**. Others were identified by matching of their mass spectra with spectra from a commercial library by more than 85% (W9N08, Wiley ver. 9.0 & NIST, ver. 8.0 library) and also with those obtained from the literature.

Semi-quantification was based on arbitrary units of the base peak ion area counts divided either by 10^4. All the determinations were carried out at least in duplicate in order to obtain a mean value with the corresponding standard deviation for each of the components studied.

Objective 1.1.2. New corn oil components detected for the first time by means of DI-SPME-GC/MS. (**Article 2**)

1. Samples subject of studies

The study was carried out on eleven different edible oils of varied vegetable origin, namely extra virgin olive oil, virgin olive oil, olive oil, refined soybean oil, virgin soybean oil, sunflower oil, virgin linseed oil and four refined corn oils (C1, C2, C3, and C4). All of them were acquired in local supermarkets. In order to have more information about the composition of the four corn oils subject of study their main components were quantified by ^{1}H NMR as in previous studies (Guillén & Ruiz, 2003a; Guillén & Uriarte, 2009). Their composition expressed as molar percentages of different kinds of acyl groups is given in Table 1 of **article 2**. It can be observed that there are no important differences among main components in these refined corn oils, C4 being the richest in oleic acyl groups followed by C1, C2 and C3 and the opposite being true for linoleic acyl groups.

2. Extraction of minor oil components

This was carried out in the same way as that described in section 2 of the experimental design of **objective 1.1.1**. (**Block 1**).

3. Gas chromatograph-Mass spectrometry

This was carried out in the same way as that described in section 3 of the experimental desing of **objective 1.1.1.** (**Block 1**).

4. Quality control of the global method and statistical analysis

The quality control of the global method was carried out by the periodic extraction of an oil sample considered as sample reference and the subsequent study of the extracted compounds by GC/MS. The detection limit was established in an abundance of 10000 area counts for the base peak of the mass spectra of the compounds. The specificity, selectivity and robustness of the GC/MS to evaluate unequivocally the compounds

included in this study is beyond all doubt as it is a recognized suitable global method for the purposes of this study.

5. Statistical Analysis

All determinations were carried out at least in duplicate. Data given in Tables 2 and 3 of **article 2** are mean values accompanied by the corresponding standard deviations. The significance of the differences among the several corn oil samples on the molar percentage of the acyl groups and on the abundances of the several nitrogenated compounds were determined by one-way variance analysis (ANOVA) followed by Tukey *b* test at $p < 0.05$ and/or by Student´s t-test at 0.05 threshold, using SPSS v.22 (IBM, NY, USA).

Objective 1.1.3. To address by means of DI-SPME-GC/MS the effect of accelerate storage process (70 ºC) on corn oil, throughout the evolution of some of its minor components. Detection of new oil oxidation markers. (**Article 3**)

1. Sample subject of studies

The studies were carried out with refined corn oils, purchased in a local supermarket.

2. Accelerated storage (AS) process

10 g of original corn oil were placed in glass Petri dishes (80 mm in diameter and 15 mm deep) and kept in an oven at 70°C with aeration. Under these conditions the original oil underwent oxidation. These experiments were performed in duplicate to obtain sound results. The evolution of the samples was followed by DI-SPME-GC/MS and ^{1}H NMR until its total polymerization.

In addition, pure squalene, α- and γ-tocopherol were subjected to the same oxidative conditions above-commented and were studied by GC/MS. To this aim, aliquots of these oxidized samples were conveniently dissolved in dichloromethane and injected directly in the chromatograph.

3. Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography/Mass Spectrometry (DI-SPME-GC/MS) experiments

This was carried out in the same way as that described in sections 2 and 3 of the experimental design of **objective 1.1.1**. (**Block 1**).

4. Quality control of the global method and statistical analysis

Quality control of the global method was carried out by periodic extraction of an oil sample considered as sample reference and the subsequent study of the extracted compounds by GC/MS. The detection limit was established in an abundance of 10000 area counts for the base peak of the mass spectra of the compounds. The specificity, selectivity and robustness of the GC/MS to evaluate unequivocally the compounds

included in this study are beyond all doubt as it is a recognized suitable global method for the purposes of this study. Data given in Tables 1, 2 and Tables S1, S2 and S3 of **article 3** are expressed as mean ± standard deviation (SD) of two measurements for the analytical determination. Microsoft Office Excel 2007 was used for the graphical representation of the obtained values. The global results here obtained have been corroborated with three other different corn oils submitted to the same oxidative conditions.

Aim 1.2. Proton Nuclear Magnetic Resonance (^{1}H NMR) as a powerful tool in the study of the edible oil oxidation processes. Advancement in the knowledge of the oxidation process. **(Manuscript 4 and Article 5)**

Objective 1.2.1. Detection and quantification of compounds previously not described during edible oil oxidation processes by means of ^{1}H NMR. A global analysis of the oxidation process.

(Manuscript 4)

Objective 1.2.2. Study by means of ^{1}H NMR the changes caused by Nixtamalization and Tortilla-making processes in the lipid composition of two corn varieties.

(Article 5)

Objective 1.2.1. Detection and quantification of compounds previously not described during edible oil oxidation processes by means of ^{1}H NMR. A global analysis of the oxidation process. (**Manuscript 4**)

1. Samples subject of study

1.1. Original oil

The study was carried out with refined corn oil, purchased in a local supermarket. Its composition in acyl groups was determined from ^{1}H NMR spectral data as in previous studies (Guillén & Ruiz, 2003, Guillén & Uriarte, 2012a). The molar percentages of the different kinds of acyl groups, regarding the total of acyl groups, were linolenic group, Ln, 0.6 ± 0.0%, linoleic group, L, 48.7 ± 0.0%, oleic group, O, 33.0 ± 0.1%, and saturated group, S, 17.7 ± 0.1%. These compositional data can be also expressed in mmol regarding to the number of moles of triglyceride, TG; in this way this oil contains, 18.8 ± 0.0 mmoles of Ln/molTG, 1,461.6 ± 0.1 mmoles of L/molTG, 990.2 ± 1.7 mmoles of O/molTG and 529.4 ± 1.5 mmoles of S/molTG.

1.2. Oxidized oil samples

In addition to the original oil, also samples derived from this oil, after submission to mild oxidative conditions, similar to those of accelerated storage for different periods of time, were subject of study. To prepare these derived samples, amounts of 10 g of original corn oil were placed in glass Petri dishes (80 mm in diameter and 15 mm deep) and kept in an oven at 70°C with aeration, during different periods of time, up to sixteen days. These experiments were performed in duplicate to obtain sound results.

2. Acquisition of the ^{1}H Nuclear Magnetic Resonance spectra (^{1}H NMR)

2.1. Operating conditions

The ^{1}H NMR spectra of the original oil and of the samples derived from this oil, after to be submitted to degradative conditions, were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. For this purpose, the above-mentioned samples (approximately 0.16 g) were dissolved in 400 µl of deuterated chloroform, which contained tetramethylsilane (TMS), as internal reference (Cortec, Paris, France). The acquisition conditions were the same as those used in previous studies (Guillén & Ruiz, 2004; Guillén & Uriarte, 2009). The relaxation delays and acquisition times allow the complete relaxation of the protons, the areas of the signals thus being proportional to the number of protons that generate them, making their use for quantitative purposes possible. The ^{1}H NMR spectra were plotted at a fixed value of absolute intensity to be valid for comparative purposes using the MestreNova program (Mestrelab Research, Santiago de Compostela, Spain).

2.2. Identification of components

The identification, of either components present in the original oil or components generated in the oxidation process, was carried out on the basis of the assignments of the ^{1}H NMR signals to the different kinds of hydrogen atoms and to the different compounds. These signals, which are shown in different figures, their chemical shifts and their assignments to the several hydrogen atoms are given in Tables S1-S8 (see Supplementary Material of **manuscript 4**). The assignments were made taken into account previous studies as it is indicated in Tables S1-S8 (Supplementary Material of **manuscript 4**), or on the basis of the signals of standard compounds acquired for this study.

2.3. Quantification of the components

This was possible because the area of each ^{1}H NMR spectral signal is proportional to the number of protons that generates it, and because the proportionality constant is the same for all kinds of protons. Taken this into account, the estimation of the concentrations of the different functional groups, or of groups of compounds, as well as of minor components, regarding the concentration of triglycerides TG can be carried out, by using the area of the corresponding spectral signals. The triglycerides can be considered as an internal reference due the low level of hydrolysis that these undergo during oxidation. To this aim, the equation $[X] = [(A_X/n)/(A_{TG}/4)]*1000$ [eq. 1] was used. In this equation A_X is the area of the signal selected for the quantification of the X functional group, n is the number of protons that generate this signal and A_{TG} the area of the protons at *sn*-1 and *sn*-3 positions in the triglyceride backbone TG (signal TG in Table S1). In this way the concentration obtained is expressed in millimol per mol of triglyceride (mmol/mol TG). The area of the signals used was determined by using the equipment software and the integrations were made three times to obtain average values.

3. Statistical analysis

Data represented in the graphical representation of the different figures and those given Table 1 of **manuscript 4** are mean values obtained as average values of at least two determinations. Microsoft Office Excel 2007 was used for the statistical analysis and for the graphical representation of the obtained values.

Objective 1.2.2. Study by means of ^{1}H NMR the changes caused by Nixtamalization and Tortilla-making processes in the lipid composition of two corn varieties. (**Article 5**)

1. Raw samples and extraction of their lipids

The study was carried out with two different varieties of corn (*Zea mays* L.) coming from Mexico. One of them is a blue native corn, B, of Tziranza variety, coming from Paracho, Michoacán, and the other is a white corn, W, of Sinaloa variety, coming from Sinaloa. Both varieties of corn have hard endosperm and nixtamalera-tortillera quality. Their grains were ground and mixed with hexane (High Performance Liquid Chromatography (HPLC) grade, 98.5%) in a proportion 1:3 and maintained under vigorous agitation for 24 hours at 25 ºC. After filtration, the solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. The extracted lipids obtained from B and W raw samples were named BR and WR, their yields being 4% and 4.5% by weight respectively.

2. Nixtamalization, tortilla manufacture and lipid extraction

Nixtamalization is a technology developed by the Mesoamerican peoples that involves alkaline thermal treatment of the corn grains with calcium hydroxide solution, keeping the grains in this solution for a certain period of time, and cleaning up the grains with water. In this case both corn samples were nixtamalized following the classical methodology. Corn grains were boiled in a solution of calcium hydroxide in a proportion (1/3) (corn/solution). The calcium hydroxide concentration was 1%. The cooking time was established, in agreement with the Mexican normative (NMX-FF-034/1-SCFI-2002), at 30 and 45 minutes for B and W corn respectively. After cooking, the grains were steeped in the cooking solution for 12 hours at room temperature. Then the cooked corn grains were washed with distilled water and ground to obtain dough with which tortillas were prepared and cooked on a griddle at 230 ºC (Gomez, Rooney, Waniska &

Pflugfelder, 1987; Rooney & Serna-Saldivar, 1987; Serna-Saldivar et al., 1991). The tortillas were dried to 6% moisture and ground to carry out the extraction of their lipids. This was carried out using hexane under the same conditions mentioned for raw corn samples before. The lipids extracted were named BT and WT, their yields being 2.8% and 3.1% by weight respectively.

3. Acquisition of the ^{1}H Nuclear Magnetic Resonance spectra (^{1}H NMR)

The ^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz. Each lipid sample, weighing 0.2 g was mixed with 400 μl of deuterated chloroform and a small proportion of TMS as internal reference; this mixture was introduced into a 5 mm diameter tube. The acquisition conditions were the same as those used in previous studies (Guillén & Ruiz, 2004; Guillén & Uriarte, 2009). The relaxation delays and acquisition times allow the complete relaxation of the protons, the signal areas thus being proportional to the number of protons that generate them, making their use for quantitative purposes possible. Two spectra were acquired from each sample. The ^{1}H NMR spectra were plotted at a fixed value of absolute intensity to be valid for comparative purposes using the MestreNova program (Mestrelab Research, Santiago de Compostela, Spain).

The identification of the compounds was made on the basis of the chemical shifts of their protons for well known compounds. In other cases their identification was confirmed by using standard compounds such as phosphatidylcholine, ethyl and isopropyl ferulate and 1,2-diglyceride, β-sitosterol, Δ5-campesterol, Δ5-avenasterol and sitostanol acquired from Sigma Aldrich (St. Louis, MO, USA), ChemFaces Biochemical Co., LTD (Wuhan, China), Larodan (Malmö, Sweeden). Table 1 of **article 5**, shows the signal assignment to the corresponding hydrogen atoms of the several compounds present in the samples subject of study.

Bearing in mind that the area of each ^{1}H NMR spectral signal is proportional to the number of protons that generates it, and that the proportionality constant is the same for all kinds of protons, it is possible, using the area of some spectral signals, to estimate the molar percentage of the several kinds of acyl groups and fatty acids, as well as the concentration of some minor components. The area of the signals used was determined by using the equipment software and the integrations were made three times to obtain average values.

4. Statistical analysis

The significance of the differences on the several determinations referred to ^{1}H NMR spectral data made among the samples was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS software v.22 (IBM, NY, USA).

BLOCK 2

Advances in the knowledge of the edible oil *in vitro* digestion process. Antioxidant effectiveness of mono- or poly-phenolic compounds. Bioaccessibility of main and minor oil components.

(Articles 6-7 and Manuscript 8)

Aim 2.1. To investigate by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the effect of the addition of *alpha*-tocopherol (α-T) and *gamma*-tocopherol (γ-T) on the lipolysis extent, advance of the oxidation reactions and bioaccessibility of main and minor compounds, during *in vitro* digestion of corn oil. **(Article 6)**

Aim 2.2. To address by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the antioxidant effectiveness of mono-, di- and tri-phenolic compounds (*gamma*-tocopherol (γ-T), hydroxytyrosol acetate (HTA) and dodecyl gallate (DG)) during the *in vitro* digestion of virgin flaxseed oil. **(Article 7)**

Aim 2.3. To study by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the behaviour of olive oil during *in vitro* digestion. Study of the lipolysis extent and pattern and oxidation reactions, as well as of the bioaccessibility of main and minor compounds. Comparison with corn oil and virgin flaxseed oil submitted to the same digestive conditions. **(Manuscript 8)**

Aim 2.1. To investigate by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the effect of the addition of *alpha*-tocopherol (α-T) and *gamma*-tocopherol (γ-T) on the lipolysis extent, advance of the oxidation reactions and bioaccessibility of main and minor compounds, during *in vitro* digestion of corn oil. **(Article 6)**

1. Samples subject of study

The study was carried out with refined corn oil (C), acquired in a local supermarket. Its composition in molar percentages of linolenic (Ln), linoleic (L), oleic (O) and saturated (S) acyl groups is, 0.6 ± 0.0%, 49.2 ± 0.5%, 34.1 ± 0.3% and 16.1 ± 0.1% respectively. This was determined from ^{1}H NMR spectral data as in previous studies (Guillén & Ruiz, 2003; Guillén & Uriarte, 2012a). The tocopherols used were *alpha*-tocopherol (αT) (purity of 98.2%) purchased in Sigma-Aldrich (St. Louis, MO, USA), and *gamma*-tocopherol (γT) (purity of ≥ 90%) provided by Eisai Food & Chemical Co. Ltd. (Tokyo, Japan).

Aliquots of the oil were enriched with *alpha*-tocopherol or *gamma*-tocopherol at 0.2, 2 and 5% by weight in each case. The samples submitted to *in vitro* digestion were the original oil C, and all samples enriched in αT (C0.2αT, C2αT and C5αT) and in γT (C0.2γT, C2γT and C5γT).

2. Digestion experiments

Aliquots (0.5 g) of the above-mentioned samples were digested following the semi-static *in vitro* gastrointestinal digestion model developed by Versantvoort, et al. (2005). This validated method was optimized, in order to improve the lipids digestion, attempting to reach lipolysis levels of a similar order to in vivo digestion (Nieva-Echevarría et al., 2016) It has three-stages which simulates digestive processes in mouth, stomach, and small intestine, by sequentially adding the corresponding digestive juices (saliva, gastric juice,

duodenal juice and bile), whose composition is given in Table S1 (see Supplementary Material of **article 6**). The first stage begins by adding 6 mL of saliva to the sample. After 5 min of incubation, 12 mL of gastric juice are added and the mixture is rotated head-over-heels at 40 rpm for 2 h at 37±2ºC. One hour after the start of the gastric stage, pH is set between 2 and 3 with HCl (37%), simulating the gradual acidification of the chyme occurring in vivo. After 2 h of the gastric stage, 2 mL of sodium bicarbonate solution (1 M), 12 mL of duodenal juice and 6 mL of bile juice are added. Subsequently, pH was set between 6 and 7, and the mixture is again rotated at 40 rpm and incubated at 37±2ºC for 4 h. All the reagents and enzymes for the preparation of digestive juices were acquired from Sigma-Aldrich (St. Louis, MO, USA): α-amylase from *Aspergillus oryzae* (10065, ~30 U/mg); pepsin from porcine gastric mucosa (P7125, ≥400 U/mg protein); amano lipase A from *Aspergillus niger* (534781, ≥120,000 U/g); pancreatin from porcine pancreas (P1750); lipase type II crude from porcine pancreas (L3126, 100-500 U/mg protein (using olive oil, 30 min incubation)) and bovine bile extract (B3883). The digested samples were named after the original samples but preceded by D (DC, DC0.2αT, DC2αT, DC5αT, DC0.2γT, DC2γT and DC5γT). Three digestion experiments, each including duplicate samples, were performed. Blank samples corresponding to the mixture of juices submitted to digestive conditions were also taken for further analysis.

3. Digestate lipid extraction

Lipids of the digestates were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich) following a methodology that also allows fatty acid extraction as in a previous studies (Nieva-Echevarría et al., 2017). This methodology involves a three-stage liquid-liquid extraction process with 20 ml of CH_2Cl_2 each. Afterwards, to ensure a complete protonation of fatty acids and/or the dissociation of the potential salts formed, the remaining water phase was acidified to pH 2 with HCl (37%) and a second extraction

was carried out in three steps. All the CH_2Cl_2 extracts of each sample were mixed and any solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. The extraction yield was in all cases near 85%. These extracts contain triglycerides, diglycerides and monoglycerides, as well as fatty acids and tocopherols and other minor lipophilic compounds either present in the original oil samples or formed from oil components in the digestion process.

4. Study by ^{1}H NMR of oil samples and lipid extracts of digestates

4.1. Operating conditions

The ^{1}H NMR spectra of the original oil (C), and of the oil samples enriched with each one of the tocopherols at the different concentrations (C0.2αT, C2αT, C5αT; C0.2γT, C2γT and C5γT), and of the lipids extracted from their digestates (DC, DC0.2αT, DC2αT, DC5αT, DC0.2γT, DC2γT and DC5γT), were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. For this purpose, the above-mentioned samples (approximately 0.16 g) were dissolved in 400 µl of deuterated chloroform, which contained tetramethylsilane (TMS), as internal reference (Cortec, Paris, France). The acquisition conditions were the same as those used in previous studies (Guillén & Uriarte, 2012b). It must be noted that the relaxation delay and acquisition time allow the complete relaxation of the protons, the signal areas thus being proportional to the number of protons that generate them, making it possible to use them for quantitative purposes.

4.2. Identification of the components

The identification of the components present in the original oil, in the oil samples enriched with tocopherol and in the lipid extracts of their digestates, was carried out on the basis of the assignments of the ^{1}H NMR signals, present in Figure 1 and Figure 2 of the **article 6**, to the different kinds of hydrogen atoms, and to the different compounds.

These signals, their chemical shifts and assignments are given in Tables S2–S5 (see Supplementary Material in the **article 6**). Their assignments were made, taking into account previous studies as indicated in each table, or on the basis of the signals of standard compounds acquired for this study. Among the latter are: *trans*-12,13-epoxy-9-keto-10(*E*)-octadecenoic acid, linolein hydroperoxides, linolein hydroxides, 9-oxo-10*E*,12*Z*-octadecadienoic acid and 13-oxo-9*Z*,11*E*-octadecadienoic acid purchased from Cayman Chemical (Ann Arbor, MI, USA), 9(S)-Hydroxy-10(*E*),12(*E*)-octadecadienoic acid (Dimorphecolic acid) acquired from Larodan (Malmö, Sweeden).

Table S2 shows ^{1}H NMR signals of specific protons of the different glyceride structures, such as triglycerides, diglycerides and monoglycerides. Table S3 shows the ^{1}H NMR signals of protons of linolenic, linoleic, oleic and saturated acyl groups and fatty acids, and the signals of methylenic protons supported on carbons atoms in *alpha* position, in relation to carbonyl-carboxyl groups. Table S4 shows ^{1}H NMR signals of protons of oxidation compounds coming from the main oil components degradation, which occurred during digestion. Finally, Table S5 gives ^{1}H NMR signals of protons of *alpha*- and *gamma*-tocopherol. The areas of some of these spectral signals were used to quantify the concentration of the different kinds of the above-mentioned structures in the corresponding samples, as it will be explained below.

4.3. Quantification made from ^{1}H NMR spectral data

This technique allows the estimation of the concentrations, expressed in different ways, of all identified compounds above mentioned. This is possible because the intensity of the ^{1}H NMR signals is proportional to the number of protons that generate the signal. The quantification of the different kinds of compounds or structures is explained below.

4.3.1. Estimation of the molar percentage of the different kinds of glycerides in the digestates

The estimation of the molar percentage of each kind of glyceride structures can be carried out by using the intensity of some signals indicated in Tables S2 and S3, which can also be observed in Figure 1 (see **article 6**). Although glycerol is formed during digestion, due to its polar nature, it is not present in the lipid extract of the digestate. However, its concentration can be estimated indirectly. This is possible because the concentration of total fatty acids plus acyl groups, of only acyl groups, and of fatty acids liberated in the formation of diglycerides and monoglycerides can be determined from ^{1}H NMR data. Thus, the estimation of the molar percentage of triglycerides (TG), 1,2-diglycerides (1,2-DG), 1,3-diglycerides (1,3-DG), 2-monoglycerides (2-MG), 1-monoglycerides(1-MG) and glycerol (Gol) in relation to the total glyceryl structures present in the digestate, was carried out using equations [eq. S1-eq. S10] given in Supplementary Material of **article 6**. They are based exclusively on the intensity of ^{1}H NMR spectral signals (Nieva-Echevarría et al., 2017).

4.3.2. Estimation of the percentages of fatty acids plus acyl groups that have linoleic structures in relation to the total of all fatty acids and acyl groups in digestates

In refined oils, the concentration of fatty acids is very small and unappreciable in comparison with the concentration of acyl groups. However, as is known, hydrolysis during oil digestion provokes the transformation of a certain number of acyl groups into fatty acids. The fatty acids formed maintain the same number of carbon atoms and unsaturation pattern as the starting acyl groups. Acyl groups and fatty acids having the same structure provide NMR spectra signals with a high degree of overlapping, which allows their joint quantification. In this study the molar percentage of the linoleic acyl groups plus linoleic fatty acids in the digestates was estimated in relation to the total

number of moles of all kinds of fatty acids plus acyl groups. This estimation was made using the equation [eq. S11] given in Supplementary Material of **article 6,** in which the areas of some signals thar are shown in Figure 1 and in Table S3 are involved. This equation is the same as employed in previous studies (Guillén & Ruiz, 2003; Guillén & Uriarte, 2012a), but using the signal of methylenic protons supported on carbon atoms in *alpha* position in relation to carbonyl-carboxyl groups, instead of the signal of triglyceride protons used in edible oil studies.

4.3.3. Estimation of the concentration of specific compounds (SC) in oil samples and in the digestates

The concentration of oxidation compounds, and of other ones, such as *gamma*- and *alpha*-tocopherol, either in oils or in digestates, can be estimated by using the general equation [eq. S12] given in Supplementary Material of **article 6** and the intensity of one of their non-overlapped ^{1}H NMR spectral signal, which are shown in Figures 1 and 2, in Figure S1 and in Tables S3, S4 and S5 (see **article 6**). This equation allows one to estimate the concentration of any compound in oils or in digestates in relation to the concentration of fatty acids plus acyl groups, which are considered the internal reference.

5. Study by SPME-GC/MS of the headspace of digestates and of the mixture of the digestive juices submitted to digestion conditions with the corn oil

The extraction of the volatile components of the several samples (0.5 g in a 10 ml screw-cap vial) was carried out automatically using a CombiPAL autosampler (Agilent Technologies, Santa Clara, CA, USA). The samples studied were the several digestates (DC, DC0.2αT, DC2αT, DC5αT, DC0.2γT, DC2γT and DC5γT) and the mixture (CDJ) of digestive juices (DJ), after submission to digestion conditions, and corn oil (C). The comparison of the headspaces of the several samples enables one to deduce differences provoked in them by *in vitro* digestion.

The fiber used for the headspace components extraction was coated with Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm film thickness, 1 cm long; acquired from Supelco (Sigma-Aldrich, St. Louis, MO, USA)). It was inserted into the headspace of the sample and maintained for 55 min at 50 °C, after a pre-equilibration time of 5 min. The fiber containing the components extracted was desorbed for 10 min in the injection port (splitless mode with 5 min purge time) of a 7890A gas chromatograph, equipped with a 5975C inert MSD with a Triple Axis Detector (Agilent Technologies, Palo Alto, CA, USA) and a computer operating with the ChemStation program. A fused silica capillary column was used (60 m length, 0.25 mm inside diameter, 0.25 μm film thickness; from Agilent Technologies Inc., Palo Alto, CA, USA), coated with a nonpolar stationary phase (HP-5MS, 5% phenyl methyl siloxane). The operation conditions were the following: the injector and interface temperatures were held at 250 °C and 305 °C respectively, and helium at a constant pressure of 117 kPa (16.9 psi) was used as the carrier gas. The oven temperature was initially held at 50 °C for 5 min, increased from 50 to 300 °C at a rate of 4 °C/min, and then held at 300 °C for 30 min. Mass spectra were recorded at an ionization energy of 70 eV, with data acquisition in Scan mode. The temperatures of the ion source and the quadrupole mass analyzer were kept at 230 and 150 °C, respectively. A reference sample of known composition was periodically analyzed, in order to verify the sensitivity of the SPME-GC/MS experiments, as in previous studies (Guillén et al., 2005).

Identification of the headspace components was performed using several commercial standard compounds, asterisked in Table 3 of **article 6**, acquired from Sigma-Aldrich (St. Louis, MO, USA). When standard compounds were not available, the identification was made by matching the spectra obtained, higher than 85%, with those of commercial libraries (Wiley W9N08, Mass Spectral Database of the National Institute of Standards

and Technology) or with those spectra provided by the scientific literature, as in previous studies (Guillén et al., 2005).

Semi-quantification of the compounds was based on the area counts of the base peak (Bp) of the mass spectrum of each compound divided by 10^6. When the Bp of a compound overlapped with some ion peak of the mass spectrum of another compound, an alternative ion peak was selected for the semi-quantification of the former (Guillén et al., 2005). Although the chromatographic response factor of each compound is different, the area counts thus determined are useful for the comparison of the abundance of each compound in the different samples. The detection limit was established at an abundance of 50,000 area counts. Data given in Table 3 of **article 6** are average values of duplicate experiments

6. Statistical analysis

The significance of the differences in the several kinds of data among samples, was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM, NY, USA).

Aim 2.2. To address by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the antioxidant effectiveness of mono-, di- and tri-phenolic compounds (*gamma*-tocopherol (γ-T), hydroxytyrosol acetate (HTA) and dodecyl gallate (DG)) during the *in vitro* digestion of virgin flaxseed oil. **(Article 7)**

1. Samples subject of study

The study was carried out with virgin flaxseed oil (F), purchased in a local supermarket. Its composition in molar percentages of linolenic (Ln), linoleic (L), oleic (O) and saturated (S) acyl groups is 55.7 ± 0.0%, 14.2 ± 0.3%, 20.5 ± 1.2% and 9.5 ± 0.9% respectively; this was determined from the ^{1}H NMR spectral data as in previous studies (Guillén & Ruiz, 2003; Guillén & Uriarte, 2012). In virgin flaxseed oil there is an important presence of terpenes and of sesquiterpenes, as has been previously shown (Uriarte et al., 2011) and the oil used in this study no exception, which will be shown later. Likewise, it is also known that the main sterols of this oil are cycloartenol and 24-methylenecyclartenol and the main tocopherol is *gamma*-tocopherol (Schwartz et al., 2008; Alberdi-Cedeño et al., 2017); the concentration of these compounds in this oil will be indicated later. The presence of these minor components is important because they have been assigned various biological activities (Jiang et al., 2001; Burbank et al., 2018; Abraham et al., 2019; Zhang et al., 2020).

Aliquots of virgin flaxseed oil containing, a natural concentration of *gamma*-tocopherol of 0.33 mmol/mol (AG+FA), were enriched separately in dodecyl gallate (DG) (purity 98%, from Alfa Aesar., GmbH & Co KG, Germany), in hydroxytyrosol acetate (HTA) (purity of 99.54%, from Seprox Biotech, Madrid, Spain), and in *gamma*-tocopherol (γT) (purity ≥ 90%, Eisai Food & Chemical Co. Ltd., Tokyo, Japan). The samples enriched with dodecyl gallate were named FDG_1 (with an enrichment of 0.14mmol DG/mol

$[FA+AG]_O$) and FDG_2 (with an enrichment of 1.35mmol DG/mol $[FA+AG]_O$). The samples enriched with hydroxytyrosol acetate, were named $FHTA_1$ (with an enrichment of 0.24mmol HTA/mol $[FA+AG]_O$) and $FHTA_2$ (with an enrichment of 2.65mmol HTA/mol $[FA+AG]_O$). Finally, the samples enriched with different additional concentrations of *gamma*-tocopherol, were named $F\gamma T_1$ (with an enrichment of 0.13mmol γT/mol $[FA+AG]_O$), $F\gamma T_2$ (with an enrichment of 1.30mmol γT/mol $[FA+AG]_O$), $F\gamma T_3$ (with an enrichment of 14.21mmol γT/mol $[FA+AG]_O$) and $F\gamma T_4$ (with an enrichment of 32.79mmol γT/mol $[FA+AG]_O$). These enrichment levels were set in function of the solubility of these compounds in the oil. The above concentrations were obtained trying to reach enrichment degrees near to 0.02 % and 0.2 % in weight for the three phenolic compounds and, in addition, near 2% and 5% in weight in the case of gamma-tocopherol due to its high solubility in oils. However, these latter levels of enrichment were not possible for dodecyl gallate and hydroxytyrosol acetate because of their limited solubility in oils.

2. Digestion experiments

Aliquots (0.5 g) of the above-mentioned samples were digested following the semi-static *in vitro* gastrointestinal digestion model developed by Versantvoort, *et al*. (2005), as described in section 2. of the experimental design of **aim 2.1.**

3. Digestate lipid extraction

Lipids of the digestates were extracted as indicated in section 3. of the experimental design of **aim 2.1.**

4. Study by ^{1}H NMR of oil samples and lipid extracts of digestates

4.1. Operating conditions

The ^{1}H NMR spectra of the original oil (F), and of the oil samples enriched with the different compounds at the different concentrations (F, FDG_1, FDG_2, $FHTA_1$, $FHTA_2$, $F\gamma T_1$, $F\gamma T_2$, $F\gamma T_3$, and $F\gamma T_4$), and of the lipids extracted from their digestates (DF, $DFDG_1$, $DFDG_2$, $DFHTA_1$, $DFHTA_2$, $DF\gamma T_1$, $DF\gamma T_2$, $DF\gamma T_3$, and $DF\gamma T_4$) were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. For this purpose, the above-mentioned samples (approximately 0.16 g) were dissolved in 400 µl of deuterated chloroform, which contained tetramethylsilane (TMS), as internal reference (Cortec, Paris, France). First, a standard ^{1}HNMR spectrum was acquired and in a second step a NOESYGPPS experiment consisting of a one-dimensional ^{1}H NMR pulse sequence with selective suppression of certain strong signals was carried out. This NOESYGPPS experiment allowed one to obtain a ^{1}H NMR spectrum with higher sensitivity than the standard single pulse ^{1}H NMR in the spectral region ranging from 5.8 to 9.8 ppm (Ruiz-Aracama et al., 2017) at the cost of suppressing some signals in other regions. The relaxation and acquisition time used allow the complete relaxation of the protons, the signal areas thus being proportional to the number of protons that generate them, making it possible to use them for quantitative purposes (Guillén & Uriarte, 2012b).

4.2. Identification of the components

The identification of the components present in the original oil, in the oil samples enriched with phenolic compounds and in the lipid extract of their digestates, was carried out on the basis of the assigments of the ^{1}H NMR signals to the different kinds of hydrogen atoms, and in short of the different compounds. Figure 1 of **article 7** gives the spectral region comprised between 0.00 and 4.9 ppm, of virgin flaxseed oil (F) ^{1}H NMR spectrum and the region comprised between 3.5 ppm and 5.10 ppm, conveniently enlarged, of the

^{1}H NMR spectra of the lipids extracted of its digestate (DF), in which signals of protons of their main components appear.

These signals, and other ones due to protons of oxidation compounds and minor components not shown in Figure 1, but present in the spectra of all the samples here studied, their chemical shifts and assignments are given in Tables S2, S3, S4 and S5 (see Supplementary Material of **article 7**). Their assignments were made taken into account previous studies as it indicated in each table, or on the basis of the signals of standard compounds acquired for this study. Among these later are: cycloartenol, hexanal, decanal, acquired from Sigma-Aldrich (St. Louis, MO, USA).

Table S2 shows ^{1}H NMR signals of specific protons of the different glyceride structures, such triglycerides, diglycerides and monoglycerides. Table S3 shows ^{1}H NMR signals of protons of linolenic, linoleic, oleic and saturated acyl groups and fatty acids, and the signals of methylenic protons supported on carbons atoms in *alpha* and *beta* position in relation to carbonyl-carboxyl groups. Table S4 shows ^{1}H NMR signals of protons of oxidation compounds coming from main oil components degradation occurred during digestion. Finally, Table S5 gives ^{1}H NMR signals, present in Figure 2, of some protons of dodecyl gallate, of hydroxytyrosol acetate, and of *gamma* tocopherol and of free and esterified cycloartenol and 24-methylenecycloartenol. The areas of some of these spectral signals were used to quantify the concentration of the different kinds of above mentioned structures in the corresponding samples, as it will be explained below.

4.3. Quantification made from ^{1}H NMR spectral data

The quantification of the different kinds of compounds or structures were estimated as indicated in section 4.3. of the experimental design of **aim 2.1.**

4.3.1. Estimation of the molar percentage of the different kinds of glycerides in the digestates

Estimation of the molar percentage of each kind of glyceride structures can be carried out by using the intensity of some signals indicated in Tables S2 and S3, which can be also observed in Figure 1 of **article 7.** This estimation was carried out as indicated in section 4.3.1. of the experimental design of **aim 2.1**.

4.3.2. Estimation of the molar percentage of fatty acids plus acyl groups that have linolenic structure in relation to the total of all types of fatty acids and acyl groups in digestates

In this study, the molar percentage of the linolenic acyl groups plus linolenic fatty acids in the digestates was estimated in relation to the total number of moles of all kinds of fatty acids plus acyl groups. This estimation was made using the equation [eq. S11], given in Supplementary Material of **article 7,** in which the areas of some signals that are shown in Figure 1 and in Table S3 of **article 7** are involved. This equation is the same employed in previous studies (Guillén & Ruiz, 2003; Guillén & Uriarte, 2012), but using the signal of methylenic protons supported on carbons atoms in *alpha* position in relation to carbonyl-carboxyl groups, instead of the signal of triglyceride protons used in edible oils studies.

4.3.3. Estimation of the concentration of specific compounds (SC) in oil samples and in the digestates

The concentration of oxidation compounds, and of other ones, such as *gamma*-tocopherol, cycloartenol plus 24-methylenecycloartenol, dodecyl gallate and hydroxytyrosol acetate, either in oils or in digestates can be estimated by using the general equation [eq. 12] given in Supplementary Material of **article 7** and the intensity of one of their non-overlapping ^{1}H NMR spectral signals, indicated in Figures 1 and 2, and in Table S3, S4 and S5.

5. Study by SPME-GC/MS of the headspace of digestates and of the mixture of the digestive juices submitted to digestion conditions with the virgin flaxseed oil

The extraction of the volatiles components constituting the headspace of the several samples was made in the same conditions and following the same GC/MS operating conditions as that described in section 5 of the experimental design of **aim 2.1.**

Identification of the headspace components and their semi-quantification was performed as indication in section 5 of the experimental design of **aim 2.1.**

6. Statistical analysis

The significance of the differences in the several kinds of data among samples, was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM, NY, USA).

Aim 2.3. To study by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the behaviour of olive oil during *in vitro* digestion. Study of the lipolysis extent and pattern and oxidation reactions, as well as of the bioaccessibility of main and minor compounds. Comparison with corn oil and virgin flaxseed oil submitted to the same digestive conditions. **(Manuscript 8)**

1. Samples subject of study

The study was carried out with two different olive oils O_1 and O_2, of the same brand, acquired in a local supermarket. The composition of both oils in molar percentages of linolenic (Ln), linoleic (L), oleic (O) and saturated (S) acyl groups, is very similar (O_1: Ln%= 0.6 ± 0.1, L%= 8.0 ± 0.4, O%= 75.5 ± 0.6, and S%= 15.8 ± 0.2; O_2: Ln%= 0.7 ± 0.1, L%= 8.0 ± 0.1, O%= 75.1 ± 0.6, and S%= 16.2 ± 0.5). This was determined from ^{1}H NMR spectral data as in previous studies (Guillén & Ruiz, 2003; Guillén & Uriarte, 2012). Both olive oils also contain a small concentration of alkanals, as Table 2 shows of **manuscript 8**. These olive oils also contain squalene and sterols, detectable by ^{1}H NMR spectroscopy, and a certain number of terpenes and sesquiterpenes, detectable by SPME-GS/MS.

Aliquots of olive oil O_1 were enriched with two different concentrations either of dodecyl gallate DG (purity 98%, from Alfa Aesar., GmbH & Co KG, Germany) or of hydroxytyrosol acetate HTA (purity of 99.54%, from Seprox Biotech, Madrid, Spain). Likewise, aliquots of the olive oil O_2 were enriched with different concentrations of *gamma*-tocopherol (γT) (purity ≥ 90%, Eisai Food & Chemical Co. Ltd., Tokyo, Japan). The samples enriched with dodecyl gallate were named, O_1DG_1 (with an enrichment of 0.12mmol DG/mol $[FA+AG]_O$) and O_1DG_2 (with an enrichment of 1.36mmol DG/mol $[FA+AG]_O$). The samples enriched with hydroxytyrosol acetate, were named O_1HTA_1

(with an enrichment of 0.28mmol HTA/mol $[FA+AG]_O$) and O_1HTA_2 (with an enrichment of 2.53mmol HTA/mol $[FA+AG]_O$). Finally, the oil O_2 samples enriched with different concentrations of *gamma*-tocopherol were named $O_2\gamma T_1$ (with an enrichment of 0.11mmol γT/mol $[FA+AG]_O$), $O_2\gamma T_2$ (with an enrichment of 1.17mmol γT/mol $[FA+AG]_O$) and $O_2\gamma T_3$ (with an enrichment of 12.58mmol γT/mol $[FA+AG]_O$). These enrichment levels were set in function of the solubility of these compounds in the oil. Thus, the above concentrations were obtained in order to reach enrichment degrees near to 0.02 % and 0.2 % in weight for the three phenolic compounds and, in addition, near 2% in weight in the case of *gamma*-tocopherol due to its high solubility in oils. However, this latter level of enrichment was not possible for dodecyl gallate and hydroxytyrosol acetate because of their limited solubility in oils. All these samples were submitted to *in vitro* digestion.

2. Digestion experiments

Aliquots (0.5 g) of the above-mentioned samples were digested following the semi-static *in vitro* gastrointestinal digestion model developed by Versantvoort, *et al*. (2005), as described in section 2. of the experimental design of **aim 2.1.**

3. Digestate lipid extraction

Lipids of the digestates were extracted as indicated in section 3. of the experimental design of **aim 2.1.**

4. Study by ^{1}H NMR of oil samples and lipid extracts of digestates

4.1. Operating conditions

The ^{1}H NMR spectra of the original oils O_1 and O_2, and of the oil samples enriched with each one of the phenolic compounds above mentioned at the different concentrations (O_1DG_1, O_1DG_2, O_1HTA_1, O_1HTA_2, $O_2\gamma T_1$, $O_2\gamma T_2$ and $O_2\gamma T_3$), and of the lipids extracted

from their digestates (DO_1, DO_1DG_1, DO_1DG_2, DO_1HTA_1, DO_1HTA_2, DO_2, $DO_2\gamma T_1$, $DO_2\gamma T_2$ and $DO_2\gamma T_3$), were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz as described in section 4.1. of the experimental design of **aim 2.2.**

4.2. Identification of the components

The identification of the components present in the original oils, in the oil samples enriched with phenolic compounds and in the lipid extracts of their digestates, was carried out on the basis of the assignments of the ^{1}H NMR signals to the different kinds of hydrogen atoms, and in short to the different compounds. Figure 1 of **manuscript 8** gives the spectral regions comprised between 0.0 and 4.9 ppm, of olive oil O_1 ^{1}H NMR spectrum, and between 3.5 ppm and 5.10 ppm, conveniently enlarged, of the ^{1}H NMR spectra of the lipids extracted from the several digestates (DO_1, DO_1DG_2, DO_1HTA_2, DO_2, and $DO_2\gamma T_2$), in which signals of protons of their main components appear.

These above mentioned signals, and others due to protons of minor components not shown in Figure 1, but present in the spectra of the above mentioned samples, their chemical shifts and assignments are given in Tables S2, S3, S4 and S5 (see Supplementary Material of **manuscript 8**). These assignments were made taken into account previous studies as is indicated in each table, or were based on the signals of standard compounds acquired for this study, which include cycloartenol, squalene, hexanal and decanal, acquired from Sigma-Aldrich (St. Louis, MO, USA) and linolein hydroperoxides purchased from Cayman Chemical (Ann Arbor, MI, USA).

Table S2 shows ^{1}H NMR signals of specific protons of the different glyceride structures, such triglycerides, diglycerides and monoglycerides. Table S3 shows ^{1}H NMR signals of protons of linolenic, linoleic, oleic and saturated acyl groups and fatty acids, and the

signals of methylenic protons supported on carbons atoms in *alpha* and *beta* position in relation to carbonyl-carboxyl groups. Table S4 shows ^{1}H NMR signals of protons of oxidation compounds coming from main oil component degradation, which occurred during digestion. Finally, Table S5 gives ^{1}H NMR signals of some protons of dodecyl gallate, hydroxytyrosol acetate, *gamma*-tocopherol, of free and esterified cycloartenol plus 24-methylenecycloartenol and of squalene. The areas of some of these spectral signals were used to quantify the concentration of the different kinds of above-mentioned structures in the corresponding samples, as will be explained below.

4.3. Quantification made from ^{1}H NMR spectral data

The quantification of the different kinds of compounds or structures were estimated as indicated in section 4.3. of the experimental design of **aim 2.1.**

4.3.1. Estimation of the molar percentage of the different kinds of glycerides in the digestates

Estimation of the molar percentage of each kind of glyceride structures can be carried out by using the intensity of some signals indicated in Tables S2 and S3, which can be also observed in Figure 1 of **manuscript 8.** This estimation was carried out as indicated in section 4.3.1. of the experimental design of **aim 2.1**.

4.3.2. Estimation of the molar percentage of fatty acids plus acyl groups that have linolenic, linoleic, oleic and saturated structures in relation to the total fatty acids and acyl groups in digestates

In this study, the molar percentage of *linolenic, linoleic, oleic and saturated structures* found in acyl groups and fatty acids in the digestates was estimated in relation to the total number of moles of fatty acids plus acyl groups. This estimation was made using the equation [eq. S11- eq. S14], given in Supplementary Material of **manuscript 8,** in which the areas of some signals that are shown in Figure 1 and in Table S3 of **manuscript 8** are

involved. These equations are the same employed in previous studies (Guillén & Ruiz, 2003; Guillén & Uriarte, 2012), but using the signal of methylenic protons supported on carbons atoms in *alpha* position in relation to carbonyl-carboxyl groups, instead of the signal of triglyceride protons used in edible oils studies.

4.3.3. Estimation of the concentration of specific compounds (X) in oil samples and in the digestates

The concentration of oxidation compounds, and of others such as squalene, cycloartenol plus 24-methylenecycloartenol, dodecyl gallate, hydroxytyrosol acetate and *gamma*-tocopherol either in oils or in digestates can be estimated by using the general equation [eq. S15] given in Supplementary Material of **manuscript 8** and the intensity of one of their non-overlapped ^{1}H NMR spectral signals, which are indicated in Figure 1, and in Tables S3, S4 and S5.

5. Study by SPME-GC/MS of the headspace of digestates and of the mixture of the digestive juices submitted to digestion conditions with the virgin flaxseed oil

The extraction of the volatiles components constituting the headspace of the several samples was made in the same conditions and following the same GC/MS operating conditions as that described in section 5 of the experimental design of **aim 2.1.**

Identification of the headspace components and their semi-quantification was performed as indication in section 5 of the experimental design of **aim 2.1.**

6. Statistical analysis

The significance of the differences in the several kinds of data among samples, was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM, NY, USA).

RESULTS AND DISCUSSION

Article 1

A NEW METHODOLOGY CAPABLE OF CHARACTERIZING MOST VOLATILE AND LESS VOLATILE MINOR EDIBLE OILS COMPONENTS IN A SINGLE CHROMATOGRAPHIC RUN WITHOUT SOLVENTS OR REAGENTS. DETECTION OF NEW COMPONENTS

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ABSTRACT

The possibilities offered by a new methodology to determine minor components in edible oils are described. This is based on immersion of a solid-phase microextraction fiber of PDMS/DVB into the oil matrix, followed by Gas Chromatography/Mass Spectrometry. It enables characterization and differentiation of edible oils in a simple way, without either solvents or sample modification. This methodology allows simultaneous identification and quantification of sterols, tocols, hydrocarbons of different natures, fatty acids, esters, monoglycerides, fatty amides, aldehydes, ketones, alcohols, epoxides, furans, pyrans and terpenic oxygenated derivatives. The broad information provided by this methodology is useful for different areas of interest such as nutritional value, oxidative stability, technological performance, quality, processing, safety and even the prevention of fraudulent practices. Furthermore, for the first time, certain fatty amides, *gamma*- and *delta*-lactones of high molecular weight, and other aromatic compounds such as some esters derived from cinnamic acid have been detected in edible oils.

KEYWORDS: edible oils, solid-phase microextraction, gas chromatography-mass spectrometry, sterols and tocols, polycyclic and steroidal hydrocarbons, acids and esters, lactones, monoglycerides, fatty amides, aldehydes and other oxygenated compounds

Chemical compounds studied in this article

Campesterol (PubChem CID: 173183); β-Sitosterol (PubChem CID: 222284); α-Tocopherol (PubChem CID: 2116); γ-Tocopherol (PubChem CID: 92729); Squalene (PubChem CID: 638072); 2-Ethylhexyl-4-methoxycinnamate (PubChem CID: 21630); γ-Palmitolactone (PubChem CID: 97747); δ-Hexadecalactone (PubChem CID: 110976); Oleamide (PubChem CID: 5283387); 2-Monolinolein (PubChem CID: 5365676).

1. INTRODUCTION

In recent years, increasing attention has been paid to edible vegetable oils due to their importance in the food industry, in nutrition, and in human health. Edible oil quality is a function of its composition, which depends, among other factors, on its vegetable origin, on what processing took place, on its oxidation status, and especially on its level of those compounds which are able to exhibit antioxidant activity and other healthy properties. The evaluation of oil quality, safety and authenticity requires as deep a knowledge of its composition as is possible. However, this is a very difficult challenge due to the great number of compounds that may be present.

The main edible oil components are triglycerides. Determination of the triglyceride composition of edible oils has been the subject of many studies. The classical methodology involves the hydrolysis of triglycerides and the transformation of the resulting fatty acids into methyl esters which are then identified and quantified by gas chromatography. Recently, the use of ^{1}H NMR has allowed this determination to be made more quickly and simply (Guillén & Uriarte, 2009).

A far more complex matter is the identification and quantification of minor edible oil components. Among these there are both volatile and less volatile components. Many different techniques which have been used in the study of volatile oil components (Cavalli, Fernandez, Lizzani-Cuvelier & Loiseau, 2003) have been evolving over time until now. In recent years one of the most frequently used techniques has been solid-phase microextraction, followed by gas chromatography/mass spectrometry (Guillén, Cabo, Ibargoitia & Ruiz, 2005; Uriarte, Goicoechea & Guillén, 2011). It is important to determine these components because they have been shown to provide useful information about botanical origin, previous processing, the oxidation status of the oil and in addition because some of them are responsible for its odour and its taste (Uriarte et al., 2011).

Knowledge of minor non-volatile components is no less important than that of the minor volatile components. Non-volatile components are very numerous and also have very varied functional groups and so may have different properties and behaviours. For these reasons, the different kinds of minor non-volatile oil components have usually to date been studied separately, grouped by families of compounds, using specific methodologies for each one of them. These methodologies involve fractionation, clean up and isolation of the compounds subject of interest for their later identification and quantification. Moreover, these methodologies usually require the use of solvents and reagents, and in occasions the chemical modification of the sample. Additionally, they involve a great deal of time and expense.

In this context, it would be of the utmost importance to have the option of a simple method which can give at the same time, in only one run, information about most of the minor edible oil components. Thus, the purpose of this work is to describe the performance of a new methodology, based on the use of Direct Immersion Solid-Phase Microextraction (DI-SPME), which is able to provide as broad as possible simultaneous qualitative and quantitative information concerning all kinds of minor volatile and non-volatile edible oil components. The principles on which this methodology is based are that it is: environmentally friendly, avoiding the use of solvents or reagents; sustainable, thus able to provide information on different classes of compounds simultaneously so avoiding the use of a method for each kind of compounds; as fast as possible; as simple as possible; able to give information on new edible oil components; and economical, avoiding the use of sophisticated and expensive equipment. SPME by immersion of the fiber in a liquid phase followed by a chromatographic study has been used mainly for the determination of some types of contaminants and toxic substances, above all in water samples (Mekiki, Kalogerakis & Psillakis, 2006). Furthermore, Mikuma and Kaneko (2010) have proved the usefulness of

this methodology to discriminate between vegetable oils based mainly on the abundance of a reduced number of compounds such as three or four sterols. A method derived from this latter is used in the present paper to prove its suitability in characterizing most of the minor volatile and less volatile edible oil components, and even to discover compounds not previously detected in edible oils. The usefulness of this method is shown through the study of five different edible oils: two different soybean oils, a corn, a sunflower and a linseed oil.

2. MATERIALS AND METHODS

2.1. Samples

The study was carried out using five commercial edible oils of different vegetable origins. These took the form of one sunflower oil, SF, one corn oil, C, two soybean oils (one refined, RSB and the other virgin, VSB) and one virgin linseed oil, VL. All were acquired in local supermarkets. It is well-known that the composition of these oils in main component is closely related to their botanical origin. This composition, expressed as molar percentages of the different kinds of acyl groups, was determined from ^{1}H NMR spectral data as in previous studies (Guillén & Uriarte, 2009) and it is given in Table S1.

2.2. Extraction of the oil minor components

The method used is based on that described by Mikuma and Kaneko (2010). The extraction of the oil components was carried out by solid-phase microextraction. To this aim a fiber of 65 μm StableFlex polydimethylsiloxane/divinylbenzene (PDMS/DVB) acquired from Supelco (Bellefonte, PA, U.S.) was immersed directly into 6 ml of edible oil at room temperature for 45 minutes. This fiber was selected on the basis of its ability to extract the widest range of minor oil components with the highest possible yield. Its selection was made after analyzing the results obtained by Mikuma and Kaneno and after testing some fibers such as 100 μm PDMS, 65 μm PDMS/DVB and 50/30 μm DVB/CAR/PDMS. The extraction

time selected provided the best results in terms of extraction yield after testing extraction times of 15, 30, 45 and 90 minutes.

No influence of the concentration of a compound in the oil on the adsorption and extraction yield of the other ones was observed in studies of enrichment of the edible oils with several proportions either of sterols, or of different tocopherols, or of other compounds.

2.3. Gas Chromatography-Mass Spectrometry

The oil components picked up by the fiber were directly injected into the port of a gas chromatograph-mass spectrometer (GC/MS). For their thermal desorption, the plunger was pushed down to expose the fiber to the GC carrier gas stream and held for 10 minutes. During the first 5 min, which constitute the effective desorption time, the split valve was closed (splitless mode) to ensure the complete introduction of the desorbed compounds into the chromatographic column, and afterwards it was opened.

After the injection of the extracted compounds, the fiber was submitted to two additional desorption processes in the gas chromatograph in order to ensure the complete removal of minor oil components traces and prevent cross-sample contamination. It was confirmed that these two fiber cleanings were sufficient to avoid carry-over effects.

The desorbed compounds were separated, identified and semi-quantified by gas chromatography-mass spectrometry. The equipment used was an Agilent gas chromatograph model 6890N equipped with a mass selective detector 5973 Network and a Hewlett-Packard Compaq Pentium 4 computer. A fused silica capillary column was used (60 m length x 0.25 mm inside diameter x 0.25 μm film thickness; from Agilent Technologies Inc., Palo Alto, CA), coated with a nonpolar stationary phase (HP-5MS, 5% phenyl methyl siloxane). The operation conditions were the following: the injector and interface temperatures were held at 250°C and 305ºC respectively, and helium at a constant pressure of 117 kPa (16.9 psi) was used as the carrier gas. The oven temperature was initially held at 50ºC for 5 min, increased

from 50 to 300ºC at a rate of 4ºC/min, and then held at 300ºC for 30 min. Mass spectra were recorded at an ionisation energy of 70 eV, with data acquisition in Scan mode. The temperatures of the ion source and the quadrupole mass analyzer were kept at 230 and 150ºC, respectively. A reference sample of known composition was periodically analyzed in order to verify the sensitivity of the GC/MS equipment.

Many components were identified by comparison of their retention times and mass spectra with those of standards acquired from Sigma-Aldrich (St. Louis, MO, USA), Larodan Fine Chemicals AB (Malmo, Sweden), Chem Faces (Wuhan, China) and Cayman Chemical (Ann Arbor, MI, USA); these are asterisked in the different tables. Others were identified by matching of their mass spectra with spectra from a commercial library by more than 85% (W9N08, Wiley ver. 9.0 & NIST, ver. 8.0 library) and also with those obtained from the literature.

Semi-quantification was based on arbitrary units of the base peak ion area counts divided by 10^4. All the determinations were carried out in duplicate in order to obtain a mean value with the corresponding standard deviation for each of the components studied.

3. RESULTS AND DISCUSSION

As mentioned before, this methodology allows the simultaneous determination of the relative abundances of the oil minor components belonging to several families of compounds. These are given in Tables 1 to 3 and in Appendix of Supplementary data. Figure S1 shows the enlargements of the different regions of the total ion chromatograms corresponding to the studied oils.

3.1. Sterols and derivatives

Sterols form an important group of minor components of edible oils. The distribution of the different sterols in edible oils is associated with their botanical origin and variety;

however, it is known that variations among oils of the same variety can be found, due to changes in climatic and other environmental conditions during plant growth (Phillips, Ruggio, Toivo, Swank & Simpkins, 2002). These compounds have been assigned several healthy properties, as well as antioxidant capability and technological properties such as the proven antipolymeric activity of some sterols showed during oil frying (Blekas & Boskou, 2016). For these reasons the determination of these compounds in edible oils has received a great deal of attention.

The classical methodology developed for their determination in edible oils involves saponification, fractionation of the unsaponifiable part by thin layer chromatography or by other fractionation techniques to isolate these compounds, posterior derivatization, and study by gas chromatography or by liquid chromatography using several detection techniques (Itoh, Tamura & Matsumoto, 1973; Zarrouk, Carrasco-Pancorbo, Zarrouk, Segura-Carretero & Fernández-Gutiérrez, 2009). As example, Itoh and coworkers (1973) determined the abundance of eight sterols in a great number of edible oils. Likewise, other research groups have also determined sterols in edible oils (Phillips et al., 2002).

Table 1. Sterols and derivatives, and tocols found in the oils studied and their abundances (sunflower, SF, corn, C, refined soybean, RSB, virgin soybean VSB and virgin linseed VL), given in area counts of the base peak (BP) of their mass spectra, multiplied by 10^{-4}, together with their standard deviations.

Compounds (MW)	BP	SF	C	RSB	VSB	VL
STEROLS AND DERIVATIVES						
DESMETHYLSTEROLS						
Brassicasterol (398)*	314	--	2.9 ± 1.2	8.8 ± 0.7	2.7 ± 0.0	3.2 ± 1.4
Campesterol (400)*	400	49.9 ± 1.5	155.8 ± 16.3	226.4 ± 14.3	82.0 ± 10.2	63.9 ± 2.8
Ergostanol (402)*	215	--	5.7 ± 1.0	7.2 ± 0.1	2.4 ± 0.2	--
(23*E*)-14-Methylergosta-8,23-dien-3-ol (412) (or iso)	397	--	--	--	--	18.6 ± 7.5
Stigmasterol (412)*	412	54.5 ± 1.5	27.3 ± 8.1	203.4 ± 12.3	49.3 ± 4.8	16.5 ± 5.4
Stigmastenol (414) (iso)	414	--	--	3.6 ± 0.7	--	--
γ-Ergostenol (400)	400	9.2 ± 0.5	--	7.1 ± 0.9	3.3 ± 0.1	--
(3β)-Stigmasta-5,23-dien-3-ol (412) (or iso)	55	27.8 ± 1.8	--	5.3 ± 0.0	--	--
β-Sitosterol (414)*	414	395.7 ± 15.7	335.2 ± 91.3	420.6 ± 30.4	126.9 ± 17.4	73.5 ± 24.0
Stigmastanol (416)*	416	1.6 ± 0.2	6.9 ± 2.5	10.8 ± 1.3	2.2 ± 0.7	--
Δ5-Avenasterol (412)*	314	36.6 ± 0.7	33.2 ± 10.7	33.2 ± 3.5	6.9 ± 1.0	18.5 ± 8.6
Δ7-Avenasterol (412)	285	41.4 ± 2.5	4.3 ± 1.5	6.6 ± 0.7	3.4 ± 0.4	--
Lanosterol (426)*	69	--	--	--	--	33.4 ± 2.3
(3β,5α,24*S*)-Stigmasta-7-en-3-ol (414) (or iso)	414	35.6 ± 2.4	5.1 ± 2.2	9.2 ± 1.5	1.7 ± 0.1	--
4-METHYL STEROLS						
Obtusifoliol (426)	411	8.4 ± 0.1	--	3.6 ± 0.4	--	--
Cyclolaudenol (440)	55	95.1 ± 0.2	--	--	--	--
Citrostadienol (426)	285	90.3 ± 1.0	2.8 ± 0.8	8.8 ± 1.1	--	--
4,4'-DIMETHYL STEROLS						
Germanicol (426)	189	--	--	--	--	5.9 ± 0.9
β-Amyrin (426)	218	27.1 ± 1.6	2.1 ± 0.5	23.5 ± 1.4	25.1 ± 1.8	--
Cycloartenol (426)	69	77.3 ± 2.8	11.5 ± 2.0	--	11.0 ± 0.4	251.9 ± 49.1
α-Amyrin (426)	218	44.1 ± 1.4	--	13.4 ± 0.9	6.0 ± 0.0	--
OXO-STEROLS AND STEROLS ESTERS						
Lupan-3-one (426)	205	--	--	4.0 ± 0.2	2.3 ± 0.1	--

Compounds (MW)	**BP**	**SF**	**C**	**RSB**	**VSB**	**VL**
Campest-4-en-3-one (398)	124	--	10.5 ± 0.9	9.6 ± 0.9	10.0 ± 1.6	10.8 ± 1.5
Sitosteryl acetate (454)*	396	3.4 ± 2.1	2.4 ± 1.2	--	--	2.1 ± 0.6
α-Spinasterone (410)	55	--	15.2 ± 2.7	8.4 ± 0.6	15.3 ± 5.5	--
Stigmast-4-en-3-one (412)	124	14.5 ± 1.1	20.8 ± 3.3	20.3 ± 3.2	13.3 ± 1.5	12.5 ± 3.0
Amyrin acetate (468)	218	5.3 ± 0.2	--	--	--	--
TOCOLS						
δ-Tocopherol (402)*	402	4.7 ± 0.6	141.9 ± 35.1	1,558.7 ± 19.7	1,044.4 ± 59.2	15.2 ± 4.3
δ-Tocomonoenol (400)	400	--	--	5.3 ± 0.0	7.5 ± 0.7	--
β-Tocopherol (416)*	416	97.5 ± 9.2	26.1 ± 6.1	87.6 ± 3.2	47.3 ± 0.6	--
γ-Tocopherol (416)*	416	24.6 ± 2.8	5,262.2 ± 1,178.9	4,759.4 ± 44.6	1,947.3 ± 99.6	1,510.0 ± 538.4
γ-Tocomonoenol (414)	414	--	9.8 ± 3.3	25.3 ± 0.4	18.9 ± 2.7	1.8 ± 1.0
α-Tocopherol (430)*	165	3,171.9 ± 159.3	1,014.1 ± 63.5	551.0 ± 16.9	195.0 ± 4.2	30.2 ± 0.4
γ-Tocotrienol (410)*	151	6.8 ± 0.4	22.2 ± 1.6	--	--	--
α-Tocomonoenol (428)	428	49.7 ± 3.4	4.8 ± 1.8	2.3 ± 0.3	2.1 ± 0.0	--
α-Tocotrienol (424)*	165	7.5 ± 0.6	26.3 ± 2.5	--	--	--

MW: Molecular weight; * Asterisked compounds were acquired commercially and used as standards for identification purposes; --: not detected

The elimination of the saponification step has been proposed for the simultaneous direct determination of sterols, tocopherols and squalene by on-line coupling of liquid chromatography and capillary gas chromatography using different transfer and coupling techniques for the interfaces of the chromatographs requiring or not the dissolution of the oil in dichloromethane. These more recent methods have many advantages in relation to the prior ones (Grob, Lanfranchi & Mariani, 1990).

As Table 1 shows, in the case of the five edible oils here studied, the method used detected more than twenty five sterols and related compounds without the need of either previous saponification or modification of the samples, or fractionation steps. Figure 1 shows a time-window of the total ion chromatogram of sterols and tocols identified in corn oil, C, the richest in these compounds of the oils here studied.

Fourteen members of the group of *desmethylsterols*, have been found, the refined soybean oil being the richest in these, followed by the sunflower and corn oils, which have higher concentrations than the virgin soybean and linseed oils. As Table 1 shows, the main one is β-sitosterol in all oils, followed by campesterol and stigmasterol, the latter usually in smaller concentration than the former. In the group of *4-methylsterols* only a reduced number of compounds have been found, cyclolaudenol and citrostadienol being the main ones in sunflower oil, the richest oil in this type of components; by contrast, they are absent in virgin soybean and linseed oils. *4,4´-dimethylsterols* are present in all the oils studied although the number is small, the main one being cycloartenol in linseed oil. Finally, *oxo-sterols* and *sterol esters* have also been found, with stigmast-4-en-3-one the only one present in all these oils. It is also worth noting the detection of sitosteryl acetate in corn, sunflower, and linseed oils, but not in soybean oils.

3.2. Tocols

An important group of edible oil components are *tocols*, all of them components of vitamin E. Although some authors include only eight substances such as α-, β-, γ-, and δ- *tocopherols* and α-, β-, γ-, and δ-*tocotrienols* in this group (Sen, Khanna & Roy, 2006), some new tocols with only a double bond (named *tocomonoenols)* have been discovered in recent years, not only in marine organisms (Ng, Wang, Ketchimenin & Yuen, 2004) but also in fruits (Piombo et al., 2006). Due to the known ability of tocols to act as antioxidants and to their effects on health they have been subject of considerable attention. Their determination in edible oils is usually carried out either by HPLC (High Pressure Liquid Chromatography) without previous sample preparation or by GC (Gas Chromatography) after a preliminary preparation step (Saini & Keum, 2016).

It is noteworthy that the method proposed here is able to detect the three kinds of tocols, that is *tocopherols*, *tocotrienols* and *tocomonoenols*. Figure 1 shows the time-window of the total ion chromatogram of these compounds. The repeatability of their determination is satisfactory, as the standard deviations given in Table 1 indicate. In total, nine tocols have been found, γ-tocopherol being the main one in all oils except in sunflower oil where the main one is α-tocopherol. The richest in tocopherols are corn oil and the refined soybean oil. Both soybean oils also contain δ-, α- and β-tocopherol in decreasing abundances in agreement with Cerretani and coworkers (2009); furthermore, they also contain α-, γ-, and δ-tocomonoenols. Corn oil, in addition to γ-tocopherol, contains in decreasing abundances α-, δ-, and β-tocopherol, as well as γ-, and α-tocotrienol and γ- and α-tocomonoenol, in agreement with the results of Cerretani and coworkers (2009). In sunflower oil, in addition to α-, β- and γ- tocopherols, which are known as sunflower oil components, δ-tocopherol, α- and γ-tocotrienol and α-

tocomonoenol have been found. Among the oils here studied linseed oil is the poorest in tocols.

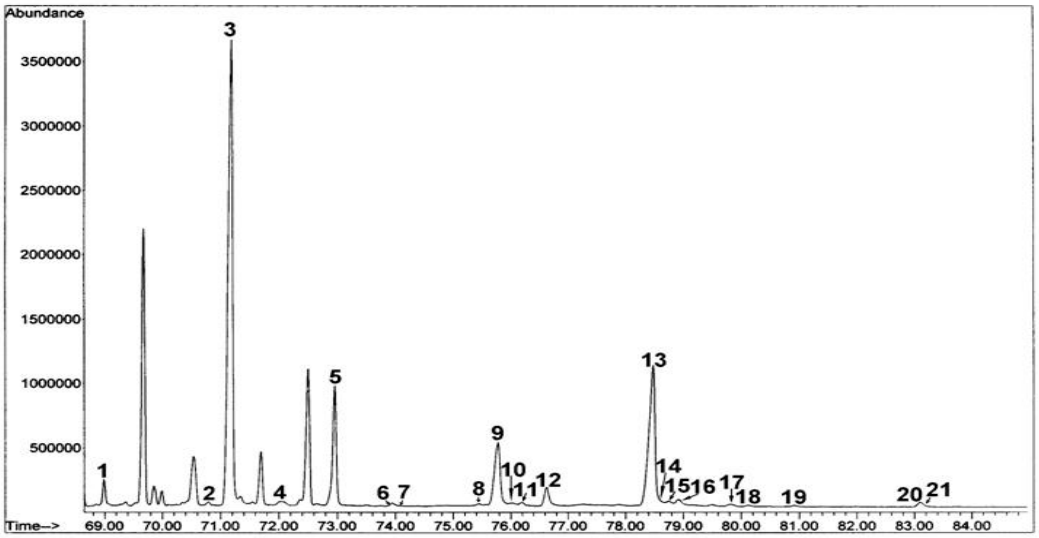

Figure 1. Total ion chromatogram time-window of sterols, sterols derivatives, and tocols found in corn oil (C). **(1)** δ-Tocopherol; **(2)** β-Tocopherol; **(3)** γ-Tocopherol; **(4)** γ-Tocomonoenol; **(5)** α-Tocopherol; **(6)** γ-Tocotrienol; **(7)** α-Tocomonoenol; **(8)** Brassicasterol; **(9)** Campesterol; **(10)** Ergostanol; **(11)** α-Tocotrienol; **(12)** Stigmasterol; **(13)** β-Sitosterol; **(14)** Stigmastanol; **(15)** Δ5-Avenasterol; **(16)** Δ7-Avenasterol; **(17)** Campest-4-en-3-one; **(18)** (3β,5α,24S)-Stigmasta-7-en-3-ol; **(19)** Cycloartenol; **(20)** α-Spinasterone and **(21)** Stigmast-4-en-3-one.

3.3. Hydrocarbons

Another very numerous groups of compounds present in edible oils is *hydrocarbons*. This methodology allows their determination simultaneously with all the other components. Although the occurrence of hydrocarbons in edible oils is well known (Cert, Moreda & Pérez-Camino, 2000), there are not many detailed studies on the hydrocarbons present in each kind of vegetable oil. However, there are exceptions, such as the determination of polycyclic aromatic hydrocarbons of well known toxicity (Guillén & Sopelana, 2004), or of steroidal hydrocarbons (Cert et al., 2000; Bezerra & Antoniosi Filho, 2014) for fraud detection. Their determination usually requires their isolation from

the unsaponifiable oil fraction and posterior separation by chromatographic techniques. As Tables 2 and S2 show, several kinds of hydrocarbons are present in these oils.

Thus, *alkanes* (Table S2) of low molecular weight such as pentane and hexane and others of high molecular weight having mainly from twenty to thirty one carbon atoms were found. The richest oils in this kind of compounds are sunflower oil followed by corn and linseed oils, the two soybean oils being the poorest. The concentrations of pentane, heptacosane, nonacosane and hentriacontane are noteworthy in sunflower oil as is that of tricosane in corn oil.

Furthermore, a reduced number of *alkenes* (Table S2) and of *aromatic hydrocarbons* (Table 2) of small size has also been detected, the soybean oils again being the poorest in these kinds of hydrocarbons. It is also worth noting the occurrence, in very low proportions, of some *polycyclic aromatic hydrocarbons* of small size, such as phenanthrene and anthracene, mainly in corn and linseed oils; this latter type of hydrocarbons has been subject of detailed studies concerning their occurrence in edible oils because some of them are carcinogenic (Guillén & Sopelana, 2004); they may come from environmental contamination of the vegetable raw material and/or from some operations carried out during their processing, such as seed drying. The information provided by this methodology about the occurrence of polycyclic aromatic hydrocarbons in edible oils can be useful to discriminate between oils by their contamination degree, and to take further decisions about the necessity of determining the absolute concentration of this kind of contaminants.

Table 2. Aromatic, terpenic, steroidal hydrocarbons in the various oils (sunflower, SF, corn, C, refined soybean, RSB, virgin soybean VSB and virgin linseed VL), and their abundances, given in area counts of the base peak (BP) of their mass spectra, multiplied by 10^{-4}, together with their standard deviations.

Compounds (MW)	BP	SF	C	RSB	VSB	VL
HYDROCARBONS						
AROMATIC HYDROCARBONS						
1,2-Dimethyl-benzene (106)	91	1.0 ± 0.5	0.8 ± 0.1	--	--	3.9 ± 0.1
Styrene (104)*	104	1.1 ± 0.1	0.6 ± 0.1	--	0.7 ± 0.2	0.2 ± 0.1
1-Ethenyl-3-ethyl-benzene (132)	117	6.7 ± 0.4	3.2 ± 1.6	1.8 ± 1.0	2.5 ± 0.1	2.3 ± 0.4
Naphthalene (128)*	128	--	--	--	--	1.4 ± 0.0
2-Methyl naphthalene (142)*	142	--	--	--	--	0.5 ± 0.1
1-Methyl naphthalene (142)*	142	--	--	--	--	0.1 ± 0.0
Phenanthrene (178)*	178	--	4.1 ± 0.0	--	--	1.9 ± 0.1
Anthracene (178)*	178	--	0.4 ± 0.0	--	--	--
Fluoranthene (202)	202	--	2.5 ± 0.2	--	--	0.9 ± 0.1
Pyrene (202)*	202	--	2.4 ± 0.2	--	--	--
TERPENIC HYDROCARBONS						
α-Ocimene (136)	93	--	--	--	--	3.0 ± 0.1
p-Cymene (134) (or iso)	119	--	--	--	--	21.8 ± 0.4
Limonene (136)*	68	3.6 ± 1.7	0.5 ± 0.1	--	9.9 ± 2.0	1.2 ± 0.4
β-Ocimene (136)	93	--	--	--	0.8 ± 0.2	--
Phytene (280)	70	1.5 ± 0.1	--	1.4 ± 0.0	0.9 ± 0.1	0.9 ± 0.2
Phytadiene (278)	68	4.1 ± 0.5	1.7 ± 0.0	11.8 ± 0.5	1.5 ± 0.3	3.7 ± 1.0
Springene (272) (iso)	69	15.1 ± 3.7	32.8 ± 4.7	106.8 ± 2.2	48.7 ± 4.8	3.5 ± 0.5
Springene (272) (iso)	69	7.0 ± 1.1	7.1 ± 0.4	43.3 ± 12.3	--	4.3 ± 0.0
Springene (272) (iso)	69	--	8.9 ± 0.8	28.5 ± 2.1	21.9 ± 0.1	--
Kaur-15-ene (272) (or iso)	94	2.5 ± 0.1	--	--	--	--
Kaur-16-ene (272) (or iso)	257	5.4 ± 0.6	1.1 ± 0.0	--	--	1.2 ± 0.4
Atisirene (272)	257	43.5 ± 1.5	1.4 ± 0.1	--	--	--
Squalene (410)*	69	2,413.1 ± 878.4	3,989.3 ± 122.1	814.1 ± 25.0	1,589.8 ± 48.6	748.8 ± 572.8
STEROIDAL HYDROCARBONS						
3,5-Campestadiene (382) (or iso)	145	1.1 ± 0.0	168.4 ± 0.5	0.6 ± 0.0	--	4.8 ± 1.5
3,5,22-Stigmastatriene (394) (or iso)	255	1.9 ± 0.5	40.5 ± 3.9	1.1 ± 0.0	--	4.9 ± 0.3

Compounds (MW)	**BP**	**SF**	**C**	**RSB**	**VSB**	**VL**
3,5-Stigmastadiene (396) (or iso)	145	4.8 ± 0.4	424.1 ± 1.6	1.7 ± 0.1	--	8.7 ± 2.5
2,5-Campestadiene (382) (or iso)	382	0.2 ± 0.0	38.2 ± 10.9	--	--	0.4 ± 0.1
2,4-Campestadiene (382) (or iso)	382	4.5 ± 0.3	130.5 ± 37.6	1.9 ± 0.1	0.9 ± 0.2	1.9 ± 0.8
2,5,22-Stigmastatriene (394) (or iso)	394	4.4 ± 0.5	3.9 ± 1.1	2.4 ± 0.1	0.7 ± 0.2	0.3 ± 0.2
2,5-Stigmastadiene (396) (or iso)	396	4.0 ± 0.5	99.0 ± 31.9	1.1 ± 0.0	--	1.2 ± 0.6
2,4,22-Stigmastatriene (394) (or iso)	394	21.2 ± 1.7	4.4 ± 1.4	--	--	--
2,4-Stigmastadiene (396) (iso)	396	28.1 ± 2.7	340.4 ± 106.5	7.4 ± 0.2	2.4 ± 1.1	4.1 ± 1.6

MW: Molecular weight; * Asterisked compounds were acquired commercially and used as standards for identification purposes; --: not detected

These edible oils also contain *terpenic hydrocarbons* (Table 2) of different sizes such as certain *terpenes*, like limonene, *linear diterpenes* such as phytene, phytadiene and springene isomers, *cyclic diterpenes* such as kaurene isomers, and, above all, squalene, which is a *triterpene*. This latter is, by far, the most abundant of these hydrocarbons and is attributed antioxidant and healthy properties (Kim & Karadeniz, 2012). Of the oils studied, as Table 2 shows, the refined soybean oil is the richest in linear diterpenic hydrocarbons, sunflower is the richest in cyclic diterpenes and corn oil is the richest in squalene.

Finally, another important group of hydrocarbons present in edible oils is made up of those derived from the dehydration of sterols, known as *steroidal hydrocarbons* (Table 2). Their presence in oils is associated with the refining process and an official method for the determination of one of them, 3,5-stigmastadiene, was established in order to detect frauds (Dobarganes, Cert & Dieffenbacher, 1999). The development of methods for the determination of this latter hydrocarbon has received great attention in recent years (Bezerra & Antoniosi Filho, 2014) due to the interest in finding methods with advantages over the official method. With the methodology here used nine *steroidal* hydrocarbons have been found and their relative abundances determined. They are derived from the main sterols, sitosterol, campesterol and stigmastadienol, three coming from each one of these. Figure 2a shows the time-window of the chromatograms of the base peaks of the three stigmastadienes (m/z= 396), (3,5-stigmastadiene, 2,5-stigmastadiene and 2,4-stigmastadiene), of the three stigmastatrienes (m/z= 394), (3,5,22-stigmastatriene, 2,5,22-stigmastatriene and 2,4,22-stigmastatriene), and of the three campestadienes (m/z=382), (3,5-campestadiene, 2,5-campestadiene and 2,4-campestadiene) found in the corn oil, which is the richest in these compounds among the oils here studied. As expected, virgin

soybean and linseed oils are the poorest in this kind of compounds. The similarity between the mass spectra of these compounds and that of the acetate of the corresponding sterol is great, although they differ considerably in retention times.

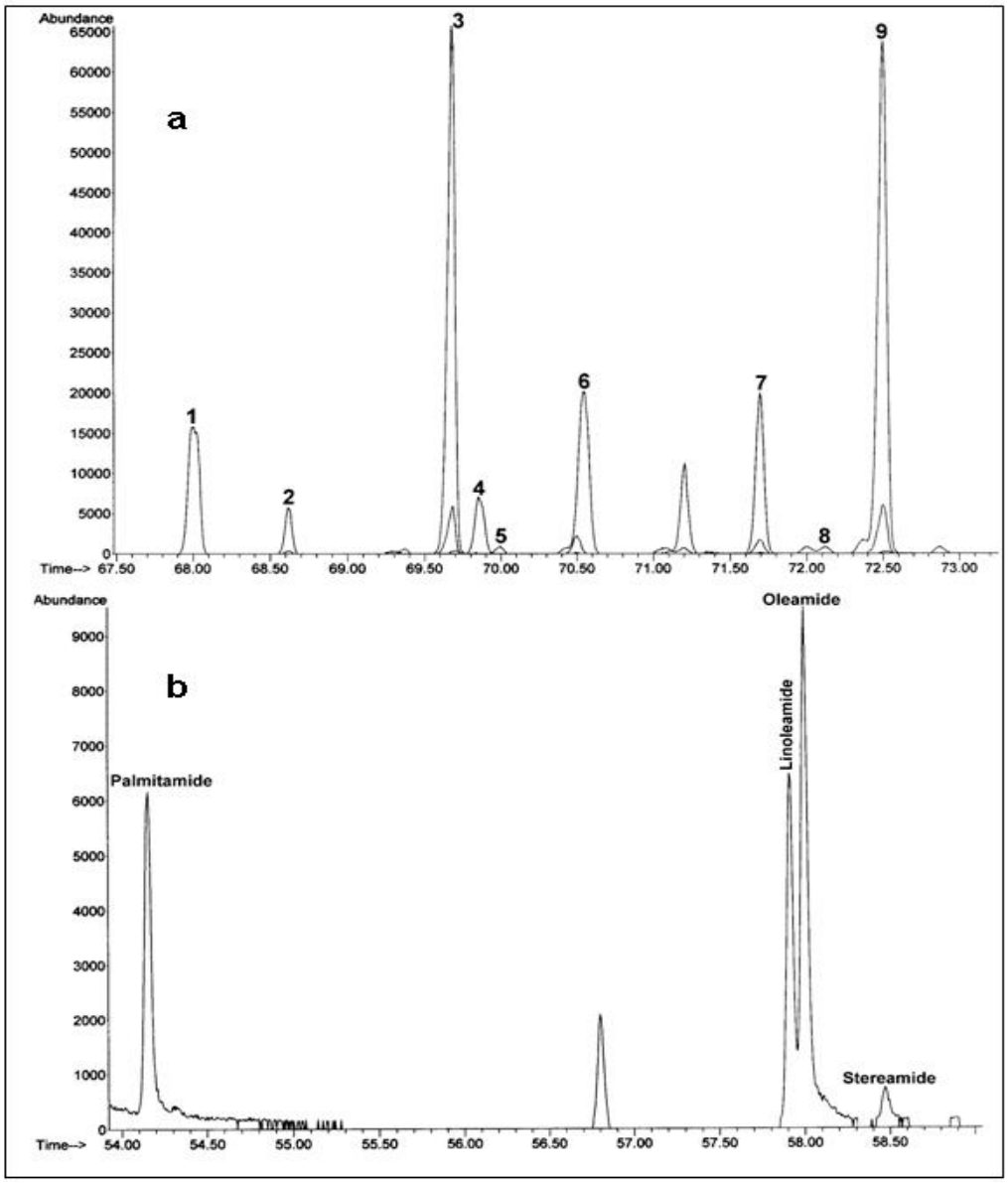

Figure 2. Base peak chromatogram time-window of: **a)** stigmastadienes (m/z= 396), stigmastatrienes (m/z= 394) and campestadienes (m/z=382) found in the corn oil (tentatively: **(1)** 3,5-campestadiene, **(2)** 3,5,22-stigmastatriene, **(3)** 3,5-stigmastadiene, **(4)** 2,5-campestadiene, **(5)** 2,4-campestadiene, **(6)** 2,5,22-stigmastatriene, **(7)** 2,5-stigmastadiene, **(8)** 2,4,22-stigmastatriene and **(9)** 2,4-stigmastadiene); **b)** amides derived from some of the fatty acids, found in the corn oil.

3.4. Acids

Other well known compounds present in edible oils which can be detected with this method are *fatty acids***.** Fatty acid content can be considered as a measure of the extent of oil triglyceride hydrolysis. It is one of the most frequently used quality parameters during oil production, storage and marketing and it is often used to classify and evaluate oils. The determination of fatty acids is of special importance for virgin oils since they are traded without any other processing than its extraction. The processing of other edible oils includes neutralization that reduces the content of fatty acids. Many specific methods have been developed for their determination (Osawa, Gonçalves & Ragazzi, 2007), even though, in most of them, this determination concerns all acids jointly and not each fatty acid individually. In these oils, acids from two to eighteen carbon atoms have been detected. The quantification of those having up to sixteen carbon atoms (palmitic acid) can be made individually in an accurate way (Table S3). However, the quantification of those of eighteen carbon atoms, namely, oleic, linoleic, linolenic and stearic acids, only can be made in an approximate way if overlapping of signals occurs. In case of signal overlapping, a global quantification of these specific acids can be made, which can be useful to discriminate between samples, and furthermore with the naked eye, it is possible to have an approximate idea of the relative concentration of each one of them. If there is not signal overlapping the relative abundance of these oils could be determined in an accurate way.

In addition to aliphatic acids, an *aromatic acid*, benzoic acid, has also been detected in all the studied oils.

3.5. Esters

A great number of *alkyl esters* (Table S3) derived from the different fatty acids are also detected in these oils and can also be quantified. They are of interest because their content is used as a quality parameter for some oils such as virgin olive oil (Bianchi, Tava, Vlahov & Pozzi, 1994). According to these authors, typical alkyl esters patterns enable the detection of certain adulterations. The determination of alkyl esters has been carried out by the use of specific methodologies for these compounds (Bianchi et al., 1994; Berardinelli et al., 2013). The classical methods involve firstly their isolation from the rest of oil components by solid-phase extraction or other methods, and later separation and quantification by gas chromatography (Bianchi et al., 1994).

The alkyl esters found in the oils here studied are derived not only from the main acids in edible oils (palmitic, oleic, linoleic, linolenic and stearic acids), but also from fatty acids having either lower or higher numbers of carbon atoms (up to twenty six carbon atoms) than the above mentioned. In turn, the alkyl radicals can have from one to nine carbon atoms. The most abundant of these are methyl and ethyl esters. In general, as may be expected, the predominant alkyl esters in each oil are those derived from the predominant fatty acids and acyl groups in the oil (see Table S3). Among the oils here studied virgin linseed is the richest in number and abundance of alkyl esters.

In addition, some *aromatic esters* (see Table 3) have also been found in the studied oils. Among them, it is worth noting the presence of ethyl-4-hydroxycinnamate, as well as of ethyl-3-methoxy-4-hydroxycinnamate and of two 2-ethylhexyl-4-methoxycinnamate isomers. As indicated in Table 3, the two latter were identified by comparison of their retention times and of their mass spectra with those of standard compounds, whereas the two former were identified by comparison of their mass spectra

with those of a commercial spectral library. Some of these compounds have been found in the essential oil of *Adenophora triphylla var. japonica* (Miyazawa, Horiuchi & Kawata, 2008), but to the best of our knowledge, this is the first time that their presence in edible oils is described. They are usually used in the cosmetic industry, to prevent damage to the skin, lips or hair, by their ability to absorb UV radiation (Chisvert, Pascual-Martí & Salvador, 2001). UV radiation absorbers, like these compounds, are also incorporated into several plastics during their manufacture for their ability to prevent the formation of free radicals (Kawamura et al., 2003). Some authors consider this kind of compounds to be potential endocrine disruptors (Kortenkamp, 2008) and others see them as emerging organic pollutants because they can bioaccumulate. In the oils here studied, the highest concentrations of the two 2-ethylhexyl-4-methoxycinnamate isomers have been found in sunflower, whereas ethyl 4-hydroxycinnamate and ethyl 3-methoxy-4-hydroxycinnamate have only been detected in corn oil. It could be thought that the presence of these compounds in these oils might be due to the migration from the plastic of the bottles in which they are packaged; however, virgin linseed and soybean oils were packed in glass receptacles and they also contain some of these compounds.

3.6. Lactones

The occurrence of *lactones*, which are *cyclic esters*, has received scarce attention in oils; however, the methodology here used enables their identification and quantification. They are present in foods of very different natures, such as fruits or cheese, and, in general, are characterized by pleasant odors. As Table 3 shows these oils contain lactones not only of small but also of high molecular weight.

Table 3. Aromatic esters, lactones, monoglycerides, fatty amides, and other unidentified compounds found in the oils studied (sunflower, SF, corn, C, refined soybean, RSB, virgin soybean VSB and virgin linseed VL), and their abundances given in area counts of the base peak (BP) of their mass spectra, multiplied by 10^{-4}, together with their standard deviations.

Compounds (MW)	BP	SF	C	RSB	VSB	VL
AROMATIC ESTERS						
Ethyl-4-hydroxycinnamate (192)	147	--	3.8 ± 0.3	--	--	--
Ethyl-3-methoxy-4-hydroxycinnamate (Ethyl ferulate) (222)	222	--	14.4 ± 0.3	--	--	--
2-Ethylhexyl-4-methoxycinnamate (iso) (290)*	178	19.4 ± 14.6	4.3 ± 2.1	2.8 ± 1.1	6.5 ± 0.7	--
2-Ethylhexyl-4-methoxycinnamate (iso) (290)*	178	27.4 ± 11.4	11.2 ± 3.8	7.0 ± 4.0	13.5 ± 7.0	16.1 ± 4.9
LACTONES						
γ-Butyrolactone (86)*	42	4.7 ± 0.2	2.6 ±1.7	--	10.5 ± 0.0	38.0 ± 5.7
5-Ethyl-2(5H)-furanone (112)	83	5.7 ± 1.2	3.5 ± 4.94	--	6.3 ± 1.8	24.8 ± 0.5
5-Ethyldihydro-2(3H)-furanone (114) (γ-Caprolactone)*	85	5.9 ± 1.0	3.1 ± 2.7	--	12.4 ± 2.9	34.2 ± 0.6
5-Propyl-dihydro-2(3H)-furanone (128) (γ-Heptalactone)*	85	--	--	--	1.1 ± 0.1	2.5 ± 0.3
5-Butyl-2(5H)-furanone (140)	84	1.9 ± 0.4	0.4 ± 0.5	--	0.5 ± 0.1	1.6 ± 0.1
5-Butyl-dihydro-2(3H)-furanone (142) (γ-Octalactone)*	85	21.2 ± 1.2	10.2 ± 5.2	6.4 ± 2.1	8.5 ± 0.2	18.2 ± 3.2
5-Pentyl-2(5H)-furanone (154)	84	36.7 ± 9.3	7.1 ± 3.2	2.9 ± 1.6	17.6 ± 1.1	20.3 ± 1.3
5-Pentyl-dihydro-2(3H)-furanone (156) (γ-Nonalactone)*	85	19.8 ± 0.3	6.5 ± 5.8	2.3 ± 0.8	54.8 ± 3.4	61.3 ±1.8
5-Hexyl-dihydro-2(3H)-furanone (170) (γ-Decalactone)*	85	3.0 ± 0.1	1.1 ± 1.0	--	0.6 ± 0.2	2.6 ± 0.9
6-Pentyl-5,6-dihydro-2H-pyran-2-one (168)	97	8.9 ± 1.0	3.2 ± 1.4	--	0.7 ± 0.0	2.3 ± 0.4
5-Heptyl-dihydro-2(3H)-furanone (184) (γ-Undecalactone)*	85	2.2 ± 0.5	0.8 ± 0.7	--	0.3 ± 0.1	0.6 ± 0.8
5-Octyl-dihydro-2-(3H)-furanone (198) (γ-Dodecalactone)*	85	1.2 ± 0.1	0.3 ± 0.4	--	--	0.7 ± 0.5
6-Hexyl-tetrahydro-2H-pyran-2-one (184) (δ-Undecalactone)	99	--	--	--	--	3.3 ± 0.2
6-Heptyl-tetrahydro-2H-pyran-2-one (198) (δ-Dodecalactone)*	99	0.5 ± 0.2	2.8 ± 0.1	--	3.2 ± 0.3	15.9 ± 2.3
5-Undecyl-dihydro-2(3H)-furanone (240) (γ-Pentadecalactone)	85	1.8 ± 0.5	1.9 ± 0.5	--	2.5 ± 0.3	5.3 ± 0.7
6-Octyl-tetrahydro-2H-pyran-2-one (212) (δ-Tridecalactone)	99	--	1.2 ± 0.3	--	2.1 ± 0.1	6.7 ± 0.6
5-Dodecyl-dihydro-2(3H)-furanone (254) (γ-Palmitolactone)	79	25.9 ± 4.5	10.5 ± 6.2	3.3 ± 2.3	8.8 ± 0.3	15.6 ± 1.8
6-Undecyl-tetrahydro-2H-pyran-2-one (254) (δ-Hexadecalactone)	99	35.6 ± 6.7	43.0 ± 0.5	10.7 ± 1.6	76.0 ± 3.5	105.6 ± 16.7
6,9-Octadecadien-4-olide (278)	79	12.6 ± 1.2	4.8 ± 1.1	2.5 ± 0.3	19.2 ± 0.9	27.3 ± 3.8
5-Tetradecyl-dihydro-2(3H)-furanone (282) (γ-Stearolactone)	85	10.9 ± 1.8	7.0 ± 2.3	1.9 ± 0.4	4.4 ± 0.4	10.0 ± 0.8

Compounds (MW)	**BP**	**SF**	**C**	**RSB**	**VSB**	**VL**
9-Octadecen-4-olide (280)	67	7.9± 0.9	6.5 ± 3.6	2.1 ± 0.2	10.1 ± 0.7	42.4 ± 3.2
6-Tridecyl-tetrahydro-2H-pyran-2-one (282) (δ-Octadecalactone)	99	5.2 ± 0.8	5.6 ± 2.0	1.9 ± 0. 5	11.3 ± 0.8	17.2 ± 1.5
δ-Oleolactone (280)	99	25.2 ± 5.7	14.7 ± 1.0	6.2 ± 3.4	64.4 ± 2.7	28.2 ± 5.4
δ-Eicosalactone (310)	99	1.1 ± 0.2	3.2 ± 0.2	2.4 ± 0.1	8.8 ± 0.3	38.0 ± 0.2
δ-Docosalactone (338)	99	3.4 ± 0.2	3.4 ± 0.5	18.9 ± 1.6	47.7 ± 1.3	9.3 ± 0.2
MONOGLYCERIDES						
1-Monopalmitin (330)	43	8.0 ± 1.7	16.4 ± 2.2	11.6 ± 1.9	6.2 ± 0.3	--
1-Monolinolein (354)*	67	10.7 ± 1.6	7.2 ± 1.2	--	--	--
2-Monolinolein (354)	67	--	105.6 ± 9.5	98.8 ± 6.0	12.8 ± 0.5	--
1-Monoolein (356)	55	--	--	66.0 ± 3.5	--	71.3 ± 9.4
1-Monolinolenin (352)	79	--	--	30.0 ± 0.4	--	86.3 ± 0.3
1-Monostearin (358)*	98	--	--	5.8 ± 0.4	--	--
FATTY AMIDES						
Palmitamide (255)*	59	2.6 ± 0.3	18.4 ± 1.9	2.7 ± 0.1	4.4 ± 0.7	1.3 ± 0.5
Linoleamide (279)	59	2.7 ± 0.7	16.3 ± 3.1	1.9 ± 0.2	1.2 ± 0.2	--
Oleamide (281)*	59	4.6 ± 1.4	24.5 ± 4.3	3.4 ± 0.1	0.6 ± 0.0	1.8 ± 1.2
Stereamide (283)	59	1.5 ± 0.9	3.2 ± 0.2	0.4 ± 0.0	--	1.4 ± 0.6
OTHER UNIDENTIFIED COMPOUDS						
Unidentified compound (386)	130	22.6 ± 0.4	35.0 ± 0.9	20.5 ± 5.6	37.4 ± 2.9	55.4 ± 12.4
Unidentified compound (410)	131	116.5 ± 2.5	121.6 ± 7.3	46.7 ± 8.9	36.2 ± 0.5	123.3 ± 34.1
Unidentified compound (412)	131	73.8 ± 1.2	9.1 ± 2.6	28.4 ± 4.6	19.4 ± 3.8	111.0 ± 34.4
Unidentified compound (408)	131	--	--	--	5.2 ± 1.2	156.6 ± 47.1
Unidentified compound (414)	130	8.4 ± 0.1	8.4 ± 1.0	4.5 ± 0.3	4.3 ± 1.0	20.9 ± 3.5

MW: Molecular weight; * Asterisked compounds were acquired commercially and used as standards for identification purposes; --: not detected

Those of small molecular weight have been previously found in undegraded oils (Ruiz del Castillo, Herraiz & Blanch, 2000) and in oils submitted to degradative conditions (Guillén et al., 2005). Among those of high molecular weight there are *gamma*- and *delta*- lactones derived from the main fatty acids present in the oil. The identification of these lactones of high molecular weight in Table 3 has been made by comparison of their mass spectra with those of the library and with others given by some authors in studies of pheromones and related compounds (Cossé, Bartelt, James & Petroski, 2001). To the best of our knowledge, to date no methods have been described to determine these latter lactones in edible oils. The abundance of these compounds in the oils here studied is in line with that of other compounds coming from oxidation processes such as esters, aldehydes, alcohols, ketones and others mentioned below.

3.7. Monoglycerides

Monoglycerides, an important group of compounds also associated with oil quality, can also be detected and quantified with this methodology. Their abundance, like that of fatty acids, is associated to the extent of the hydrolysis undergone by the oil triglycerides. These compounds can act as emulsifiers, and some authors have indicated that at high concentrations they can also act as pro-oxidants (Mistry & Min, 1988). Several specific methods have been described to determine these compounds in edible oils (Liu, Lee, Bobik Jr, Guzman-Harty & Hastilow, 1993; Schoenfelder, 2003). They can even be determined by ^{1}H NMR, if they are in high enough concentrations (Nieva-Echevarría, Goicoechea, Manzanos & Guillén, 2014). The monoglycerides found in the oils here studied are given in Table 3. Like fatty acids, monoglycerides having the same number of carbon atoms elute very close to each other and, depending on their concentration, their peaks overlap. Those derived from palmitic acid have m/z 43 as base

peak; those derived from oleic have m/z 55 as base peak, the ones derived from linoleic have m/z 67, those derived from linolenic m/z 79, and those from stearic acid m/z 98. Likewise, the separation of 1-monoglycerides from 2-monoglycerides is not possible in some occasions; however, the mass spectra of the former have a distinguishable m/z 98 ion, which is not relevant in the second ones. In case of overlapping, a global quantification of them can be made, which can be useful to discriminate between samples. Among the oils here studied the refined soybean oil is the richest in this kind of compounds.

3.8. Fatty amides

It is also noteworthy that this methodology is able to detect the occurrence in these oils of some *amides* derived from fatty acids, such as palmitamide, linoleamide, oleamide and steareamide. As indicated in Table 3, some of them (palmitamide and oleamide) have been identified by using commercially available standard compounds and the remaining ones by comparison of their mass spectra with those of a commercial spectral library. Their relative abundance, in the oils studied, is given in Table 3. As this table shows, the corn oil is the richest in these compounds. Figure 2b shows the abundances of base peak m/z 59 of some of these compounds in their chromatographic time-window.

These compounds occur naturally in the body of animals and it has been proved that they have several biological activities. They accumulate in the cerebrospinal fluid during sleep deprivation and induce sleep in animals (Mendelson & Basile, 2001). Their usefulness in medical treatments for mood and sleep disorders and in cannabinoid-regulated depression (Sakagami et al., 2015) has been studied. As far as we know, this type of compounds has not been previously described as components of common vegetable edible oils; only oleamide has been found in Camelina oil (Miura, Kida &

Nojima, 2007). However, some of these amides have been found in vegetable sources such as in the essential oil of mountain celery seeds (Cheng, Ker, Yu, Lin, Peng & Peng, 2010).

The fact that this kind of compounds is used in the fabrication of plastics as slip agents to provide lubrication to the film surface may suggest that their presence in oils could be due to their migration from the receptacle wall in which the oil is contained to the oil matrix (Bhunia, Sablani, Tang & Rasco, 2013). Nevertheless, the virgin linseed and soybean oils here studied were contained in glass receptacles and these compounds are also present in these oils. For this reason, it might be thought that the fatty amides here detected are natural components of these oils.

3.9. Aldehydes, Ketones, Alcohols, Epoxides, Furan and Pyran derivatives, and some Terpenic oxygenated derivatives

In addition to the above mentioned compounds, this method enables one to determine some of the *aldehydes, ketones, alcohols, epoxides,* and *furan and pyran derivatives* present in the oils. Those found are shown in Table S4. Many of these compounds have been found in previous studies not only in edible oils submitted to oxidation conditions (Guillén et al., 2005), but also in oils not submitted to degradative conditions (Uriarte et al., 2011).

With regard to *aldehydes*, a large number of *alkanals, alkenals, alkadienals,* certain *aromatic aldehydes* and some *oxygenated alpha,beta unsaturated aldehydes,* have been detected. Many of them result from oil oxidation and have been found in previous studies on edible oils submitted to degradative conditions (Guillén et al., 2005). It is worth highlighting the occurrence of the toxic *oxygenated alpha,beta unsaturated aldehydes* (see Table S4) which are considered responsible for degenerative diseases such as cancer,

Alzheimer or Parkinson (Zarkovic, 2003); their precursors are linolenic and linoleic acids and acyl groups. For this reason, their abundance in oils having similar proportions of their precursors gives information about the oxidation level reached by the oils. As Table S4 shows, the highest abundances of these compounds have been found in linseed virgin oil. Furthermore, *aliphatic* and a reduced number of *aromatic ketones* and *alcohols* have also been detected.

It is also worth mentioning the presence of *terpenic* derivatives having *alcohol*, *aldehyde, ketone,* or *epoxy* groups; these are showed in Table S4. They are derived from terpenes, such as *myrtenol*, from sesquiterpenes, such as *farnesol* and *farnesal*, from diterpenes, such as *phytol* and *kauranols*, and from triterpenes, such as *2,3-epoxysqualene* and as *squalen-22-one*. Among the oils here studied, sunflower oil is the richest in this kind of compounds.

3.10. Other unidentified compounds

Apart from all the above mentioned compounds some others have not been identified. Among these, it must be highlighted the presence in all the oils studied of a group of compounds with mass spectrum having as base peak m/z 131 and as molecular ions m/z 408, 410, 412, or base peak m/z 130 and molecular ions m/z 386 or 414, the difference between both kinds of ions being the mass of linolenic, linoleic, and oleic acyl groups respectively, and in the two second ones the mass of palmitic and stearic acyl groups respectively (see Table 3). To the best of our knowledge compounds with these mass spectra have not been previously described as edible oil components.

4. CONCLUSIONS

It has been proved that solid-phase microextraction of the liquid matrix of edible oils and the study of the extracted compounds by gas chromatography-mass spectrometry constitutes a powerful tool able to provide qualitative and quantitative data on minor edible oils components. This method enables a deep and comprehensive insight into oil composition which is unobtainable by other methodologies. As far as we know, this is the first time that such a high number of minor oil components, belonging to a great variety of chemical families, have been determined in different types of vegetable oils in one only run and without sample modification; the occurrence of some of these compounds in edible oils has not been described before. It is worth noticing that the data obtained about the relative proportions of different types of minor components such as sterols and tocopherols match well with the data coming from other studies where these types of compounds have been quantified in an absolute way. The potential of this technique is reinforced by the fact that it completely avoids the use of any type of solvent or reagent, and that all the information is obtained in the same chromatographic run, representing a considerable saving in time and cost. Moreover, the simultaneous detection of all the reported compounds allows one to obtain a great deal of information on diverse aspects of the oils such as nutritional value, oxidative stability, technological performance, quality, processing, safety and even fraudulent practices. The methodology proposed permits the characterization of edible oils in a simple way on the basis of their minor components and, as a result, the discrimination between different types of oils, even those from the same vegetable origin.

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Supplementary Material of

Article 1

A NEW METHODOLOGY CAPABLE OF CHARACTERIZING MOST VOLATILE AND LESS VOLATILE MINOR EDIBLE OILS COMPONENTS IN A SINGLE CHROMATOGRAPHIC RUN WITHOUT SOLVENTS OR REAGENTS. DETECTION OF NEW COMPONENTS

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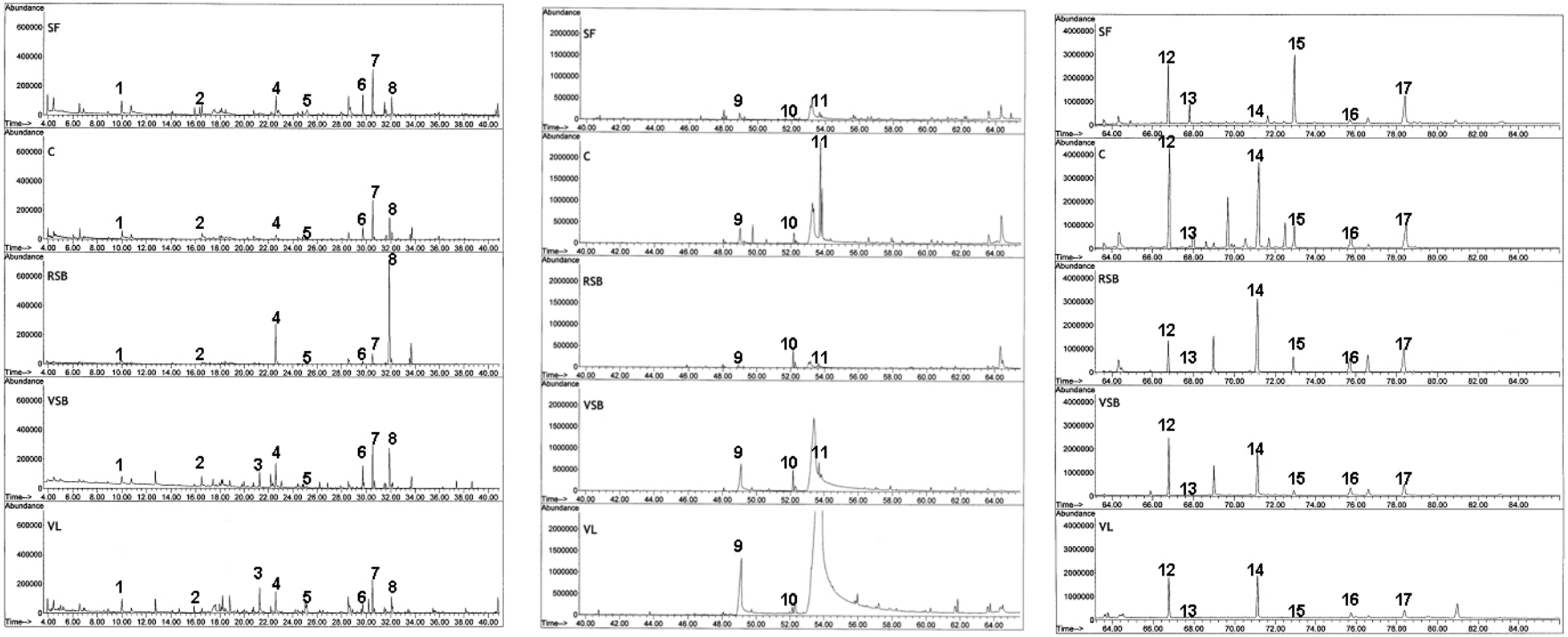

Figure S1. Enlargements of the different regions of the total ion chromatograms corresponding to the studied oils: sunflower (SF), corn (C), refined soybean (RSB), virgin soybean (VSB) and virgin linseed (VL). **(1)** Hexanal; **(2)** *E*-2-Heptenal; **(3)** (*E*,*E*)-3,5-Octadien-2-one; **(4)** Nonanal; **(5)** Octanoic acid; **(6)** (*Z*,*E*)-2,4-Decadienal; **(7)** (*E*,*E*)-2,4-Decadienal; **(8)** *E*-2-Undecenal; **(9)** Palmitic acid; **(10)** Methyl linoleate; **(11)** Ethyl linoleate; **(12)** Squalene; **(13)** Nonacosane; **(14)** γ-Tocopherol; **(15)** α-Tocopherol; **(16)** Campesterol; **(17)** β-Sitosterol.

Table S1. Proportions of the main acyl groups in the various oils (sunflower, SF, corn, C, refined soybean, RSB, virgin soybean VSB and virgin linseed VL), determined by ^{1}H nuclear magnetic resonance, as has been described in previous studies (Guillén & Ruiz, 2003; Guillén & Uriarte, 2009). They are expressed as molar percentage of the different kinds of acyl groups: polyunsaturated groups omega-3 and omega-6, monounsaturated and saturated.

ACYL GROUPS	SF	C	RSB	VSB	VL
% Omega-3	--	0.83	6.26	7.51	50.12
% Omega-6	46.70	47.02	48.51	45.81	16.60
% Monounsaturated	40.99	37.35	26.41	30.39	22.89
% Saturated	12.31	14.80	18.81	16.29	10.39

Table S2. Aliphatic hydrocarbons, found in the oils studied and their abundances in these oils (sunflower, SF, corn, C, refined soybean, RSB, virgin soybean VSB and virgin linseed VL), given in area counts of the base peak (BP) of their mass spectra, multiplied by 10^{-4} together with their standard deviations.

Compounds (MW)	**BP**	**SF**	**C**	**RSB**	**VSB**	**VL**
HYDROCARBONS						
ALIPHATIC HYDROCARBONS						
Alkanes						
Pentane (72)*	43	227.8 ± 19.8	59.1 ± 2.8	28.7 ± 5.7	57.4 ± 10.6	67.1 ± 9.4
Hexane (86)*	57	9.8 ± 0.8	6.6 ± 0.9	5.8 ± 1.4	4.1 ± 2.0	9.2 ± 1.9
Undecane (156)*	57	--	--	--	12.9 ± 4.9	--
Dodecane (170)*	43	--	--	--	17.3 ± 1.4	11.3 ± 0.3
Tridecane (184)*	57	--	--	--	21.7 ± 1.8	5.5 ± 0.7
Tetradecane (198)*	57	--	--	--	1.8 ± 0.1	6.6 ± 0.8
Pentadecane (212)*	57	3.4 ± 0.6	1.4 ± 1.0	--	0.9 ± 0.1	2.7 ± 0.5
Eicosane (282)*	57	3.9 ± 2.1	5.2 ± 0.5	--	--	7.2 ± 3.4
Heneicosane (296)*	57	--	72.4 ± 1.5	--	--	--
Docosane (310)*	57	--	17.2 ± 1.7	--	--	--
Tricosane (324)*	57	5.1 ± 1.5	66.6 ± 3.0	--	6.3 ± 0.7	21.8 ± 1.0
Pentacosane (352)*	57	17.6 ± 3.2	37.6 ± 0.1	--	6.2 ± 0.6	28.1 ± 0.3
Hexacosane (366)*	57	8.1 ± 0.1	11.3 ± 0.8	1.20 ± 0.1	5.6 ± 0.3	11.7 ± 0.2
Heptacosane (380)	57	127.4 ± 17.4	--	--	8.3 ± 0.8	41.8 ± 2.3
Octacosane (394)	57	30.9 ± 3.0	15.6 ± 2.0	2.1 ± 0.0	6.5 ± 1.1	16.8 ± 1.9
Nonacosane (408)	57	423.1 ± 29.5	41.3 ± 2.8	4.8 ± 0.2	14.5 ± 0.6	89.7 ± 1.1
Triacontane (422)	57	42.2 ± 4.2	--	--	--	--

Compounds (MW)	**BP**	**SF**	**C**	**RSB**	**VSB**	**VL**
Hentriacontane (436)	57	217.7 ± 9.5	--	9.5 ± 0.2	16.1 ± 1.2	31.5 ± 7.1
Alkenes						
Hexene (84) (iso)	56	6.1 ± 0.7	2.9 ± 0.6	2.2 ± 0.3	1.6 ± 0.5	6.5 ± 0.3
Octene (112) (iso)	55	5.7 ± 0.3	4.1 ± 0.6	--	3.9 ± 0.8	5.5 ± 0.3
Heptadecene (238) (iso)	43	5.3 ± 1.6	2.4 ± 1.5	--	0.7 ± 0.1	4.4 ± 2.1

MW: Molecular weight; * Asterisked compounds were acquired commercially and used as standards for identification purposes; --: not detected

Table S3. Acids and Alkyl esters found in the oils studied and their abundances (sunflower, SF, corn, C, refined soybean, RSB, virgin soybean VSB and virgin linseed VL), given in area counts of the base peak of their mass spectra, multiplied by 10^{-4}, together with their standard deviations.

Compounds (MW)	**BP**	**SF**	**C**	**RSB**	**VSB**	**VL**
ACIDS						
Acetic acid (60)*	60	12.2 ± 3.4	11.8 ± 1.8	3.7 ± 1.0	16.0 ± 7.2	39.7 ± 5.4
Butanoic acid (74)*	60	3.9 ± 0.2	2.0 ± 1.7	--	1.1 ± 1.1	14.0 ± 0.4
Pentanoic acid (102)*	60	45.0 ± 0.1	7.0 ± 6.9	1.1 ± 0.8	2.0 ± 0.3	16.3 ± 4.4
Hexanoic acid (116)*	60	145.8 ± 13.0	53.6 ± 39.7	10.9 ± 9.2	29.5 ± 6.4	180.3 ± 48.0
Heptanoic acid (130)*	60	39.0 ± 0.4	12.0 ± 10.7	2.6 ± 2.0	0.5 ± 0.4	15.2 ± 8.2
Benzoic acid (122)*	105	12.1 ± 2.8	5.9 ± 2.5	1.5 ± 0.1	4.3 ± 0.8	7.3 ± 2.8
Octanoic acid (144)*	60	65.4 ± 3.9	32.4 ± 20.7	4.0 ± 2.1	2.5 ± 0.0	46.3 ± 17.3
Nonanoic acid (158)*	60	52.1 ± 0.8	25.2 ± 15.8	46.2 ± 20.4	5.5 ± 0.0	24.4 ± 12.9
Decanoic acid (172)*	73	4.8 ± 0.4	2.8 ± 0.6	--	2.2 ± 0.0	3.9 ± 0.8
Dodecanoic acid (200)*	73	3.8 ± 0.1	2.2 ± 1.2	2.0 ± 0.1	1.4 ± 0.0	10.9 ± 2.9
Tetradecanoic acid (228)*	73	2.5 ± 1.4	8.4 ± 5.4	1.3 ± 0. 4	1.7 ± 0.1	21.7 ± 8.5
Pentadecanoic acid (242)	73	2.1 ± 0.6	5.3 ± 3.8	--	0.4 ± 0.0	8.3 ± 5.2
Palmitoleic acid (254)	55	--	--	--	--	17.0 ± 5.7
Palmitic acid (256)*	73	80.1 ± 34.8	229.9 ± 77.3	34.1 ± 5.1	398.8 ± 46.0	1,100.5 ± 124.3
Linoleic acid (280)*	67	Overlapped	Overlapped	Overlapped	Overlapped	Overlapped
Oleic Acid (282)*	55	Overlapped	Overlapped	Overlapped	Overlapped	Overlapped
Linolenic acid (278)*	79	--	--	5.0 ± 1.9	--	Overlapped
Stearic acid (284)*	73	--	--	4.2 ± 0.1	Overlapped	Overlapped

Compounds (MW)	**BP**	**SF**	**C**	**RSB**	**VSB**	**VL**
ALKYL ESTERS						
Fatty Acids Esters						
Ethyl dodecanoate (228)	88	--	--	--	3.2 ± 0.3	--
Ethyl myristate (256)	88	--	0.5 ± 0.1	--	1.2 ± 0.2	0.4 ± 0.1
Methyl palmitate (270)*	74	4.5 ± 2.6	11.2 ± 0.2	15.2 ± 1.9	37.8 ± 1.3	39.9 ± 3.9
Ethyl palmitate (284)	88	9.0 ± 9.7	200.9 ± 23.0	3.6 ± 0.4	30.8 ± 1.3	34.2 ± 3.1
Propyl palmitate (298)	61	--	--	--	1.4 ± 0.0	14.4 ± 1.2
Methyl linoleate (294)*	67	16.5 ± 3.9	37.2 ± 2.4	80.9 ± 10.3	108.4 ± 4.1	42.4 ± 4.5
Methyl oleate (296)*	55	11.5 ± 5.5	17.0 ± 2.5	19.9 ± 1.1	19.9 ± 1.7	37.9 ± 2.2
Methyl linolenate (292)*	79	1.9 ± 0.3	--	14.9 ± 1.7	22.2 ± 0.4	137.8 ± 15.5
Methyl stearate (298)	74	2.8 ± 1.2	2.6 ± 0.0	5.9 ± 0.7	7.9 ± 0.1	16.7 ± 2.3
Ethyl linoleate (308)	67	56.9 ± 16.7	631.5 ± 17.8	21.3 ± 1.4	178.8 ± 3.2	Overlapped
Ethyl oleate (310)	55	58.2 ± 23.8	243.9 ± 8.6	10.5 ± 0.3	23.9 ± 2.8	Overlapped
Ethyl linolenate (306)	79	--	40.4 ± 3.3	--	28.5 ± 3.2	Overlapped
Ethyl stearate (312)	88	5.0 ± 2.9	27.8 ± 2.7	1.3 ± 0.0	7.8 ± 0.3	14.7 ± 2.3
Propyl linoleate (322)	67	--	16.0 ± 1.1	3.7 ± 0.8	--	106.8 ± 2.1
Propyl oleate (324)	55	--	--	--	--	22.7 ± 3.0
Propyl linolenate (320)	79	--	--	--	--	165.5 ± 15.6
Pentyl palmitate (326)	70	--	--	0.7 ± 0.0	--	17.5 ± 3.9
Propyl stearate (326)	61	--	--	--	--	10.0 ± 2.1
Hexyl palmitate (340)	84	--	--	3.1 ± 0.1	--	25.5 ± 1.7
Pentyl linoleate (350)	67	6.2 ± 2.9	8.1 ± 0.0	2.3 ± 0.3	8.9 ± 0.0	24.3 ± 3.8

Compounds (MW)	**BP**	**SF**	**C**	**RSB**	**VSB**	**VL**
Pentyl oleate (352)	55	3.0 ± 0.6	3.6 ± 0.3	--	--	10.5 ± 0.8
Pentyl linolenate (348)	79	--	--	--	--	35.8 ± 3.0
Heptyl palmitate (354)	257	1.3 ± 0.2	--	--	--	0.7 ± 0.2
Methyl docosanoate (354)	74	28.2 ± 3.9	1.5 ± 0.2	1.3 ± 0.1	--	2.0 ± 0.0
Hexyl linoleate (364)	67	12.1 ± 1.8	14.5 ± 0.0	8.7 ± 0.7	16.5 ± 0.3	41.1 ± 3.2
Hexyl oleate (366)	55	12.3 ± 1.0	--	5.4 ± 0.4	10.9 ± 0.6	41.6 ± 0.6
Hexyl linolenate (362)	79	--	--	1.7 ± 0.1	5.0 ± 0.2	92.5 ± 8.1
Octyl palmitate (368)	257	0.8 ± 0.2	--	--	--	1.5 ± 0.3
Methyl tricosanoate (368)	74	5.4 ± 0.9	--	--	--	--
Nonyl palmitate (382)	257	--	--	--	--	1.3 ± 0.1
Methyl tetracosanoate (382)	74	53.1 ± 7.1	2.1 ± 0.0	--	--	2.8 ± 0.0
Hexyl linoleate (362)	79	--	--	--	3.5 ± 0.5	17.5 ± 4.3
Methyl hexacosanoate (410)	74	16.2 ± 1.6	--	--	--	--

MW: Molecular weight; * Asterisked compounds were acquired commercially and used as standards for identification purposes; --: not detected

Table S4. Aldehydes, ketones, alcohols and furan and pyran derivatives found in the oils subject of study (sunflower, SF, corn, C, refined soybean, RSB, virgin soybean VSB and virgin linseed VL), and their abundances given in area counts of the base peak (BP) of their mass spectra, multiplied by 10^{-4}, together with their standard deviations.

Compounds (MW)	BP	SF	C	RSB	VSB	VL
ALDEHYDES						
ALKANALS						
3-Methyl butanal (86)	44	--	25.4 ± 2.3	--	--	8.1 ± 0.4
Pentanal (86)*	44	48.9 ± 1.5	34.1 ± 8.2	21.6 ± 6.3	26.0 ± 1.0	30.7 ± 0.1
Hexanal (100)*	44	70.0 ± 0.7	52.2 ± 5.9	26.8 ± 3.6	51.0 ± 7.4	49.9 ± 12.8
Heptanal (114)*	70	10.9 ± 0.9	10.6 ± 0.8	8.7 ± 1.8	4.6 ± 0.3	4.7 ± 3.3
Octanal (128)*	43	13.1 ± 0.1	10.9 ± 0.0	7.5 ± 1.0	5.3 ± 0.1	6.7 ± 4.4
Nonanal (142)*	57	54.9 ± 8.5	35.6 ± 19.3	136.5 ± 34.3	54.9 ± 18.2	33.7 ± 31.1
Decanal (156)*	57	7.0 ± 0.8	3.2 ± 1.9	1.4 ± 0.2	1.0 ± 0.2	4.1 ± 2.1
Undecanal (156)	41	4.0 ± 0.7	1.7 ± 0.8	0.5 ± 0.1	0.4 ± 0.3	2.7 ± 0.0
Dodecanal (184)	43	6.3 ± 0.9	2.5 ± 2.0	0.4 ± 0.1	1.4 ± 0.0	3.4 ± 2.0
Tetradecanal (212)	57	3.3 ± 1.0	1.4 ± 1.4	--	--	2.0 ± 1.5
Pentadecanal (226)	82	1.1 ± 0.5	1.0 ± 0.4	--	--	0.7 ± 0.4
Hexadecanal (240)	43	3.8 ± 0.9	1.6 ± 1.4	--	--	--
ALKENALS						
E-2-Butenal (70)*	70	1.1 ± 0.2	1.7 ± 1.4	0.9 ± 0.8	2.3 ± 0.5	13.2 ± 3.6
E-2-Pentenal (84)*	55	3.7 ± 0.9	--	--	5.8 ± 0.6	11.9 ± 0.1
E-2-Hexenal (98)*	41	7.6 ± 0.2	3.7 ± 0.1	--	3.5 ± 0.6	5.3 ± 0.3
E-2-Heptenal (112)	83	29.4 ± 3.7	22.8 ± 8.2	10.8 ± 2.6	33.9 ± 4.2	11.1 ± 1.9

Compounds (MW)	**BP**	**SF**	**C**	**RSB**	**VSB**	**VL**
E-2-Octenal (126)	41	16.5 ± 2.8	19.0 ± 4.5	--	14.0 ± 3.6	11.0 ± 4.3
E-2-Nonenal (140)*	41	9.2 ± 0.3	8.4 ± 2.5	2.0 ± 0.6	5.9 ± 0.0	5.1 ± 1.8
E-2-Decenal (154)*	70	48.3 ± 9.4	25.6 ± 11.7	17.7 ± 7.1	15.1 ± 1.7	22.6 ± 7.8
E-2-Undecenal (168)	70	39.5 ± 5.0	23.0 ± 10.8	13.2 ± 3.2	8.2 ± 1.3	22.2 ± 8.7
Z-11-Hexadecenal (238)	55	3.3 ± 0.8	--	--	--	2.6 ± 0.7
ALKADIENALS AND ALKATRIENALS						
(E,E)-2,4-Hexadienal (96)	81	--	--	--	0.9 ± 0.3	4.2 ± 0.3
(Z,E)-2,4-Heptadienal (110)	81	8.3 ± 1.1	16.4 ± 9.4	6.0 ± 1.7	48.6 ± 0.3	116.5 ± 8.1
(E,E)-2,4-Heptadienal (110)*	81	7.5 ± 0.8	38.0 ± 12.4	32.5 ± 3.7	50.3 ± 4.5	125.8 ± 3.0
(Z,E)-2,4-Nonadienal (138)*	81	14.0 ± 1.5	8.5 ± 3.4	2.1 ± 1.7	35.3 ± 7.1	11.4 ± 0.5
(E,E)-2,4-Nonadienal (138) (iso)	81	--	--	--	3.0 ± 0.5	--
(E,E,E)-2,4,6-Nonatrienal (136)	79	--	--	--	2.9 ± 0.1	8.2 ± 0.4
(Z,E)-2,4-Decadienal (152)	81	141.5 ± 16.6	84.5 ± 6.8	33.0 ± 10.1	153.0 ± 13.4	62.7 ± 4.1
(E,E)-2,4-Decadienal (152)*	81	363.5 ± 58.1	308.5 ± 9.0	110.4 ± 25.1	345.4 ± 60.2	250.9 ± 40.6
(Z,E)-2,4-Undecadienal (166)	81	--	--	--	3.1 ± 0.1	--
(E,E)-2,4-Undecadienal (166) (iso)	81	--	--	--	8.2 ± 0.5	--
Hexadecadienal (236) (iso)	67	3.1 ± 0.8	1.7 ± 0.2	1.2 ± 1.0	1.0 ± 0.2	3.1 ± 0.1
Octadecadienal (264) (iso)	67	8.4 ± 0.3	8.7 ± 0.3	2.3 ± 0.4	--	--
OXIGENATED, α,β-UNSATURATED ALDEHYDES						
4-oxo-2-Hexenal (112)	83	--	--	--	--	1.7 ± 0.4
4-oxo-*E*-2-Nonenal (154)	55	12.6 ± 0.7	6.4 ± 0.9	3.0 ± 1.4	8.0 ± 1.0	7.7 ± 0.7
4-Hydroxy-*E*-2-nonenal (156)*	57	20.6 ± 0.4	14.5 ± 1.2	7.4 ± 3.1	10.1 ± 0.7	19.6 ± 5.7

Compounds (MW)	**BP**	**SF**	**C**	**RSB**	**VSB**	**VL**
4,5-Epoxy-2-heptenal (126)	68	1.4 ± 0.1	4.1 ± 0.6	--	1.8 ± 0.1	12.2 ± 0.5
4,5-Epoxy-2-heptenal (126) (iso)	68	--	1.3 ± 0.6	--	1.5 ± 0.2	4.4 ± 0.2
4,5-Epoxy-2-decenal (168)	68	4.4 ± 1.1	4.2 ± 0.2	0.8 ± 0.4	--	4.5 ± 0.5
4,5-Epoxy-2-decenal (168) (iso)	68	12.2 ± 3.1	11.7 ± 1.0	2.4 ± 0.8	--	11.6 ± 1.0
AROMATIC ALDEHYDES						
Benzaldehyde (106)*	106	1.9 ± 0.2	5.3 ± 2.1	0.8 ± 0.2	4.4 ± 0.6	5.2 ± 0.6
Benzeneacetaldehyde (120)	91	2.2 ± 0.5	17.5 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	4.0 ± 0.1
2-Hydroxy-4-methylbenzaldehyde (136)	135	--	--	--	--	4.9 ± 0.4
2-Phenylpropenal (132)	131	2.3 ± 0.0	1.1 ± 0.1	0.4 ± 0.1	1.0 ± 0.0	0.9 ± 0.3
3-Hydroxy-4-methoxybenzaldehyde (152) (Vanillin)*	151	--	0.5 ± 0.2	--	0.8 ± 0.1	22.9 ± 2.4
KETONES						
2-Heptanone (114)*	43	7.1 ± 0.9	4.8 ± 2.6	5.0 ± 0.2	4.8 ± 0.2	6.4 ± 4.0
3-Octen-2-one (126)	55	--	--	--	27.8 ± 4.5	5.9 ± 0.4
6-Octen-2-one (126)	43	1.9 ± 0.4	1.5 ± 0.1	--	--	12.9 ± 0.7
4-Ethyl-cyclohexanone (126)	55	2.6 ± 1.3	--	1.1 ± 0.0	2.1 ± 0.3	6.4 ± 1.0
(E,E)-3,5-Octadien-2-one (124)	95	--	--	--	61.9 ± 10.4	108.7 ± 6.0
3,5-Octadien-2-one (124) (iso)	95	--	--	--	65.5 ± 13.3	31.8 ± 2.2
(Z,E)-3,5-Octadien-2-one (124)	95	--	--	--	10.1 ± 2.4	--
6-Dodecanone (184)	58	3.1 ± 0.0	0.6 ± 0.2	--	0.1 ± 0.0	0.6 ± 0.4
1-Phenyl-1-hexanone (176)	105	4.8 ± 0.6	2.2 ± 2.0	--	1.0 ± 0.0	2.0 ± 1.6
2,6-Di-*tert*-butylbenzoquinone (220)	177	3.5 ± 1.6	1.5 ± 1.1	--	--	1.3 ± 0.9
6-Tetradecanone (212)	43	3.9 ± 0.3	1.2 ± 1.5	--	--	1.5 ± 0.8

Compounds (MW)	BP	SF	C	RSB	VSB	VL
2-Pentadecanone (226)	58	--	2.2 ±2.1	0.2 ± 0.1	0.5 ± 0.0	2.4 ± 1.9
2-Hexadecanone (240)	58	1.8 ± 0.8	3.2 ±0.7	4.5 ± 1.1	0.3 ± 0.1	1.4 ± 0.8
2-Nonadecanone (282)	58	3.4 ± 0.5	13.7 ± 1.0	0.4 ± 0.0	1.5 ± 0.0	--
n-Heneicosanone (310)	58	--	6.1 ± 0.2	--	--	--
Chalcone (208)	207	--	6.3 ± 0.0	--	--	--
ALCOHOLS						
Propanol (60)	59	--	--	--	--	11.2 ± 0.2
1-Pentanol (88)*	42	21.6 ± 1.1	9.1 ± 3.7	6.1 ± 2.8	16.0 ± 1.6	18.8 ± 2.2
2-Hexen-1-ol (100)	57	--	--	--	--	4.9 ± 0.7
1-Hexanol (102)	56	8.0 ± 1.1	2.9 ± 0.5	--	102.2 ± 18.4	90.2 ± 0.0
1-Heptanol (116)	70	3.8 ± 0.7	3.1 ± 0.5	2.8 ± 1.3	5.4 ± 0.4	4.3 ± 0.0
1-Octen-3-ol (128)	57	17.2 ± 2.1	21.8 ± 6.0	5.1 ± 1.6	34.5 ± 2.1	16.4 ± 3.3
Benzyl alcohol (108)*	79	0.7 ± 0.4	--	--	9.6 ± 1.6	6.0 ± 0.8
2-Octen-1-ol (128)	57	4.1 ± 0.7	3.8 ± 2.8	--	3.2 ± 0.6	7.7 ± 0.6
1-Octanol (130)*	56	5.5 ± 0.7	3.8 ± 0.6	4.9 ± 0.4	4.1 ± 0.9	5.9 ± 1.2
Benzeneethanol (122)	91	--	--	--	45.4 ± 4.3	13.6 ± 2.8
6-Undecanol (172)	83	5.6 ± 0.1	--	--	1.5 ± 0.1	5.8 ± 0.6
1-Dodecanol (186)	55	--	--	2.1 ± 0.2	1.8 ± 0.1	2.5 ± 0.1
FURAN AND PYRAN DERIVATIVES						
2-Ethylfuran (96)	81	1.4 ± 0.0	2.1 ± 3.0	0.5 ± 0.8	1.5 ± 0.0	23.0 ± 2.1
3-Methyl-2,5-furandione (112)	68	113.1 ± 23.0	1.3 ± 0.3	2.6 ± 0.4	--	--
2-Pentylfuran (138)	81	36.5 ± 7.0	43.2 ± 8.8	8.0 ± 2.5	38.7 ± 0.1	66.4 ± 8.6

Compounds (MW)	**BP**	**SF**	**C**	**RSB**	**VSB**	**VL**
Z-2-(2-Pentenyl)furan (136) (iso)	107	--	--	--	--	1.5 ± 0.1
2-Hydroxy-3-methyl-4H-pyran-4-one (126) (Maltol)*	126	3.7 ± 0.2	--	--	7.3 ± 1.4	6.3 ± 0.5
2-Heptylfuran (166)	81	4.1 ± 0.2	2.4 ± 1.2	--	3.9 ± 0.1	3.1 ± 0.8
2-Octylfuran (180)	81	2.4 ± 0.0	0.4 ± 0.1	--	0.2 ± 0.1	1.4 ± 0.6
TERPENIC DERIVATIVES						
Myrtenol (152)	79	2.5 ± 0.6	1.6 ± 0.4	0.3 ± 0.4	2.6 ± 0.1	2.1 ± 0.0
Farnesal (220)*	69	0.4 ± 0.3	--	--	--	1.1 ± 0.3
Farnesyl acetaldehyde (248)	69	32.1 ± 13.6	2.7 ± 0.2	5.6 ± 0.6	3.1 ± 0.4	6.4 ± 1.2
Phytone (268)	43	7.8 ±1.1	4.6 ± 0.5	2.7 ± 0.8	5.4 ± 0.0	5.1 ± 0.7
Phytol (296)*	82	2.8 ± 0.4	1.4 ± 0.1	7.5 ± 0.7	--	--
Farnesol (222)	69	29.4 ± 7.4	2.6± 0.0	4.6 ± 0.4	2.4 ± 0.0	5.4 ± 0.3
Kauran-16-ol (290) (or iso)	123	12.3 ± 0.6	--	--	--	--
Kauran-16-ol (290) (or iso)	272	3.1 ± 0.1	--	--	--	--
Isophytol (296) (or iso)	71	0.7 ± 0.3	--	--	5.2 ± 0.3	1.4 ± 0.0
Phytol acetate (338)	95	--	--	2.9 ± 0.1	9.5 ± 0.8	--
2,3-Epoxysqualene (426)*	69	36.4 ± 2.8	--	--	11.2 ± 0.2	25.9 ± 5.3
Squalen-22-one (426)	69	11.9 ± 4.5	11.5 ± 1.7	--	--	11.5 ± 5.7

MW: Molecular weight; * Asterisked compounds were acquired commercially and used as standards for identification purposes; --: not detected

Article 2

BIOACTIVE COMPOUNDS DETECTED FOR THE FIRST TIME IN CORN OIL. CYCLIC DIPEPTIDES AND OTHER NITROGENATED COMPOUNDS

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ABSTRACT

Eleven edible oils, namely extra virgin olive, virgin olive, olive, sunflower, virgin soybean, refined soybean, linseed and four corn oils were studied in order to analyze the occurrence in them of minor nitrogenated components. The study was carried out by using Solid Phase Microextraction (SPME) followed by Gas Chromatography/Mass Spectrometry (GC/MS). Apart from fatty amides no other nitrogenated compound was found in the first seven oils cited. However, it has been discovered that the four corn oils studied contain cyclic dipeptides or 2,5-diketopiperazines (DKPs), and other nitrogenated compounds such as oxazolines, pyrrolidines and other pyrrol derivatives, as well as indol, pyrazol, pyridine and β-phenylethylamine derivatives. DKPs were the most numerous and some of them were the most abundant of all nitrogenated compounds detected. All these compounds are bioactive and for this reason they can give corn oil interesting properties from a technological, nutritional and health points of view. Cyclic dipeptides could be considered as corn oil markers in authentication and fraud studies and their concentration can be used for differentiation and classification of corn oils. This is the first time that the presence of these bioactive compounds has been reported in corn oils.

KEYWORDS: edible oils; corn oil; bioactive components; cyclic dipeptides or DKPs; nitrogenated compound; food analysis; food composition.

CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE

Cyclo (Pro-Leu) (PubChem CID: 7074739); cyclo (Leu-Phe) (PubChem CID: 7076347); cyclo (Pro-Phe) (PubChem CID: 99895); 4,4-dimethyl-2-(1-hydroxy-heptadec-8-enyl)-2-oxazoline (PubChem CID: 5372667); acetyl-beta-carboline (PubChem CID: 638667); 3,5-diphenylpyridine (PubChem CID: 66692).

1. INTRODUCTION

Edible vegetable oils are highly complex mixtures of a large number of components whose nature is very varied. They have great importance in the food industry due to their widespread use as food ingredients, as media for culinary preparations and as carriers of flavor and taste components, among other uses. They have also great importance in nutrition, mainly for their influence on health; this influence is attributed to their composition in both major and minor components. Regarding main components, many studies have related the unsaturation degree of edible oils with effects on health. Likewise, some minor edible oil components have also been related to healthy properties, given the ability of some of them to act as antioxidants (Kamal-Eldin & Appelqvist, 1996; Singh 2013; Alberdi-Cedeño et al., 2017). As an example, the health claims of extra virgin olive oil having a certain concentration of some phenolic compounds have recently been approved in the European Union (EFSA, 2012). It is noteworthy that not only oil saturation degree but also the content and nature of minor edible oil components play a very important role in food industry because they are together responsible for the oil oxidative stability (Guillén & Ruiz, 2005a, b, c) and for the oil behavior in processing at industrial and at the home level.

For the above mentioned reasons, the composition of edible oils has been extensively studied and many efforts have been made in the development of new methods with this aim, using even the most sophisticated and expensive technologies. The study of major oil components has been traditionally carried out by gas chromatography after transformation of the triglycerides into methyl esters, although in recent years ^{1}H NMR (^{1}H Nuclear Magnetic Resonance) has provided a much simpler and faster method (Guillén & Ruiz, 2003a, b; Guillén & Uriarte, 2012; Martínez-Yusta et al., 2014) not requiring chemical modification of the sample. The study of minor oil components is much more difficult than that of the main ones not only because they are very numerous but also because they have very different

functional groups. Their study has been traditionally carried out by extraction of the compounds of interest by a specific extraction technique for each kind of compound and their subsequent separation, identification and quantification by chromatography using diverse detectors (Cert et al., 2000). In this way, phenols, tocols, sterols and other minor oxygenated components have been quantified in several oils. Taking into account that the composition of edible oils is very complex, it might be thought that some of their minor components still remain to be discovered.

A group of compounds that until now has received very scarce attention in oils is that of nitrogenated compounds. Nevertheless, some of them, such as cyclic dipeptides, have been found in certain foods such as bread, beer, coffee and cocoa among others (Gautschi et al., 1997; Ginz & Engelhardt, 2000, 2001; Stark & Hofman, 2005; Ryan et al., 2009), and some of them have been attributed bioactive abilities (Kumar et al., 2013; Ser et al., 2015; Vázquez-Rivera et al., 2015). Although, the occurrence of compounds of this nature has not been described in edible oils to date, it cannot be discounted.

In this context, the aims of this study are to study the occurrence in edible oils of nitrogenated compounds not described as oil components before and to show the potential relevance of their presence. To reach the first aim a new methodology, developed in our research group, will be used, (Alberdi-Cedeño et al., 2017). In relation to the second aim some aspects regarding the occurrence of these compounds in other foods, their possible origin, their contribution to the oil sensory and bioactive properties, as well as their possible use as oil markers will be considered.

2. EXPERIMENTAL

2.1. Samples

The study was carried out on eleven different edible oils of varied vegetable origin, namely extra virgin olive oil, virgin olive oil, olive oil, refined soybean oil, virgin soybean oil, sunflower oil, virgin linseed oil and four refined corn oils (C1, C2, C3, and C4). All of them were acquired in local supermarkets. In order to have more information about the composition of the four corn oils subject of study their main components were quantified by ^{1}H NMR as in previous studies (Guillén & Ruiz, 2003a; Guillén & Uriarte, 2009). Their composition expressed as molar percentages of different kinds of acyl groups is given in Table 1. It can be observed that there are no important differences among main components in these refined corn oils, C4 being the richest in oleic acyl groups followed by C1, C2 and C3 and the opposite being true for linoleic acyl groups.

Table 1. Molar percentage of the different kinds of acyl groups in C1, C2, C3 and C4, corn oils. Different letters within each row indicate a significant difference ($p < 0.05$).

ACYL GROUPS	C1	C2	C3	C4
% Linolenic	0.6 ± 0.0a	0.7 ± 0.3a	1.0 ± 0.1a	0.8 ± 0.3a
% Linoleic	47.9 ± 0.3a	48.3 ± 0.9a	50.2 ± 0.2b	46.5 ± 0.2a
% Oleic	34.9 ± 0.2a	33.5 ± 0.6b	32.5 ± 0.1b	35.6 ± 0.0a
% Saturated	16.5 ± 0.2a	17.4 ± 0.7a	16.2 ± 0.2a	17.0± 0.2a

2.2. Extraction of minor oil components

The oils were submitted to extraction directly without any previous sample preparation. The method used for extraction is based on that described by Mikuma & Kaneko (2010). This was carried out by solid phase micro extraction. To this aim a fibre of 65 μm StableFlex polydimethylsiloxane/divinylbenzene (PDMS/DVB) acquired from Supelco (Bellefonte, PA, U.S.) was immersed directly into 6 ml of edible oil at room temperature for 45 minutes. The selection of the type of fiber (polarity and thickness of the coating) was made on the basis of its ability to extract the widest range of minor oil components with the highest

possible yield, after analyzing the results obtained by Mikuma and Kaneko and after testing several polymer-coated fused silica fibers, like in a previous study (Alberdi-Cedeño et al., 2017). Likewise, extraction conditions such as sample amount, extraction time and temperature were also tested, to find those considered optimal on the basis of the extraction results, as in a previous study (Alberdi-Cedeño et al., 2017).

2.3. Gas Chromatography-Mass Spectrometry

The oil components picked up by the fiber were directly injected into the port of a gas chromatograph–mass spectrometer (GC–MS). For their thermal desorption, the plunger was pushed down to expose the fiber to the GC carrier gas stream and held for 10 minutes. The desorbed compounds were separated, identified and semi-quantified by gas chromatography-mass spectrometry.

The equipment used was an Agilent gas chromatograph model 6890N equipped with a mass selective detector 5973 Network and a Hewlett-Packard Compaq Pentium 4 computer. A fused silica capillary column was used (60 m length x 0.25 mm inside diameter x 0.25 μm film thickness; from Hewlett-Packard, Palo Alto, CA), coated with a nonpolar stationary phase (HP-5MS, 5% phenyl methyl siloxane). The injector and interface temperatures were held at 250°C and 305ºC respectively and helium at a constant pressure of 117 kPa (16.9 psi) was used as the carrier gas. The oven temperature was initially held at 50ºC for 5 min, increased from 50 to 300ºC at a rate of 4ºC/min, and then held at 300ºC for 30 min. The injections were carried maintaining the fiber in the injection port for 10 min, the first 5 in splitless mode. Mass spectra were recorded at an ionisation energy of 70 eV, with data acquisition in Scan mode. The temperatures of the ion source and the quadrupole mass analyzer were kept at 230 and 150ºC respectively. After the first desorption, the fibre was routinely submitted to desorption conditions for a second time both for its clean up and to

determine if the first process had been completed. A reference sample of known composition was periodically analyzed in order to verify the reproducibility of the chromatographic runs.

The identification of the several nitrogenated compounds was made using standards in some cases and on the basis of their mass spectra and retention times in others. The standards used, asterisked in Table 2, acquired from Bachem AG and Carbosynth Ltd (Cymit Quimica S.L, Barcelona, Spain) and ChemFaces Biochemical Co., LTD (Wuhan, China). The identification of the other ones was made by matching their mass spectra with spectra from commercial libraries by more than 85% (Wiley 275.L, and Mass Spectral Database, Rev. D.01.00, June 2000 and NIST) and also with other obtained from the literature (Benítez et al., 2012; Fabbri et al., 2012; Poerschmann et al., 2016) and taking also into account their retention times as in previous studies (Guillén et al., 2005; Guillén & Abascal, 2012; Alberdi-Cedeño et al., 2017). Mass spectral data of the detected compounds are given in Table S1. This way of identification is accepted for food studies in which, as is the case here, the composition is very complex, the number of components is very high, the components subject of study are present in very low concentration, the molecules are not new, and their mass spectra are well known and established (Guillén et al., 2005, Cabrita et al., 2007; Guillén & Abascal, 2012, Alberdi-Cedeño et al., 2017, Balzano et al., 2017)..

The semi-quantification of the identified compounds was based on arbitrary units of the base peak ion area counts divided by 10^5. The purpose of this study was not the determination of absolute but of relative concentrations that are valid for comparative purposes. This kind of quantification is accepted for comparative studies such as this one.

2.4. Quality control of the global method and statistical analysis

The quality control of the global method was carried out by the periodic extraction of an oil sample considered as sample reference and the subsequent study of the extracted compounds by GC/MS. The detection limit was established in an abundance of 10000 area

counts for the base peak of the mass spectra of the compounds. The specificity, selectivity and robustness of the GC/MS to evaluate unequivocally the compounds included in this study is beyond all doubt as it is a recognized suitable global method for the purposes of this study.

All determinations were carried out at least in duplicate. Data given in Tables 2 and 3 are mean values accompanied by the corresponding standard deviations. The significance of the differences among the several corn oil samples on the molar percentage of the acyl groups and on the abundances of the several nitrogenated compounds were determined by one-way variance analysis (ANOVA) followed by Tukey *b* test at $p < 0.05$ and/or by Student´s t-test at 0.05 threshold, using SPSS v.22 (IBM, NY, USA).

3. RESULTS AND DISCUSSION

As has been commented before, the minor components of eleven oils were studied looking for nitrogenated compounds. The only common nitrogenated compounds found in all kind of oils were fatty acid amides, as reported in a previous paper (Alberdi-Cedeño et al.,2017). Among the oils studied, other minor nitrogenated compound different than the before mentioned were detected only in corn oils.

Tables 2 and 3, show the nitrogenated compounds detected exclusively in the four corn oils studied together with their relative abundances. Among them there are cyclic dipeptides and other nitrogenated compounds.

3.1. Cyclic dipeptides or 2,5-diketopiperazines (DKPs)

These are the smallest dipeptides known. Their presence in edible oils has not been described before. However, with the methodology used in this study, twenty five compounds of this nature were detected in corn oil but not in any of the other edible oils studied (extra virgin olive, virgin olive, olive, sunflower, refined soybean, virgin soybean and linseed oils).

The DKPs here found are derived mainly from proline (Pro), leucine/isoleucine (Leu/Ile), phenylalanine (Phe) and other amino acids.

Table 2. Cyclic dipeptides detected in, C1, C2, C3 and C4 corn oils, and their abundances, expressed as area counts of their mass spectra base peak (Bp) divided by 10^5, obtained as average value of two determinations together with their standard deviations. Different letters within each row indicate a significant difference ($p < 0.05$).

Nº	Cyclic dipeptides	Bp	C1	C2	C3	C4
1	Cyclo (Pro-Gly)	83	-	-	-	2.5 ± 0.0
2	Cyclo (Pro-Ala)* I	70	-	-	0.4 ± 0.0a	2.6 ± 0.0b
3	Cyclo (Pro-Ala) II	70	-	-	-	2.2 ± 0.0
4	Cyclo (Pro-Pro)*	70	-	-	0.8 ± 0.0a	6.5 ±7.6b
5	Cyclo (Pro-Val)* I	154	-	-	-	1.8 ± 0.0
6	Cyclo (Pro-Val) II	154	-	-	-	0.7 ± 0.0
7	Cyclo (Pro-PyroGlu)	70	-	-	-	8.0 ± 8.4
8	Cyclo (Pro-Leu)* I	154	12.3 ± 1.0a	6.1 ± 0.7b	0.3 ± 0.1c	3.6 ± 0.0bc
9	Cyclo (Pro-Leu/Ile) II	154	9.1 ± 0.9a	1.6 ± 0.0b	0.4 ± 0.1b	2.6 ± 0.0b
10	Cyclo (Pro-Phe)* I	125	4.6 ± 0.5a	1.8 ± 0.1b	0.9 ± 0.4b	2.8 ± 1.1ab
11	Cyclo (Pro-Phe) II	125	6.5 ± 0.5a	4.3 ± 0.5a	1.0 ± 0.3b	6.6 ± 1.1a
12	Cyclo (Leu-Val)* I	156	2.9 ±0.2a	0.2 ± 0.0b	-	0.5 ± 0.0b
13	Cyclo (Leu/Ile-Val) II	156	2.9 ± 0.2a	0.3 ± 0.1b	-	0.3 ± 0.2b
14	Cyclo (Ile-Val)* III	156	0.9 ± 0.1	-	-	-
15	Cyclo (Leu-Leu)* I	170	3.5 ± 0.1a	0.4 ± 0.1b	-	0.4 ± 0.3b
16	Cyclo (Ile-Leu)* II	170	3.2 ± 0.3a	-	0.4 ± 0.1b	-
17	Cyclo (Leu/Ile-Leu/Ile) III	170	4.7 ± 2.1a	0.8 ± 0.0a	0.4 ± 0.0a	0.6 ± 0.6a
18	Cyclo (Leu/Ile-Leu/Ile) IV	170	3.8 ± 0.1a	0.7 ± 0.1b	-	0.3 ± 0.3b
19	Cyclo (Leu-Phe)* I	91	6.3 ± 0.5a	2.6 ± 0.7b	1.1 ± 0.1b	2.9 ± 0.6b
20	Cyclo (Leu/Ile-Phe) II	91	5.7 ± 0.2a	2.8 ± 0.3b	1.4 ± 0.2c	3.0 ± 0.2b
21	Cyclo (Leu/Ile-Phe) III	91	7.8 ± 0.5a	3.7 ± 0.8b	3.2 ± 0.2b	6.1 ± 0.6a
22	Cyclo (Leu/Ile-Phe) IV	91	4.1 ± 0.2a	2.7 ± 0.6b	2.1 ± 0.0b	4.2 ± 0.0a
23	Cyclo (Phe-Gly)*	91	-	-	-	2.5 ± 2.4
24	Cyclo (Phe-Val)* I	91	4.8 ± 0.2a	1.8 ± 0.7b	0.8 ± 0.1c	2.6 ± 0.4bc
25	Cyclo (Phe-Val) II	91	4.6 ± 0.5a	2.8 ± 0.9bc	1.1 ± 0.0c	3.6 ± 0.7a

*Asterisked compounds were acquired commercially and used as standards for identification purposes. -, no detected

3.1.1. DKPs detected. Figure 1 show the time-windows of the chromatograms of the mass spectra base peaks and the structures of the DKPs detected in the corn oil studied and Table S1 shows the main fragments of their mass spectra.

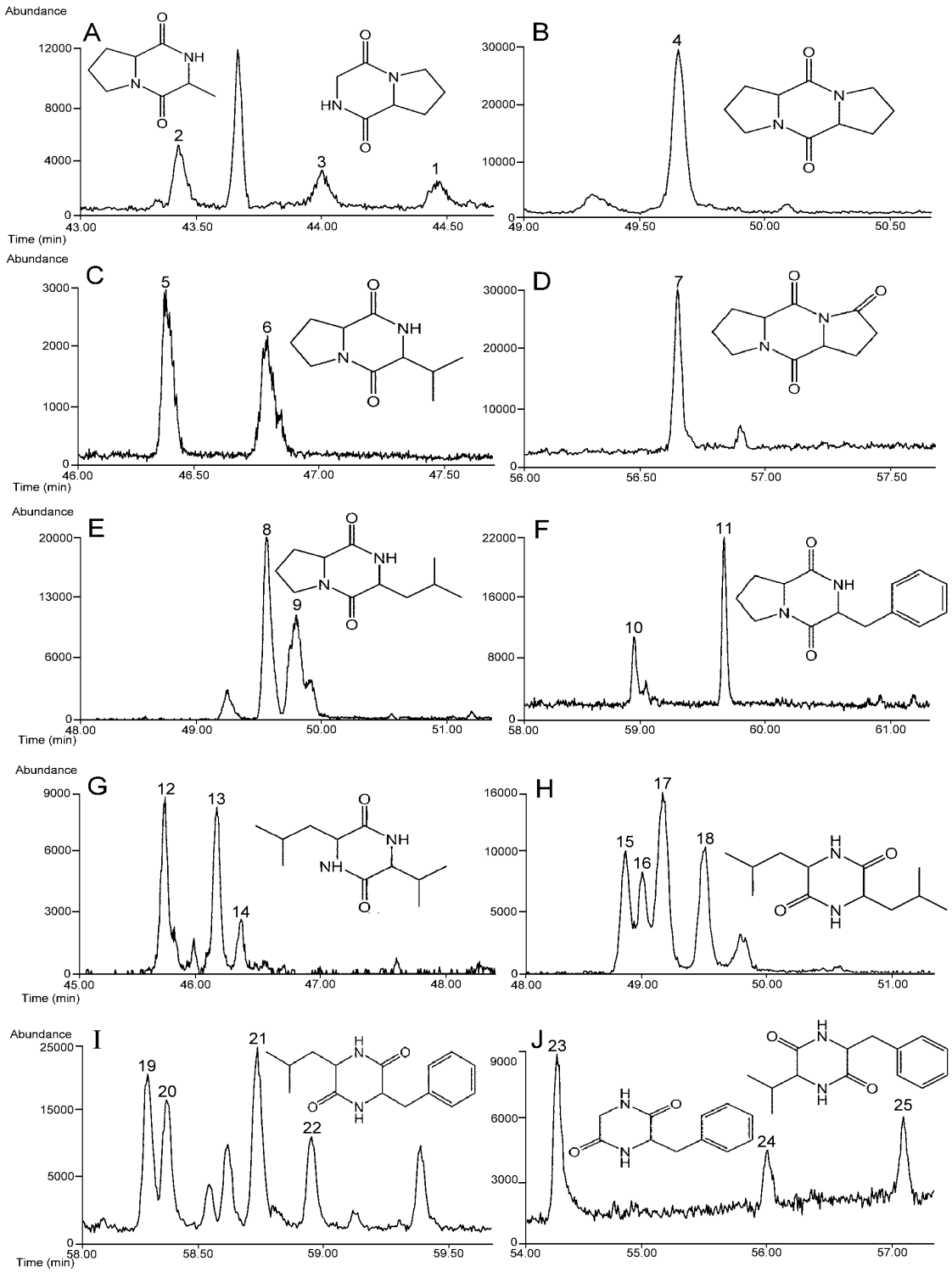

Figure. 1. Time-windows of the chromatograms of the base peaks of the mass spectra of cyclic dipeptides and structures found in corn oil samples, C1, C2, C3 and C4. **A)** (1) cyclo (Pro-Gly), (2),(3) cyclo (Pro/Ala), **B)** (4) cyclo (Pro-Pro), **C)** (5),(6) cyclo (Pro-Val), **D)** (7) cyclo (Pro-Pyroglu), **E)** (8),(9) cyclo (Pro-Leu/Ile), **F)** (10),(11) cyclo (Pro-Phe), **G)** (12),(13),(14) cyclo (Leu/Ile-Val), **H)** (15),(16),(17),(18) cyclo (Leu/Ile-Leu/Ile), **I)** (19),(20),(21),(22) cyclo (Leu/Ile-Phe) and **J)** (23) cyclo (Phe-Gly), (24),(25) cyclo (Phe-Val).

Among them there are: one derived from proline-glycine (Pro-Gly) (peak 1 in Figure 1A) having as base peak of its mass spectrum m/z 83; two isomers derived from proline-alanine (Pro-Ala) (peaks 2 and 3 in Figure 1A) with base peak m/z 70; one derived from proline-proline (Pro-Pro) (peak 4 in Figure 1B) with base peak m/z 70; two isomers derived from proline-valine (Pro-Val) (peaks 5 and 6 in Figure 1C), with base peak m/z 154; one derived from proline-pyroglutamic (Pro-PyroGlu) (peak 7 in Figure 1D) with base peak m/z 70; two isomers derived from proline-leucine/isoleucine (Pro-Leu/Ile) (peaks 8 and 9 in Figure 1E) with base peak m/z 154; and two isomers derived from proline-phenylalanine (Pro-Phe) (peaks 10 and 11 in Figure 1F) with base peak 125; three isomers derived from leucine/isoleucine-valine (Leu/Ile-Val) (peaks 12, 13 and 14 in Figure 1G) with base peak m/z 156; four isomers derived from leucine/isoleucine-leucine/isoleucine (Leu/Ile-Leu/Ile) (peaks 15, 16, 17 and 18 in Figure 1H) with base peak m/z 170; and four isomers derived from leucine/isoleucine-phenylalanine (Leu/Ile-Phe) (peaks 19, 20, 21 and 22 in Figure 1I) with base peak m/z 91; one derived from phenylalanine-glycine (Phe-Gly) (peak 23 in Figure 1J) with base peak m/z 91; and two isomers derived from phenylalanine-valine (Phe-Val) (peaks 24 and 25 in Figure 1J) with base peak m/z 91. As mentioned before some of them have been identified using standard compounds and others by matching their mass spectra with those provided by several authors (Fabbri et al., 2012; Benítez et al., 2012; Poerschmann et al., 2016) or with the mass spectra of the libraries mentioned together with their retention times.

3.1.2. Occurrence of these DKPs in other foods and potential origin. Some of the DKPs detected have also been recently found in some foods such as bread (derived from Leu/Ile-Val, Leu/Ile-Pro and Phe-Pro; Ryan, et al., 2009), chicken essence (derived from Leu/Ile-Val, and Leu/Ile-Leu/Ile; Chen et al., 2004), Japanese sake (derived from Leu/Ile-Phe and Leu/Ile-Leu/Ile; Takahashi et al., 2012, 2016), beer (derived from Leu/Ile-Pro, Pro-Pro and

Phe-Pro; Gautschi et al., 1997), beef (derived from Leu/Ile-Pro, Phe-Val and Phe-Pro; Chen et al., 2009), roasted coffee (derived from Leu/Ile-Phe, Phe-Val, Pro-Val, Pro-Pro, Pro-Gly, Pro-Ala and Phe-Pro; Ginz & Engelhardt, 2000, 2001), and cocoa (derived from Leu/Ile-Val, Pro-Val, Pro-Ala and Phe-Gly; Stark & Hofman, 2005). The cyclic dipeptide Pro-Pyroglu (Figure 1D and number 7 in Tables 2 and S1) has not been found previously in foods. This compound has been described for the first time as forming during the pyrolysis of collagen and bovine serum albumin as well as in the pyrolisis of dipeptides containing proline and glutamic acid or glutamine (Fabbri et al., 2012).

The origin and presence of DKPs in the above mentioned foods has been attributed to their formation in: fermentation processes (Takahashi et al., 2012, 2016); in thermal (Adamiano et al., 2014) or hydrothermal (Poerschmann et al., 2016) treatments of proteins; in baking (Ryan et al., 2009), or roasting (Ginz & Engelhardt, 2000, 2001) or in Maillard reactions. Their presence in corn oil could be due firstly to their occurrence in corn; in fact some cyclic dipeptides have been found in corn steep water (Dansi et al., 1970), this being the only reference found in relation to cyclic dipeptides and corn. Furthermore, they could also have formed during oil processing because thermal treatments are involved. However, it is not possible to discard other origins because it is also known that some fungus of several genus such as, *Phellinus (P igniarius, linteus, baumii*, etc; Airong et al., 2013), *Aspergillus* (*A fumigates*; Furtado et al., 2007), or *Pleurotus* (*P djamor*; Benítez et al., 2012) are able to synthesize this kind of compounds. Likewise, it has been shown that yeasts and bacteria such as *Lactobacillus* (Yang & Chang, 2010), *Bacillus subtilis* (Elkahoui et al., 2013), *Bacillus pumilus* (Brack et al., 2014), and *Bacillus cereus* subsp. *thuringiensis* (Kumar et al., 2013), and specifically some found in corn oil, are able to synthesize DKPs. At this point one should highlight the existence of a genetically modified corn, called "*Bacillus thuringiensis* corn" (BT corn), able to generate one or more proteins from the bacterium *Bacillus thuringiensis*

which preserve the crop against insect pests. Likewise, another genetically modified corn including some genes from the bacterium *Bacillus subtilis* with the aim to provide resistance of the crop against drought is available on the market.

3.1.3. Importance of the occurrence of DKPs in corn oil. The importance of the presence of DKPs in corn oil is due to their activities. Among them can be cited sensory attributes and bioactive abilities.

Regarding sensory attributes, their taste has been broadly described as bitter but also as metallic, astringent, salty, and grainy (Gautschi et al., 1997). All DKPs contain a group donor of protons and another acceptor of protons which is a requirement in the Shallenberger & Acre (1971) molecular theory on bitter taste. These compounds will contribute to the sensory properties of the corn oil whenever their concentrations in the oil are higher than their recognition thresholds (Chen et al., 2009).

Furthermore, studies about certain bioactive abilities of some DKPs have been carried out. Thus, it has been described that: cyclo (Pro-Gly) exhibits a significant antioxidant activity (Ser et al., 2015); cyclo (Pro-Ala) is able to inhibit the production of aflatoxin B1 and G1 of *A. parasiticus* and aflatoxin B1 of *A. flavus* (Jermnak et al., 2013); cyclo (Pro-Pro) shows antibacterial activity against *Micrococcus luteus* and *Pseudomonas aeruginosa* (Huberman et al., 2007); cyclo (Pro-Val) also inhibits the production of aflatoxin by *A. parasiticus and flavus.* (Jermnak et al., 2013) and in addition has been shown to have antibacterial and antilarval effect (Qi et al., 2009); cyclo (Pro-Leu) has antifungal activity (Kumar et al., 2013); cyclo (Pro-Phe) is able to inhibit the proliferation of Influenza A virus and some pathogenic fungi (Kwak et al., 2013, 2014), and has proved to be a good anticancer and anti-mutagenic compound (Vázquez-Rivera et al., 2015), having also showed antilarval and antibacterial activity (Qi et al., 2009); cyclo (Leu-Val) has activity against Gram-positive bacteria (Kumar et al., 2014); cyclo (Leu/Ile-Leu) has antioxidant (Furukawa et al., 2012)

and antifungal activities (Yang & Chang, 2010); cyclo (Leu-Phe) exhibits a certain radical scavenging activity (Takaya et al., 2007); cyclo (Phe-Gly) produces a moderate inhibition of the cancer cells growth (Coursindel et al., 2010) and has antimalarial activity (Peréz-Picaso et al., 2012); cyclo (Phe-Val), also shows antimalarial activity (Peréz-Picaso et al., 2012). It is to be expected that, in addition, the DKPs found in corn oil can exhibit other bioactive activities which have not been studied yet and moreover synergic effects could also occur.

The bioactivity of these compounds is a consequence of their chemical structure being able to interact with different receptors and this is reinforced by their stability with regard to enzymatic proteolysis (Peréz-Picaso et al., 2009) and their easy absorption, without modification of their structure, by the digestive tract membrane due to their high permeability (Amidon & Lee, 1994).

Due to their antioxidant ability, the presence of DKPs in corn oil contributes to the oxidative stability of this oil. In fact, it has been proved that corn oil has higher oxidative stability than sunflower oil (Guillén & Ruiz, 2005b) in spite of both oils having similar unsaturation degrees, and the DKPs contained in corn oil could contribute to this higher stability.

3.1.4. DKPs as potential corn oil markers and tool to differentiate between corn oils. It is noteworthy that all the corn oils studied contain DKPs. However, using the same methodology, no DKP was detected in the other edible oils studied (extra virgin olive, virgin olive, olive, sunflower, virgin soybean, refined soybean and virgin linseed oils). This fact suggests that these compounds could be considered as corn oil markers as far as present-day knowledge can tell, making this valuable tool to authenticate corn oil and to differentiate from other oils with similar molar percentages of the different kinds of acyl groups.

Furthermore, data in Table 2 show that many of the DKPs found are common to all four corn oils. However, differences between corn oils are also found due not only to the

concentration of the common DKPs but also to the absence or presence of some of them. Thus, as Table 2 shows, two kinds of corn oils can be distinguished clearly: those in which the main DKPs are those derived from leucine/isoleucine and from phenylalanine, many of the DKPs derived from proline being absent and there being none derived from glycine, such as C1 and C2 oils; and those in which the main DKPs are those derived from proline and phenylalanaline, such as C4, which also contain many of those DKPs derived from leucine/isoleucine although in smaller abundances. Corn oil C3 has the smallest concentrations of DKPs of the four studied and could be considered to occupy an intermediate position regarding the other two groups. It is noteworthy that a main DKP in C4 oil is cyclo (Pro-PyroGlu) which has been recently identified for the first time as a novel DKP in studies of pyrolysates of dipeptides and proteins (Fabbri et al., 2012). From these results it is evident that the corn oils can also be differentiated and classified on the basis of their main DKPs. Further studies to find relationships between corn variety and presence and abundance of certain DKPs in the derived oils could be of interest because these compounds might also be markers of the corn variety used to obtain the oil.

3.1.5. Contribution to current knowledge of peptide presence in edible oils. The results of this study open a new perspective in relation to knowledge of corn oil composition because, to the best of our knowledge, the presence of DKPs in corn oils is shown for the first time in this study. Furthermore, the bioactivity and molecular characteristics of the DKPs increase the added value of this oil and its interest from the technological, nutritional and healthy points of view.

In addition, it should also be commented on that until now little attention has been paid to the occurrence of proteins and peptides in edible oils mainly due to the lack of established methods for their determination (Hidalgo & Zamora, 2006; Martín-Hernández et

al., 2008). Earlier methods proposed to this aim included extraction of the proteic fraction and posterior global quantification of the amino acids in them contained by HPLC after total hydrolysis, which involve chemical modification of the sample. However, to the best of our knowledge, no identification or quantification of the existing real molecules in the oils has been carried out. This fact increases the importance of this study because the method here used is very simple and fast, does not involves chemical modification of the sample, and provides information which is both qualitative and quantitative about the specific cyclic dipeptides present in corn oil simultaneously with that of other nitrogenated compounds present in this oil and in any other edible oil if these compounds are present.

3.2. Other nitrogenated compounds

In addition to the DKPs above mentioned other nitrogenated compounds have also been found in corn oils but not in the other oils studied. These can be grouped into several groups such as oxazolines, pyrrolidines, other pyrrol derivatives, indol derivatives and miscellaneous compounds. These are indicated in Table 3 and in Figure S1 which shows the structures of these compounds. Furthermore Table S2 gives the main fragments of their mass spectra.

Table 3. Nitrogenated compounds detected in corn oil samples, C1, C2, C3 and C4, and their abundances, expressed as area counts of their mass spectra base peak (Bp) divided by 10^5, obtained as average of two determinations together with their standard deviations. Different letters within each row indicate a significant difference ($p < 0.05$).

Compound (molecular weight)	Bp	C1	C2	C3	C4
Oxazoline derivatives					
4,4-dimethyl-2-(1-hydroxy-pentadecyl)-2-oxazoline (325)	129	4.0 ± 0.1a	4.3 ± 0.5a	-	6.1 ± 0.3b
4,4-dimethyl-2-(1-hydroxy-heptadeca-8,11-dienyl)-2-oxazoline (349)	142	3.6 ± 0.1a	2.8 ± 0.3b	0.8 ± 0.1c	6.0 ± 0.2d
4,4-dimethyl-2-(1-hydroxy-heptadec-8-enyl)-2-oxazoline (351)	142	3.8 ± 0.5a	2.7 ± 0.1b	1.2 ± 0.0c	5.4 ± 0.3d
Pyrrolidine derivatives					
Hexadecanoyl pyrrolidine (309)	113	2.3 ± 0.4a	2.6 ± 0.2a	0.9 ± 0.1b	2.7 ± 0.3a
Octadecadienoyl pyrrolidine (333)	113	2.5 ± 0.0a	1.3 ± 0.0b	1.6 ± 0.2b	3.6 ± 0.4a
Octadecenoyl pyrrolidine (335)	113	2.9 ± 0.1a	1.6 ± 0.1b	1.5 ± 0.2b	3.5 ± 0.3a
Pyrrol derivatives					
1-furfuryl-2-formylpyrrole (175)	81	3.3 ± 0.7	-	-	-
N-(2-phenylethenyl)-pyrrolidin-2-one (187)	132	4.4 ± 0.3a	0.6 ± 0.1b	-	-
1-(2-phenylethyl)-2-formylpyrrol (199)	104	4.8 ± 0.3a	0.5 ± 0.0b	-	-
Indole derivatives					
1H-indole-3-carboxaldehyde (145)	144	2.7 ± 0.2a	0.5 ± 0.1b	-	0.1 ± 0.0c
3-phenylindole (193)	193	5.6 ± 0.3a	2.1 ± 0.2b	-	3.0 ± 0.2c
1H-indole-3-acetic acid, ethyl ester (203)	130	0.8 ± 0.1	-	-	-
Acetyl-beta-carboline (210)	168	1.6 ± 0.1	-	-	-
Miscellaneous compounds					
3,5-diphenylpyrazole (220)	220	7.0 ± 0.4a	0.9 ± 0.0b	0.8 ± 0.0b	1.0 ± 0.0b
3,5-diphenylpyridine (231)	231	4.0 ± 0.4a	-	-	2.5 ± 0.1b
Alatamide (253)	105	2.9 ± 0.2a	2.9 ± 0.3a	1.3 ± 0.0b	1.0 ± 0.2b

-, no detected

3.2.1. Fatty Oxazolines and Pyrrolidines. Compounds belonging to these groups have been synthesized, by derivatization, in the past for hydroxy fatty acids and fatty acid analysis (Spitzer, 1996; Christie, 1998; Hamilton & Christie, 2000). However, in this study no synthesis has been carried out. The *oxazolines* (see Tables 3 and S2 and Figure S1A) found in the corn oils here studied are 4,4-dimethyl-2-(1-hydroxy-heptadec-8-enyl)-2-oxazoline and the homologous derived from 1-hydroxy-octadecen-8,11-oic acid. Furthermore, another tentatively identified as being derived from 1-hydroxyhexadecenoic acid has been found (base peak of its mass spectrum m/z 129). The fatty *pyrrolidine*s (see Tables 3 and S2 and Figure S1B) found are those derived from palmitic, linoleic and oleic acids. It is known that the mass spectra of these pyrrolidines coincide with those of 4,4-dimethyl oxazolines derived from the same acids. However, the former cannot be confused with the latter because they elute at a later point.

Although certain oxazolines of small sizes, such as those formed in Maillard reactions, have been found in some food, to the best of our knowledge the occurrence of the found in this study has not been described in foods. Regarding the pyrrolidines found, some of them (hexadecanoyl pyrrolidine and octadecenoyl pyrrolidine) have been found as components of piper genus fruits (Parmar et al., 1997). Certain biological activities have been attributed to these latter components, such as larvicidal activity (Marques & Kaplan, 2015) and ability in depressing levels of cholesterol in the blood (Toki & Nakatani, 1969). Both kinds of compounds are found in the four corn oil studied though in somewhat higher concentrations in C4 oil.

3.2.2. Other pyrrol derivatives. Other compounds found in these corn oils containing the pyrrolic ring in their structure are N-furfuryl-2-formylpyrrole, 1-(2-phenylethyl)-2-formylpyrrol, and N-(2-phenylethenyl)-pyrrolidin-2-one (see Tables 3 and S2 and Figure S1C). The first is present in several foods such as coffee (Lee et al., 2016) and sweet

worts (Hoff et al., 2014), and it can be formed during fermentation of coffee beans or even during roasting. It has also been found among the products of Maillard reaction of soy protein hydrolysates (Liu et al., 2012). This compound has been detected in only one of the corn oils studied (C1). The second compound of this group has been detected in two of the corn oils (C1 and C2) and is a well-known component of *Boletus edulis* (Thomas, 1973).

3.2.3. Indole derivatives. Indoles are very common in the body and diet and participate in many biochemical processes. Compounds belonging to this family have also been found in some of the corn oils studied. These are, as Tables 3 and S2 and Figure S1D show, 1H-indole-3-carboxaldehyde, acetyl-beta-carboline, 1H-indole-3-acetic acid ethyl ester, and 3-phenylindol, or isomers of these four compounds.

1H-indole-3-carboxaldehyde displays a well known anticarcinogenic activity (Lin et al., 2007). It has also been found in rice plants (Xuan et al., 2003) and in citrus peels (Jeong et al., 2004), as well as in *Pleurotus citrinopileatus* (Li et al., 2013) and is present in three of the four corn oils studied.

Acetyl-beta-carboline is a very interesting indole derivative with some proven biological, physiological, antibacterial and pharmacological activities (Airaksinen & Kari, 1981; Cao et al., 2007; Hemamalini et al., 2014; Kim et al., 2015). It can be synthesized by plants (Hemamalini et al., 2014) and microorganisms such as *Streptomyces kasugaensis* (Proksa et al., 1990) and can also be formed in non-enzymatic reactions such as Maillard and Pictet-Spengler reactions (Rönner et al., 2000; Nemet & Varga-Defterdarović, 2008). For the above mentioned reasons its presence in corn oil could be due either to natural origin or to oil processing or to both. This compound has only been detected in C1 oil.

Likewise, 1H-indole-3-acetic acid, ethyl ester only detected in C1 oil and 3-phenylindol (detected in C1, C2 and C4 oils) are compounds with known biological activities. The first is considered a fitohormone (Epstein et al., 1989; Ludwig-Müller, 2000) and the second has been proved to have antimicrobial activity among other activities (Sinha & Smejtek, 1983).

3.2.4. Miscellaneous compounds. This group includes the detected compounds having different functional groups such as (see Tables 3 and S2 and Figure S1E) 3,5-diphenylpyrazole, whose bioactivity has been subject of study (Armstrong et al., 2016) and alatamide (Pasqua et al., 2014) a natural compound also isolated from certain parts of plant of piper genus (*Piper guayranum*) like some pyrrolidines already mentioned. Furthermore, 3,5-diphenylpyridine, previously found in the pyrolysates of the D-glucose/L-phenylalanine mixture when formed by the Maillard reaction (Yaylayan et al., 1997) has also been found; its activity as an inhibitor of interleukin-6 has also been tested (Tagat et al., 1995).

In addition, a group of unknown compounds, supposedly nitrogenated derivatives, with well defined mass spectra have been also found. For further information about this matter, Table S3 gives the fragments of their mass spectra.

In short, in addition to the above mentioned DKPs, various other nitrogenated compounds having different biological activities, whose origins may lie in the corn plant metabolisms or in the oil processing have been found in the corn oils studied. In many of them certain biological activities have been studied and it is expected that others will be tackled in the near future to establish the consequences of their intake for humans through the corn oil.

4. CONCLUSIONS

The occurrence in corn oils of a great number of minor nitrogenated components has been demonstrated. This lends the oil interesting added value from technological, nutritional and healthy points of view. The compounds detected in this study, for the first time, as corn oil components, are an important number of DKPs, derived mainly from proline, leucine/isoleucine and phenylalanine and of other amino acids. The fact that these nitrogenated compounds have not been detected in the other edible oils studied, namely extra virgin olive, virgin olive, olive, sunflower, virgin soybean, refined soybean and linseed oils, suggests that they could be considered as corn oil markers. In addition the presence or absence of certain DKPs as well as the different concentration of them in the corn oils makes it possible to differentiate and classify these latter. These differences could be associated to the corn variety. Most DKPs are considered antioxidants and will contribute to the greater oxidative stability observed in corn oil than in other oils having similar unsaturation degree, such as sunflower oil, although other antioxidants may also play a role. Furthermore, the presence of other nitrogenated components in corn oils has been shown. These include oxazolines, pyrrolidines and other pyrrol derivatives, indol derivatives as well as pyrazol, pyridine and β-phenylethylamine derivatives. The importance of these findings lies in that not only DKPs but also most of the other nitrogenated compounds found in corn oils are bioactive. The fact that they are not present in other edible oils gives special characteristics to these oils. Once the presence of these compounds is known in corn oil, and because they are consumed daily by humans, further studies on the bioactivity of these minor components and the existence of possible synergic effects may be considered of great interest. To the best of our knowledge this is the first time that specific peptides of known structures are reported in edible oils. Fatty

amides, the only nitrogenated compounds also detected in the other six edible oils studied, were also detected in the corn oils.

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Supplementary Material of

Article 2

BIOACTIVE COMPOUNDS DETECTED FOR THE FIRST TIME IN CORN OIL. CYCLIC DIPEPTIDES AND OTHER NITROGENATED COMPOUNDS

Jon Alberdi-Cedeño, María L. Ibargoitia, María D. Guillén

Table S1. Cyclic dipeptides detected in corn oil samples, C1, C2, C3 and C4, and the main fragments of their mass spectra.

Nº	**Cyclic dipetides** (molecular weight)	Fragments (m/z)
1	Cyclo (Pro-Gly) (154)	41, 55, 70, 83(100), 111, 154
2	Cyclo (Pro-Ala)* I (168)	70(100), 97, 125, 168
3	Cyclo (Pro-Ala) II (168)	70(100), 97, 125, 168
4	Cyclo (Pro-Pro)* (194)	70(100), 96, 110, 138, 166, 194
5	Cyclo (Pro-Val)* I (196)	70, 72, 125, 154(100), 196
6	Cyclo (Pro-Val) II (196)	70, 72, 125, 154(100), 196
7	Cyclo (Pro-PyroGlu) (208)	55, 70(100), 84, 96, 124, 152, 180, 208
8	Cyclo (Pro-Leu)* I (210)	43, 70, 86, 96, 125, 154(100), 167, 195, 210
9	Cyclo (Pro-Leu/Ile) II (210)	43, 70, 86, 96, 125, 154(100), 167, 195, 210
10	Cyclo (Pro-Phe)* I (244)	55, 70, 91, 125(100), 153, 201, 244
11	Cyclo (Pro-Phe) II (244)	55, 70, 91, 125(100), 153, 201, 244
12	Cyclo (Leu-Val)* I (212)	72, 86, 113, 126, 156(100), 170, 197
13	Cyclo (Leu/Ile-Val) II (212)	72, 86, 113, 126, 156(100), 170, 197
14	Cyclo (Ile-Val)* III (212)	72, 86, 113, 126, 156(100), 170, 197
15	Cyclo (Leu-Leu)* I (226)	43, 86, 113, 140, 170(100), 211, 226
16	Cyclo (Ile-Leu)* II (226)	43, 86, 113, 140, 170(100), 211, 226
17	Cyclo (Leu/Ile-Leu/Ile) III (226)	43, 86, 113, 140, 170(100), 211, 226
18	Cyclo (Leu/Ile-Leu/Ile) IV (226)	43, 86, 113, 140, 170(100), 211, 226
19	Cyclo (Leu-Phe)* I (260)	65, 85, 86, 91(100), 113, 120, 141, 169, 204, 260
20	Cyclo (Leu/Ile-Phe) II (260)	65, 85, 86, 91(100), 113, 120, 141, 169, 204, 260
21	Cyclo (Leu/Ile-Phe) III (260)	65, 85, 86, 91(100), 113, 120, 141, 169, 204, 260
22	Cyclo (Leu/Ile-Phe) IV (260)	65, 85, 86, 91(100), 113, 120, 141, 169, 204, 260
23	Cyclo (Phe-Gly)* (204)	65, 85, 91(100), 113, 204
24	Cyclo (Phe-Val)* I (246)	91(100), 99, 113, 127, 155, 204, 246
25	Cyclo (Phe-Val) II (246)	91(100), 99, 113, 127, 155, 204, 246

*Asterisked compounds were acquired commercially and used as standards for identification purposes.

Table S2. Nitrogenated compounds detected in corn oil samples, C1, C2, C3 and C4, and the main fragments of their mass spectra.

Compound (molecular weight)	Fragments (m/z)
Oxazoline derivatives	
4,4-dimethyl-2-(1-hydroxy-pentadecyl)-2-oxazoline (325)	73, 86, 114, 129(100), 142, 310, 325
4,4-dimethyl-2-(1-hydroxy-heptadeca-8,11-dienyl)-2-oxazoline (349)	114, 129, 142(100), 182, 184, 196, 198, 306, 349
4,4-dimethyl-2-(1-hydroxy-heptadec-8-enyl)-2-oxazoline (351)	114, 129, 142(100), 184, 196, 198, 252, 294, 308, 336, 351
Pyrrolidine derivatives	
Hexadecanoyl pyrrolidine (309)	55, 70, 85, 98, 113(100), 126, 168, 182, 210, 266, 280, 309
Octadecadienoyl pyrrolidine (333)	55, 70, 72, 85, 98, 113(100), 126, 140, 168, 182, 196, 248, 262, 290, 333
Octadecenoyl pyrrolidine (335)	55, 70, 85, 98, 113(100), 126, 140, 168, 182, 236, 250, 264, 292, 320, 335
Pyrrol derivatives	
1-furfuryl-2-formyl pyrrole (175)	53, 65, 81(100), 94, 107, 117, 118, 146, 175
N-(2-phenylethenyl)-pyrrolidin-2-one (187)	51, 77, 91, 103, 117, 130, 132(100), 158, 187
1-(2-phenylethyl)-2-formylpyrrol (199)	53, 65, 77, 91, 95, 104(100), 108, 199
Indole derivatives	
1H-indole-3-carboxaldehyde (145)	58, 63, 89, 116, 144(100), 145
3-phenylindole (193)	63, 89, 90, 96, 115, 139, 161, 165, 191, 192, 193(100), 194, 195
1H-indole-3-acetic acid, ethyl ester (203)	51, 77, 103, 130(100), 203
Acetyl-beta-carboline (210)	63, 88, 114, 140, 167, 168(100), 169, 182, 210
Miscellaneous compounds	
3,5-diphenylpyrazole (220)	63, 89, 110, 139, 165, 189, 176, 191, 192, 220(100)
3,5-diphenylpyridine (231)	102, 116, 202, 230, 231(100), 232
Alatamide (253)	77, 105(100), 121, 148, 253

Table S3. Unidentified compounds detected in corn oil samples, C1, C2, C3 and C4 with the main fragments of their mass spectra.

Fragments (m/z)
51, 79, 104, 105, 117, 118, 132, 160, 161(100), 162
53, 81, 95, 109, 111(100), 137, 138, 166
51, 77, 104, 117, 118, 130, 131, 132, 144, 160, 161(100), 162
57, 85, 101, 117(110), 127, 129, 130, 201
53, 77, 91, 104(100), 105, 108, 199, 236
51, 65, 78, 91, 105, 117, 132, 133, 160, 161(100), 192
81(100), 82, 187, 205, 208
53, 80(100), 107, 120, 187
43, 71, 77, 102, 103(100), 104, 115, 131, 160, 202, 203
41, 55(100), 69, 83, 97, 98, 99, 125, 152, 171, 213
53, 77, 91, 96, 107, 108, 120(100), 121, 215
69, 77, 91, 93, 105, 107, 108, 117, 119, 135(100), 136, 272
79, 91, 93, 105, 107, 122, 135(100), 136, 272
130(100), 131,143, 210
156(100), 157, 247, 248
77, 103, 115, 130, 143(100), 144, 238
72, 87, 95, 100, 130, 143(100), 144, 252, 307
43, 55, 57, 69, 71, 98, 101, 117, 129, 130(100), 131, 173, 190, 203, 239, 315, 371, 386
130, 143(100), 252

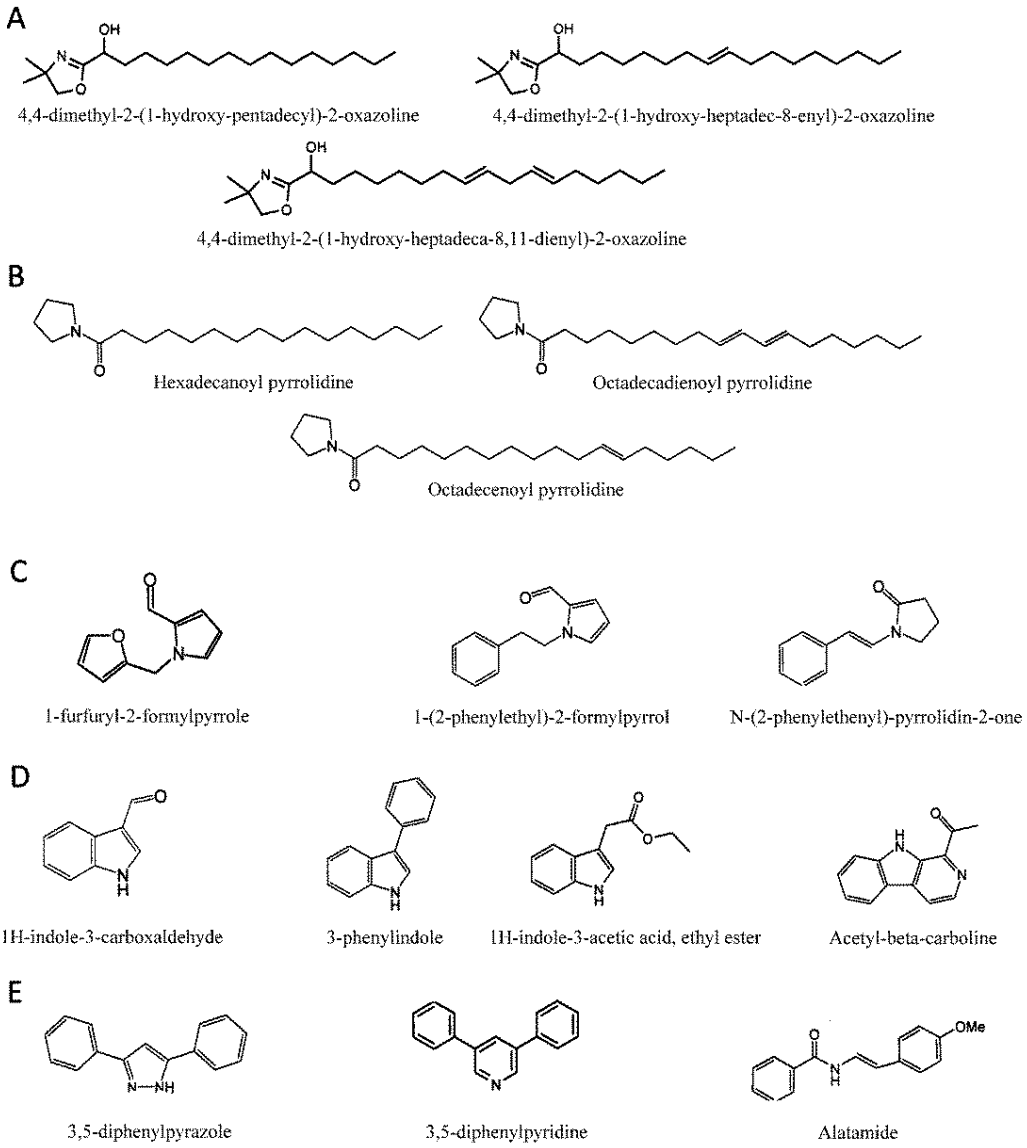

Figure S1. Structures of nitrogenated compounds found in C1, C2, C3 and C4 corn oil samples. **A)** oxazolines derivatives, **B)** pyrrolidines derivatives, **C)** other pyrrol derivatives, **D)** indole derivatives and **E)** miscellaneous compounds.

Article 3

MONITORING OF MINOR COMPOUNDS IN CORN OIL OXIDATION BY DIRECT IMMERSION-SOLID PHASE MICROEXTRACTION-GAS CHROMATOGRAPHY/MASS SPECTROMETRY. NEW OIL OXIDATION MARKERS

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ABSTRACT

The aim of this study is to shed light on the evolution of the minor compounds in the corn oil oxidation process, through the information provided by Direct Immersion-Microextraction in Solid Phase followed by Gas Chromatography/Mass Spectrometry (DI-SPME-GC/MS). This methodology enables one, in a single run, to establish the identity and abundance both of original oil minor components, some with antioxidant capacity, and of other compounds coming from both main and minor oil components oxidation. For the first time, some of the compounds formed from oil minor components degradation are proposed as new markers of oil incipient oxidation. Although the study refers to corn oil, the methodology can be applied to any other edible oil and constitutes a new approach to characterizing the oxidation state of edible oils.

KEY WORDS: corn oil, oxidation, direct immersion-solid phase microextraction (DI-SPME), gas chromatography/mass spectrometry (GC/MS), minor compounds, oxidation markers.

Chemical compounds studied in this article: α-Tocopherol (PubChem CID: 14985); β-Tocopherol (PubChem CID: 6857447); γ-Tocopherol (PubChem CID: 14986); δ-Tocopherol (PubChem CID: 92094); Squalene (PubChem CID: 638072); β-Sitosterol (PubChem CID: 222284); 6,10,14-trimethylpentadecan-2-one (PubChem CID: 10408); α-tocopherylquinone-5,6-epoxide (PubChem CID: 14753697); 3,7,11-trimethyl-3-dodecanol (PubChem CID: 138824); 4,8,12,16-tetramethylheptadecan-4-olide (PubChem CID: 567149)

1. INTRODUCTION

Vegetable oils are well known food ingredients and cooking media all over the world. They undergo great changes in their composition under oxidative conditions. These are due to the degradation of some of their components and to the formation of other new compounds. As a consequence, the safety, nutritional value and sensory characteristics of the oil may be affected.

For these reasons, it is of great importance to know the evolution of the original oil components and the identity and characteristics of the new formed compounds, when the oil is submitted to oxidative conditions.

The most common methods used to evaluate the oxidation degree of edible oils require chemical modification of the sample, and provide limited information about the identity of compounds whose functional groups are measured. For example, peroxide or conjugated diene values measure the abundance of both kinds of functional groups; however, there are both primary and secondary oxidation compounds that can have hydroperoxide groups and the same can be said of the conjugated diene group, as has been widely discussed (Frankel, 2005). These approaches are valid, to some extent, for certain objectives but provide no useful information to further knowledge of the different types of reactions which occur, simultaneously or successively, in the process, or about their mechanisms. Nor do they give specific information concerning the nature of the different kind of compounds that may form during oil oxidation.

Another approach with the same aim is based on the use of ^{1}H NMR spectroscopy. This technique permits the qualitative and quantitative characterization of main edible oil components (Guillén & Ruiz, 2003; Guillén & Uriarte, 2012a; Martínez-Yusta, Goicoechea & Guillén, 2014) and it is very useful in monitoring their oxidation because it allows one to evaluate the rate of degradation of the oil main components as well as the formation rate of

primary and of many secondary oxidation products (Guillén & Ruiz, 2004; Guillén & Uriarte, 2012a; Martínez-Yusta et al., 2014).

In this context, this paper addresses the characterization of the oxidative status of edible oils, specifically of corn oil, as well as their evolution under oxidative conditions with a new approach based exclusively on the minor compounds contained in the oil. To this end, a fairly new methodology that employs direct immersion solid-phase microextraction to extract oil minor compounds up to a certain molecular weight, followed by gas chromatography/mass spectrometry (Alberdi-Cedeño, Ibargoitia, Cristillo, Sopelana & Guillén, 2017a) will be used. Both approach and methodology have not been used previously in edible oil oxidation studies. They permit the simultaneous monitoring of the original minor oil components and of other compounds coming from the oxidation of main and minor oil components, formed under accelerated storage conditions. Furthermore, this methodology does not require chemical modification of the sample, nor the use of solvents, reagents or fractionation steps.

2. MATERIALS AND METHODS

2.1. Samples subject of study.

2.1.1. The study was carried out with refined corn oil, purchased in a local supermarket. Its composition in acyl groups was determined from ^{1}H NMR spectral data as in previous studies (Guillén & Ruiz, 2003, Guillén & Uriarte, 2012a). The molar percentages of the different kinds of acyl groups were linolenic group (Ln) 0.6 ± 0.0 %, linoleic group (L) 48.7 ± 0.0 %, oleic group (O) 33.0 ± 0.1 %, and saturated groups (S) 17.7 ± 0.1 %. The peroxide value of this oil was < 0.5 meq O_2/Kg. It was determined by an adaptation of the official method described in EC2568/91, by using the HI 83730 equipment from Hanna Instruments (range: 0.0-25 meq O_2/Kg; resolution: 0.5 meq O_2/Kg; accuracy: ±0.5 meq O_2/Kg). The volatile and less volatile minor components of this oil were extracted by means of Direct Immersion-

Solid Phase Microextraction and were separated, identified and semi-quantified by Gas Chromatography/Mass Spectrometry as indicated in section 2.2.

2.1.2. In addition to the original oil, samples also derived from this oil, after subjected to accelerated storage conditions for different periods of time, were subject of study. To prepare these derived samples amounts of 10 g of original corn oil were placed in glass Petri dishes (80 mm in diameter and 15 mm deep) and kept in an oven at 70°C with aeration for three, six, nine and twelve days. Under these conditions the original oil underwent oxidation. These experiments were performed in duplicate to obtain sound results. The volatile and less volatile minor compounds of these oxidized oil samples were extracted by means of Direct Immersion-Solid Phase Microextraction and were separated, identified and semi-quantified by Gas Chromatography/Mass Spectrometry, as indicated in section 2.2.

2.1.3. Pure squalene, α- and γ-tocopherol were subjected to the same oxidative conditions as above and were studied by GC/MS. To this aim, aliquots of these oxidized samples were conveniently dissolved in dichloromethane and injected directly in the chromatograph. The chromatographic conditions were the same as described in section 2.2.2. In this way the origin of some new compounds found in the oxidized corn oil samples could be confirmed.

2.2. Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography/Mass Spectrometry (DI-SPME-GC/MS) experiments

2.2.1. Extraction of the minor compounds contained in the samples subject of study

The minor compounds contained either in the original oil or in its derived samples were extracted by direct immersion-solid phase microextraction without any previous sample preparation. To this aim a 65 μm StableFlex polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber acquired from Supelco (Bellefonte, PA, U.S.) was immersed directly into 5.4 g of edible oil, at room temperature, for 45 minutes, maintained under continuous

stirring. The selection of the type of fiber (polarity and thickness of the coating) was made on the basis of its ability to extract the widest range of minor oil components with the highest possible yield, after analyzing the results obtained by Mikuma and Kaneko (2010) and after testing several polymer-coated fused silica fibers, as in a previous study (Alberdi-Cedeño et al., 2017a).

2.2.2. Gas Chromatography/Mass Spectrometry study (GC/MS)

The oil components picked up by the fiber were directly injected into the port of a gas chromatograph/mass spectrometer (GC/MS). For their thermal desorption, the plunger was pushed down to expose the fiber to the GC carrier gas stream and held for 10 minutes. The desorbed compounds were separated, identified and semi-quantified by gas chromatography-mass spectrometry.

The equipment used was an Agilent gas chromatograph model 6890N equipped with a mass selective detector 5973 Network and a Hewlett-Packard Compaq Pentium 4 computer. A fused silica capillary column was used (60 m length x 0.25 mm inside diameter x 0.25 µm film thickness; from Hewlett-Packard, Palo Alto, CA), coated with a nonpolar stationary phase (HP-5MS, 5% phenyl methyl siloxane). The injector and interface temperatures were held at 250°C and 305ºC respectively and helium at a constant pressure of 117 kPa (16.9 psi) was used as the carrier gas. The oven temperature was initially held at 50ºC for 5 min, increased from 50 to 300ºC at a rate of 4ºC/min, and then held at 300ºC for 30 min. The injections were carried maintaining the fiber in the injection port for 10 min, the first 5 in splitless mode. Mass spectra were recorded at ionisation energy of 70 eV, with data acquisition in Scan mode. The Scan range was 40-550 amu. The temperatures of the ion source and the quadrupole mass analyzer were kept at 230 and 150ºC respectively. After the first desorption, the fibre was routinely submitted to desorption conditions for a second time both for its clean up and to determine if the first process had been completed. A reference

sample of known composition was periodically analyzed in order to verify the reproducibility and sensitivity of the chromatographic runs.

The identification of the several compounds was made using standards in some cases and on the basis of their mass spectra and retention times in others. The standards used, asterisked in Tables 1, 2, S1, S2 and S3, were acquired from Sigma Aldrich (St. Louis, MO, USA), Bachem AG and Carbosynth Ltd (Cymit Quimica S.L, Barcelona, Spain), Larodan (Malmö, Sweden), Cayman Chemical (Ann Arbor, MI, USA), Toronto Research Chemicals, (Canada; Commercial Rafer, Spain), LGC Standards, (Barcelona, Spain) and ChemFaces Biochemical Co., LTD (Wuhan, China). The identification of the others was made by matching their mass spectra with spectra from commercial libraries by more than 85% (W9N08, Wiley ver. 9.0 & NIST, ver. 8.0 library) with spectra obtained from the literature (Cornwell, Kim, Mazzer, Jones & Hatcher, 2003; Rontani, Nassiry & Mouzdahir, 2007; Rontani, Nassiry, Michotey, Guasco & Bonin, 2010; Nassiry, Aubert, Mouzdahir & Rontani, 2009) and taking also into account their retention times as in previous studies (Guillén, Cabo, Ibargoitia & Ruiz, 2005; Goicoechea & Guillén, 2014; Alberdi-Cedeño et al., 2017a; Alberdi-Cedeño, Ibargoitia & Guillén, 2017b). This identification method is accepted for food studies where, as in this case, their composition is very complex, the number of components is very high, the components subject of study can be present in very low concentration, the molecules are not new, and their mass spectra are well known and established (Guillén et al., 2005; Goicoechea & Guillén, 2014; Alberdi-Cedeño et al., 2017a,b).

The semi-quantification of the identified compounds was based on arbitrary units of area counts, of the corresponding mass spectra base peaks, multiplied by 10^{-5}. The purpose of this study was not the determination of absolute but rather of relative concentrations that are valid for comparative purposes.

2.3. Quality control of the global method and statistical analysis

Quality control of the global method was carried out by periodic extraction of an oil sample considered as sample reference and the subsequent study of the extracted compounds by GC/MS. The detection limit was established in an abundance of 10000 area counts for the base peak of the mass spectra of the compounds. The specificity, selectivity and robustness of the GC/MS to evaluate unequivocally the compounds included in this study are beyond all doubt as it is a recognized suitable global method for the purposes of this study. Data given in Tables 1, 2 and Tables S1, S2 and S3 are expressed as mean ± standard deviation (SD) of two measurements for the analytical determination. Microsoft Office Excel 2007 was used for the graphical representation of the obtained values. The global results here obtained have been corroborated with three other different corn oils submitted to the same oxidative conditions.

3. RESULTS AND DISCUSSION

3.1. Minor compounds present in the original corn oil

As mentioned above, characterization of the original corn oil minor components was the starting point of this study. It provided information about the identity and abundance of a broad range of compounds, some of which can have antioxidant ability, others which come from oil oxidation and provide information about its oxidation status, and others which are of varied origin and nature.

3.1.1. Compounds with antioxidant ability. These include squalene, tocols, sterols, and some cyclic dipeptides or DKPs (2,5-diketopiperazines) (Alberdi-Cedeño et al., 2017a,b). Most of these, except DKPs, eluted in the chromatographic run after sixty-five minutes, as can be seen in Figure 1.

The main *tocol* is γ-tocopherol (γ-T; peak 27 in Figure 1) followed by α- and δ-tocopherols (α-T and δ-T; peaks 28 and 25 respectively, in Figure 1) and in much smaller abundances β-tocopherol (β-T; peak 26 in Figure 1), as well as some tocomonoenols and tocotrienols. All of them elute in the chromatographic run between sixty-nine and seventy five minutes (Figure 1).

Like most edible oils, corn oil contains several *sterols*, mainly β-sitosterol (β-S; peak 30 in Figure 1), followed by campesterol (C; peak 29 in Figure 1) and in much smaller abundance stigmasterol (STG), Δ5-avenasterol (Δ5-A) and one stanol, sitostanol (STN) (see Table 1). All these compounds elute after seventy six minutes (Figure 1).

The abundances, within each one of these two groups of compounds above mentioned, measured in area counts, follow a fairly similar relative order to those of their absolute concentrations (Ostlund, Racette, Okeke & Stenson, 2002; Phillips, Ruggio, Toivo, Swank & Simpkins, 2002; Shahidi & de Camargo, 2016). This is the case within each kind of compound because of the similarity in functional groups and structure and also due to the proximity of their retention times. However, as commented above, and as is well known, the abundance values thus determined are only valid to compare the evolution of the same compound in several samples.

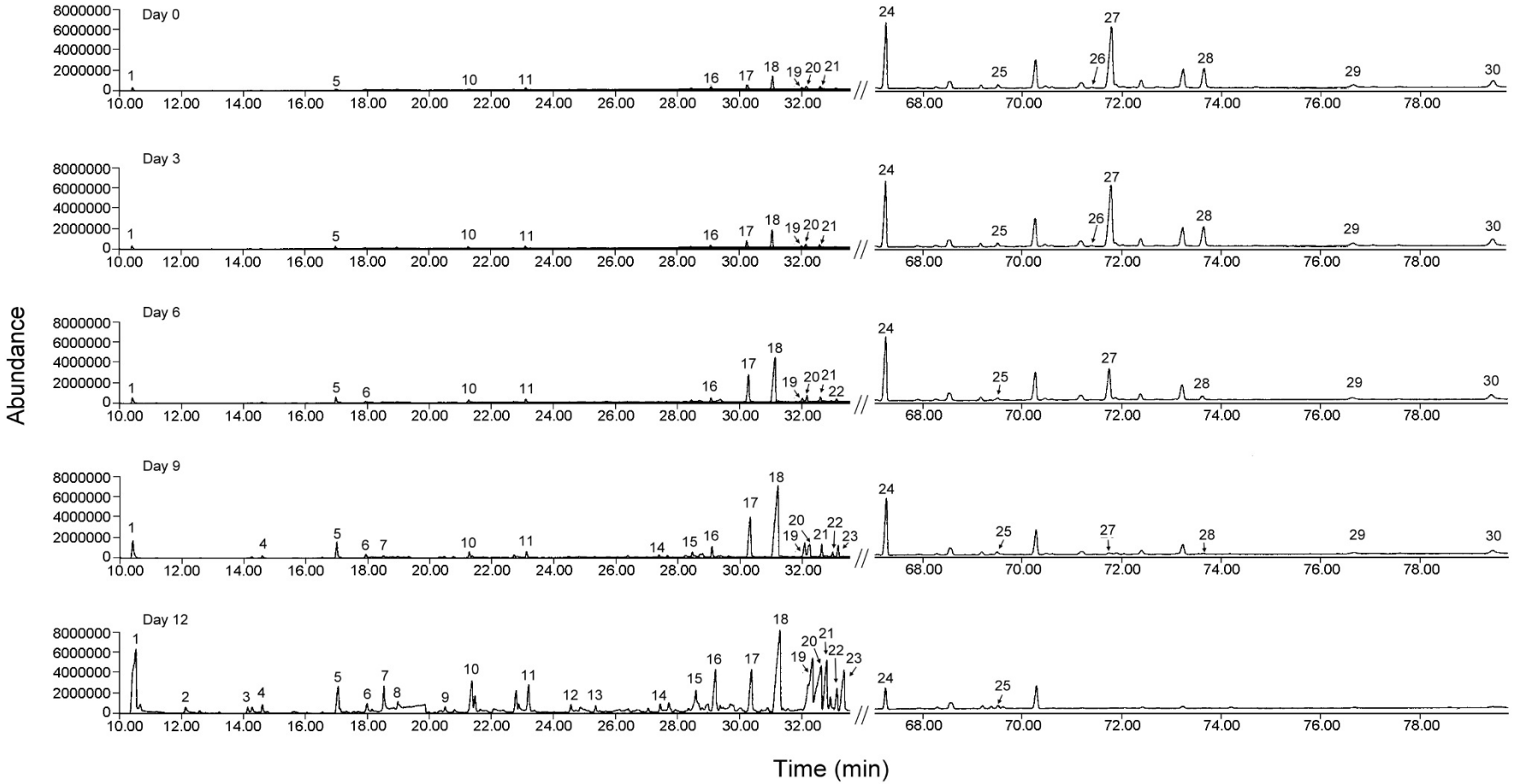

Figure 1. Regions between 10-32 minutes and 67-80 minutes of the total ion chromatograms obtained by DI-SPME-GC/MS of corn oil submitted during different periods of times to the same oxidative conditions. Peaks identified as: **(1)** hexanal; **(2)** (*E*)-2-hexenal; **(3)** 2-heptanone; **(4)** heptanal; **(5**) (*E*)-2-heptenal; **(6)** 1-octen-3-ol; **(7)** 2-pentylfuran; **(8)** octanal; **(9)** 3-octen-2-one; **(10)** (*E*)-2-octenal; **(11)** nonanal; **(12)** 3-nonen-2-one; **(13)** (*E*)-2-nonenal; **(14)** (*E,E*)-2,4-nonadienal; **(15)** 4-oxo-(*E*)-2-nonenal; **(16)** (*E*)-2-decenal; **(17)** (*Z,E*)-2,4-decadienal; **(18)** (*E,E*)-2,4-decadienal; **(19)** 5-pentyl-2-(5H)-furanone; **(20)** 4-hydroxy-(*E*)-2-nonenal; **(21)** (*E*)-2-undecenal; **(22)** 4,5-epoxy-2-decenal (isomer); **(23)** 4,5-epoxy-(*E*)-2-decenal; **(24)** squalene; **(25)** δ-tocopherol; **(26)** β-tocopherol; **(27)** γ-tocopherol; **(28)** α-tocopherol; **(29)** campesterol and **(30)** β-sitosterol.

In addition to the above-mentioned corn oil components, this oil also contains *cyclic dipeptides or DKPs*. To the best of our knowledge, the only edible oil in which the presence of this type of compounds has been described to date is corn oil (Alberdi-Cedeño et al., 2017b). They are, as usual, in very small abundance. The DKPs found in this corn oil derive from phenylalanine (Phe) and other amino acids such as valine (Val) (isomers Cyclo(Phe-Val)I and Cyclo(Phe-Val)II, in Table 1), leucine (Leu), isoleucine (Ile) (isomers Cyclo(Leu-Phe)I, Cyclo(Leu/Ile-Phe)II, Cyclo(Leu/Ile-Phe)III and Cyclo(Leu/Ile-Phe)IV, in Table 1) and proline (Pro) (isomers Cyclo(Pro-Phe)I and Cyclo(Pro-Phe)II, in Table 1). Those found here are among the most abundant found in other corn oils (Alberdi-Cedeño et al., 2017b). They elute between 56 and 60 minutes in the chromatographic run and due to their low concentration, their chromatographic peaks are not clearly visible with the naked eye, for which reason they are not indicated in Figure 1.

3.1.2. Compounds coming from oil oxidation. This oil also contains a basal concentration of some compounds whose origin could be in the oxidation of main corn oil components. These elute in the first thirty-three minutes of the chromatographic run, as can be seen in Figure 1 day 0. Among them there are aldehydes, of which the most abundant are some *alkanals* such as pentanal, hexanal (peak 1 in Figure 1) and nonanal (peak 11 in Figure 1), some *(E)-2-alkenals* such as (*E*)-2-heptenal (peak 5 in Figure 1), (*E*)-2-octenal (peak 10 in Figure 1), (*E*)-2-decenal (peak 16 in Figure 1), and (*E*)-2-undecenal (peak 21 in Figure 1), some *2,4-alkadienals* such as (*Z,E*)- and (*E,E*)-2,4-decadienal (peaks 17 and 18 respectively, in Figure 1) and some *oxygenated alpha,beta unsaturated aldehydes* like, 4-hydroxy-(*E*)-2-nonenal (peak 20 in Figure 1) and 4,5-epoxy-(*E*)-2-decenal (peak 23 in Figure 1) (see Table S1). Likewise, small abundances of *2-pentylfuran* (peak 7 in Figure

1), a reduced number of *alcohols*, *ketones*, some *furanones* and *lactones* were also found (see Table S2 and Figure 1).

3.1.3. Other minor compounds. Finally, a third group of compounds was found in this corn oil (see Table S3). They are a reduced number of *glycidyl fatty acid esters* (GEs). They are considered toxic contaminants (Hoogenboom, 2016), supposedly formed in the refining process (Cheng, Liu & Liu, 2016). The abundance of these compounds is, as expected, very low.

3.2. Evolution under oxidative conditions of the antioxidant compounds present initially in the corn oil

When oil is subjected to oxidative conditions, it is to be expected that the antioxidants, initially present, undergo degradation, thus decreasing in concentration. The rate of this degradation will depends on the oxidative stability of each one of these compounds under these conditions.

3.2.1. Degradation kinetics of tocols. The abundances of the various *tocopherols* in the oil, at different days under oxidative conditions, are given in Table 1 and represented in Figure S1A. It can be observed that they remained practically constant during an initial period of time (approximately up to day 3 under degradative conditions). However, from day 3 until day 9, they underwent a rapid degradation. The abundance of each one of these tocopherols [**T**] and time, **t**, under degradative conditions (considering day 3 the starting point (time = 0) and day 9 the end point (time = 6)) fitted, with a high correlation coefficient, to the general equation [**T**] = **m t** + **b**, being **m** and **b** specific of each tocopherol. The equations obtained were: [**γ-T**] = -174.7 **t** + 1037.8 (R=0.9961, n=3); [**α-T**] = -32.8 **t** + 180.3 (R=0.9401, n=3); [**δ-T**] = -5.7 **t** + 40.4 (R=0.9960, n=3); and [**β-T**]

= -1.8 **t** + 11.4 (R=0.9934, n=3). In these equations the abundance [**T**] is given in area counts of the base peak of the mass spectra of the corresponding tocol multiplied by 10^{-5} and **t** is given in days. The degradation rate **m** of each tocopherol, derived from these equations, is defined by **m** = d**[T]**/d**t**. The highest degradation rate was shown by γ-T, followed by α-T, δ-T and β-T. Furthermore, it was also observed that there is a very close relationship between the degradation rate **m** of each tocopherol thus obtained and its abundance at time 0. In fact, both latter parameters, including the four tocopherols, are linearly related with a correlation coefficient of 1. In summary, it can be said that the degradation of these four tocopherols contained in corn oil fits well with a kinetic model of zero-order, under the oxidative conditions of this study.

Likewise, a first-order kinetic model $Ln[\mathbf{T}] = Ln[\mathbf{T}]_0 + \mathbf{k}\,\mathbf{t}$ was tested**.** In this model **T** and **t** meaning is the same as above, **k** being the degradation rate constant. The equations obtained (Ln[γ-**T**] = 7.4 - 0.7 **t** (R=0.9454, n=3); Ln[α-**T**] = 5.4 - 0.7 **t** (R=0.9942, n=3); Ln[δ-**T**] = 3.8 - 0.3 **t** (R=0.9657, n=3); Ln[β-**T**] = 2.4 -0.2 **t** (R=1.0000, n=2) also fit fairly well, however the correlation coefficients were somewhat worse than those obtained with the zero-order kinetic model. With this new approach the degradation rate of each tocopherol at time **t**, $(\mathbf{r})_t$, is a function of its abundance **T**, at his same time, $[\mathbf{T}]_t$, as indicated in the equation $(\mathbf{r})_t = k\,[\mathbf{T}]_t$, where **k** has the same meaning as above. In this approach γ-T, also shows the highest degradation rate followed by α-T, δ-T and β-T.

A comparison of the results here obtained with those obtained by other authors is not easy because the systems involved, degradative conditions, analytical tools employed and data treatment are usually different.

Regarding studies in model systems, the degradation of α-T in methyl linoleate, during storage, fitted well to a zero-order kinetic model (Widicus & Kirk, 1981) like here. However, a first-order kinetic model was considered the best to describe the degradation

of α-, γ- and δ-T separately in glycerol (Chung, 2007); in this latter study δ-T and γ-T showed higher degradation rate than α-T.

With respect to the studies of edible oils, it can be mentioned that it refers to the degradation of the same three tocopherols cited above in soybean oil stored in bottles sealed at 50 ° C. (Player, Kim, Lee & Min, 2006). Although no kinetic study was made, the results indicated that α-T disappeared from the oil before γ- and δ-T. Nevertheless, it should be mentioned that in soybean oil the concentration of γ- T and δ-T, is much higher than that of α-T, and this could influence on its early disappearance (Cerretani, Lerma-García, Herrero-Martínez, Gallina-Toschi & Simó-Alfonso, 2010; Alberdi-Cedeño et al., 2017a). Other authors studied the rate of degradation of the same three tocopherols above mentioned contained in perilla and corn oils maintained at different temperatures (20, 40, 60 and 80ºC) in bottles closed with Korean paper (Wang, Hwang, Yoon & Choe, 2010). No clear effect of temperature on the degradation rate of the same tocopherol in the same oil was found, and the order in the degradation rate of the three tocopherols was not the same in the same oil at different temperatures.

The evolution of *tocomonoenol* and *tocotrienol* abundances followed a similar path as tocopherols, under the oxidative conditions of this study, and after 9 days all of them disappear from the corn oil, as Table 1 shows.

3.2.2. Degradation kinetics of sterols. The abundance of these compounds remained, like that of tocols, practically unchanged until day 3 (see Table 1 and Figure S1B). Abundance and time, from day 3 (**t**=0) to day 12 (**t**=9) under oxidative conditions, fitted well to linear equations. The equations found for the main sterols, campesterol (**C)**, and β-sitosterol (**β-S)**, were [**C**] = -1.2 **t** + 13.5 (R=0.9945, n=4) and [**β-S**] = -2.8 **t** + 30.9 (R=0.9992, n=4). These high correlation coefficients indicate that also in this case the degradation of sterols

fits well to a zero-order kinetic model, the degradation rate of β-sitosterol being higher than that of campesterol. The equations obtained considering a first-order kinetic model also have high correlation coefficients although somewhat smaller than those of the zero-order kinetic model (Ln[**C**]= -0.2 **t** + 2.7 (R=0.9814, n=4) and Ln[**β-S**]= -0.2 **t** + 3.5 (R=0.9815, n=4)); in this second model the degradation rate of β-sitosterol is also higher than that of campesterol. As in the case of tocols, the degradation rate of sterols is strongly affected by the oxidative conditions. No degradation of sterols in oils has been observed under very mild oxidation conditions (low temperature, dark and absence of oxygen), whereas under stronger oxidative conditions a relevant degradation level has been detected (Oehrl, Hansen, Rohrer, Fenner & Boyd, 2001; Thanh, Vergnes, Kaloustian, El-Moselhy, Amiot-Carlin & Portugal, 2006; Gawrysiak-Witulska, Rudzińska, Siger & Bartkowiak-Broda, 2015). However, to the best of our knowledge, kinetic studies on sterols degradation in vegetable oils have not been carried out.

3.2.3. Degradation kinetics of squalene. Another important minor corn oil component is squalene, Sq. As Table 1 and Figure S1C show, the abundance of this compound decreases very slowly from day 0 to day 9 and then very sharply from day 9 to day 12. In the first stage (days 0- 9), the abundance [**Sq**] and time **t** fit, with a high correlation coefficient, to the linear equation [**Sq**] = -11.1 **t** + 527.9 (R=0.9583, n=4) and also to the equation Ln[**Sq**]= -0.02 **t** + 6.3 (R=0.9660, n=4). This indicates that squalene degradation is fairly well described by both either a zero-order or a first-order kinetic model. In a previous study, of olive oil subjected to storage conditions, it was proposed that α-tocopherol exerts a protective effect on squalene by avoiding or retarding its degradation (Rastrelli, Passi, Ippolito, Vacca & De Simone, 2002). This could explain the slow degradation rate of this compound found here from day 0 to day 9, although this could also be due to the oxidative stability of squalene exclusively.

Table 1. Evolution of the abundances of tocols, sterols, squalene and DKPs present in corn oil submitted to oxidative conditions during different periods of time, expressed as area counts of their mass spectra base peak (Bp) multiplied by 10^{-5}, obtained as average of two determinations together with their standard deviation.

		Time (days)				
Compound (molecular weight)	**Bp**	**0**	**3**	**6**	**9**	**12**
Tocols						
Tocopherols						
δ-tocopherol (402)*	402	41.5 ± 0.2	40.1 ± 0.0	23.7 ± 2.4	5.6 ± 1.0	0.6 ± 0.1
β-tocopherol (416)*	416	11.1 ± 0.1	11.0 ± 0.3	6.6 ± 0.6	-	-
γ-tocopherol (416)*	416	1,135.1 ± 54.4	1,064.7 ± 21.9	459.9 ± 12.5	16.5 ± 1.6	4.0 ± 0.5
α-tocopherol (430)*	165	220.1 ±13.6	200.9 ± 6.3	40.5 ± 9.9	3.9 ± 0.0	2.6 ± 0.4
Tocomonoenols						
γ-tocomonoenol (414)	414	4.3 ± 0.1	3.8 ± 0.1	1.3 ± 0.1	-	-
α-tocomonoenol (428)	428	4.0 ± 0.2	3.4 ± 0.2	0.5 ± 0.1	-	-
Tocotrienols						
γ-tocotrienol (410)*	151	7.2 ± 0.3	6.7 ± 0.5	2.6 ± 0.3	-	-
α-tocotrienol (424)*	165	11.0 ± 1.0	9.7 ± 0.6	3.1 ± 0.1	-	-
Sterols-stanol						
Campesterol (400)*	400	14.3 ± 0.1	13.9 ± 0.6	9.1 ± 0.9	6.2 ± 0.1	2.7 ± 0.0
Stigmasterol (412)*	412	2.8 ± 0.1	2.6 ± 0.3	1.6 ± 0.3	1.2 ± 0.0	0.4 ± 0.1
β-sitosterol (414)*	414	32.7 ± 0.5	31.3 ± 1.9	22.2 ± 2.9	14.2 ± 0.4	6.5 ± 0.5
Sitostanol (416)*	215	1.1 ± 0.1	1.0 ± 0.0	0.8 ± 0.0	0.5 ± 0.0	-
Δ5-avenasterol (412)*	314	2.5 ± 0.0	2.4 ± 0.2	1.4 ± 0.1	1.3 ± 0.0	-
Terpenic hydrocarbon						
Squalene (410)*	69	539.8 ± 24.0	476.8 ± 1.5	461.3 ± 22.7	433.9 ± 28.4	117.5 ± 22.0
DKPs						
Cyclo (Phe-Val)I (246) *	91	0.8 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	-	-
Cyclo (Phe-Val)II (246)	91	0.9 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	-	-
Cyclo (Leu-Phe)I (260) *	91	0.7 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	-	-
Cyclo (Leu/Ile-Phe)II (260)	91	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	-	-
Cyclo (Pro-Phe)I (244) *	125	0.6 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	-	-
Cyclo (Leu/Ile-Phe)III (260)	91	1.2 ± 0.2	1.0 ± 0.0	0.9 ± 0.1	-	-
Cyclo (Leu/Ile-Phe)IV (260)	91	0.8 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	-	-
Cyclo (Pro-Phe)II (244)	125	1.3 ± 0.0	1.2 ± 0.1	0.9 ± 0.0	0.6 ± 0.1	-

*Asterisked compounds were acquired commercially and used as standards for identification purposes; -: no detected.

3.2.4. Evolution of DKPs. As mentioned before, this oil, like other corn oils, also contains *DKPs* in very small abundance. Table 1 shows the evolution of their abundances over time under oxidative conditions. The degradation of these compounds appears to begin from day 0, and on day 9 they are totally degraded. Due to their low abundance the equations relating their abundance and time under degradative conditions are more affected by experimental errors than those of the components in higher abundance mentioned above, for which reason no kinetic data are given.

In summary, it is evident that under oxidative conditions, from day 3 to day 9, the abundance of tocols has been reduced nearly ten times, whereas that of sterols has been reduced about a half and that of squalene by a factor of around 0.8. From day 9 onwards the degradation rate of all of these oil components increases considerably and on day 12 the abundance of many of them is small and that of others null.

3.3. Formation of compounds derived from the antioxidant components of corn oil and evolution of their abundance

The degradation of each one of the above-mentioned minor corn oil components can lead to the formation of several derived compounds which were not present in the original oil or that were present in very low abundance. As the parent compounds are minor oil components, the abundance of their derived compounds will be very small. For this reason, their detection is not easy. This may be the reason why, in most of the studies dedicated to analyzing the evolution, under oxidative conditions, of oil antioxidant components, their derived compounds were not contemplated (Player et al., 2006; Thanh et al., 2006; Wang et al., 2010). Nevertheless, the methodology here used allows one to identify and determine the abundance of some of these derived compounds.

3.3.1. Compounds coming from tocopherols degradation. Compounds found coming from tocopherol degradation can be placed in two groups: one constituted by compounds derived from any of the four tocopherols and a second group constituted by those derived specifically from α-T.

The first group comprises prist-1-ene (P), 6,10,14-trimethylpentadecan-2-one (TrMPD), 3,7,11-trimethyl-3-dodecanol (TrMD) and 4,8,12,16-tetramethylheptadecan-4-olide (TeMHD) (see Figure 2A and Table 2). The first two compounds (P and TrMPD) were present, in very low abundance, in the original corn oil, due to the basal oxidation level present in edible oils. Some authors have reported that these two compounds can be formed from tocopherols under very different degradative conditions (Goossens, Leeuw, Schenck & Brassell, 1984; Nassiry et al., 2009). The formation of TrMD and TeMHD as coming from tocopherol degradation has been proved in our laboratory, in parallel studies of pure tocopherol oxidation submitted to 70ºC with aeration. To the best of our knowledge, this is the first time that the formation of these two latter compounds has been described in edible oil oxidation.

The abundance of these tocopherol derivatives increases with time under oxidative conditions (see Table 2). As mentioned, two of them, P and TrMPD, were present in the original oil, and TeMHD was detected from day 3 onwards (Table 2 and Figure S2); the three show increasing abundances until day twelve. Likewise, the abundance of TrMD, which was detected from day 6, shows a growth path to day twelve. The abundance [**DT**] of each one of these four compounds and time **t** under oxidative conditions fit well to equations like [**DT**] = **a** $e^{\mathbf{kt}}$, where **a** is a coefficient specific of each compound, and **k** the formation rate constant. The equations corresponding to each one of the above mentioned tocopherol derivatives, are: [**P**] = 1.0 $e^{0.2\mathbf{t}}$ (R=0.9751, n=5); [**TrMPD**] = 0.5 $e^{0.3\mathbf{t}}$ (R=0.9740, n=5); [**TrMD**]= 1.4 $e^{0.3\mathbf{t}}$ (R=0.9808, n=3); and [**TeMHD**]= 2.7 $e^{0.4\mathbf{t}}$

(R=0.9898, n=4). From these results it is evident that TeMHD has the highest formation rate among these four tocopherol derivatives.

The presence of these four tocopherol derivatives in the oil indicates that tocopherols have undergone degradation and their presence and abundance are associated with advancing corn oil oxidation. For this reason, they can be considered as corn oil oxidation markers. The detection of P and TrMPD in the original oil in very low abundances corresponds to a basal oxidation level which may occur in many edible oils before submission to oxidative conditions. However, the detection of TeMHD (see Table 2 and Figure S2) at a very low abundance at day 3 is an indication that the oil has begun its oxidation process. It should be noticed that this lactone has not been detected in unoxidized edible oils such as sunflower oil, virgin and refined soybean oil, linseed oil, and several corn oils (Alberdi-Cedeño et al., 2017a, b). Furthermore, as its abundance increases very quickly throughout the oxidation process, this compound can be considered a good marker of incipient oxidation in edible oils containing tocopherols (see Figure S2). Finally, the detection of TrMD in the corn oil only occurs after six days under oxidative conditions. For this reason, it can also be considered a corn oil oxidation marker but not from an early stage.

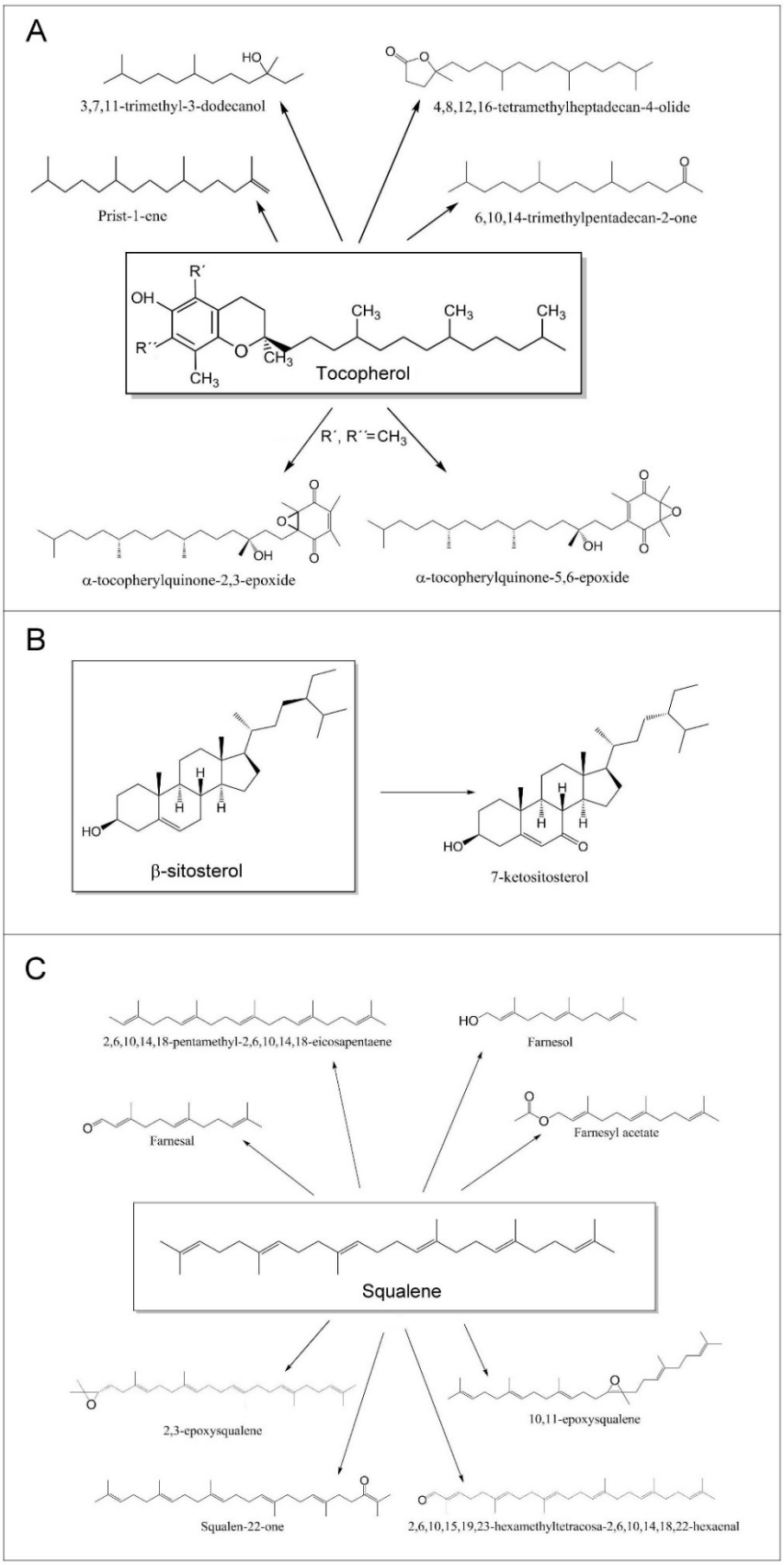

Figure 2. Detected oxidation compounds derived from: A) tocopherols, B) β-sitosterol and C) squalene.

Table 2. Abundances of the compounds formed by the oxidation of minor corn oil components at different periods of time under oxidative conditions, expressed as area counts of their mass spectra base peak (Bp) multiplied by 10^{-5}, obtained as average of two determinations together with their standard deviations.

		Time (days)				
Compound (molecular weight)	**Bp**	**0**	**3**	**6**	**9**	**12**
Tocopherol oxidation derived compounds						
Prist-1-ene (266) (P)	56	1.0 ± 0.1	1.7 ± 0.1	4.1 ± 0.4	10.2 ± 0.5	10.6 ± 1.6
6,10,14-trimethylpentadecan-2-one (268) (TrMPD)	43	0.6 ± 0.0	1.4 ± 0.0	1.6 ± 0.2	5.0 ± 0.5	15.2 ± 3.8
3,7,11- trimethyl-3-dodecanol (228) (TrMD)	73	-	-	1.6 ± 0.0	3.0 ± 0.2	10.9 ± 0.2
4,8,12,16-tetramethylheptadecan-4-olide (324) (TeMHD)	99	-	2.2 ± 0.2	13.2 ± 1.7	35.5 ± 3.1	105.3 ± 25.8
α-tocopherylquinone-2,3-epoxide (462) (TQ23E)	237	-	-	1.9 ± 0.6	2.9 ± 0.3	2.2 ± 0.6
α-tocopherylquinone-5,6-epoxide (462) (TQ56E)	237	-	-	1.1 ± 0.2	1.6 ± 0.1	-
β-sitosterol oxidation derived compounds						
7-ketositosterol (428)*	428	-	-	-	-	0.8 ± 0.1
Squalene oxidation derived compounds						
Farnesal (220)*	69	-	-	-	1.4 ± 0.2	28.8 ± 9.4
Farnesal (isomer) (220)	69	-	-	-	-	4.4 ± 0.3
Farnesyl acetate (264)	69	-	-	-	3.3 ± 0.9	13.3 ±4.8
2,6,10,14,18-pentamethyl-2,6,10,14,18-eicosapentaene (342)	69	-	-	-	-	4.1 ± 1.5
Farnesol (isomer) (222)	69	-	-	-	-	4.1 ± 1.1
10,11-epoxysqualene (426)	69	-	-	-	5.6 ± 0.6	13.2 ± 1.5
2,3-epoxysqualene (426)*	69	-	-	-	9.1 ± 0.0	17.5 ± 1.7
Squalen-22-one (426)	69	-	-	-	5.6 ± 0.9	13.1 ± 1.5
2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaenal (424)	69	-	-	-	-	7.9 ± 0.2

*Asterisked compounds were acquired commercially and used as standards for identification purposes; -: no detected.

The second of the groups mentioned above includes the compounds coming exclusively from α-T. Two are the members of this group: α-tocopherylquinone-2,3-epoxide (TQ23E) and α-tocopherylquinone-5,6-epoxide (TQ56E) (see Table 2 and Figure 2A). Their formation from α-T degradation has been described in model systems constituted by α-T, triolein and tripalmitin or by α-T and triolein submitted to thermoxidation or to deodorization simulated conditions respectively (Verleyen, Kamal-Eldin, Dobarganes, Verhé, Dewettinck & Huyghebaert, 2001; Verhé, Verleyen, Van Hoed & De Greyt, 2006). The evolution of the abundance of these two compounds follows a different path than to the above mentioned tocopherol derivatives. These are not detected before day 6, reach a maximum on day 9, after which their abundance decreases, probably because these compounds evolve to form others. Their presence in the oil also indicates that an oxidation process has taken place.

In summary, all of these tocopherol derivatives can be considered corn oil oxidation markers and this can be extrapolated to all edible oils containing tocopherols. To the best of our knowledge, this is the first time that these compounds have been proposed as edible oil oxidation markers. It should taken into account that in this same oil the hydroperoxides and their associated conjugated dienic systems (primary oxidation compounds coming from main corn oil components) are not detectable by ^{1}H NMR before day four under the same oxidative conditions (see Figure S2), and the aldehydes (secondary oxidation compounds coming from main corn oil components) are not detected until day eleven.

3.3.2. Compounds coming from sterols degradation. The detection of sterols derived compounds was much more difficult than that of tocopherol derivatives. Of the various sterol oxidation derivatives described (O´Callaghan, McCarthy & O´Brien, 2014) only 7-ketositosterol, which is derived from β-sitosterol (the main sterol in this oil), has been detected, but in very small abundance. This compound was detected after 12 days under oxidative conditions, coinciding with an important degree of β-sitosterol degradation (see

Table 2 and Figures 2B and S1B). The formation of 7-ketositosterol in the oxidation of β-sitosterol at different temperatures has been proved previously by several authors (Zhang et al., 2005; Hu et al., 2015).

3.3.3. Compounds coming from squalene degradation. As mentioned before the squalene degradation rate is very slow before day 9. However, as Table 2 shows, some of its derived compounds are already detected on day 9 and others on day 12. The derived compounds found, as Table 2 and Figure 2C shows, are: four oxygenated derivatives of farnesene; one unsaturated branched hydrocarbon of 25 carbon atoms; and four oxygenated derivatives of squalene, among which there are two epoxides, one ketone and one aldehyde. The formation of these compounds from squalene oxidation has been proved in studies carried out in our laboratory by submitting pure squalene to the same oxidative conditions as here. Of all squalene derivatives, the two epoxy-squalene isomers, squalen-22-one, farnesal and farnesyl acetate were the first formed and the most abundant. Although these compounds were not present initially in this corn oil, some of them have been detected, by using this methodology, in various edible oils not submitted to oxidative conditions (Alberdi-Cedeño et al., 2017a). Furthermore, 2,3-epoxysqualene has also been identified as an intermediate in the biosynthesis of terpenoids in plants, animals, and fungi (Abe, 2014). For these reasons its presence in edible oils in general could have various origins.

3.3.4. Compounds coming from cyclic dipeptides degradation. None of these compounds could be detected. This could be expected because these antioxidant compounds are in very low abundance in the original oil.

3.4. Evolution with time of compounds coming from corn oil main components oxidation

It is well known that the main corn oil components are triglycerides, having linoleic as main acyl group, followed by oleic, saturated and linolenic acyl groups, this latter in a very small molar percentage, as mentioned in the experimental section. Their oxidation first generates hydroperoxides, also named primary oxidation compounds; these are intermediate compounds that in turn degrade forming different kinds of secondary oxidation compounds. These latter include a great number of small compounds, which are detectable by this technique, among which aldehydes, furan derivatives, alcohols, ketones and lactones are.

3.4.1. Aldehydes. These compounds are the most numerous and abundant secondary oxidation compounds of those detected here. All of them have been previously found in different edible oils submitted to oxidative conditions (Guillén et al., 2005; Guillén & Uriarte, 2012b; Goicoechea & Guillén, 2014). It can be observed in Table S1 that some alkanals, (*E*)-2-alkenals, (*E,E*)-2,4-alkadienals, certain aromatic aldehydes and even some oxygenated *alpha,beta* unsaturated aldehydes are present in this original corn oil, in very low abundance. This indicates again that this oil has a basal oxidation level, which was also evidenced by the presence of two tocopherol derivatives (P and TrMPD), before mentioned.

The abundance of aldehydes increases, as time under oxidative conditions increases. This increase follows a general path of two stages. In the first stage (from day 0 to day 6) the formation rate is much slower than in the second stage (from day 6 onwards) (see Table S1 and Figure S3). In each one of these stages the abundance fits well with storage time through linear or exponential equations that relate both variables with very high correlation coefficients. It is noteworthy that the point between these two stages occurs after six days under oxidative conditions, that is to say when tocopherols have been degraded to a great extent. Since linoleic is the main acyl group in corn oil, its derived aldehydes are the most abundant. All aldehydes are known oxidation markers and the greater their abundance the greater the oil oxidation level.

3.4.2. Other secondary oil oxidation compounds. In addition to aldehydes, some furan derivatives, alcohols, ketones, as well as furanones and lactones were also detected in the corn oil after its submission to accelerated storage conditions. All of them are generated in the oxidation of oil main components. They are shown in Table S2, and some of them, such as 2-pentylfuran, 1-octen-3-ol, 3-nonen-2-one, 2,3-octanedione and 5-pentyl-2(5H)-furanone, are well known oil oxidation markers (Guillén et al., 2005; Guillén & Uriarte, 2012b; Goicoechea & Guillén, 2014). The evolution of their abundance, like that of aldehydes, shows two stages of different growth rate. In both stages, abundance fits well with storage time to linear or exponential equations (see Figure S3). The point between both stages also occurs at day six as in the case of the aldehydes mentioned above.

3.5. Evolution of glycidyl fatty acid esters

As mentioned before, three glycidyl fatty acid esters, GEs, were present in the original oil. These are glycidyl linoleate, glycidyl oleate and glycidyl palmitate. Their abundance seems to increase with time under oxidative conditions until day 9, after which they begin to decline until their total disappearance on day twelve (Table S3 shows this evolution). Their increase could be attributed to heating. It has been described that they are formed from mono- and di-acylglycerides by heating during oil deodorization (Cheng et al., 2016). Their decrease after a certain time under oxidative conditions is in agreement with observations made by Aniołowska & Kita (2015, 2016).

3.6. Comparison of the performance of this methodology in the study of oil oxidation with that of other methodologies that do not require chemical changes in the sample either

In addition to the methodology used here, (DI-SPME-GC/MS), there are others which also provide information on the oxidation status of edible oils, without requiring chemical

modification of the sample. One of these involves the study of the oil headspace components, by gas chromatography/mass spectrometry, after their extraction by means of solid phase microextraction (HS-SPME-GC/MS). The other studies the oil sample as a whole by means of ^{1}H NMR spectroscopy.

3.6.1. Performance of HS-SPME-GC/MS. This methodology provides information about the oil oxidation status through the abundance of volatile secondary oxidation compounds. The oxidation markers in this case are the volatile compounds generated in the main oil components oxidation. This has been shown in previous papers (Guillén et al., 2005; Guillén & Uriarte, 2012b; Goicoechea & Guillén, 2014). Nevertheless, all these compounds are also detected by DI-SPME-GC/MS as has been described in point 3.4. Furthermore, it should be noticed that extraction by SPME of a compound from a headspace (HS) requires greater abundance of the compound in the oil than does its extraction from the oil matrix directly by means direct immersion (DI). For this reason, volatile compounds in very small abundance in the oil can be detected by using direct immersion-solid phase microextraction (DI-SPME) but not by headspace solid phase microextraction (HS-SPME). Likewise, in the study of an oil oxidation process the minor volatile components newly formed are detected slightly earlier by direct immersion than through the study of the oil headspace. In addition it should be remembered that DI-SPME-GC/MS, in addition to the above mentioned secondary oxidation compounds, also provides information about the oil antioxidant components and of some of their derived compounds, as shown in points 3.2.and 3.3.

3.6.2. Performance of ^{1}H NMR spectroscopy. This technique provides qualitative and quantitative information not only of the oil main components (molar percentage of the several kinds of acyl groups), but also of their primary and secondary oxidation derivatives. The information of primary oxidation compounds refers to total hydroperoxides and to their *Z,E*-

and *E,E*-conjugated dienic systems. The information of secondary oxidation compounds refers mainly to keto- and hydroxy-dienes as well as to epoxy derivatives and to several kinds of aldehydes (Martin-Rubio, Sopelana, Ibargoitia, & Guillén, 2018).

Of all those compounds which may be studied by ^{1}H NMR spectroscopy, DI-SPME-GC/MS is only able to provide information of some secondary oxidation compounds as indicated in point 3.4. However, as mentioned above, this latter technique provides information of the oil antioxidant components and of some of their derived compounds (points 3.2.and 3.3), which cannot be obtained directly from ^{1}H NMR. Furthermore, the information provided by this latter technique in most cases does not refer to individual compounds but to groups of compounds having the same functional group and protons with similar electronic environment (Guillén & Ruiz, 2004; Guillén & Ruiz, 2005; Martin-Rubio et al., 2018). In addition, its sensitivity is low, for which reason the compounds are only detected from a certain abundance level onwards.

In summary, HS-SPME-GC/MS only provides information about volatile secondary oxidation compounds derived from oil main components. ^{1}H NMR provides information concerning main oil components, and their primary and secondary oxidation compounds. DI-SPME-GC/MS provides information about oil antioxidant components, some compounds coming from antioxidant components oxidation, and some secondary oxidation compounds coming from main oil component oxidation.

4. CONCLUSIONS

Study by direct immersion-solid phase microextraction followed by gas chromatography/mass spectrometry of corn oil and of this oil submitted to storage accelerated conditions provides a great deal of information concerning its volatile and less volatile minor components. Among these latter there are very important antioxidant corn oil components,

such as tocols, sterols, squalene and cyclic dipeptides that play an essential role in the oil oxidation process. This methodology provides information about the degradation rate of these compounds and also about the identity and formation rate of compounds coming from their degradation during storage. The compounds formed in the degradation of tocopherols are proposed here, for the first time, as new oxidation markers of corn oil and of any edible oils containing tocopherols. Among these are 3,7,11-trimethyl-3-dodecanol and 4,8,12,16-tetramethylheptadecan-4-olide, whose formation in edible oils from tocopherols, is described for the first time in this paper. The latter can be considered a very good corn oil oxidation marker because it is formed very early after oil subjection to oxidative conditions and because it has a high formation rate. The formation and evolution of secondary oxidation volatile compounds derived from main corn oil components, all them well known oxidation markers, can also be analyzed by this methodology. The abundance of these becomes noticeable coinciding with the effective decline of those compounds having antioxidant ability. Likewise, this methodology enables the detection and study of the evolution, under these storage conditions, of contaminants present in the original oil such as various glycidyl esters. The usefulness of this technique for the study of edible oil oxidation processes is shown for the first time, providing new information which would be difficult, if not impossible, to obtain by other methods.

CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

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Supplementary Material of

Article 3

MONITORING OF MINOR COMPOUNDS IN CORN OIL OXIDATION BY DIRECT IMMERSION-SOLID PHASE MICROEXTRACTION-GAS CHROMATOGRAPHY/MASS SPECTROMETRY. NEW OIL OXIDATION MARKERS

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Table S1. Aldehydes (alkanals, (*E*)-2-alkenals, 2,4-alkadienals, oxygenated and aromatic) detected in corn oil submitted to oxidative conditions during different periods of time and their abundances, expressed as area counts of their mass spectra base peak (Bp) multiplied by 10^{-5}, obtained as average of two determinations together with their standard deviations.

		Time (days)				
Compound (molecular weight)	**Bp**	**0**	**3**	**6**	**9**	**12**
Alkanals						
Butanal (72)*	44	-	-	3.9 ± 0.8	7.7 ± 2.9	60.3 ± 9.1
Pentanal (86)*	44	6.1 ± 3.1	11.7 ± 0.5	15.4 ± 1.5	32.4 ± 4.6	231.6 ± 12.7
Hexanal (100)*	44	4.2 ± 0.8	17.9 ± 2.1	29.6 ± 2.8	133.8 ± 16.0	1,443.1 ± 44.9
Heptanal (114)*	70	0.5 ± 0.0	2.0 ± 0.3	4.9 ± 1.2	14.5 ± 0.5	91.5 ± 6.9
Octanal (128)*	41	-	-	2.4 ± 0.5	4.6 ± 0.2	91.5 ± 6.9
Nonanal (142)*	57	6.1 ± 2.0	8.2 ± 0.1	11.8 ± 2.7	24.0 ± 2.5	217.7 ± 1.9
Decanal (156)*	57	-	-	0.6 ± 0.3	1.7 ± 0.2	16.4 ± 4.6
(E)-2-Alkenals						
(*E*)-2-butenal-2-methyl (84)	55	-	0.5 ± 0.1	2.4 ± 0.3	2.9 ± 0.5	26.2 ± 1.4
(*E*)-2-pentenal (84)	55	-	0.7 ± 0.5	2.7 ± 0.1	3.2 ± 0.5	11.4 ± 1.5
(*E*)-2-hexenal (98)*	41	-	0.9 ± 0.1	1.2 ± 0.2	4.4 ± 0.8	33.6 ± 2.2
(*E*)-2-heptenal (112)*	83	3.8 ± 1.6	10.4 ± 1.5	24.5 ± 5.6	89.1 ± 10.1	321.2 ± 0.9
(*E*)-2-octenal (126)*	55	2.1 ± 1.9	5.2 ± 1.5	7.6 ± 2.3	22.9 ± 3.4	294.3 ± 2.0
(*E*)-2-nonenal (140)*	41	0.4 ± 0.0	1.0 ± 0.2	1.5 ± 0.3	3.4 ± 0.1	28.7 ± 2.2
(*E*)-2-decenal (154)*	70	3.6 ± 0.8	6.5 ± 1.0	9.5 ± 2.2	29.7 ± 2.5	306.5 ± 0.9
(*E*)-2-undecenal (168)*	70	3.0 ± 0.6	6.9 ± 1.4	12.1 ± 2.3	38.4 ± 3.5	402.5 ± 7.7
2,4-Alkadienals						
(*Z,E*)-2,4-heptadienal (110)	81	-	2.2 ± 0.4	7.3 ± 0.9	12.2 ± 2.1	61.5 ± 25.7
(*E,E*)-2,4-heptadienal (110)*	81	1.3 ± 1.1	2.5 ± 0.1	9.5 ± 0.9	20.1 ± 1.6	31.1 ± 1.0
(*Z,E*)-2,4-octadienal (124)	81	-	-	0.5 ± 0.0	1.4 ± 0.2	7.4 ± 0.2
(*E,E*)-2,4-octadienal (124)	81	-	-	-	-	3.2 ± 0.1
(*Z,E*)-2,4-nonadienal (138)	81	-	1.2 ± 0.2	2.3 ± 0.6	6.8 ± 0.9	38.5 ± 2.0

Compound (molecular weight)	Bp	Time (days)				
		0	3	6	9	12
	81	-	4.9 ± 1.1	9.4 ± 1.9	32.7 ± 2.7	163.2 ± 1.0
(*E*,*E*)-2,4-nonadienal (138)						
(*Z*,*E*)-2,4-decadienal (152)	81	14.1 ± 1.4	59.6 ± 7.9	339.1 ± 31.7	675.7 ± 50.4	975.5 ± 10.0
(*E*,*E*)-2,4-decadienal (152)*	81	50.3 ± 6.8	209.4 ± 35.9	872.5 ± 89.0	2,130.8 ± 168.5	3,853.9 ± 2.1
Oxigenated aldehydes						
4,5-epoxy-(*E*)-2-heptenal (126)	68	-	0.4 ± 0.0	1.1 ± 0.2	3.8 ± 0.7	14.2 ± 3.7
4,5-epoxy-2-heptenal (isomer) (126)	68	-	-	-	1.3 ± 0.1	6.4 ± 0.2
4-oxo-(*E*)-octenal (140)	55	-	0.4 ± 0.1	1.1 ± 0.0	3.7 ± 0.2	25.3 ± 3.8
4-oxo-(*E*)-2-nonenal (154)*	55	1.0 ± 0.2	5.5 ± 0.0	7.9 ± 1.0	26.3 ± 3.7	179.2 ± 36.0
4-oxononanal (156)	43	1.5 ± 0.3	3.1 ± 0.3	3.2 ± 0.5	10.3 ± 1.9	73.6 ± 13.9
4-hydroxy-(*E*)-2-nonenal (156)*	57	2.7 ± 0.4	14.2 ± 3.4	29.1 ± 0.8	100.9 ± 6.4	1,227.1 ± 3.9
4,5-epoxy-2-decenal (isomer) (168)	68	0.6 ± 0.0	2.6 ± 0.5	11.8 ± 1.6	59.5 ± 4.1	617.8 ± 0.1
4,5-epoxy-(*E*)-2-decenal (168)*	68	1.6 ± 0.0	8.0 ± 1.5	38.9 ± 5.1	173.8 ± 8.2	1,560.3 ± 0.1
Aromatic aldehydes						
Benzaldehyde (106)*	106	0.4 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	1.4 ± 0.0	24.7 ± 0.9
2-phenylpropenal (132)	131	0.2 ± 0.0	0.4 ± 0.0	0.8 ± 0.2	1.1 ± 0.1	1.1 ± 0.0
Vanillin (152)*	151	-	0.8 ± 0.1	5.2 ± 0.6	8.2 ± 0.3	39.2 ± 10.4

*Asterisked compounds were acquired commercially and used as standards for identification purposes; -: no detected.

Table S2. Furan derivatives, alcohols, ketones and lactones and furanones detected in corn oil submitted to oxidative conditions during different periods of time and their abundances, expressed as area counts of their mass spectra base peak (Bp) multiplied by 10^{-5}, obtained as average of two determinations together with their standard deviations.

		Time (days)				
Compound (molecular weight)	**Bp**	**0**	**3**	**6**	**9**	**12**
Furan derivatives						
2-ethylfuran (96)	81	-	0.2 ± 0.0	0.4 ± 0.0	1.0 ± 0.0	4.6 ± 0.5
2-propylfuran (110)	81	-	-	-	0.7 ± 0.1	2.5 ± 0.5
2-butylfuran (124)	81	-	-	-	2.0 ± 0.0	22.5 ± 0.8
2-pentylfuran (138)*	81	2.5 ± 0.1	13.6 ± 3.1	14.6 ± 2.0	37.2 ± 2.8	721.1 ± 36.5
2-hexylfuran (152)	81	-	-	-	-	4.5 ± 1.5
2-heptylfuran (166)	81	-	-	-	0.8 ± 0.2	11.2 ± 3.2
Alcohols						
1-pentanol (88)*	42	2.6 ± 0.1	4.6 ± 1.0	6.4 ± 2.2	17.9 ± 4.5	116.7 ± 13.4
1-hexanol (102)*	56	-	-	0.6 ± 0.1	2.0 ± 0.6	22.4 ± 5.4
1-heptanol (116)*	70	-	-	0.7 ± 0.3	2.2 ± 0.3	20.0 ± 7.8
1-octen-3-ol (128)*	57	3.7 ± 0.9	6.6 ± 1.0	13.3 ± 2.5	47.8 ± 5.8	195.0 ± 47.0
(*E*)-2-octenol (128)	57	-	3.6 ± 0.9	5.2 ± 1.2	16.9 ± 1.8	153.4 ± 43.3
1-octanol (130)	56	-	-	-	1.3 ± 0.1	17.3 ± 3.3
6-undecanol (172)	83	-	0.8 ± 0.0	3.6 ± 2.5	8.5 ± 2.6	57.1 ± 20.9
Ketones						
2-pentanone (86)	43	-	-	-	5.4 ± 2.4	20.2 ± 8.1
2-hexanone (100)	43	-	-	-	0.9 ± 0.2	10.6 ± 1.1
2-heptanone (114)*	43	0.4 ± 0.1	1.5 ± 0.2	3.4 ± 0.8	13.0 ± 1.4	114.9 ± 41.8
1-octen-3-one (126)	55	-	0.7 ± 0.3	1.4 ± 0.4	4.5 ± 1.1	21.1 ± 2.4
2,3-octandione (142)	43	-	1.6 ± 0.2	3.6 ± 0.8	14.1 ± 1.2	105.0 ± 2.2
2-octanone (128)	43	-	0.9 ± 0.0	1.4 ± 0.4	5.4 ± 1.3	41.5 ± 0.7
3-octen-2-one (126)	55	-	0.9 ± 0.1	2.9 ± 0.6	15.8 ± 1.3	73.1 ± 23.2
3-nonen-2-one (140)	55	-	0.9 ± 0.5	3.8 ± 0.8	16.0 ± 1.9	121.2 ± 21.6

		Time (days)				
Compound (molecular weight)	**Bp**	**0**	**3**	**6**	**9**	**12**
2-decanone (156)	43	-	-	0.4 ± 0.2	0.6 ± 0.0	7.8 ± 2.6
2-undecanone (170)	43	-	-	-	-	15.4 ± 5.9
2-dodecanone (184)	43	-	0.9 ± 0.2	0.9 ± 0.1	3.8 ± 1.2	48.2 ± 8.3
1-phenyl-1-hexanone (176)	105	-	0.9 ± 0.1	0.9 ± 0.0	2.1 ± 0.1	61.5 ± 0.0
6-tridecanone (198)	43	-	-	-	1.9 ± 0.4	17.0 ± 5.4
6-tetradecanone (212)	43	-	-	-	-	6.7 ± 1.7
10-nonadecanone (282)	43	-	-	-	0.9 ± 0.1	9.7 ± 2.7
2-pentadecanone (226)	58	-	-	1.5 ± 1.0	3.4 ± 1.5	36.3 ± 7.6
2-heptadecanone (254)	58	-	-	0.3 ± 0.1	0.7 ± 0.1	6.2 ± 1.5
2-nonadecanone (282)	58	-	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	1.8 ± 0.4
Lactones and furanones						
γ-caprolactone (114)*	85	0.6 ± 0.3	1.1 ± 0.0	2.1 ± 0.8	5.4 ± 0.2	79.2 ± 15.3
γ-heptalactone (128)*	85	0.1 ± 0.0	0.3 ± 0.0	0.5 ± 0.3	0.6 ± 0.1	11.6 ± 0.1
5-butyl-5H-furan-2-one (140)	84	-	0.3 ± 0.1	0.9 ± 0.1	3.7 ± 0.9	37.2 ± 6.1
γ-octalactone (142)*	85	2.0 ± 1.0	3.1 ± 0.9	8.9 ± 4.4	27.4 ± 9.6	196.8 ± 3.3
5-pentyl-2(3H)-furanone (154) (or isomer)	98	-	-	2.2 ± 0.1	4.0 ± 0.1	52.8 ± 11.0
(*E*)-4-hydroxy-3-methyloctanoic acid lactone (156) (or isomer)	99	-	-	-	-	53.8 ± 24.6
5-pentyl-2(5H)-furanone (154)	84	0.8 ± 0.1	7.4 ± 1.6	25.8 ± 8.8	84.2 ± 15.4	848.8 ± 151.5
γ-nonalactone (156)*	85	0.3 ± 0.0	1.7 ± 0.1	7.0 ± 4.7	8.7 ± 1.9	80.3 ± 14.9
δ-nonalectone (156)	99	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	2.0 ± 0.0	51.7 ± 29.1
6-pentyl-5,6-dihydro-2H-pyran-2-one (168) (or isomer)	97	-	1.9 ± 0.6	4.6 ± 1.5	8.5 ± 0.2	55.6 ± 7.7
β-methyl-γ-decalactone (184)*	99	-	-	0.6 ± 0.2	1.2 ± 0.0	26.8 ± 3.0
γ-undecalactone (184)*	85	-	-	-	0.5 ± 0.1	13.6 3.1
δ-undecalactone (184)*	99	-	-	0.9 ± 0.2	4.8 ± 0.9	45.0 ± 16.7
(*Z*)-4-hydroxy-3-methylundecanoic acid lactone (198) (or isomer)	99	-	-	-	0.6 ± 0.2	23.8 ± 6.5
6-hexyl-hexan-6-olide or ε-dodecalactone (198)	85	0.5 ± 0.3	0.5 ± 0.1	0.6 ± 0.0	0.9 ± 0.1	12.8 ± 0.3
γ-palmitolactone (254)	85	0.7 ± 0.0	1.6 ± 0.4	2.1 ± 0.5	7.4 ± 2.3	83.5 ± 0.1
δ-palmitolactone (254)	99	5.0 ± 0.8	4.8 ± 0.2	5.4 ± 0.0	8.8 ± 0.7	81.9 ± 25.2

		Time (days)				
Compound (molecular weight)	**Bp**	**0**	**3**	**6**	**9**	**12**
6,9-octadecadien-4-olide (278)	79	1.6 ± 0.3	1.6 ± 0.2	1.6 ± 0.2	2.5 ± 0.5	14.1 ± 3.9
γ-stearolactone (282)	85	-	-	0.5 ± 0.2	1.4 ± 0.2	15.9 ± 4.3
9-octadecen-4-olide (280)	67	-	1.9 ± 0.0	2.3 ± 0.6	3.6 ± 0.2	22.5 ± 6.3
δ-oleolactone (280)	99	0.8 ± 0.2	0.7 ± 0.1	-	-	-
δ-stearolactone (282)	99	-	-	0.4 ± 0.2	0.7 ± 0.1	15.4 ± 1.7
γ-eicosalactone (310)	85	-	-	-	-	5.9 ± 2.0
δ-eicosalactone (310)	99	0.5 ± 0.2	0.6 ± 0.0	0.6 ± 0.0	0.9 ± 0.0	4.4 ± 1.1

*Asterisked compounds were acquired commercially and used as standards for identification purposes; -: no detected.

Table S3. Glycidyl esters detected in corn oil submitted to oxidative conditions during different periods of time and their abundances, expressed as area counts of their mass spectra base peak (Bp) multiplied by 10^{-5}, obtained as average of two determinations together with their standard deviations.

		Time (days)				
Glycidyl esters (molecular weight)	**Bp**	**0**	**3**	**6**	**9**	**12**
Glycidyl palmitate (312)*	98	1.2 ± 0.2	1.2 ± 0.3	1.6 ± 0.0	1.8 ± 0.3	-
Glycidyl linoleate (336)*	67	3.1 ± 0.3	3.6 ± 0.6	4.2 ± 0.0	4.6 ± 0.5	-
Glycidyl oleate (338)*	55	2.6 ± 0.4	3.0 ± 0.4	3.5 ± 0.1	3.8 ± 0.1	-

*Asterisked compounds were acquired commercially and used as standards for identification purposes; -: no detected.

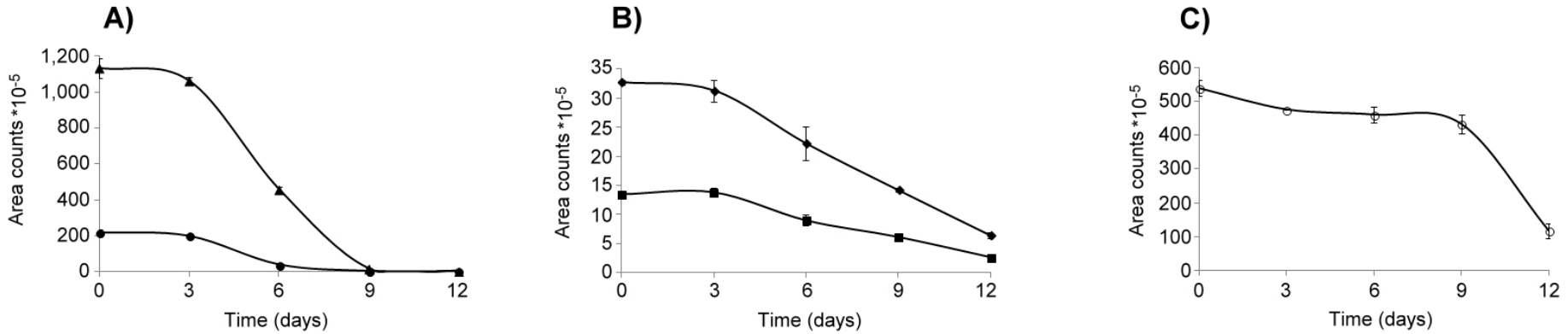

Figure S1. Evolution of the abundances, expressed as area counts of their mass spectra base peak multiplied by 10^{-5}, of some tocopherols, sterols and squalene present in corn oil submitted to oxidative conditions, *versus* time given in days. **(A)** γ- (▲) and α- (●) tocopherols, **(B)** β-sitosterol (♦) and campesterol (■), **(C)** squalene (○).

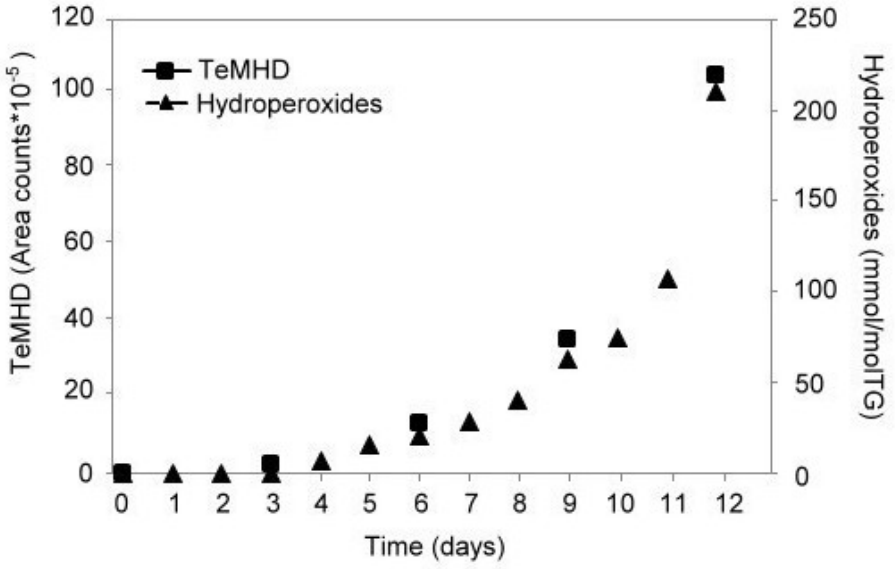

Figure S2. Evolution of the abundance of TeMHD (■, expressed as area counts of their mass spectra base peak multiplied by $10^{-5;}$ data given in Table 2) and of the concentration of Hydroperoxides (▲, expressed as mmol/mol triglyceride, determined from the ^{1}HNMR spectral data, as in previous studies (Martínez-Yusta, A., Goicoechea, E., & Guillén, M. D. (2014). ***Comprehensive Reviews in Food Science and Food Safety***, *13*, 838-859; Martin-Rubio, A. S., Sopelana, P., Ibargoitia, M. L., & Guillén, M. D. (2018). ***Food Chemistry***, 245, 312-323) in corn oil submitted for different periods of time (given in days) to oxidative conditions.

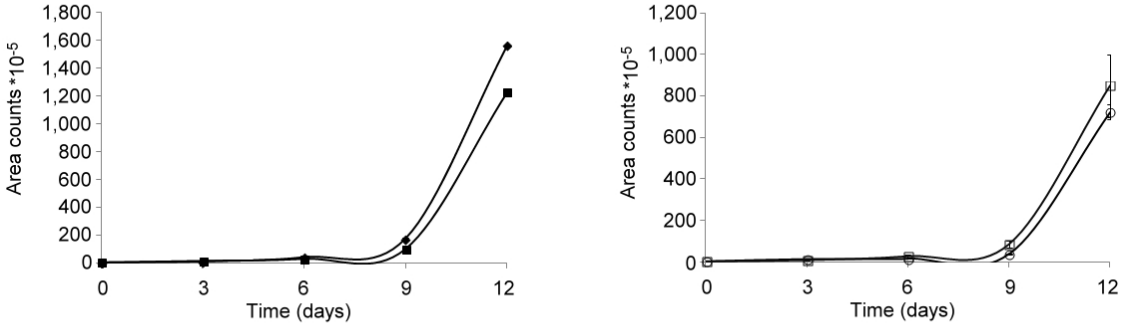

Figure S3. Evolution of the abundances, expressed as area counts of their mass spectra base peak multiplied by 10^{-5}, of some oxidation compounds derived from corn oil main components *versus* time, given in days, under oxidative conditions. 4-hydroxy-(*E*)-2-nonenal (■), 4,5-epoxy-(*E*)-2-decenal, (♦) 2-pentylfuran (○) and 5-pentyl-2(5H)-furanone (□).

Manuscript 4

TOXIC OXYLIPINS ASSOCIATED TO CURRENT DISEASES DETECTED FOR THE FIRST TIME IN AN EDIBLE OIL RICH IN LINOLEIC ACYL GROUPS SUBMITTED TO OXIDATIVE CONDITIONS. A GLOBAL, BROAD AND IN-DEPTH STUDY BY ^{1}H NMR SPECTROSCOPY

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ABSTRACT

For the first time an important number of oxylipins have been identified and quantified in an oil, rich in omega-6 acyl groups, submitted to mild oxidative conditions at each time of their oxidation process. The study was carried out using ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR), which does not requires chemical modification of the sample. These newly detected oxylipins, which can also be formed in cells, include dihydroperoxy-non-conjugated-dienes, hydroperoxy-epoxy-, hydroxy-epoxy- and keto-epoxy-monoenes as well as *E*-epoxy-monoenes, some of which have been associated to several diseases. Furthermore, the formation of other functional groups such as poly-formates, poly-hydroxy and poly-ether groups has also been proved for the first time. These are responsible for the increased viscosity of the oil as a consequence of its oxidation and polymerization. Simultaneously, monitoring of the formation of well-known oxylipins, such as hydroperoxy-, hydroxy-, and keto-dienes, and of different kinds of oxygenated-*alpha,beta*-unsaturated aldehydes such as 4-hydroperoxy-, 4-hydroxy-, 4-oxo-2*E*-nonenal and 4,5-epoxy-2*E*-decenal which are also related to different degenerative diseases, has been carried out. The data provided about the compounds identified constitute valuable information for other researchers in future studies in which lipid oxidation is involved both in the food and other scientific fields.

KEYWORDS: corn oil; linoleic acyl groups; oxidation; oxylipins; hydroproxy-, hydroxy-, keto-, epoxy-derivatives; aldehydes oxygenated *alpha,beta*-unsaturated; poly-formates; poly-hydroxy; poly-ethers; ^{1}H NMR.

1. INTRODUCTION

Lipid oxidation is one of the main degradative process occurring in foods in general and in oil and fats in particular, with serious repercussions for food shelf-life, nutrition and health. Oils in food processing are submitted to very varied conditions, in both industrial and culinary processes, during which their main and minor components might be degraded [1-14]. Likewise, some studies have also suggested that during gastrointestinal digestion oxidation reactions take place, in which lipidic components undergo degradation [15-21]. Furthermore, it is well known that inside humans many endogenous processes take place at cellular level in which lipid oxidation occurs [22-24].

Many factors influence lipid oxidation, among which can be cited: unsaturation degree of lipids, oxygen concentration in the system, temperature, light irradiation and the presence and concentration of minor compounds or molecular species able to exhibit either antioxidant or prooxidant activities [9,13,24,25]. In addition to the many influencing factors, the lipid oxidation reaction itself is a very complex process, which triggers a set of reactions, some simultaneous others successive [26], giving rise to the formation of a large number of compounds of different nature some of which remain still today unidentified. The great number of influencing factors and the numerous simultaneous and successive reactions involved have made it very difficult to know in depth the predominant mechanisms through which lipid oxidation evolves in each system [26]. Due to the great importance of this degrading process, not only because it produces the deterioration of food but also because it generates toxic compounds [27-32] it has been the subject of many studies in both food and biological systems. However, in spite of this, many aspects of this highly complex reaction, such as the mechanisms through which it evolves, the identity and concentration of many of the compounds formed as well as the evolution of some of their minor components are not known nowadays.

Among lipidic foods, edible oils can be considered very appropriate models to study the above-mentioned subjects concerning lipid oxidation. They are of primordial importance for the food industry from technological, economical, nutritional and safety points of view and the study of their oxidation processes can help to enter in depth into the oxidation that takes place in biological systems. Many of the studies regarding lipid oxidation in foods have been carried out by using classical methods that require chemical modification of the sample, and provide very limited information about the identity of compounds whose functional groups are measured. Among these, both peroxide value (PV) as well as conjugated dienes (CD) to evaluate the occurrence of primary oxidation compounds, and thiobarbituric acid reactive substances (TBARS) test to evaluate the occurrence of secondary oxidation products, are widely used. These methods only give information about certain compounds or functional groups, and do not provide information about the specific nature of the compounds involved in each determination, which sometimes could give rise to inaccurate conclusions [33,34].

Furthermore, the study of the oxidation of edible oils has also been tackled by means of chromatographic techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC), coupled to different detectors. With these techniques, the study of volatile and non-volatile compounds, even of dimers, oligomers and polymers formed during different thermo-oxidative processes [13,35-40] has been carried out.

In addition, the use of ^{1}H NMR spectroscopy to these ends has been gaining prominence in the last twenty years. From the early studies in which its usefulness to characterize edible oils and to quantify their unsaturation degree and their molar percentage of the different kinds of acyl groups [41,42] was proved, notable advances have been made in the study of edible oil oxidation processes. These advances refer not only to the evolution of the edible oil oxidation process under different degradative conditions but also to the identification and quantification of new oxidation compounds many of which are very relevant due to their

negative bioactive properties [5-9,12,29,30,38,43-45]. Despite these important advances, there are still many unknown aspects related to the complex process of oxidation of edible oils that can be discovered and understood using this technique.

In this context, the aim of this study is to analyze in a global, broad and in-depth way, by means of ^{1}H NMR spectroscopy, the evolution of corn oil oxidation when it is submitted to mild oxidative conditions, similar to those of accelerated storage. Attention will be paid to the degradation rate not only of their main but also of their minor components. Moreover, simultaneously, the course of the oxidation will be addressed through the identification and formation rate of newly formed oxidation compounds as well as the degradation of some of these to give rise to secondary or further oxidation compounds. It is to be expected that new information could be extracted, that will provide new insights into the oxidation of corn oil, which could be applied to the oxidation process of edible oils in general and also of biological systems. In addition, information provided in previous studies on oxidation of pure compounds, and also on certain processes applied to vegetable oils will be taken into account. The interest of the methodology used lies in that it does not involve chemical modification of the sample and in just one run allows monitoring of a great number of compounds involved in the process, in a very short period of time, providing information about their concentration at any step of the process.

2. MATERIALS AND METHODS

2.1. Samples subject of study

2.1.1. Original oil

The study was carried out with refined corn oil, purchased in a local supermarket. Its composition in acyl groups was determined from ^{1}H NMR spectral data as in previous studies

[42,44,46]. The molar percentages of the different kinds of acyl groups, regarding the total of acyl groups, were linolenic group, Ln, 0.6 ± 0.0%, linoleic group, L, 48.7 ± 0.0%, oleic group, O, 33.0 ± 0.1%, and saturated group, S, 17.7 ± 0.1%. These compositional data can be also expressed in mmol regarding to the number of moles of triglyceride, TG; in this way this oil contains, 18.8 ± 0.0 mmoles of Ln/molTG, 1,461.6 ± 0.1 mmoles of L/molTG, 990.2 ± 1.7 mmoles of O/molTG and 529.4 ± 1.5 mmoles of S/molTG.

2.1.2. Oxidized oil samples

In addition to the original oil, samples derived from this oil, after their submission to mild oxidative conditions, similar to those of accelerated storage for different periods of time, were also subject of study. To prepare these derived samples, amounts of 10 g of original corn oil were placed in glass Petri dishes (80 mm in diameter and 15 mm deep) and kept in an oven at 70°C with aeration, over different periods of time, of up to sixteen days. Under these conditions, the progress of the oxidative process in each sample increased in line with the time during which each sample was kept under oxidative conditions, thus allowing a continuous view of the process from its beginning to the total polymerization of the sample. These experiments were performed in duplicate to obtain sound results.

2.2. Acquisition of the ^{1}H Nuclear Magnetic Resonance spectra (^{1}H NMR)

2.2.1. Operating conditions

The ^{1}H NMR spectra of the original oil and of the samples derived from this oil, after being subjected to degradative conditions, were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. For this purpose, the above-mentioned samples (approximately 0.16 g) were dissolved in 400 µL of deuterated chloroform, which contained tetramethylsilane (TMS), as internal reference (Cortec, Paris, France). The acquisition conditions were the same as those used in previous studies [3,47]. The relaxation delays and

acquisition times allow the complete relaxation of the protons, the areas of the signals thus being proportional to the number of protons that generate them, making their use for quantitative purposes possible. The ^{1}H NMR spectra were plotted at a fixed value of absolute intensity to be valid for comparative purposes using the MestreNova program (Mestrelab Research, Santiago de Compostela, Spain).

2.2.2. Identification of components

Identification, both of components present in the original oil and of components generated in the oxidation process, was carried out on the basis of the assignments of the ^{1}H NMR signals to the different kinds of hydrogen atoms and to the different compounds. These signals, which are shown in different figures, their chemical shifts and their assignments to the several hydrogen atoms are given in Tables S1-S8 (see Supplementary material). The assignments were made taking into account previous studies as indicated in Tables S1-S8 (Supplementary material), or on the basis of the signals of standard compounds acquired for this study. Among these latter are: Δ5-avenasterol, sitostanol, acquired from ChemFaces Biochemical Co., LTD (Wuhan, China), β-sitosterol, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-decenal, (*E*,*E*)-2,4-hexadienal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4decadienal, 4,5-epoxy-(*E*)-2-decenal, 12,13-epoxy-9(*Z*)-octadecenoic acid methyl ester (isoleukotoxin methyl ester), 2-pentylfuran, 2-ethylfuran, amylformate and octylformate acquired from Sigma–Aldrich (St. Louis, MO, USA); 9,10-Epoxy-12-*Z*-octadecenoic acid (leukotxin), 4-hydroxy-(*E*)-2-nonenal, 4-hydroperoxy-(*E*)-2-nonenal, 4-oxo-(*E*)-2-nonenal, 9,10-dihydroxy-12-(*Z*)-octadecenoic acid (leukotoxin diol), 12,13-dihydroxy-9-(*Z*)-octadecenoic acid (isoleukotoxin diol), *trans*-12,13-epoxy-9-keto-10(*E*)-octadecenoic acid, 9-keto-10(*E*),12(*E*)-octadecadienoic acid, linolein hydroperoxides, linolein hydroxides, 9-oxo-10*E*,12*Z*-octadecadienoic acid, 13-oxo-9*Z*,11*E*-octadecadienoic acid and 12R-hydroxy-9(*Z*)-octadecenoic acid methyl ester (ricinoleic acid methyl ester), purchased from Cayman

Chemical (Ann Arbor, MI, USA), Δ5-campesterol, Δ7-avenasterol, 9(S)-Hydroxy-10(*E*),12(*E*)-octadecadienoic acid (Dimorphecolic acid), Methyl 9(S),10(R)-epoxy-13(S)-hydroxy-11-(*E*)-octadecenoate, 9(S),10(S)-epoxy-11(S)-hydroxy12-(*Z*)-octadecenoic acid methyl ester, 11(S),12(S)-epoxy-13(S)-hydroxy-9(*Z*)-octadecenoic acid methyl ester, 9-hydroxy-10-oxo-12(*Z*)-octadecenoic acid; 9,10-12,13-diepoxyoctadecanoic acid and 13-hydroxy-12-oxo-9(*Z*)-octadecenoic acid acquired from Larodan (Malmö, Sweeden).

2.2.3. Quantification of the components

This was possible because the area of each ^{1}H NMR spectral signal is proportional to the number of protons that generates it, and because the proportionality constant is the same for all kinds of protons. Taking this into account, the estimation of the concentrations of the different functional groups, or of groups of compounds, as well as of minor components, regarding the concentration of triglycerides TG can be carried out, by using the area of the corresponding spectral signals. Triglycerides can be considered as an internal reference due the low level of hydrolysis that these undergo during oxidation. To this aim, the equation, $[X] = [(A_X/n)/(A_{TG}/4)]*1000$ [eq. 1] was used. In this equation A_X is the area of the signal selected for the quantification of the X functional group, n is the number of protons that generate this signal and A_{TG} the area of the protons at *sn*-1 and *sn*-3 positions in the triglyceride backbone TG (signal TG in Table S1). In this way the concentration obtained is expressed in millimole per mol of triglyceride (mmol/mol TG). The area of the signals used was determined by using the equipment software and the integrations were made three times to obtain average values.

2.3. Statistical analysis

Data represented in the different figures and those given in Table S9 (Supplementary Material) are mean values obtained as average values of at least two determinations.

Microsoft Office Excel 2007 was used for the statistical analysis and for the graphical representation of the obtained values.

3. RESULTS

As mentioned before, this methodology allows one to monitor, throughout all the oxidation process, the degradation rate of the original main oil components and also of minor ones. Furthermore, and simultaneously, this methodology permits the detection of new compounds formed as consequence of the degradation of the former. In this study the identification of the newly formed compounds will be addressed as will the estimation of their concentration and evolution during the oxidation process, with the aim of obtaining as complete and integrated as possible a picture of the whole oxidation process of this oil.

3.1. Evolution of original oil components.

3.1.1. Corn oil main components.

As is well known, the main edible oil components are triglycerides supporting acyl groups having different unsaturation degrees and chain lengths. In corn oil the main acyl group is *linoleic*, as already indicated. For this reason and due to its unsaturation degree, this acyl group can be considered an appropriate representative for the study of the degradation rate of corn oil main components. The hydrogen atoms of this acyl group, as Table S1 and Figures 1a and 1b show, give several ^{1}H NMR spectral signals some of them specific to its methylic, *mono*-allylic and *bis*-allylic protons (in Figure 1b, signals A2, E2, and G respectively). As may be expected, the intensity of these signals decreases as the oxidation process advances (see Figure 1b) and from the area of signal G the evolution of the concentration of linoleic acyl group (or linoleate group) at different times under degradative conditions can be estimated by using [eq. 1]. This evolution has been represented *versus* time in Figure 1b´. It

can be observed that after 16 days under degradative conditions, the corn oil has reached a very high polymerization degree, and the linoleic group is in a very low concentration (near 291.2 ± 29.5 mmol/molTG).

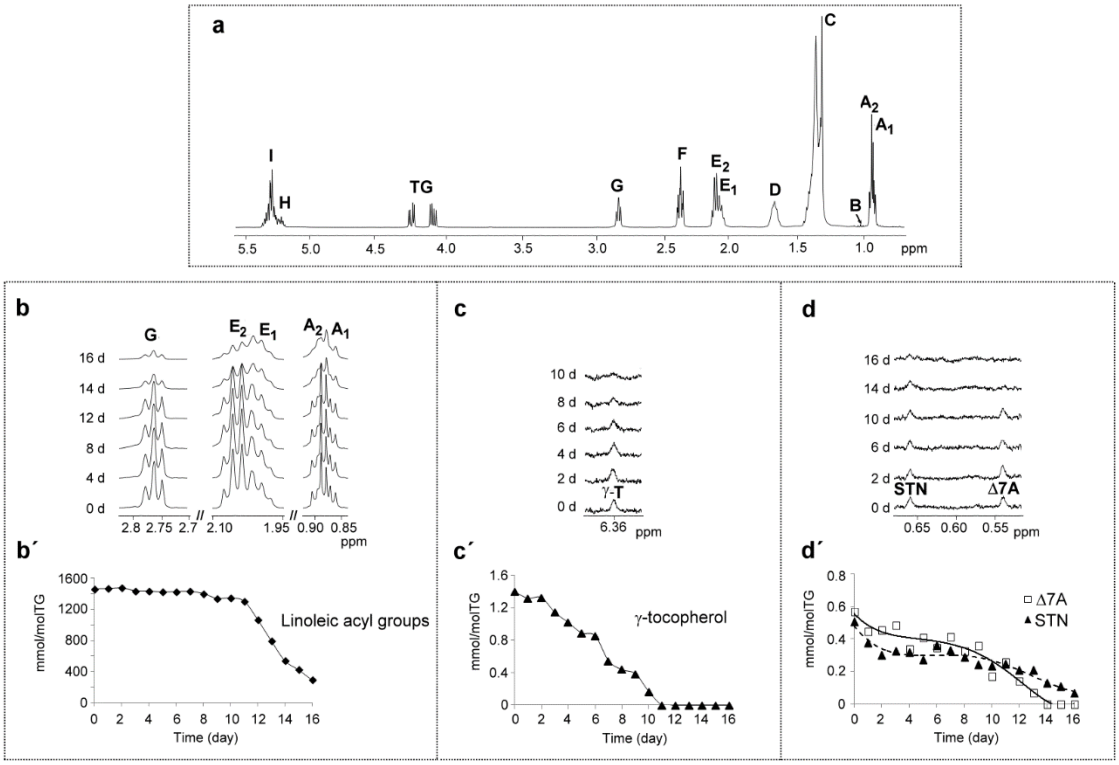

Figure 1. a) Region between 0.5 and 5.5 ppm of corn oil, C, ^{1}H NMR spectrum. Spectral regions, conveniently enlarged, of the signals of: **b)** methylic, *mono*-allylic and *bis*-allylic; **c)** *gamma*-tocopherol (γ-T); **d)** Δ7-avenasterol (Δ7A) and sitostanol (STN). The signal letters agree with those of Table S1 of Supplementary Material. Evolution of the concentration, expressed as mmol/molTG, present in corn oil submitted to oxidation process, *versus* time given in days of: **b´)** linoleic acyl groups; **c´)** *gamma*-tocopherol (γ-T); **d´)** Δ7-avenasterol (Δ7A) and sitostanol (STN).

3.1.2. Corn oil minor components.

As is well known, corn oil has different kinds of minor components [48,49]. Among them *tocopherols* and *sterols-stanols* are important because they have been attributed antioxidant activity and also because their concentrations are higher than those of other minor corn oil components.

a) Regarding tocopherols.

It is known that corn oil contains *α-, β-, γ-* and *δ*-tocopherol [48]. Two conditions are required for their study to be carried out by ^{1}H NMR: that their spectra have at least one signal that does not overlap with any other; and that their concentrations in the oil are high enough to be able to be detected by ^{1}H NMR spectroscopy. Among the tocopherols present in this corn oil only *γ-tocopherol* meets these two required conditions. The ^{1}H NMR spectrum of this compound has a signal of its aromatic proton [50] (see signal γT at 6.360 ppm in Figure 1c and in Table S1) that not overlaps with any other. It can be observed in Figure 1c that the intensity of this signal decreases during the oxidation process until its total disappearance. The concentration of this compound, determined by using [eq. 1], is represented *versus* time in Figure 1c´. It can be observed that the degradation of *γ-tocopherol* throughout this oxidative process has two stages, which is in agreement with observations from in a previous study carried out by direct immersion-solid phase microextraction followed by gas chromatography / mass spectrometry (DI-SPME-GC/MS) [13]. In the first stage (days 0-2) the concentration of this compound, initially near 1.4 ± 0.0 mmol/mol TG, remains almost unchanged, its rate of degradation being near to 0.04 mmol/mol TG per day. However, in a second stage, from day 2 to 11, a significant decrease takes place with an estimated degradation rate near 0.14 mmol/mol TG per day. On day 11, its presence cannot be detected by ^{1}H NMR, for which reason it could be said that it has practically disappeared from the oil. These results suggest that in the period of time in which there is *γ-tocopherol* in the oil sample, the degradation rate of linoleic group is low (near 14 mmol/mol TG per day) and when this compound disappears this rate increases very sharply. However, it must be pointed out that the presence of *γ-tocopherol* in the oil does not prevent the degradation of either the linoleic group or indeed of the corn oil, although its presence slows down its degradation.

b) Regarding sterols-stanols.

It is well known that corn oil can contain at least brassicasterol, Δ5-stigmasterol, β-sitosterol, Δ5-campesterol, sitostanol and Δ7-avenasterol [48]. Among all these compounds, only sitostanol (STN) and Δ7-avenasterol (Δ7A) have, at least, a ^{1}H NMR signal that does not overlaps with any other (see Figure 1d and Table S1) and are in enough concentration to be detected by ^{1}H NMR. This signal, which is centered at 0.651 ppm in the case of STN [51] and at 0.540 ppm in the case of Δ7A, [51-53] (see Figure 1d and Table S1), is due in both cases to their methylic protons on the C18. It can be observed in Figure 1d that the intensity of these signals remains almost unchanged up to the last days of the experiment. The concentration of these compounds at different times under the degradative conditions was determined also by using [eq. 1] and is depicted in Figure 1d´. The results indicate that these compounds, which are in much lower concentration than γ-tocopherol, have a higher oxidative stability than the latter, and as a consequence they show a very small degradation rate. This increases somewhat from day 9 onwards although both compounds are present in the sample up to days 13 (Δ7A) and 16 (STN) respectively. The higher oxidative stability of STN regarding Δ7A could be attributed to its lower unsaturation degree. In summary, at the end of this experiment the only totally undegraded minor corn oil component of the two here studied is STN even though its concentration has been significantly reduced. These results are in general agreement with those observed in this kind of compounds in a previous study using a very different technique [13].

3.2. Formation of new compounds.

As is well known, alongside the degradation of original oil main and minor components, new derived compounds are formed. In this study, attention is first paid to the formation of compounds derived from main components, and secondly to some compounds derived from

oil minor components. Tables S2-S8 give the ^{1}H NMR signals found in the spectra of corn oil at different times under degradative conditions, their chemical shifts and multiplicities and their assignment to protons of different functional groups, structures supporting several functional groups or compounds that will be commented on below. Likewise, the quantification of the different functional groups, or of structures supporting several functional groups will be made in all cases by using [eq. 1].

3.2.1. Compounds derived from corn oil main components.

The degradation of oil main components gives rise to the formation of so-called primary oxidation compounds and the evolution of these latter gives rise to the formation of so-called secondary oxidation compounds and so on. ^{1}H NMR spectroscopy allows one to detect them, to estimate their concentration and to follow their evolution through the oxidation process.

(a) ***Monohydroperoxides (mHPOs).***

These are the first compounds detected by ^{1}H NMR spectroscopy as being new compounds formed by from the degradation of corn oil main components under these conditions. As above mentioned, the concentration of linolenic groups in this oil is very small, almost negligible, linoleic being the main unsaturated acyl group in this oil. For this reason, and taking into account the low oxidative stability of linoleic group, the first main hydroperoxides detected should come from this latter acyl group. Indeed the first structures detected as new, in this corn oil submitted to oxidative conditions, are monohydroperoxy-conjugated dienes, mHPO-c-dEs, derived from linoleic group; the well known 9- and 13-hydroperoxy-*Z*,*E*-conjugated dienes (mHPO-c(*Z*,*E*)-dEs) as well as 9- and 13-hydroperoxy-*E*,*E*-conjugated dienes (mHPO-c(*E*,*E*)-dEs) (see Figure 2 and Table S2). They can be easily identified and quantified from ^{1}H NMR spectral data. Their identification is based on the signals of their

conjugated olefinic protons and of the corresponding OOH group proton, which are shown in Figure 2a. Figure 2b shows the evolution of these spectral signals in the corn oil at different times. Their concentration can be determined separately over time, using the area of their signals centered at 6.55 ppm in the case of *Z,E*-isomers and at 6.24 ppm in the case of *E,E*-isomers. Figure 2c shows the evolution of their concentrations over time. It can be observed that from day 4 to day 8 the concentration of the *Z,E*-isomers is somewhat higher than that of the *E,E*-isomers and the opposite happens from day 8 onwards. Both kinds of isomers reached the maximum concentration near day 13 (*E,E*-isomers near 140.9 ± 3.4 mmol/molTG and *Z,E*-isomers near 48.5 ± 2.4 mmol/molTG). After this maximum, the concentration of these compounds decreased very sharply until day 16, *E,E*- and *Z,E*-isomers reaching 37.9 ± 3.7 and 12.3 ± 3.0 mmol/molTG, respectively. This diminution evidences their role as intermediate compounds.

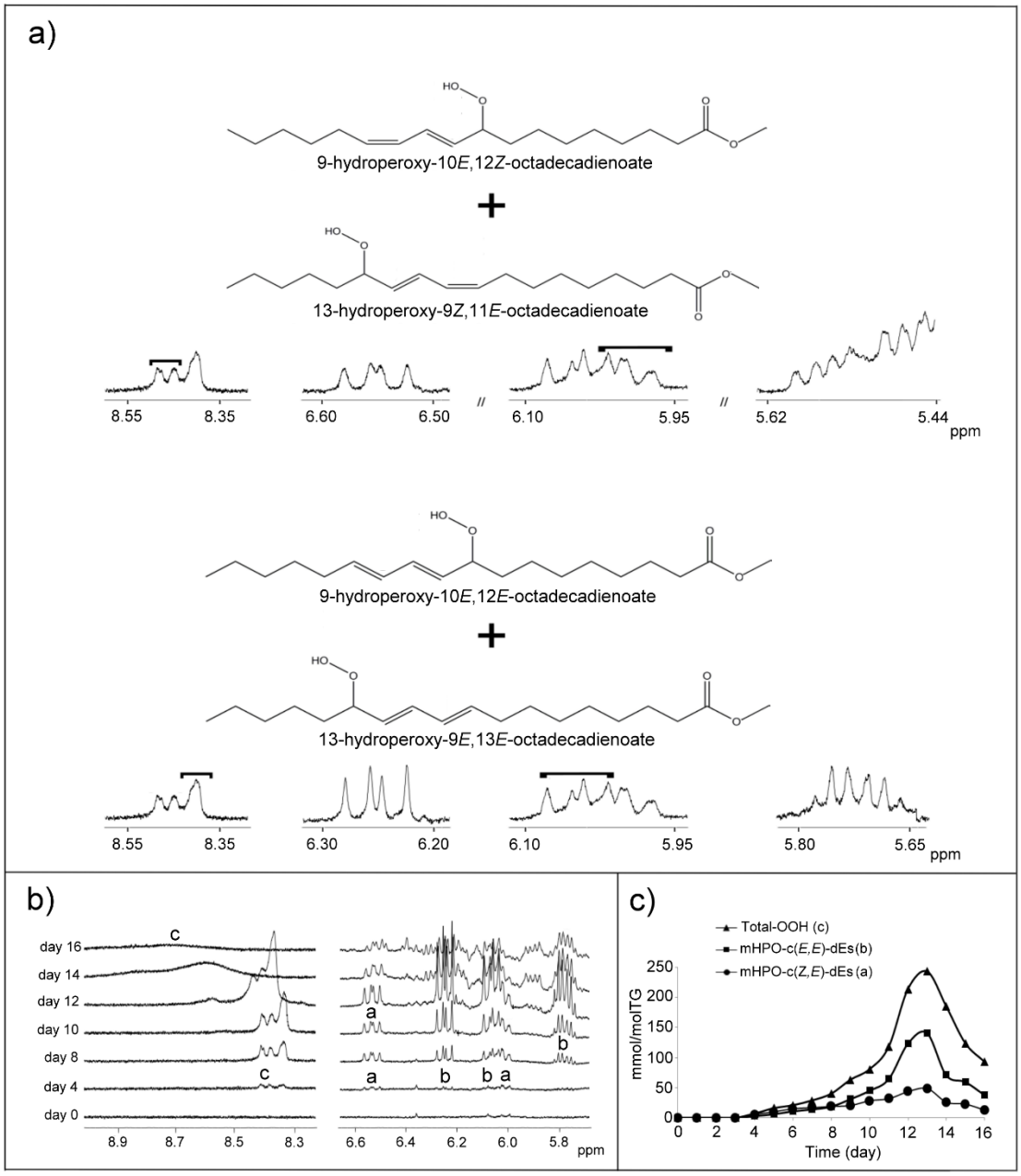

Figure 2. a) Chemical structure of the *monohydroperoxy-conjugated dienes* (mHPO-c-dEs) detected in corn oil submitted to oxidative conditions, together with the enlargement of some regions of the ^{1}H NMR spectra in which the signals of *monohydroperoxy-conjugated dienes* appear. **b)** Enlargements of some spectral regions between 5.8 and 6.6 ppm and 8.3 and 8.9 ppm, where changes occur throughout the oxidation process and their evolution with time. **c)** Evolution of the concentration of mHPO-c(*Z*,*E*)-dEs, mHPO-c(*E*,*E*)-dEs and Total-OOH, expressed as mmol/molTG *versus* time given in days.

Furthermore, from the beginning of the process up to day 8 the total concentration of hydroperoxy groups (Total-OOH), determined from the area of the signal of the OOH protons

(between 8.38 and 8.52 ppm), was coincident with the sum of the mHPO-c-dEs above mentioned. However, from day 9 onwards, new signals of hydroperoxy protons appear clearly in the spectrum and as consequence the concentration of total hydroperoxy groups is higher than the sum of the above-cited mHPO-c-dEs (see Figures 2b and 2c). The new signals appear at higher and also at lower ppm values than those of the OOH protons of the aforementioned *Z,E*- and *E,E*-isomers. All these signals, in advanced oxidation stages, become broader and appear from 8.3 to 9.3 ppm, and they can be attributed, in addition to the above-mentioned conjugated isomers, to other compounds also supporting hydroperoxy groups.

In this context, it could be thought that monohydroperoxy-non conjugated dienes, mHPO-nc-dEs could also be formed in this process. In fact, the formation of mHPO-nc-dEs supporting the hydroperoxy group in carbon atoms numbers 8 or 14, maintaining the double bonds (9*Z*,12*Z*) of linoleic group (8-hydroperoxy-9*Z*,12*Z*-octadecadienoate and 14-hydroperoxy-9*Z*,12*Z*-octadecadienoate) has been described in autoxidation processes of linoleate, although in very low concentrations (Figure S1a) [54]. It should be noted that although ^{1}H NMR spectral data of these mHPO-nc-dEs have not been given, they maintain the *bis*-allylic protons of the original linoleic group and the chemical shifts of their spectral signals should be very similar to those of the original linoleic group. The proportion of these compounds in autoxidation processes has been estimated as about 1% of the total of mHPO formed [55]. For this reason, their detection, if they are formed under the conditions of this study, is very difficult, if not impossible by the technique here used.

In addition, other mHPO-nc-dEs such as 10-hydroperoxy-8*E*,12*Z*-octadecadienoate and 12-hydroperoxy-9*E*,13*Z*-octadecadienoate (Figure S1a) have been reported as forming from linoleate groups only under photoinduced oxidative conditions [56], for which reason they

could not be expected to form in the process here studied. This is in agreement with the absence in the spectra of the samples subject of study of proton signals of the hydroperoxy groups near 7.8-7.9 ppm described for these kind of compounds [56] and is the reason for which their formation can be discarded in the process here studied.

Finally, the mHPO-nc-dE, 11-hydroperoxy-9Z,12Z-octadecadienoate (Figure S1a) has been described in the oxidation of linoleate groups, together with 9- and 13-mHPO-c-dEs, only when the oxidation occurs in presence of *alpha-tocopherol* [57], for which reason their occurrence should not be expected in this study.

(b) *Dihydroperoxides (dHPOs).*

In addition to mHPO-dEs, dihydroperoxydienes, dHPO-dEs, either conjugated (dHPO-c-dEs) and/or non conjugated (dHPO-nc-dEs) could also be formed by subsequent oxidation of mHPO-dEs. In fact, both kinds of dHPOs have been described as intermediate compounds in several formation pathways of aldehydes [58,59].

Regarding dihydroperoxy-non conjugated dienes dHPO-nc-dEs, some such as 9,12-dihydroperoxy-10*E*,13*E*-octadecadienoate (9,12-dHPO-10*E*,13*E*-dE) and 10,13-dihydroperoxy-8*E*,11*E*-octadecadienoate (10,13-dHPO-8*E*,11*E*-dE) have been described [60] (see Figure 3a). Among their ^{1}H NMR signals given in the literature [60] there is a double doublet near 4.82 ppm (see Figure 3b) of one of the methine carbinol protons, being the signals of the rest of protons overlapping with those of other oxidation compounds. This signal appears in the spectra of the oil studied here from day 9 onwards and reaches its maximum intensity on day 13 like the mHPO-c-dEs, after which their intensity decreases, thus indicating their role as intermediate compounds. The estimated concentration of these potential dHPO-nc-dEs on day thirteen is near 19.2 ± 0.2 mmol/molTG (see Figure 3b).

These compounds have been postulated as intermediate steps in the formation of different compounds among which are certain dioxo derivatives [60]. As far as we know, this is the first time that dihydroperoxy-non conjugated dienes have been detected and quantified in an edible oil submitted to oxidative conditions.

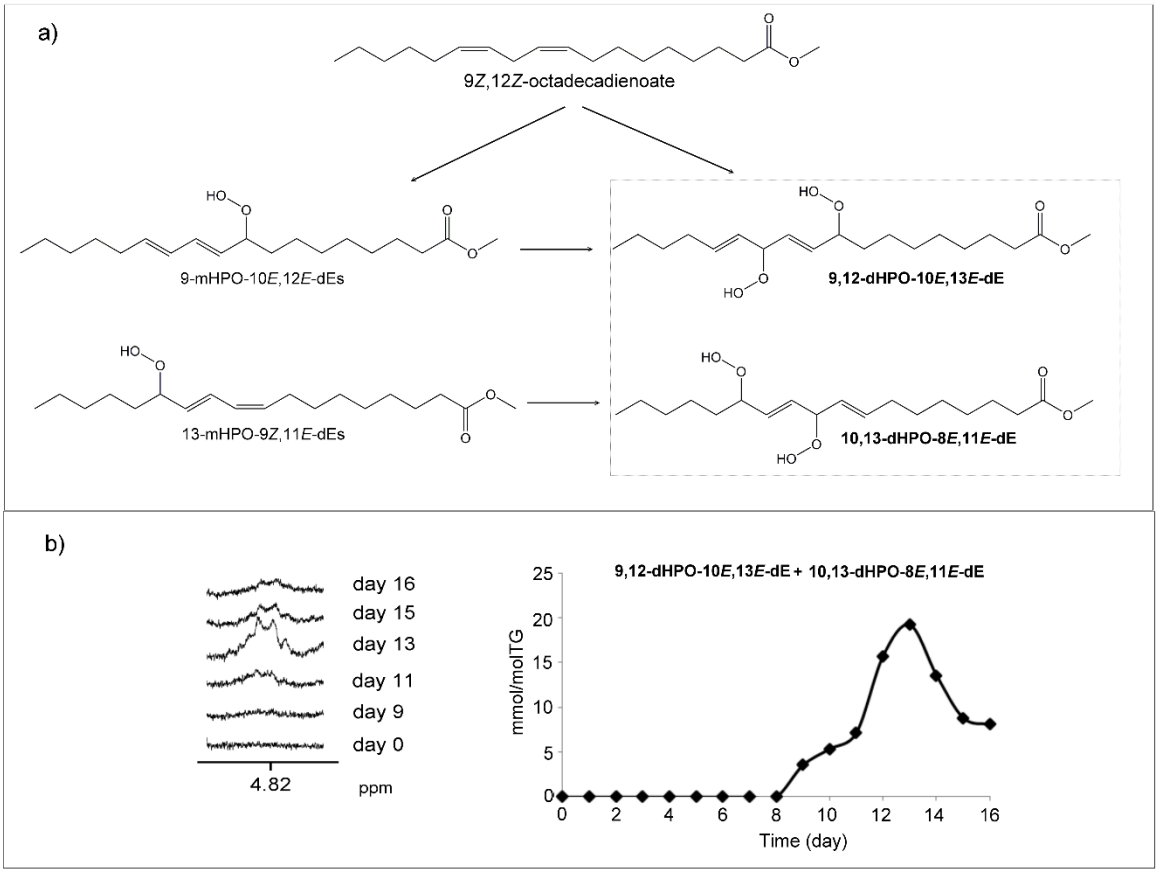

Figure 3. a) Pathways of formation of *dihydroperoxy-non conjugated dienes* (dHPO-nc-dEs) proposed by some authors under certain oxidation conditions [56,60]. **b)** Enlargements of the spectral region where changes occur throughout the accelerated storage process and their evolution with time, together with the graphical representation of the evolution of the concentration of 9,12-dHPO-10*E*,13*E*-dE + 10,13-dHPO-8*E*,11*E*-dE, expressed as mmol/molTG, *versus* time given in days.

In addition to the above mentioned dHPO-nc-dEs, others could be formed by oxidation of mHPO-c-dEs coming from linoleate. It has been described that one of them, 10,12-dihydroperoxy-8*E*,13*E*-octadecadienoate (10,12-dHPO-8*E*,13*E*-dE) (Figure S1b), gives

signals in the ^{1}H NMR spectrum [56] near 8.05 and 8.13 ppm attributable to protons of the OOH groups. In the spectra of the oil here studied, from day 12 onwards signals appear overlapping with other ones included in a very broad signal between 8.00 and 8.17 ppm, for which reason it could be thought that these hydroperoxides could be present in this corn oil. However, these signals are not due to hydroperoxy groups because they do not disappear in the spectra when deuterated water is added, making it evident that these hydrogen atoms are not exchanged for deuterium as occurs in hydroperoxy groups. Furthermore, other signals of this structure such as a multiplet at 5.83 ppm and a double doublet near 4.45 ppm are not clearly observed in the spectra. For all these reasons, the formation of this dHPO-nc-dE in this oxidation process cannot be guaranteed.

Likewise, the formation of *dihydroperoxy-conjugated dienes*, dHPO-c-dEs, could be possible [61]. 8,13-dihydroperoxy-10*E*,12*E*-octadecadienoate (8,13-dHPO-10*E*,12*E*-dE) and 9,14-dihydroperoxy-11*E*,13*E*-octadecadienoate (9,14-dHPO-11*E*,13*E*-dE) (see Figure S1b) coming from mHPO-c-dEs of linoleic groups has been described as possible, under induced oxidation conditions. Some of these compounds have also been proposed as intermediates in the formation of toxic aldehydes, some of which are formed in the oxidation of corn oil, which will be discussed later. However, the ^{1}H NMR signals of specific protons of these compounds provided by some authors [61] are not clearly distinguishable (8.25-7.82 (br, s), 4.87 (m), 4.57 (m), 4.40 (m), 4.36 (m), 4.30 (m) ppms) in the spectra of the samples here studied. This indicates that if they are formed the concentration is not enough as to be detected by the technique here used.

Closely related with the above compounds are those having both hydroperoxy and peroxy cyclic groups of six members, also named hydroperoxy-epidioxy-monoene compounds (mHPO-EPIdO-mE). These could be formed in this oxidation process from linoleic group.

Some of them, such as 13-hydroperoxy-9,12-epidioxy-10-octadecenoate (13-HPO-9,12-EPIdO-10-octadecenoate) and 9-hydroperoxy-10,13-epidioxy-11-octadecenoate (9-HPO-10,13-EPIdO-11-octadecenoate) (see Figure S1c), whose ^{1}H NMR signals have been described [61], either are absent in the samples subject of study or are in very small concentrations. This is deduced because although signals near 4.61 and 4.66 ppm could be present overlapped with other ones in the spectra, the other signals due to the proton bonded to the carbon atom supporting the hydroperoxy group centered at 4.14-4.12 ppm, and 4.17 ppm respectively, are not clearly visible in the spectra of this oil submitted to mild degradative conditions.

(c) *Hydroperoxy-epoxy-monoenes (HPO-EPO-mEs).*

Other compounds which may be formed as a consequence of the oxidation of mHPO-c-dE are hydroperoxy-epoxy-monoenes (HPO-EPO-mEs) (see Figure 4a). Studies about the formation of this kind of compounds in the oxidation of linoleic group [62] and of the analysis of their ^{1}H NMR spectral signals are very scarce. In fact, to the best of our knowledge, only the ^{1}H NMR signals of 9-HPO-12,13-*E*-EPO-10*E*-octadecenoate have been reported previously [62]. As Figure 4a and Table S2 show, signals of epoxydic protons (3.11 and 2.84 ppm), of olefinic proton in position *alpha* to the C-OOH group (double doublet at 5.85 ppm), and of hydroperoxy protons near 8.6-8.8 ppm are typical of the HPO-EPO-mE above mentioned [62]. All these signals, as Figure 4a shows, are present in the spectra of the corn oil here studied. They appear simultaneously from day 10 onwards (Figure 4b). It has been described that this compound is derived from the above mentioned mHPO-c-dE, 13-mHPO-9Z,11*E*-octadecadienoate and the possible formation mechanisms have also been explained [62].

Furthermore, the simultaneous appearance of a signal of epoxydic proton near 3.13 ppm is probably due to another isomer of the above mentioned HPO-EPO-mE, because both signals have the same evolution over time. These signals are visible in the ^{1}H NMR spectra from day 10 onwards and appear almost simultaneously to that of dHPO-nc-dEs at 4.82 ppm, both being the first kinds of secondary oxidation compounds formed.

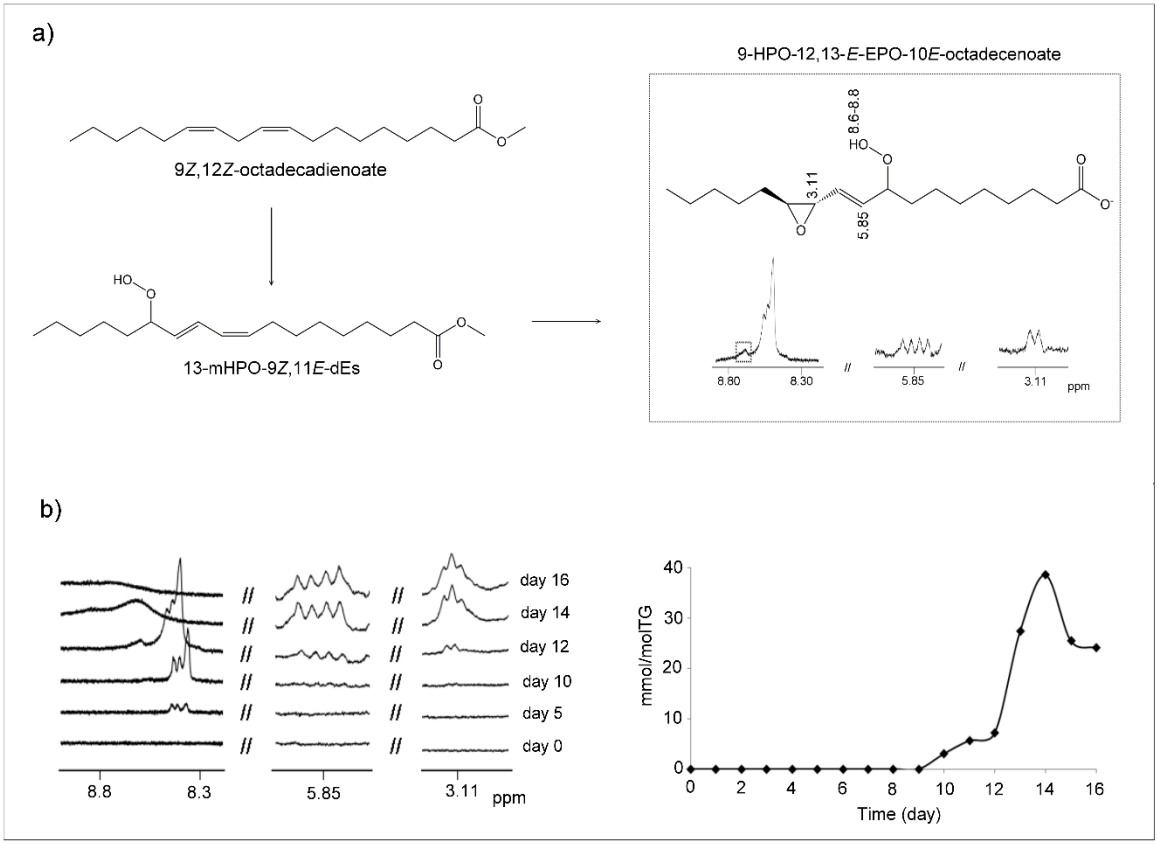

Figure 4. a) Pathway of formation of *hydroperoxy-epoxy-monoenes* (HPO-EPO-mEs) proposed by some authors under certain oxidation conditions [62] together with some chemical shifts (ppm) of the ^{1}H NMR signals of some of their hydrogen atoms. **b)** Enlargements of some spectral regions where changes occur throughout the accelerated storage process and their evolution with time, together with the graphical representation of the evolution of the concentration of 9-HPO-12,13-*E*-EPO-10*E*-octadecenoate, expressed as mmol/molTG, present in corn oil submitted to accelerate storage conditions, *versus* time given in days.

The confirmation of the assignment of the above signals to the afore cited structure (see Figure 4a), is corroborated by a more recent study in which several isomers having this structure have been found in the autoxidation of mHPO-c-dEs derived from arachidonic

group [63]. Nevertheless, the mechanism proposed in this latter study is somewhat different from that of other authors [62], because although both cases involve an intermediate dimer, in the latter case the hydroperoxy group remain in the same position in HPO-EPO-mEs as in its corresponding precursor mHPO-c-dEs. In addition, these authors inform that the HPO-EPO-mEs formed constitute near 20-30 % of the total polar compounds derived from the oxidation of mHPO-c-dEs of arachidonic group. This shows the relevance of these compounds as secondary oxidation compounds.

The concentration of all these HPO-EPO-mEs over time was determined from the area of the double doublet centered near 5.85 ppm. The results indicate that these compounds that appear from day 10 onwards reach their highest concentration (near 38.7 ± 0.6 mmol/mol TG) near day 14 with a very small decrease on day 16 up to 24.2 ± 1.2 mmol/mol TG (see Figure 4b).

It only remains to add that, to the best of our knowledge, these oxidation compounds, which are considered inhibitors-uncouplers of mitochondrial respiration [64] have not been described before as forming in the oxidation of vegetable oils, except briefly in previous studies of our group [45].

(d) ***Monohydroxy-conjugated dienes (mHO-c-dEs).***

From day 8 to day 13 signals of monohydroxy-conjugated-*Z,E*-dienes, centered at 6.48 ppms, with very low intensity, are observed. They can come from direct oxidation of linoleic groups or from reduction of mHPO-c-dEs or from peroxyl radicals by loss of an oxygen atom (Figure 5a). These are clearly intermediate compounds and their signals are not visible from day 13 onwards. The evolution of their concentration is represented in Figure 5b. It must be noted that their low concentration requires a very detailed observation of the spectra in order to recognize the presence of these compounds. *E,E*-isomers may also form in very low

concentrations as their precursors are present; however, their signals are not clearly visible in the spectra due to overlapping of their signals with others, for which reason they were not quantified. As far as we know, this is the first time that these well-known oxylipins have been detected and quantified directly in edible oils submitted to oxidative conditions, using ^{1}H NMR spectroscopic data, without chemical modification of the sample [40]. It should be added that these compounds has been attributed cytotoxicity [65], and together with other oxylipins have been associated to rheumatoid arthritis [66].

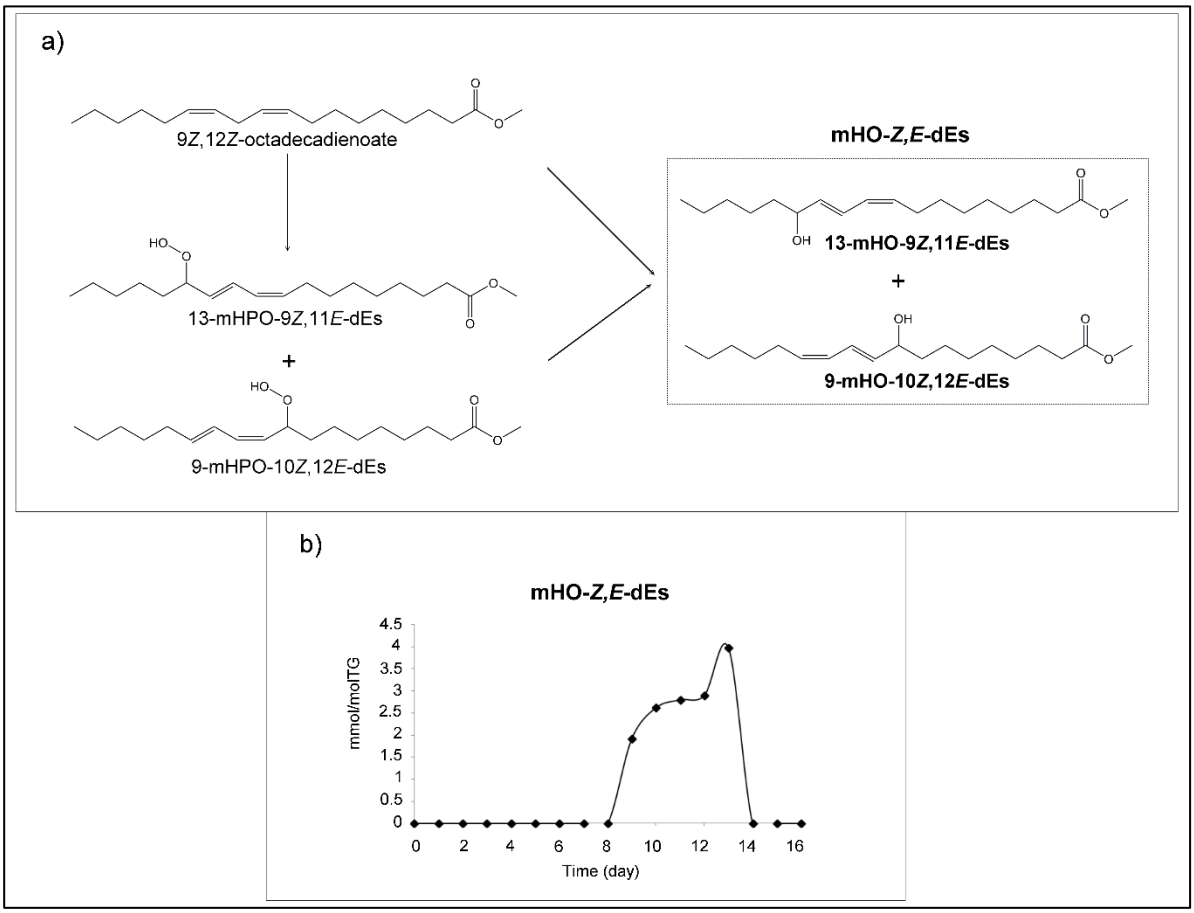

Figure 5. a) Pathway of *monohydroxy-conjugated-Z,E-dienes* (mHO-c(*Z*,*E*)-dEs) formation proposed by some authors under certain oxidation conditions [67]. **b)** Evolution of its concentration, expressed as mmol/molTG *versus* time given in days.

(e) *Hydroxy-epoxy-monoenes (HO-EPO-mEs).*

The formation of several kinds of HO-EPO-mEs has been described in the oxidation of linoleic acid or linoleate structures from HPO-EPO-mEs [62,68] as Figure 6a shows. In the oxidation process of this corn oil signals assignable to two different groups of these compounds appear after 13 days under oxidative conditions. Nevertheless, some of their spectral signals overlap to certain extent.

The first group includes those HO-EPO-mEs having the double bond between the epoxy and hydroxy groups, such as 9-HO-12,13-*E*-EPO-10*E*-octadecenoate or/and 13-HO-9,10-*E*-EPO-11*E*-octadecenoate (see Figure 6a$_1$). Among their ^{1}H NMR spectral signals, there are: one near 3.09 ppm, due to one of epoxydic protons; and other one near to 5.94 ppm, due to the olefinic proton in *alpha* position in relation to the carbon atom that supports the OH group (see Figure 6a$_1$ and Table S3). These signals are in agreement with those reported by Gardner et al., (1978) [62], Schieberle et al., (1988) [68]. In addition to the above mentioned HO-EPO-mEs, other isomers such as 13-HO-9,10-*Z*-EPO-11*E*-octadecenoate (Table S3) could also be formed in this process because their signals at 3.07 (dt), 3.41 (dd), 5.95 (dd) [69] are also present in the spectra of this oil from day 13 onwards.

In the second group of HO-EPO-mEs there are those having vicinal hydroxy and epoxy groups. Signals attributable to 11-HO-12,13-*E*-EPO-9*Z*-octadecenoate and to 11-HO-9,10-*E*-EPO-12*Z*-octadecenoate, such as those at 4.63 (dd) ppm of its methine carbinol proton, and at 2.98 (m) and 2.77 (d) ppm of the epoxydic protons [70] are observed in the spectra from day 13 onwards. Nevertheless, it should be mentioned that Ramsden et al., (2017) [32] assign to these compounds signals somewhat different to the above mentioned (see Table S3). Likewise, signals attributable to 11-HO-12,13-*E*-EPO-9*E*-octadecenoate and to 11-HO-9,10-*E*-EPO-12*E*-octadecenoate such as those at 4.25 (dd) ppm of the methine carbinol

protons in the erythro isomer, or at 3.96 (q) ppm in the threo isomer, and signals of epoxydic protons at 2.93 (dtr) and 2.78 (dd) ppm [68,70-72] are also observable in the spectra (see Table S3).

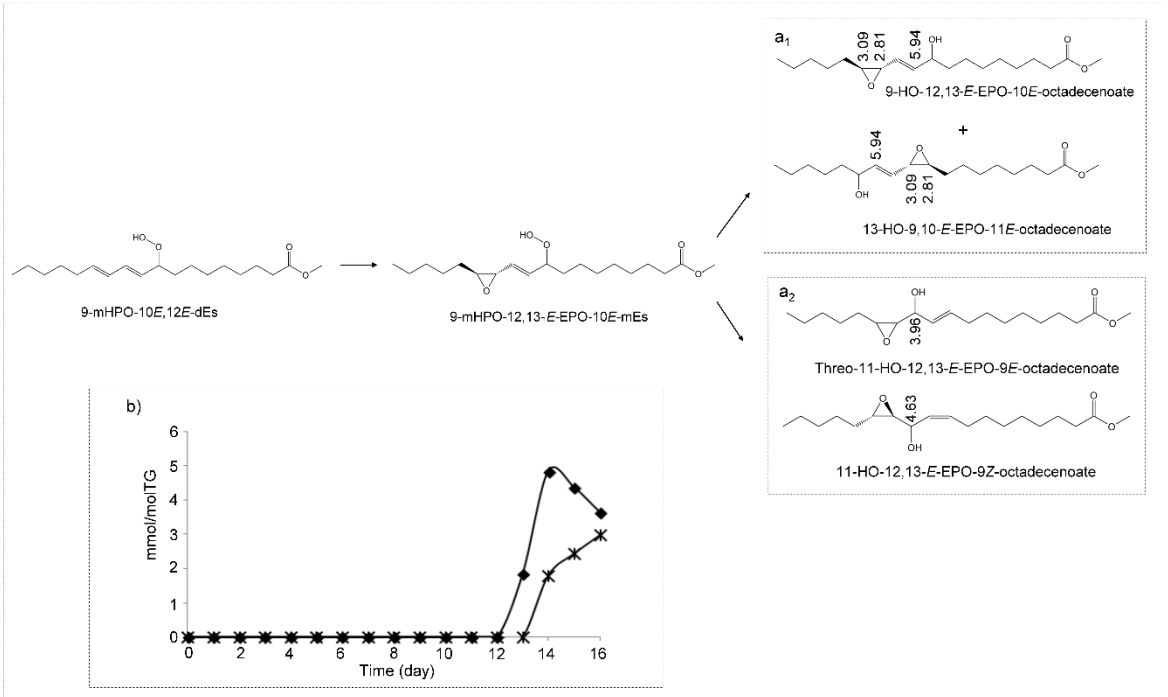

Figure 6. a) Pathways of formation of *hydroxy-epoxy-monoenes* (HO-EPO-mEs) proposed by some authors under certain oxidation conditions [68,71] for: (**a$_1$**) mHO-EPO-mEs having the double bond between the epoxy and hydroxy groups together with some chemical shifts (ppm) of the ^{1}H NMR signals of some of their hydrogen atoms; (**a$_2$**) mHO-EPO-mEs having hydroxy and epoxy group vicinal together with some chemical shifts (ppm) of the ^{1}H NMR signals of some of their hydrogen atoms. **b)** Evolution of the concentration, expressed as mmol/molTG *versus* time given in days of: 9-HO-12,13-*E*-EPO-10*E*-octadecenoate + 13-HO-9,10-*E*-EPO-11*E*-octadecenoate + 13-HO-9,10-*Z*-EPO-11*E*-octadecenoate (♦); *threo*-11-HO-12,13-*E*-EPO-9*E*-octadecenoate + *threo*-11-HO-9,10-*E*-EPO-12*E*-octadecenoate (*).

The concentration of the HO-EPO-mEs above mentioned belonging to the first group was estimated jointly using the area of the signal near 5.95-5.94 ppm. Regarding the second group of HO-EPO-mEs, due to the overlapping of signals, only the concentration of the threo isomers of 11-HO-12,13-*E*-EPO-9*E*-octadecenoate and of 11-HO-9,10-*E*-EPO-12*E*-octadecenoate was estimated using the area of the signal near 3.96 ppm. In both cases, it was

assumed that these signals are due exclusively to the cited protons of this kind of compounds. The evolution of the concentration of these compounds *versus* time is given in Figure 6b. It can be observed that concentrations of HO-EPO-mEs of the first group reach their maximum (near to 4.8 ± 1.2 mmol/molTG) on day 14 and those of the second group of HO-EPO-mEs show increasing concentrations up to day 16 (near 3.7 ± 0.2 mmol/molTG).

To the best of our knowledge, this is the first time that the formation of HO-EPO-mEs has been described in the oxidation of edible oils and that the concentration of some of them has also been estimated from ^{1}H NMR spectral data. It should be noticed that these compounds are also formed in living beings and some of them have been associated to human psoriatic skin lesions [32]. Furthermore, some of these compounds have also been associated with the production of paint and itch in rodents' skin [32]. In addition, it has been reported that 11-HO-12,13-*E*-EPO-9*Z*-linoleate and 11-HO-9,10-*E*-EPO-12*Z*-linoleate increase trigeminal neuron excitability, suggesting a potential role in headache or facial pain [73].

(f) ***Hydroxy-keto-monoenes (HO-KO-mEs).***

These kinds of structures are also formed after 14 days under degradative conditions. Those found belong to two different groups. In one group, both oxygenated groups are on vicinal carbon atoms (see Figure 7a), such as 13-HO-12-KO-9Z-octadecenoate and in 9-HO-10-KO-12*Z*-octadecenoate, with ^{1}H NMR signals at 2.00 (m), 3.24 (t), 4.23 (dd), 5.54 (m) ppm [74], which are visible, with very low intensity, partially overlapped with other ones (for assignments see table S3). The other group of HO-KO-mEs is made up of structures that do not have the hydroxy and keto groups in vicinal carbon atoms. One example is 9-HO-11-KO-12*E*-octadecenoate, depicted in Figure 7b. Some of their ^{1}H NMR signals, whose assignments are given in Table S3, appear at 2.58 (dd), 3.24 (d), 3.98-4.04 (m), 6.05 (dt) 6.83 (dt) ppm [75]. These are also observable in the spectra of this oil after 14 days under oxidative

conditions. The concentration of all these compounds was estimated jointly, using the area of the signal at 3.24 ppm due to the methylenic protons in *alpha* position regarding either the keto group and the double bond, or regarding both oxygenated groups (see Figure 7a and 7b), assuming that these signals are due exclusively to the cited protons of these kinds of compounds (see Table S3).

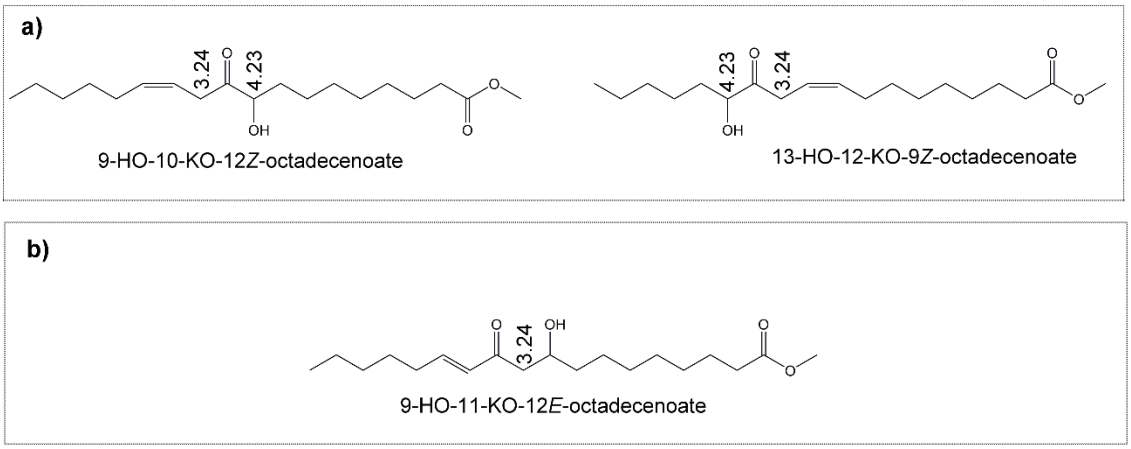

Figure 7. Chemical structures of the *hydroxy-keto-monoenes* (HO-KO-mEs) involved in this study together with some chemical shifts (ppm) of the ^{1}H NMR signals of some of their hydrogen atoms. **a)** Structures in which keto group and the hydroxy group are on vicinal carbon atoms; **b)** Structure in which the hydroxy group is in *beta* position regarding the keto group.

To the best of our knowledge, this is also the first time that the formation of HO-KO-mEs has been described in the oxidation of edible oils and that their concentration has been estimated from ^{1}H NMR spectral data. The concentrations found are very small, between 1-2 mmol/molTG.

(g) *Monoketo-conjugated dienes (mKO-c-dEs).*

Monoketo-conjugated dienes, which are well known oxylipins, were detected and quantified in this corn oil submitted to degradative conditions. Monoketo conjugated-*E*,*E*-dienes (mKO-c(*E*,*E*)-dEs) appear in the spectra from day 11 onwards and the *Z*,*E*-isomers (mKO-c(*Z*,*E*)-dEs) from day 12 onwards. Their formation has been described as coming from the corresponding monohydroperoxy-conjugated dienes (mHPO-c-dEs) [67] (see Figure 8a).

The identification of these compounds has been made taking into account ^{1}H NMR spectral data given in the literature and with the use of standard compounds. The assignment of their signals is given in Figure 8a and in Table S4 and their quantification has been made using the area of signals centered near 7.49 ppm in the case of *Z,E*-isomers and centered near 7.13 ppm in the case of *E,E*-isomers. The concentration of the *E,E* isomers is more than double than that the *Z,E* isomers (see Figure 8b) and both are in line with that of their corresponding precursors mHPO-c(*E,E*)-dES and mHPO-c(*Z,E*)-dES at this time, but much lower. The maximum concentration of the *Z,E* isomers is reached on day 13 (near 5.0 ± 0.8 mmol/molTG) and that of the *E,E* isomers on day 15 (near 11.2 ± 0.1 mmol/molTG), decreasing slightly up to day 16 (see Figure 8b).

These results are of great interest because they show that monoketo-conjugated dienes are important secondary oxidation compounds derived from edible oils rich in linoleic groups, to which little attention has been paid until now, possibly due to the difficulty involved in their determination. Nevertheless, it should be noted that they have been attributed cytotoxicity, that of the *E,E* isomers more than that of the *Z,E* isomers [67,77].

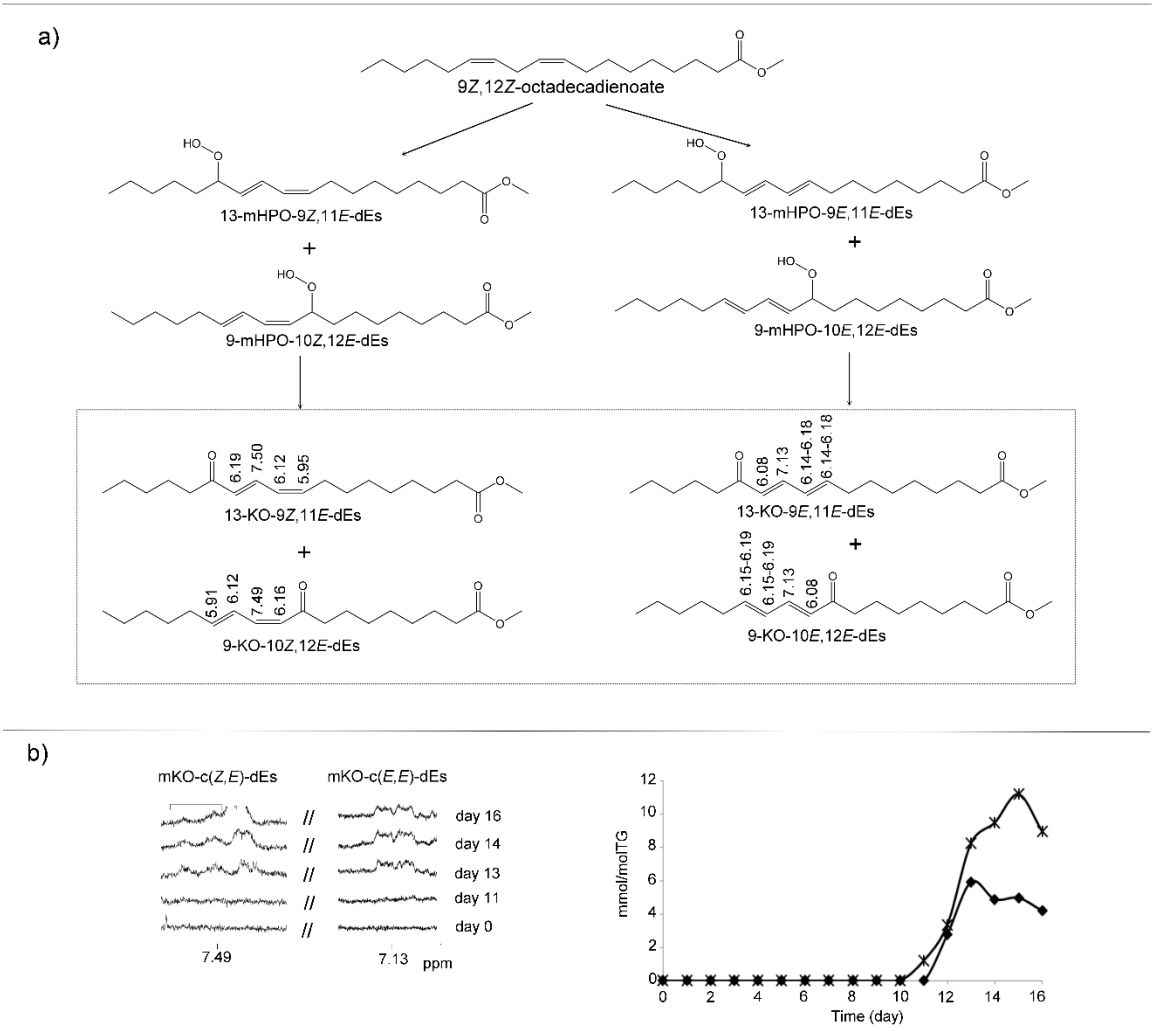

Figure 8. a) Pathways of *monoketo-conjugated dienes* (mKO-c-dEs) formation proposed by some authors under certain oxidation conditions [67,76] together with some chemical shifts (ppm) of the ^{1}H NMR signals of some of their hydrogen atoms**. b)** Enlargements of some spectral regions where changes occur throughout the accelerated storage process and their evolution with time, together with the graphical representation of the evolution of the concentration, expressed as mmol/molTG *versus* time given in days of: mKO-c(*Z,E*)-dEs (♦); mKO-c(*E,E*)-dEs (*).

(h) *Keto-epoxy-monoenes (KO-EPO-mEs).*

The formation of this kind of compounds can be clearly observed in the spectra of this oil at advanced stages of the degradation process. Their signals appear from day 14 onwards, later than those of mKO-c-dEs, as may be expected. Bearing in mind the spectral signals, two groups of possible isomers are present.

One group of KO-EPO-mEs has the double bond between the keto and epoxy groups such as in 13-keto-9,10-*E*-epoxy-11*E*-octadecenoate and in 9-keto-12,13-*E*-epoxy-10*E*-octadecenoate. These structures have ^{1}H NMR signals at 2.53 (t), 2.91 (td), 3.20 (dd), 6.38 (d) and 6.52 (dd) ppm. This group also includes 13-keto-9,10-*Z*-epoxy-11*E*-octadecenoate and/or 9-keto-12,13-*Z*-epoxy-10*E*-octadecenoate which have ^{1}H NMR signals at 2.55 (t), 3.20 (dd), 3.52 (dd), 6.40 (d) and 6.66 (dd) ppm. This has been confirmed with literature data [32,69,75] and with some standard compounds. Figure 9a$_1$ gives the structures of these compounds, a potential formation pathway and the ^{1}H NMR signals of some of their protons. For the assignments of signals see Table S4. The determination of their concentrations can be carried out using the intensity of several signals such as those that appear: at 3.20 ppm referring to both *E,E* plus *Z,E* isomers; at 3.52 ppm that refers to *Z,E* isomers only; or at 6.38 ppm and 6.66 ppm due to *E,E* and *Z,E* isomers respectively. The concentrations obtained and their evolution *versus* time are given in Figure 9b. Again, the concentration of *E,E* isomers is higher than that of *Z,E* isomers in line with the observed before in mHPO-c-dEs and mKO-c-dEs and also with the higher rate of decrease of the concentration of mKO-c(*E,E*)-dEs than of mKO-c(*Z,E*)-dEs at advanced oxidation stages (see Figure 8b). These results demonstrate that these biologically potent oxylipins can be formed at advanced stages of the oxidation process of edible oils rich in omega 6 groups.

In addition, the formation in the oxidation of linoleic acid of other KO-EPO-mEs characterized because their oxygenated groups are supported on vicinal carbon atoms has also been described [32,75]. These compounds, like the above, come from mHPO-c-dEs and have been synthesized, by either enzymatic or non-enzymatic pathways *in vivo* or *in vitro*, from linoleic acid [75]. Signals attributable to this kind of compounds, such as 11-keto-12,13-*E*-epoxy-9*E*-octadecenoate and/or 11-keto-9,10-*E*-epoxy-12*E*-octadecenoate, at 3.04-2.98 (ddd) ppm, 3.34-3.28 (d) ppm, 6.23-6.16 (dt) ppm and 7.02 (dt) ppm [75], are present, with

very low intensity, in the ^{1}H NMR spectra of this oil, after 14 days under degradative conditions (Figure 9a$_2$). The assignment of these signals to the corresponding hydrogen atoms is indicated in Table S4. The appearance of these signals indicates that this kind of KO-EPO-mEs is also formed in this oil oxidation process. The estimation of their concentrations was made using the intensity of the signal at 7.02 ppm. Figure 9a$_2$ shows their structure and Figure 9b shows the evolution of their concentration in this process, which as can be observed, is very low.

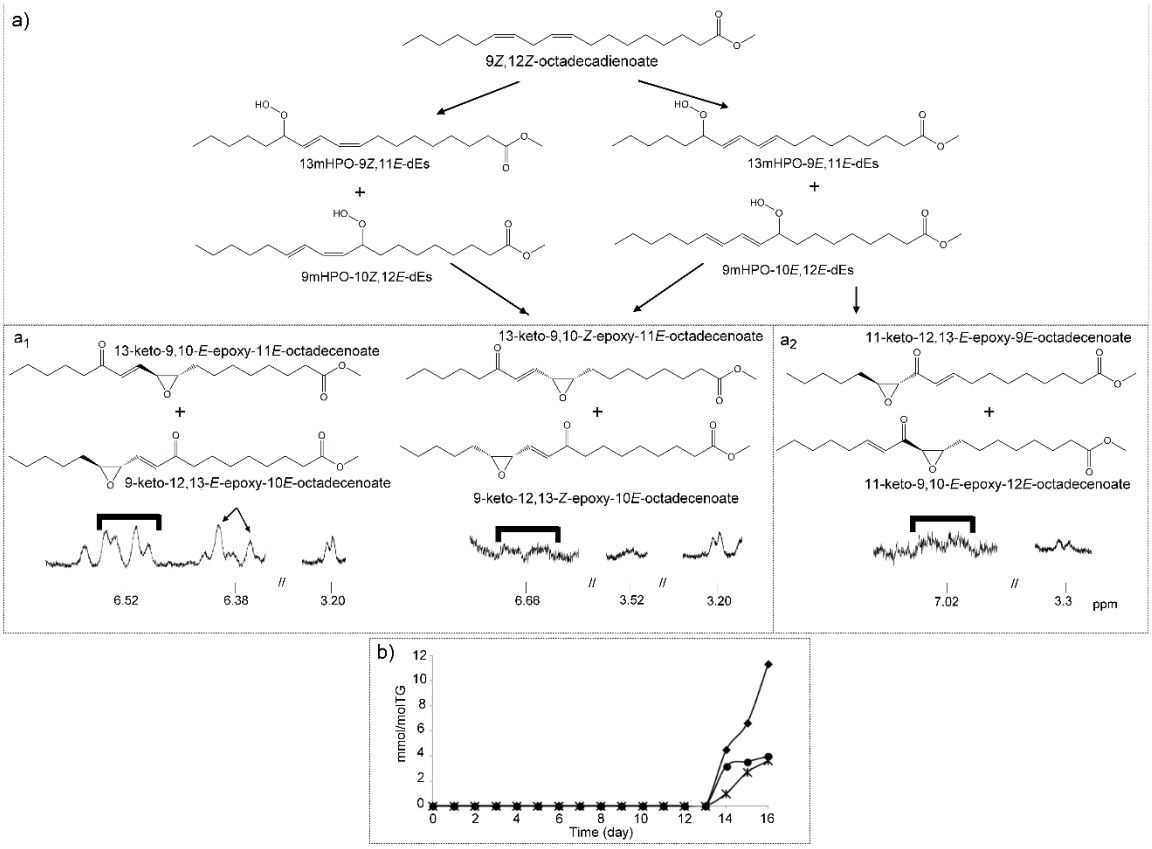

Figure 9. Pathways of formation of *keto-epoxy-monoenes* (KO-EPO-mEs) formation proposed by some authors under certain oxidation conditions [32,69,75]. **(a$_1$)** KO-EPO-mEs having in their chains keto-double bond-epoxy, together with the enlargement of some regions of the ^{1}H NMR spectra in which the signals of these compounds appear; **(a$_2$)** KO-EPO-mEs having in their chains double bond-keto-epoxy together with the enlargement of some regions of the ^{1}H NMR spectra in which the signals of these compounds appear. **b)** Evolution of the concentration, expressed as mmol/molTG *versus* time given in days of: 13-keto-9,10-*E*-epoxy-11*E*-octadecenoate + 9-keto- 12,13-*E*-epoxy-10*E*-octadecenoate (♦); 11-keto-12,13-*E*-epoxy-9*E*-octadecenoate + 11-keto- 9,10-*E*-epoxy-12*E*-octadecenoate (●); 13-keto-9,10-*Z*-epoxy-11*E*-octadecenoate + 9-keto-12,13-*Z*-epoxy-10*E*-octadecenoate (*).

As far as we know this is the first time that the formation of all these compounds has been reported in the oxidation of edible oils. In a previous paper about soybean oil oxidation the formation of some of them was briefly described [12]. The importance of the presence of these compounds in oxidized edible oils is great, due to their great reactivity. They are able to modify proteins through covalent bonds, have been related with numerous pathophysiological processes associated to inflammatory pain, and to respiratory diseases [78]. They have also been related with inflammation in skin [32] and with psoriatic lesions [32]. Moreover, it has been demonstrated that intradermal injection of these compounds induced itch-related scratching behavior in mice [32]. Finally, other authors have reported that some of these compounds stimulate aldosterone production [79] which is related with some types of human hypertension [80].

(i) *Epoxy-keto-hydroxy derivatives (EPO-KO-HOs).*

From day 12 onwards under oxidative conditions, signals appear in the ^{1}H NMR spectra of this oil near 2.42 (dd), 2.51 (dd), 3.02-3.08 (ddd), 3.16 (d) ppm and 3.98-4.04 ppm [75], which are overlapped with other ones. All these signals have been attributed to 12,13-*E*-EPO-11-KO-9-HO-octadecanoate (Figure 10) [74]. For this reason, it could be thought that structures with these three oxygenated functional groups could also have been formed in this oil.

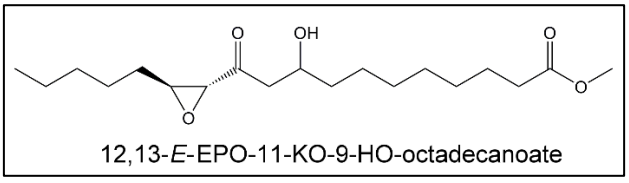

Figure 10. Chemical structure of *epoxy-keto-hydroxy derivatives* (EPO-KO-HOs), 12,13-EPO- *E*-11-KO-9-HO-octadecanoate.

(j) ***Monoepoxy-monoenes (mEPO-mEs) and diepoxy (dEPO) structures*****.**

The appearance in the oil spectra from day 13, of signals at 2.98-2.88 ppm and 2.73-2.66 ppm together with other ones (see Table S5), suggests the formation of mono unsaturated epoxy structures (mEPO-mEs). Signals with these chemical shifts are due to protons of different kinds of monoepoxy derivatives.

The first signal (2.98-2.88 ppm) and other ones present in the spectra agree with those of standard compounds such as 9,10-*Z*-EPO-12*Z*-octadecenoate (leukotoxin) and 12,13-*Z*-EPO-9*Z*-octadecenoate (isoleukotoxin) and also with those provided by Nilewski et al., (2015) [81], for the first compound. Likewise, there are other structures having epoxy groups whose protons give signal at this same chemical shift, which have been cited before. A pathway for the formation of this kind of epoxides [82], assumes that it is formed directly from intact linoleic chains with the participation of chains supporting hydroperoxy groups or peroxyl radicals (see Figure 11a$_1$). These latter could supply an oxygen atom to linoleic chains to form epoxy groups. At the same time hydroperoxy groups or peroxyl radicals may evolve to give hydroxy groups or alkoxyl radicals, or keto groups [76]. In other words, these epoxides could derive directly from intact linoleic chains although their formation would require the presence of hydroperoxides or peroxyl radicals, which would act as prooxidant systems. This reaction could lead to the formation of monoepoxides with the epoxy group just at the original position of the double bonds in the linoleic chain, forming leukotoxin or isoleukotoxin structures (see Figure 11a$_1$). These epoxidation reactions could be considered similar to those produced by oxidant systems such hydrogen peroxide in acidic medium or by peroxycarboxylic acids [83-85]. All edible oils, including this one, contain acids as minor components [48] and the presence of high concentrations of hydroperoxides or of peroxyl radicals could give rise to this epoxidation reaction. It has also been proved that this kind of

epoxides can be formed in cells. The concentration of *Z*-EPO-*Z*-mEs, increases continuously from day 13 reaching its maximum value (27.3 ± 3.1 mmol/molTG) on day 16 (Figure 11b). This was determined using the area of the signal at 2.98-2.88 ppm, subtracting the contribution of the above mentioned structures that also give this signal. Both leukotoxin and isoleukotoxin can also be formed endogenously in cells, and are known toxic compounds. They have been clinically associated with acute respiratory distress syndrome (ARDS), with circulatory shock and disseminated intravascular coagulation, and with multiple organ failure. This toxicity can be related to their great reactivity [86-90]. Nevertheless, it has been reported that these compounds, rather than being themselves toxic could be considered as prototoxic, exhibiting toxicity in the presence of epoxide hydrolase, which opens the epoxy ring to give leukotoxin or isoleukotoxin diols, these latter being the toxic compounds able to provoke the above mentioned diseases.

The second signal (at 2.73-2.66 ppm) above mentioned and other ones present in the spectra agree with those of the standard compound 9,10-*E*-EPO-12*Z*-octadecenoate and with those provided by Nilewski et al., (2015) [81] for 12,13-*E*-epoxy-9*Z*-octadecenoic acid. The pathway proposed for the formation of *E*-EPO-*Z*-mEs, as Figure 11a$_2$ shows, involves an isomerization before the epoxidation reaction. The presence of these *E* epoxy structures is detectable in the ^{1}H NMR spectrum from day 13 onwards and reaches maximum concentration (17.0 ± 1.6 mmol/molTG) on day 16 (see Figure 11b). This was determined using the area of the signal at 2.73-2.66 ppm, assuming that only the protons of this kind of compounds contribute to this signal. It should be noted that *Z*-EPO-*Z*-mE isomers are formed in greater concentration than *E*-EPO-Z-mEs isomers (Figure 11b). This may be because in the first case a previous isomerization is not required. It should be noted that, to the best of our knowledge, this is the first time that the formation of *E*-EPO-Z-mEs isomers has been reported in the oxidation of edible oils.

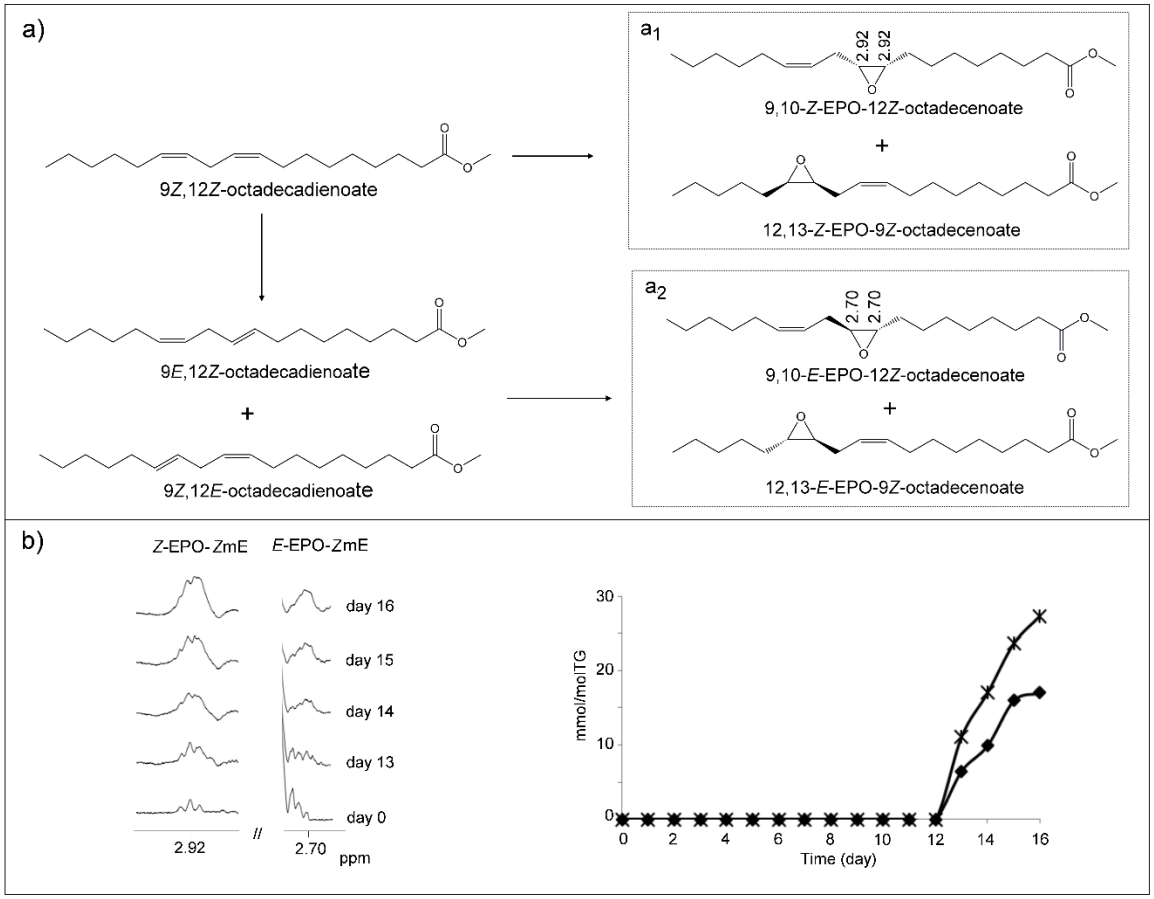

Figure 11. Pathway of formation of *monoepoxy-monoenes* (mEPO-mEs) proposed by some authors under certain oxidation conditions [81]. **(a_1)** Formation of *Z*-EPO-mEs together with some chemical shifts (ppm) of the ^{1}H NMR signals of their epoxydic hydrogen atoms; **(a_2)** Formation of *E*-EPO-mEs together with some chemical shifts (ppm) of the ^{1}H NMR signals of their epoxydic hydrogen atoms**. b)** Enlargement of regions of the ^{1}H NMR spectra in which the signals of these compounds appear together with evolution of their concentration, expressed as mmol/molTG *versus* time given in days of: *Z*-EPO-ZmEs (*); *E*-EPO-ZmEs (♦).

Finally, it could be thought that the formation of diepoxy structures (dEPO) could also occur in the oxidation process undergone by this oil, as described previously by other authors [85]. However, the absence of spectral signals of the standard, 9,10-EPO-12,13-EPO-octadecanoic acid, such as those at 2.98 and 1.73 ppm in the oil spectra throughout the oxidation process raises doubts about this possibility. In Supplementary Material, (Figure S2) shows the following spectral regions in which these signals appear, of: corn oil after 14 days under oxidative conditions; the same sample enriched with 9,10-EPO-12,13-EPO-octadecanoic acid; and of pure 9,10-EPO-12,13-EPO-octadecanoic acid. It is evident that if diepoxides are

formed in the oxidation process of this oil they are in such very low concentrations that they cannot be detected by ^{1}H NMR.

(k) ***Dihydroxy (dHO) and/or polyhydroxy (pHO) structures.***

The formation of dHO structures in the oxidation of this corn oil is also possible (Figure 12a$_1$). In this study, as Figure 12a$_2$ depicted, one broad signal centered at 3.42 ppm, attributable to the methine carbinol protons of 9,10-dHO-12*Z*-octadecanoate (leukotoxin diol) and/or 12,13-dHO-9*Z*-octadecanoate (isoleukotoxin diol) (see Table S6) [81,91] appears in the spectra from day 12 onwards. For this reason, the presence of dHO structures cannot be discarded. Figure 12 a$_2$ represents the evolution of the concentration of methine carbinol protons that give this signal *versus* time after subtracting the contribution of HO-*Z*-EPO-*E*-mEs from day 13. The formation of hydroxy groups from epoxide ring opening, in acyl group chains of vegetable oils, can be produced by hydrolysis [92]. As already mentioned, dihydroxy structures, such as leukotoxin and isoleukotoxin diols, are well known toxic oxylipins, associated with multiple organ failure, and related with adult respiratory distress syndrome [86,93,94].

Furthermore, the formation of pHO can also occur in epoxidized triglycerides by oxirane ring opening by acids or by alcohols, as described previously [95], which will be commented on later. The methine carbinol proton of pHO could also contribute to the signal at 3.42 ppm. For this reason, data in Figure 12a$_2$ also include these hydroxyl groups if there are any.

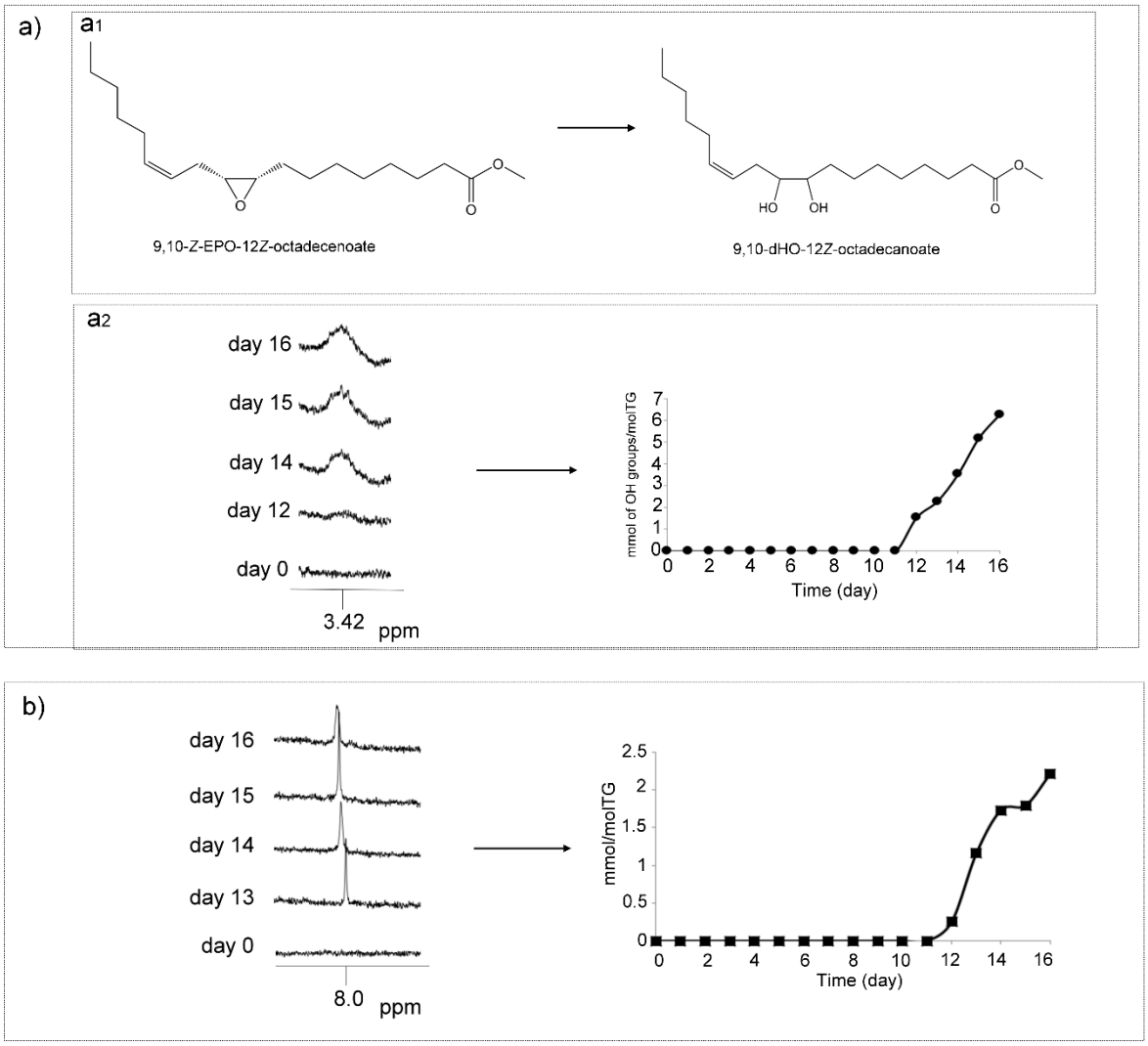

Figure 12. a) (a_1) Formation pathway of *dihydroxy monoenes* (dHO-mEs) proposed by some authors under enzymatic conditions [81]; **(a_2)** Enlargement of region of the ^{1}H NMR spectra in which the signals of these compounds appear together with evolution of their concentration, expressed as mmol/molTG *versus* time given in days; **b)** Enlargement of region of the ^{1}H NMR spectra in which the signals of formic acid appear together with evolution of their concentration, expressed as mmol/molTG *versus* time given in days.

(l) *Acids and formic acid*

It is known that all unoxidized edible oils contains fatty acids as minor components in small concentrations and also that, when they are submitted to oxidative conditions as here, a numerous group of acids of short chain length from formic to decanoic or further, are formed [13,38,96,97]. Of these, it has been reported that formic acid is formed in significant concentrations and in fact several pathways have been proposed for its formation either as

coming from recurrent oxidation of aldehydes [96,97] or even in the formation of 4-hydroperoxy-2*E*-nonenal from m-HPO-c-dEs [59]. How could it be otherwise in the oxidation process of this corn oil also the formation of formic acid has been observed from day 12 onwards, simultaneously with the appearance of some aldehydes such as it will be mentioned later. This was detected by the appearance in the ^{1}H NMR spectra of the singlet signal at 8.01 ppm (see Figure 12b and Table S6). The evolution of its singlet signal and of its concentration, estimated by ^{1}H NMR throughout the oxidation process from day 12 up to day 16 are depicted in Figure 12b. The formation of this acid and of others is of interest because they react with compounds which are present in the system to generate further derived compounds with important repercussions. It is worth noting that although the formation of formic acid had been described in lipid oxidation process [96], this is the first time that this compound has been identified by ^{1}H NMR spectroscopy in an oxidized oil. It must be remembered that the appearance of this signal has been described for a long time in previous studies on edible oil oxidation but without being assigned to any compound [4-7,98].

(m) ***Poly-formate (pF), poly-ester (pEst) and poly-hydroxy (pHO) structures.***

In addition to the above mentioned possibility of opening the oxirane ring to give diols, ring opening can also occur in the presence of acids. Formic acid is able to open the epoxydic rings yielding one hydroxy group and one formate group as Figure 13a$_1$ shows. The existence of this reaction in the oxidation process of this oil under the conditions of this study is confirmed by the appearance of signals between 8.03 and 8.17 ppm (see Figure 13b and Table S6) of the proton of formate groups and at 5.18 ppm of methine protons of the ester group [99]. To the best of our knowledge, this is the first time that the formation of formates has been proved in the oxidation process of edible oils. This has been confirmed on the basis of

previous studies concerning the preparation of polymers derived from vegetable oils [99,100] and also with the use of a standard compound. It is worth highlighting the similarities between the formate signals in the spectra of this study and those published by other authors in studies of poly-hydroxy and poly-formate structures formation [101]. The appearance of formate signals in the spectrum occurs on day 13 and the concentration of these groups increases progressively up to the end of the polymerization process (Figure 13b). As above mentioned, other acids present in the oil could also open the oxirane rings in triglycerides forming poly-hydroxy and poly-ester groups (Figure 13a_2) [102].

It should be added that it has also been described that epoxy, hydroxy and formate groups can be directly formed, in the unsaturated chains of the oil triglycerides, in presence of formic acid and potent oxidants such as hydroperoxides, as Figure 13a_3 shows. If the oxidative conditions are maintained all epoxy groups can be opened to give more formate and hydroxyl groups [103]. In turn, some of the hydroxy groups formed can be esterified by formic acid (or by other acids) present, thus increasing the ratio between formate group (or ester group) and hydroxy groups.

These reactions increase the viscosity of the oil and to the best of our knowledge, they have not been previously described in the oxidation of edible oils, but rather in polymerization studies to obtain precursor of polymers based on edible oils. All oxidation compounds found in this oil and mentioned before can also be formed endogenously in cells, for which reason it could be thought that these poly-hydroxy and poly-formate structures could also be formed in cells and tissues, contributing to the loss of elasticity in membranes and even in blood vessels.

(n) ***Poly-ether (pEt) and poly-hydroxy (pHO) structures.***

It is known that not only acids are able to open the oxirane rings but so too can both primary and secondary alcohols [104]. This reaction can also take place during the oxidation of this oil because both kinds of alcohol groups are present. The occurrence of primary alcohols supported on small size molecules is well known in edible oils submitted to oxidation conditions [13,38]. Likewise, secondary alcohols supported on large sized molecules such as the before mentioned mono-hydroxy, di-hydroxy and even poly-hydroxy structures are also present. The oxirane ring opening provoked by alcohols yields, as Figure 13a$_4$ depicts, one hydroxy group on the one side and on the other side one ether group, which incorporates into the molecule the structure that supports the alcohol group (see Figure 13a$_4$). Furthermore, if this reaction takes place between epoxides of one triglyceride and the secondary hydroxy groups of another triglyceride, polymerization is produced by the formation of C-O-C bridges between fatty chains. In addition, reactions between hydroxyl groups of different structures are also possible to again generate ether groups contributing also to generate further ether groups and polymerization of the sample.

From days 12-13 onwards signals appear at near 3.62, 3.98 and 4.23 ppm, with increasing intensity to the end of the experiment, which can be assignable to methine protons of alcohols or to ether groups, in agreement with several authors. Thus, Caillol et al., (2012) [102] assign all signals comprised between 3.3 to 4.1 ppm either to methine protons of secondary hydroxyl groups or of ether bonds. De Souza et al., (2012) [101], also attribute the signal at 3.64 ppm to methine protons of secondary hydroxyl groups, and Lligadas et al., (2006) [105], to protons of the poly-ether backbone. Nevertheless, this signal could also be attributed to primary alcohols whose concentration increases, like acids, with oxidation time and are present in oxidized oils [38]. Likewise, the high increase observed in the intensity in the overlapped

signals near 3.1 ppm, from day 12 onwards, could also be attributed to protons supported on carbon atoms of ether linkages.

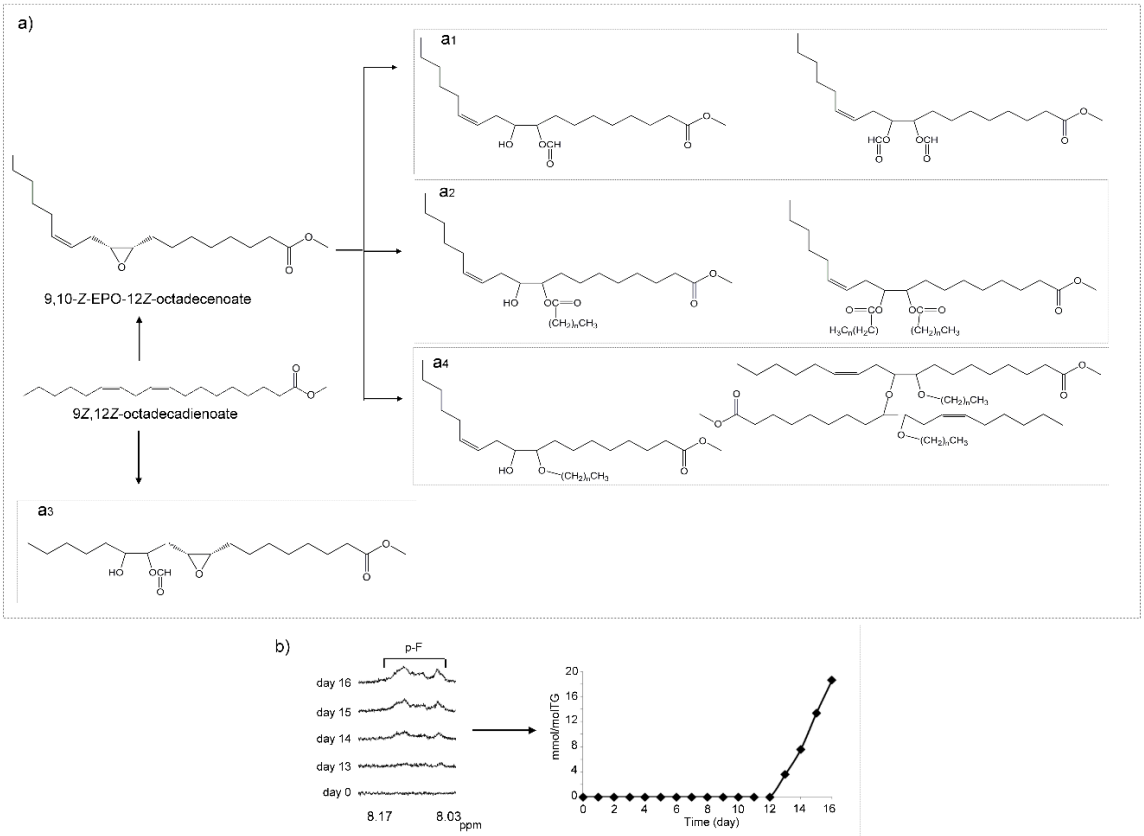

Figure 13. a) Pathways of formation of *poly-formates, poly-ester and poly-hydroxy structures* (**a_1**, **a_2** and **a_3**), *and poly-ether and poly-hydroxy structures* (**a_4**), proposed by some authors [100,102,105]; **b)** Enlargement of region of the ^{1}H NMR spectra in which the signals of poly-formates appear together with evolution of their concentration, expressed as mmol/molTG *versus* time given in days.

As before mentioned concerning poly-esters, it could not be discarded that the poly-ether structures that produce polymerization in the oil, could also be formed endogenously contributing to the hardening of membranes, to the clogging of blood vessels and to the atherosclerotic plaque.

(o) ***Structures supporting furan ring (Frs)*.**

Among these, two types can be distinguished, alkyl-furans and furanones. The formation of furan derivatives from methyl linoleate submitted to oxidative conditions has been described

before [69]. Likewise, the formation of pentyl- and other alkyl-furans has also been reported previously in the oxidation of vegetable oils rich in diunsaturated acyl groups in several studies [13,29,30,38]. In this oil signals belonging to protons of alkyl-furans at 7.27 (dd) ppms appear (Figure 14a and Table S6) from day 14, growing in concentration up to the end of the experiment as Figure 14b$_1$ shows.

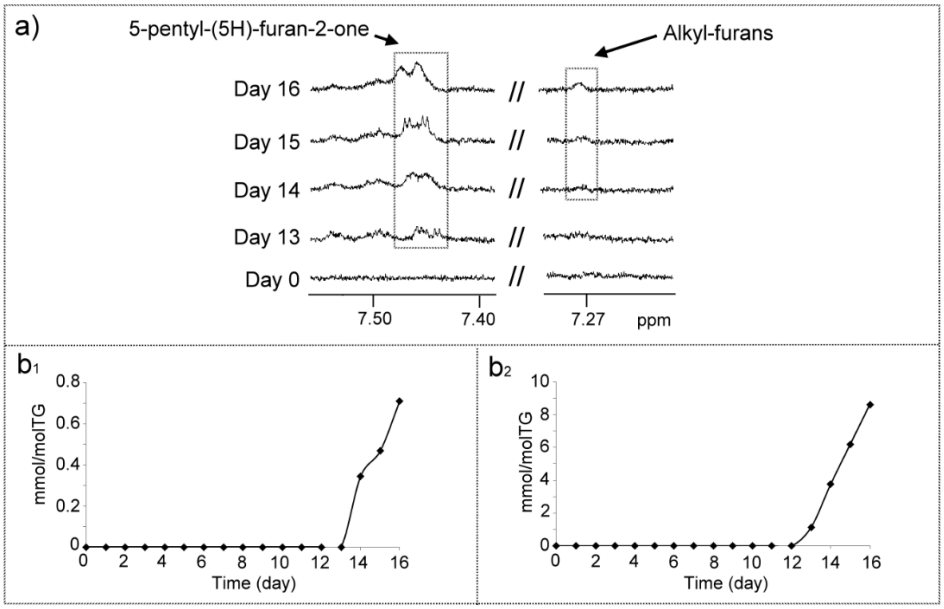

Figure 14. a) Enlargement of region of the ^{1}H NMR spectra region in which the signals of alkyl-furans and 5-pentyl-(5H)-furan-2-one appear. **b)** Evolution of the concentration of alkyl-furans (**b$_1$**) and 5-pentyl-(5H)-furan-2-one (**b$_2$**), expressed as mmol/molTG *versus* time given in days.

Likewise, ^{1}H NMR signals attributable to 5-pentyl-(5H)-furan-2-one (see Figure 14a and Table S6) and to other compounds of this family appear from day 13 onwards with increasing intensity up to the end of the process. Among these there is a characteristic double doublet signal of their unsaturated protons centered near 7.47 ppm [106-108] that overlaps partially with that of m-KO-c(*Z*,*E*)-dEs, which appears one day before. This kind of compounds has been found previously in several vegetable oils rich in diunsaturated acyl groups submitted to oxidative conditions [13,29,30,38]. Their formation has been described as coming from the fragmentation of hydroperoxy- and hydroxy-endoperoxides derived from

polyunsaturated fatty acids [108]. The increasing concentration of these structures up to day 16 suggests that they are oxidation end products (see Figure 14b$_2$).

(p) ***Aldehydes (A).***

Like hydroperoxides, aldehydes are well known oxidation compounds. Several monitoring classical methods have been developed for their estimation over the years, such as *p*-Anisidine, but they do not provide accurate information. However, techniques such as ^{1}H NMR spectroscopy allows the identification of the different kinds of aldehydes formed in oil oxidation processes and also their quantification [3-5,109], and SPME-GC/MS also permits the monitoring of the individually volatile aldehydes [38].

The chemical shifts of the ^{1}H NMR signals of the different kinds of aldehydes are given in Table S7. In the oxidation process undergone by this corn oil the first aldehydes formed are 2*E*-alkenals and 4-hydroperoxy-2*E*-alkenals which are detected from day 11 onwards. However, n-alkanals and, 2*E*,4*E*-alkadienals appear in the spectra from day 12, whereas 4-hydroxy-2*E*-alkenals, 4-oxo-2*E*-alkenals and 4,5-epoxy-2*E*-alkenals appear on day 13. Finally, from day 15 the presence of 2,3-epoxyalkanals is observed (see Figure 15a and 15b). It is noteworthy that the formation of 4-oxo-2*E*-alkenals and that of 2,3-epoxyalkanals, has been detected by ^{1}H NMR spectroscopy, in an oxidized edible oil for the first time. These latter kinds of aldehydes have been detected before by SPME-GC/MS in oxidation studies of polyunsaturated oils submitted to similar oxidative conditions to those here [38], never however by ^{1}H NMR. It is of interest to note that the same functional groups (hydroperoxy, hydroxy, oxo (or keto) and epoxy) that are formed in the long chains of acyl groups are also present in these smaller aldehydic structures.

The structure of these aldehydes and the evolution of their concentration with time are given in Figure 15. It can be observed that the concentration of aldehydes reaches near 69 mmol/mol TG at the end of the experiment, the main ones being 4-hydroxy-2*E*-alkenals (19.8 ± 0.9 mmol/mol TG) and 2*E*-alkenals (15.6 ± 0.7 mmol/mol TG). All these detected structures can be supported either on small volatile molecules or in truncated acyl groups of TG and agree with findings of previous studies of edible oils oxidation [3-5,8,13,38,110].

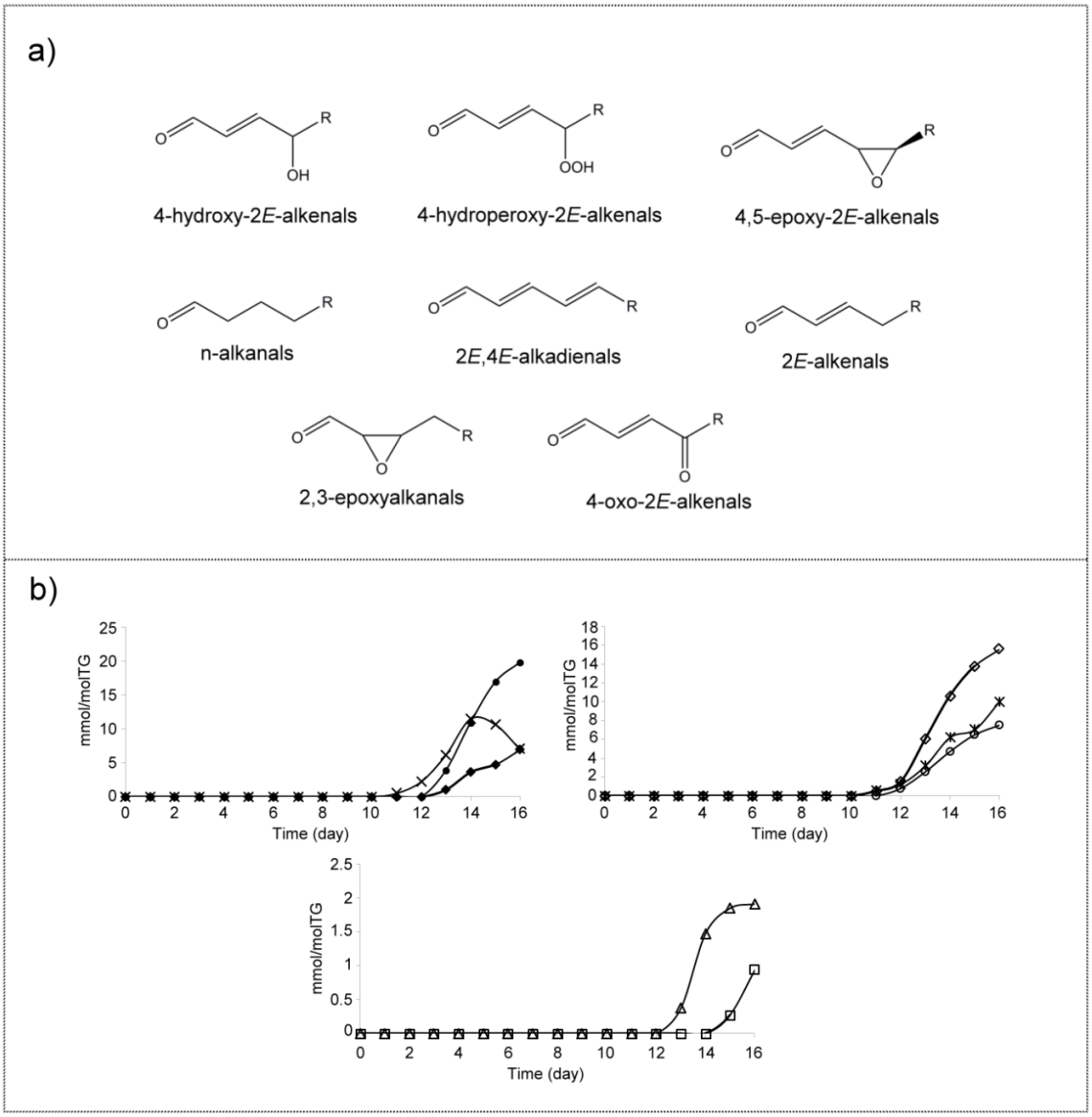

Figure 15. a) Chemical structures of aldehydes detected in this study. **b)** Evolution of their concentration, expressed as mmol/molTG *versus* time given in days of: 4-hydroxy-2*E*-alkenals (●); 4-hydroxyperoxy-2*E*-alkenals (x); 4,5-epoxy-2*E*-alkenals (♦); 2*E*-alkenals (◊); 2*E*,4*E*-alkadienals (○); n-alkanals (*); 4-oxo-2*E*-alkenals (Δ); 2,3-epoxyalkanals (□).

The reactivity and toxicity of some of them such as those of oxygenated-*alpha,beta*-unsaturated as 4-hydroperoxy- and 4-hydroxy-2*E*-nonenal, as well as of 4,5-epoxy-2*E*-decenal among others, have been subject of attention [27,29,30]. It must be remembered that these compounds have been held responsible for different degenerative diseases such as cancer, Alzheimer, or Parkinson among others [111-113].

3.2.2. Compounds derived from corn oil minor components.

As has been commented above, not only main but also minor components present in the oil undergo degradation under these conditions. Due to the low concentration of minor components, their derived oxidation compounds will be in very low concentration, a reason to expect that only a reduced number of them could be detected by ^{1}H NMR spectroscopy.

The main sterols of this oil are sitosterol+campesterol [48], and the only oxidation compounds derived from oil minor components detected are coming from them. They are 5*α*,6*α*-epoxysitosterol+campesterol and 5*β*,6*β*-epoxysitosterol+campesterol which have singlet signals at 0.61 ppm and at 0.64 ppm respectively, in agreement with previous studies [114,115] (see Table S8 and Figure 16a). They appear from day 14, in very low concentrations, 0.18 ± 0.04 mmol/molTG and 0.14 ± 0.06 mmol/molTG, for 5α,6α-epoxysitosterol+campesterol and 5β,6β-epoxysitosterol+campesterol, respectively as Figure 16a´ shows. It should be pointed out that this is the first time that the presence of some sterols oxidation products has been detected directly in oxidized edible oil by the ^{1}H NMR technique.

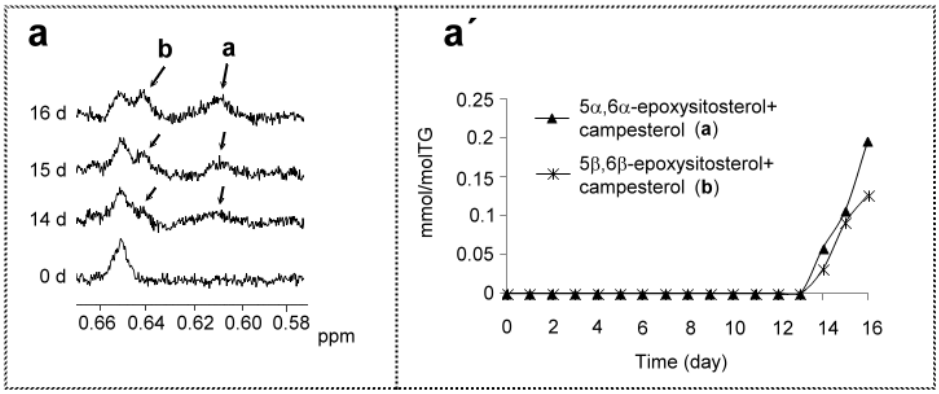

Figure 16. a) Enlargement of region of the ^{1}H NMR spectra region in which the signals of 5α,6α-epoxysitosterol+campesterol and 5β,6β-epoxysitosterol+campesterol appear. **a´)** Evolution of their concentration, expressed as mmol/molTG *versus* time given in days.

3.3. View of the evolution over time of the oxidation process

As mentioned, the process begins with the degradation of the oil main and minor components. The evolution of the degradation of oil main components is well represented by that of linoleic groups. With this premise in mind, Figure 1b´ shows four different stages can be observed in the process. In the first stage, the linoleic groups degradation is slow from day 0 to day 8 (rate of degradation is near -7.6 mmol/mol TG per day). In the second stage from day 8 to day 11 the degradation is somewhat faster than in the first (near -28.9 mmol/mol TG per day are loss). In the third stage, from day 11 to day 14, the degradation reaches its highest rate (near -256.7 mmol/mol TG per day). Moreover, in the last stage that goes to day 16 the degradation rate remains high (-123.7 mmol/mol TG per day). At the end of the process, from the initial 1,461.6 ± 0.1 mmol/mol TG of linoleic groups, near 291.2 ± 29.5 mmol/mol TG only remains without modification.

At the same time, the concentration of *gamma*-tocopherol, which is the main antioxidant of this oil, decreases at an almost constant rate from day 2 onwards (0.14 mmol/molTG per day) up to day 11, being totally degraded on day 12 (see Figure 1c´), just when the degradation of linoleic groups reaches the highest rate.

As linoleic groups are degraded so other structures are formed. The first to be formed are mHPO-c-dEs which can be detected and quantified from day 4 onwards (see Figure 2b and 2c and Table S9). With detailed study of the concentrations it is evident that the transformation of linoleic groups, up to day 8, is mainly due to the formation of mHPO-c-(*Z,E*)-dEs (rate of concentration increase of 3.3 mmol/mol TG per day) and of mHPO-c-(*E,E*)-dEs (rate of concentration increase of 3.9 mmol/mol TG per day). This stage coincides with the first stage of degradation of linoleic groups. In the following stage, from day 8 to day 11, the degradation rate of linoleic is somewhat higher than that of the increase of the concentration of mHPO-c-dEs which is near 19.8 mmol/mol TG per day. From these, 4.7 mmol /mol TG per day are of mHPO-c-(*Z,E*)-dEs and near 15.1 mmol/mol TG per day are of mHPO-c-(*E,E*)-dEs). In other words, the increase in the concentration of mHPO-c-dEs (mmol/mol TG) represents only 68% of the mmol/mol TG of linoleic groups lost. This means that at least 32% of the linolenic groups degraded has generated other compounds that are not mHPO-c-dEs. This difference is much more marked in the following days, under degradative conditions, in such a way that from day 11 to day 13 the increase in the concentration of mHPO-c-dEs only represents 18% of the linolenic groups degraded in this period.

Likewise, it has also been proved that the increase in the concentration of hydroperoxy groups determined from the signal of the proton of the hydroperoxy groups between 8.3 and 9.3 ppm, agrees exactly with that of mHPO-c-dEs from day 4 up to day 8. However, from this day onwards, the concentration of hydroperoxy groups is higher than that of mHPO-c-dEs. This fact proves that other hydroperoxy groups than these are present in the sample. Although the concentration of mHPO-c-dEs decreases strongly from day 13 onwards, as they are clearly intermediate compounds, they do not disappear totally from the sample, remaining to the end of the experiment in a significant concentration.

After mHPO-c-dE, as Table S9 shows, the following formed compounds detected by ^{1}H NMR are mHO-c-(*Z*,*E*)-dEs. They appear on day 8, then reach very low concentration and disappear on day 13 showing their character of intermediate compounds. On day 9 dHPO-nc-*E*,*E*-dEs appear, presumably derived from mHPO-c-dE, reach their maximum concentration on day 13, which diminishes, but they do not disappear, clearly showing their role as intermediate compounds. The formation of HPO-*E*-EPO-*E*-mEs is also fairly early; these compounds appear on day 10 reach a maximum important concentration on day 14 decreasing slowly to day 16.

On day 11, in addition to m-KO-c(*E*,*E*)-dEs, two kinds of aldehydes appear in the sample, whose formation involves the break-up of the acyl group chain, namely 4-HPO-2*E*-alkenals and 2*E*-alkenals. These compounds reach their maximum concentration on days 15, 14 and 16 respectively, the concentration of the first two decreasing slightly at the end of the process (Table S9).

On day 12 there appear in the spectra, in addition to signals of m-KO-c(*Z*,*E*)-dEs, and of methine carbinol protons of secondary alcohols which are either vicinal or not, signals of compounds formed from breaks in chains of acyl groups, such as n-alkanals, 2*E*,4*E*-alkadienals and formic acid. Except for the first structure (m-KO-c(Z,E)-dEs), which reaches its maximum concentration on day 13, the concentrations of the others increase up to the end of the experiment, albeit most of them in low concentrations, except the group due to the signal at 3.98 ppm attributable to methine carbinol protons of secondary alcohols (see Table S9).

A numerous group of different structures appear on day 13. Four of them reach important concentrations at the end of the experiment, as Table S9 shows. These are *Z*-EPO-*Z*-mEs, *E*-EPO-*Z*-mEs, pF, and 4-HO-2*E*-alkenals. In addition to these, 5-pentyl-(5H)-furan-2-one, 4,5-

EPO-2*E*-alkenals, 4-KO-2*E*-alkenals also appear on day 13 with increasing concentration to day 16. HO-*E*-EPO-*E*-mEs, HO-*Z*-EPO-*E*-mEs and *E*-EPO-KO-HOs, also appear on day 13, reaching very low concentrations. And finally, on day 13 there appear signals at 3.62 and 4.24 ppm, associated to methine carbinol protons, in increasing concentrations up to the end of the process probably associated to polymerized structures, although the first signal could also be attributed to primary alcohols, which are also formed in the oxidation of edible oils.

Day 14 also brings new oxidation compounds such as HO-*E*-EPO-*E*-mEs, HO-KO-*Z*-mEs, HO-KO-*E*-mEs, KO-*E*-EPO-*E*-mEs, KO-*Z*-EPO-*E*-mEs, alkyl-furans all of them with increasing concentrations up to the end of the experiment, the concentration of KO-*E*-EPO-*E*-mEs being the main one. The last compounds to be detected were 2,3-EPO-alkanals on day 15 (see Table S9).

4. CONCLUSIONS

^{1}H NMR spectroscopy has allowed one, without chemical modification of the sample, to evaluate the oxidation state of this edible oil at all times under degrading conditions and thus to study its oxidation process globally. The estimation of the concentration of main and minor components of the oil, their degradation kinetic, and the identification and quantification of the new compounds formed during the oxidation process, as well as the estimation of the kinetic of their formation, and even of their further degradation, if it is the case, has been possible. Well-known oxylipins, which are able to form in edible oil oxidation, such as hydroperoxy- hydroxy- and keto-conjugated-dienes, as well as all kinds of oxygenate-*alfa,beta*-unsaturated aldehydes, such as 4-hydroperoxy-2*E*-nonenal, 4-hydroperoxy-2*E*-nonenal and 4,5-epoxy-2*E*-decenal, have been detected in the oxidation of this oil, and their kinetic of formation has been established, and in some cases, that of their subsequent degradation. It is noteworthy that these aldehydes, and also 4-oxo-2*E*-nonenal, which has

been detected for the first time in an oxidized oil by this methodology, are associated to different degenerative diseases such as cancer, Alzheimer or Parkinson among others. In addition, and also for the first time, the formation, in an edible oil subjected to degradative conditions, of an important number of oxylipins, among which are dihydroperoxy-non-conjugated-dienes, hydroperoxy-epoxy-monoenes, hydroxy-epoxy-monoenes, keto-epoxy-monoenes, hydroxy-keto-monoenes, keto-hydroxy-epoxy-structures, and epoxy-monoenes has been proved. Furthermore, their concentration has been estimated, and their evolution *versus* time has been followed. These findings are very important because most of these oxylipins have been found also in cells and have been related to several diseases, such as acute respiratory distress syndrome (ARDS), circulatory shock, disseminated intravascular coagulation, and with multiple organ failure. Moreover, by means of ^{1}H NMR has been detected the presence of a certain concentration of formic acid and for the first time, has been proved the subsequent formation in the triglycerides of poly-formates (and probably of other poly-esters), and also of poly-hydroxides and poly-ethers provoking the increase of the oil viscosity and its polymerization degree. Although there are no previous indications, it could be thought that these latter oxidation compounds could also be formed endogenously, and could be related to the hardening of cell membranes and the obstruction of blood vessels due to the increase in atherosclerotic plaque. Finally, it only remains to add that the large volume of data provided in this study regarding the ^{1}H NMR signal assignments to the different oxidation compounds, and the global and depth insight provided on the oxidation process of this oil, rich in omega 6 groups, which is extendable to other oils, constitute valuable information for other researchers in future studies in which lipid oxidation is involved both in food and in other fields of science..

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: ^{1}H NMR signals, obtained in $CDCl_3$, of protons of main and of some minor corn

oil components, their chemical shifts, multiplicities and assignments to protons of different functional groups present in the corn oil; Table S2: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some hydroperoxides coming from the oxidation of main components detected in the corn oil during the oxidation process; Table S3: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some hydroxy derivatives coming from the oxidation of main components detected in the corn oil during the oxidation process; Table S4: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some keto derivatives coming from the oxidation of main components detected in the corn oil during the oxidation process; Table S5: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some epoxy derivatives coming from the oxidation of main components detected in the corn oil during the oxidation process; Table S6: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of other oxidation compounds coming from the oxidation of main components detected in the corn oil during the oxidation process; Table S7: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of aldehydes (A) coming from the oxidation of main components detected in the corn oil during the oxidation process; Table S8: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some sterols oxidation products coming from the oxidation of minor components detected in the corn oil during the oxidation process; Table S9: Some oxidation compounds or structures detected in corn oil submitted to accelerated storage conditions, together with their detection time (day), the moment (day) in which they reach the maximum concentration and the maximum concentration reached (mmol/molTG); Figure S1: Chemical structures of some oxidation compounds having hydroperoxy groups in their structures, that could be formed during linoleic acyl groups oxidation but that have not been observed in the oxidation of corn oil under the conditions of this study as has been commented on. **a)** monohydroperoxy-non conjugated dienes; **b)** dihydroperoxy-non conjugated dienes and dihydroperoxy-conjugated dienes; **c)** hydroperoxy-epidioxy-monoene; Figure S2: Enlargement of some regions of the ^{1}H NMR spectra of pure 9,10-EPO-12,13-EPO-octadecanoic acid, corn oil after 14 days under oxidative conditions enriched with 9,10-EPO-12,13-EPO-octadecanoic acid and corn oil after 14 days under oxidative conditions.

AUTHOR CONTRIBUTIONS

J.A-C.; performed the experimental work, contributed to data interpretation and to manuscript preparation. M.L.I.; supervised the analyses performed and contributed to data interpretation and to manuscript preparation. M.D.G.; conceived the work, supervised the whole work and the results obtained, and contributed to data interpretation and to the manuscript preparation. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Supplementary Material of

Manuscript 4

TOXIC OXYLIPINS ASSOCIATED TO CURRENT DISEASES DETECTED FOR THE FIRST TIME IN AN EDIBLE OIL RICH IN LINOLEIC ACYL GROUPS SUBMITTED TO OXIDATIVE CONDITIONS. A GLOBAL, BROAD AND IN-DEPTH STUDY BY ^{1}H NMR SPECTROSCOPY

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Table S1. ^{1}H NMR signals, obtained in $CDCl_3$, of protons of main and of some minor corn oil components shown in Figures 1a**,** 1b, 1c and 1d, their chemical shifts, multiplicities and assignments to protons of different functional groups present in the corn oil. The signal letters agree with those given in the above mentioned Figures.

Signal	Chemical shift (ppm)	Multiplicity		Functional group
		Main components[a]		
A_1	0.879	t	-C**H**$_3$	Saturated, monounsaturated ω-9 and/or ω-7 acyl groups
A_2	0.889	t	-C**H**$_3$	Unsaturated ω-6 acyl groups
B	0.972	t	-C**H**$_3$	Unsaturated ω-3 acyl groups
C	1.221-1.419	m	-(C**H**$_2$)$_n$-	acyl groups
D	1.522-1.700	m	-OCO-CH$_2$-C**H**$_2$-	acyl groups
E1+E2	1.941-2.139	m	-C**H**$_2$-CH=CH-	Monounsaturated ω-9 acyl groups Unsaturated ω-6 acyl groups
F	2.305	dt	-OCO-C**H**$_2$-	Acyl groups
G	***2.765***	t	=HC-C**H**$_2$-CH=	Unsaturated ω-6 acyl groups
TG	***4.139, 4.303***	dddd	-C**H**$_2$OCOR	Glyceryl groups
H	5.225-5.296	m	>C**H**OCOR	Glyceryl groups
I	5.296-5.470	m	-C**H**=C**H**-	Acyl groups
		Some minor components		
Δ7A[b]	***0.540***	s	-C**H**$_3$ (C-18)	Δ7-avenasterol**
STN[b]	***0.651***	s	-C**H**$_3$ (C-18)	Sitostanol**
S+C+Δ5A[b]	0.684	s	-C**H**$_3$ (C-18)	β-sitosterol, campesterol and Δ5-avenasterol**
ST+B[b]	0.704	s	-C**H**$_3$ (C-18)	Δ5-stigmasterol and brassicasterol
γ-T[c]	***6.360***	s	-C**H** (Aromatic C-5)	γ-tocopherol**

Abbreviations: s: singlet; d: doublet; t: triplet; m: multiplet; dddd: double of double doublet.

*Area of the signals due to the protons in bold, were used for the quantification of each compound, using the [eq. 1] showed in the Materials and Methods.

**The assignment of the ^{1}H NMR signals of the protons was made with the aid of standard compounds.

***The assignment of the ^{1}H NMR signals of the protons was made with the data taken from the literature:

[a]Assignments of main components taken from:

Guillén, M.D., & Ruiz, A. (2003). *Eur. J. Lipid Sci. Tech. 105*(11), 688-696.

[b]Assignments of sterols-stanols taken from:

Sopelana, P., Arizabaleta, I., Ibargoitia, M.L., & Guillén, M.D. (2013). *Food Chem. 141*(4), 3357-3364.

Ibargoitia, M.L., Sopelana, P., & Guillén, M.D. (2014). *Food Chem. 165*, 119-128.

[c]Assignments of γ-tocopherol taken from:

Baker, J.K., & Myers, C.W. (1991). *Pharm. Res.* 8(6), 763-770.

Table S2. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some hydroperoxides coming from the oxidation of main components detected in the corn oil during the oxidation process.

Chemical shift (ppm)	Multiplicity	Functional group	Compounds and/or family of compounds
Monohydroperoxides (mHPOs)			
8.48	dd	-OO**H**	
6.55[a]	dddd	-C**H**=CH-	9-hydroperoxy-10*E*,12*Z*-octadecadienoate
5.99	ddtd	-C**H**=C**H**-	13-hydroperoxy-9*Z*,11*E*-octadecadienoate
5.57	ddm	-C**H**=CH-	(mHPO-c(*Z*,*E*)-dEs)[**]
5.48	dtm	-CH=C**H**-	
8.42	br	-OO**H**	
6.24[a]	ddm	-C**H**=CH-	9-hydroperoxy-10*E*,12*E*-octadecadienoate
6.03	ddtd	-C**H**=C**H**-	13-hydroperoxy-9*E*,11*E*-octadecadienoate
5.72	dtm	-C**H**=CH-	(mHPO-c(*E*,*E*)-dEs)[**]
5.47	ddm	-CH=C**H**-	
Dihydroperoxides (dHPOs)			
5.36-5.50	m	-C**H**=CH-	9,12-dHPO-10*E*,13*E*-dE + 10,13-dHPO-8*E*,11*E*-dE (dHPO-nc(*E*,*E*)-dEs)
4.82[b]	dd	-C**H**-OOH	
4.30-4.39	m	-C**H**-OOH	
2.33	t	-C**H**$_2$-	
Hydroperoxy-epoxy-monoenes (HPO-EPO-mEs)			
5.85[c]	dd	-C**H**=CH-	9-HPO-12,13-*E*-EPO-10*E*-octadecenoate (HPO-*E*-EPO-mEs)
5.47	dd	-C**H**=C**H**-	
4.33	m	-C**H**-OOH	
3.11	dd	-**H**COCH-	
2.84	m	-HCOC**H**-	
2.30	t	-C**H**$_2$-	
Total hydroperoxides			
8.3-9.3[a]	br	-OO**H**	Total hydroperoxide groups Total-OOH

Abbreviations: d: doublet; t: triplet; m: multiplet, br: broad singlet, dddd: double of double doublet

[*] area of the signals due to the protons in bold, together with the area of the sn-1 and sn-3 signals of TG shown, in Table S1 and in Figure 1, were used for the quantification of each compound, using the [eq. 1] showed in the Materials and Methods.

[**]The assignment of the ^{1}H NMR signals of the protons was made with the aid of standard compounds.

[***]The assignment of the ^{1}H NMR signals of the protons was made with the data taken from the literature:

[a]Assignments of monohydroperoxides (mHPOs) taken from:

Guillén, M.D., & Ruiz, A. (2005a). *Eur. J. Lipid Sci. Tech. 107*(1), 36-47.

Guillén, M.D., & Ruiz, A. (2005b). *J. Sci. Food Agri. 85*(14), 2413-2420.

[b]Assignments of dihydroperoxides (dHPOs) taken from:

Zhang, W. (2008). Synthesis and Fragmentation Reactions of Linoleic Acid-Derived Hydroperoxides (Doctoral dissertation, Case Western Reserve University).

Zhang, W., Sun, M., & Salomon, R.G. (2006). *J. Org. Chem.* 71(15), 5607-5615.

[c]Assignments of hydroperoxy-epoxy-monoenes (HPO-EPO-mEs) taken form:

Gardner, H.W., Weisleder, D., & Kleiman, R. (1978). *Lipids, 13*(4), 246-252.

Table S3. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some hydroxy derivatives coming from the oxidation of main components detected in the corn oil during the oxidation process.

Chemical shift (ppm)	Multiplicity	Functional group	Structures
Monohydroxy-conjugated dienes (mHO-c-dEs)			
6.48[a]	dd	-C**H**=CH-	(*Z*,*E*)-conjugated double bonds associated with hydroxides (OH) mHO-c(*Z*,*E*)-dEs[**]
5.97	dd	-CH=CH-	
5.66	dd	-CH=CH-	
5.45	dt	-CH=CH-	
4.15	m	-CH-OH	
Hydroxy-epoxy-monoenes (HO-EPO-mEs)			
5.94[b]	dd	-C**H**=CH-	9-HO-12,13-*E*-EPO-10*E*-octadecenoate / 13-HO-9,10-*E*-EPO-11*E*-octadecenoate[**] (HO-*E*-EPO-*E*-mEs)
5.54	ddd	-CH=CH-	
4.13	m	-CH-OH	
3.09	dt,br	-CHOHC-	
2.81	dt	-CHOHC-	
5.95[c]	dd	-C**H**=CH-	9-HO-12,13-*Z*-EPO-10*E*-octadecenoate / 13-HO-9,10-*Z*-EPO-11*E*-octadecenoate (HO-*Z*-EPO-*E*-mEs)
5.54	ddd	-CH=CH-	
3.41	dd	-CHOHC-	
3.07	dt	-CHOHC-	
5.65[d]	dt	-CH=CH-	11-HO-12,13-*E*-EPO-9*Z*-octadecenoate / 11-HO-9,10-*E*-EPO-12*Z*-octadecenoate[**] (HO-*E*-EPO-*Z*-mEs)
5.32	dd	-CH=CH-	
4.63	dd	-CH-OH	
2.98	m	-CHOHC-	
2.77	d	-OH-HC-CHOHC-	
5.54[e]	m	-CH=CH-	Erythro-11-HO-12,13-*E*-EPO-9*E*-octadecenoate/ Erythro-11-HO-9,10-*E*-EPO-12*E*-octadecenoate (Erythro- HO-*E*-EPO-*E*-mEs)
4.25	dd	-CH-OH	
2.92	m	-CHOHC-	
2.78	dd	-CHOHC-	
5.78	dtr	-CH=CH-	Threo-11-HO-12,13-*E*-EPO-9*E*-octadecenoate Threo-11-HO-9,10-*E*-EPO-12*E*-ctadecenoate (Threo- HO-*E*-EPO-*E*-mEs)
5.53	ddtr	-CH=CH-	
3.96[e]	q	-C**H**-OH	
2.93	dtr	-CHOHC-	
2.78	dd	-CHOHC-	
Hydroxy-keto-monoenes (HO-KO-mEs)			
6.83[f]	dt	-CH=CH-	9-HO-11-KO-12*E*-octadecenoate (HO-KO-*E*-mEs)
6.05	dt	-CH=CH-	
3.98-4.04	m	-CH-OH	
3.24	d	C=O-CH_2-	
2.58	dd	-CH_2-	
5.54	m	-CH=CH-	9-HO-10-KO-12*Z*-octadecenoate / 13-HO-12-KO-9*Z*-octadecenoate[**] (HO-KO-*Z*-mEs)
4.23	dd	-CH-OH	
3.24[g]	t	C=O-C**H**$_2$-	
2.00	m	-CH_2-	

Abbreviations: s: singlet; d: doublet; t: triplet; m: multiplet, br: broad singlet, ddd: double of double doublet; q: quadruplet.

[*]area of the signals due to the protons in bold, together with the area of the sn-1 and sn-3 signals of TG shown, in Table S1 and in Figure 1, were used for the quantification of each compound, using the [eq. 1] showed in the Materials and Methods.

[**]The assignment of the ^{1}H NMR signals of the protons was made with the aid of standard compounds.

[***]The assignment of the ^{1}H NMR signals of the protons was made with the data taken from the literature:

[a]Assignments of mHO-c(*Z,E*)-dEs taken from:

Manini, P., Camera, E., Picardo, M., Napolitano, A., & d'Ischia, M. (2005). *Chem. Phys. Lipids*, *134*(2), 161-171.

[b]Assignments of 9-HO-12,13-*E*-EPO-10*E*-octadecenoate / 13-HO-9,10-*E*-EPO-11*E*-octadecenoate taken from:

Gardner, H.W., Weisleder, D., & Kleiman, R. (1978). *Lipids*, *13*(4), 246-252.

Gardner, H.W., & Kleiman, R. (1981). *BBA-Lipid Lipid Met. 665*(1), 113-125.

Schieberle, P., Trebert, Y., Firl, J., & Grosch, W. (1988). *Chem. Phys. Lipids*, *48*(3-4), 281-288.

Ramsden, C.E., Domenichiello, A.F., Yuan, Z.X., Sapio, M.R., Keyes, G.S., Mishra, S. K., ... & Davis, J.M. (2017). *Sci. Sign. 10*(493), eaal5241.

[c]Assignments of 9-HO-12,13-*Z*-EPO-10*E*-octadecenoate / 13-HO-9**,**10-*Z*-EPO-11*E*-octadecenoate taken from:

Hidalgo, F.J., Zamora, R., & Vioque, E. (1992). *Chem. Phys. Lipids*, *60*(3), 225-233.

[d]Assignments of 11-HO-12,13-*E*-EPO-9*Z*-octadecenoate / 11-HO-9,10-*E*-EPO-12*Z*-octadecenoate taken from:

Gardner, H.W., Kleiman, R., & Weisleder, D. (1974). *Lipids*, *9*(9), 696-706.

Ramsden, C.E., Domenichiello, A.F., Yuan, Z.X., Sapio, M.R., Keyes, G.S., Mishra, S. K., ... & Davis, J.M. (2017). *Sci. Sign. 10*(493), eaal5241.

[e]Assignments of Erythro-11-HO-12,13-*E*-EPO-9*E*-octadecenoate / Erythro-11-HO-9,10-*E*-EPO-12*E*-octadecenoate and Threo-11-HO-12,13-*E*-EPO-9*E*-octadecenoate / Threo-11-HO- 9,10-*E*-EPO-12*E*-ctadecenoate taken from:

Gardner, H.W., Kleiman, R., & Weisleder, D. (1974). *Lipids*, *9*(9), 696-706.

Gardner, H.W., & Kleiman, R. (1981). *BBA-Lipid Lipid Met. 665*(1), 113-125.

Gardner, H.W., & Crawford, C.G. (1981). *BBA-Lipid Lipid Met. 665*(1), 126-133.

Schieberle, P., Trebert, Y., Firl, J., & Grosch, W. (1988). *Chem. and Phys. Lipids*, *48*(3-4), 281-288.

[f]Assignments of 9-HO-11-KO-12*E*-octadecenoate taken from:

Lin, D., Zhang, J., & Sayre, L.M. (2007). *J. Org. Chem. 72*(25), 9471-9480.

[g]Assignments of 9-HO-10-KO-12*Z*-octadecenoate / 13-HO-12-KO-9*Z*-octadecenoate taken from: Gardner, H.W., Kleiman, R., Christianson, D.D., & Weisleder, D. (1975). *Lipids*, *10*(10), 602-608.

Table S4. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some keto derivatives coming from the oxidation of main components detected in the corn oil during the oxidation process.

Chemical shift (ppm)	Multiplicity	Functional group	Structures
Monoketo-conjugated dienes (mKO-c-dEs)			
7.13[a]	dm	-C**H**=CH- (C-11)	*(E,E)*-conjugated double bonds associated with ketodiene of linoleic acyl groups mKO-c(*E,E*)-dEs[**]
6.15-6.19	m	-CH=CH-(C-12,13)	
6.07	d	-CH=CH- (C-10)	
2.54	t	-CH_2-CO	
7.49[a]	ddd	-CH=C**H**- (C-11)	*(Z,E)*-conjugated double bonds associated with ketodiene of linoleic acyl groups mKO-c(*Z,E*)-dEs[**]
6.16	d	-CH=CH- (C-10)	
6.12	m	-CH=CH- (C-12)	
5.91	dt	-CH=CH- (C-13)	
2.54	t	-CH_2-CO	
Keto-epoxy-monoenes (KO-EPO-mEs)			
6.52	dd	-CH=CH-	13-keto-9,10-*E*-epoxy-11*E*-octadecenoate / 9-keto-12,13-*E*-epoxy-10*E*-octadecenoate[**] (KO-*E*-EPO-*E*-mEs)
6.38[b]	d	-CH=C**H**-	
3.20	dd	-HCOCH-	
2.91	td	-HCOCH-	
2.53	t	-CH_2-	
6.66[b]	dd	-C**H**=CH-	13-keto-9,10-*Z*-epoxy-11*E*-octadecenoate / 9-keto-12,13-*Z*-epoxy-10*E*-octadecenoate (KO-*Z*-EPO-*E*-mEs)
6.40	d	-CH=CH-	
3.52	dd	-HCOCH-	
3.20	dd	-HCOCH-	
2.55	t	-CH_2-	
7.02[c]	dt	-C**H**=CH-	11-keto-12,13-*E*-epoxy-9*E*-octadecenoate / 11-keto-9,10-*E*-epoxy-12*E*-octadecenoate (KO-*E*-EPO-*E*-mEs)
6.23-6.16	dt	-CH=CH-	
3.34-3.28	d	-HCOCH-	
3.04-2.98	ddd	-HCOCH-	
2.25	t	-CH_2-	

Abbreviations: s: singlet; t: triplet; d: doublet; m: multiplet; ddd: double of double doublet

[*]area of the signals due to the protons in bold, together with the area of the sn-1 and sn-3 signals of TG shown, in Table S1 and in Figure 1, were used for the quantification of each compound, using the [eq. 1] showed in the Materials and Methods.

[**]The assignment of the ^{1}H NMR signals of the protons was made with the aid of standard compounds.

[***]The assignment of the ^{1}H NMR signals of the protons was made with the data taken from the literature:

[a]Assignments of mono-keto-conjugated dienes (m-KO-c-dEs) taken from:

Dufour, C., & Loonis, M. (2005). *Chem. Phys. Lipids. 138*(1), 60-68.

[b]Assignments of 13-keto-9,10-*E*-epoxy-11*E*-octadecenoate / 9-keto-12,13-*E*-epoxy-10*E*-octadecenoate and 13-keto-9,10-*Z*-epoxy-11*E*-octadecenoate / 9-keto-12,13-*Z*-epoxy-10*E*-octadecenoate taken from:

Hidalgo, F.J., Zamora, R., & Vioque, E. (1992). *Chem. Phys. Lipids. 60*(3), 225-233.

Lin, D., Zhang, J., & Sayre, L.M. (2007). *J. Org. Chem. 72*(25), 9471-9480.

Ramsden, C.E., Domenichiello, A.F., Yuan, Z.X., Sapio, M.R., Keyes, G.S., Mishra, S. K., ... & Davis, J.M. (2017). *Sci. Sign., 10*(493), eaal5241.

[c]Assignments of 11-keto-12,13-*E*-epoxy-9*E*-octadecenoate / 11-keto-9,10-*E*-epoxy-12*E*-octadecenoate from:

Lin, D., Zhang, J., & Sayre, L.M. (2007). *J. Org. Chem. 72*(25), 9471-9480.

Table S5. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some epoxy derivatives coming from the oxidation of main components detected in the corn oil during the oxidation process.

Chemical shift (ppm)	Multiplicity	Functional group	Structures
Monoepoxy-monoenes (mEPO-mEs)			
5.56-5.47	m	-CH=C<u>H</u>-	9,10-*E*-EPO-12*Z*-octadecenoate / 12,13-*E*-EPO-9*Z*-octadecenoate (*E*)-EPO-*Z*-mE**
5.42-5.33	m	-C<u>H</u>=CH-	
2.73-2.66[a]	m	-C<u>**H**</u>O<u>**H**</u>C-	
2.30	t	-C<u>H$_2$</u>-	
5.46-5.55	m	-CH=C<u>H</u>-	9,10-*Z*-EPO-12*Z*-octadecenoate / 12,13-*Z*-EPO-9*Z*-octadecenoate *Z*-EPO-*Z*-mE**
5.43-5.34	m	-C<u>H</u>=CH-	
2.98-2.88[a]	m	-C<u>**H**</u>O<u>**H**</u>C-	

Abbreviations: t: triplet; m: multiplet.

*area of the signals due to the protons in bold, together with the area of the sn-1 and sn-3 signals of TG shown, in Table S1 and in Figure 1, were used for the quantification of each compound, using the [eq. 1] showed in the Materials and Methods.

**The assignment of the ^{1}H NMR signals of the protons was made with the aid of standard.

***The assignment of the ^{1}H NMR signals of the protons was made with the data taken from the literature:

[a]Assignments of mono-epoxy-monoenes (m-EPO-mEs) taken from:

Nilewski, C., Chapelain, C.L., Wolfrum, S., & Carreira, E.M. (2015). *Org. Lett. 17*(22), 5602-5605.

Table S6. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of other oxidation compounds coming from the oxidation of main components detected in the corn oil during the oxidation process.

Chemical shift (ppm)	Multiplicity	Functional group	Structures
Dihydroxy (dHO)/polyhydroxy (p-HO)			
5.61-5.52	m	-C<u>H</u>=CH-	9,10-dHO-12*Z*-octadecanoate /
5.46-5.36	m	-C<u>H</u>=C<u>H</u>-	12,13-dHO-9*Z*-octadecanoate
<u>3.48-3.37</u>[a]	m	-OHC<u>**H**</u>-C<u>**H**</u>OH-	dHO-mE[**]
			p-HO
Formic acid			
<u>8.01</u>[b]	s	<u>**H**</u>-COOH	Formic acid
Formates or poly-formates (p-F)			
<u>8.17-8.03</u>[c]	m	-H_2C-O-C<u>**H**</u>=O	Polyformates
			pF[**]
Furane groups (Frs)			
<u>7.45</u>[d]	dd	-CH=C<u>**H**</u>- (ar.C-4)	
6.11	dd	-C<u>H</u>=CH- (ar.C-3)	5-pentyl-(5H)-furan-2-one
5.04	m	-C<u>H</u>- (ar.C-5)	
<u>7.27</u>	dd	O-C<u>**H**</u>=CH- (ar.C-5)	
6.24	m	-CH=C<u>H</u>-(ar.C-4)	Alkyl-furans[**]
5.94	m	-C<u>H</u>=C- (ar.C-3)	

Abbreviations: s: singlet; d: doublet; m: multiplet.

[*]area of the signals due to the protons in bold, together with the area of the sn-1 and sn-3 signals of TG shown, in Table S1 and in Figure 1, were used for the quantification of each compound, using the [eq. 1] showed in the Materials and Methods.

[**]The assignment of the ^{1}H NMR signals of the protons was made with the aid of standard compounds.

[***]The assignment of the ^{1}H NMR signals of the protons was made with the data taken from the literature:

[a]Assignments of dihydroxy monoenes (dHO-mEs) taken from:

Nilewski, C., Chapelain, C.L., Wolfrum, S., & Carreira, E.M. (2015). *Org. Lett. 17*(22), 5602-5605.

[b]Assignments of formic acid taken from:

Babij, N. R., McCusker, E. O., Whiteker, G. T., Canturk, B., Choy, N., Creemer, L. C., ... & Li, F. (2016). *Org. Process Res. Dev. 20*(3), 661-667.

[c]Assignments of poly-formates taken from:

Abdullah, B.M., Zubairi, S. I., Huri, H.Z., Hairunisa, N., Yousif, E., & Basu, R.C. (2016). *PloS one*, *11*(3), e0151603.

Harry-O'kuru, R.E., Biresaw, G., Tisserat, B., & Evangelista, R. (2016). *J. Lipids, ID 3128604,* 12.

[d]Assignments of 5-pentyl-(5H)-furan-2-one taken from:

Bonete, P., & Najera, C. (1994). *J. Org. Chem. 59*(11), 3202-3209.

Braukmüller, S., & Brückner, R. (2006). *Eur. J. Org. Chem. 2006*(9), 2110-2118.

Table S7. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of aldehydes (A) coming from the oxidation of main components detected in the corn oil during the oxidation process.

Chemical shift (ppm)	Multiplicity	Functional group	Compounds and/or family of compounds
9.00[a]	d	-C**H**O	2,3-epoxyalkanals (2,3-EPO-alkanals)
3.20	m	-HCOCH-	
3.10	dd	-HCOCH-	
9.49[b]	d	-C**H**O	2*E*-alkenals[**]
6.86	tt	CHO-CH=CH-	
6.11	qt	-CH=CH-	
2.32	q	-CH_2-	
9.52[b]	d	-C**H**O	2*E*,4*E*-alkadienals[**]
7.09	m	CHO-CH=CH-	
6.33	m	-CH=CH-	
6.08	dd	CHO-CH=CH-	
2.22	m	-CH_2-	
9.55[b]	d	-C**H**O	4,5-epoxy-2*E*-alkenals[**] (4,5-EPO-2*E*-alkenals)
6.55	dd	CHO-CH=CH-	
6.39	dd	CHO-CH=CH-	
3.33	dd	-HCOCH-	
2.96	td	-HCOCH-	
9.57[b]	d	-C**H**O	4-hydroxy-2*E*-alkenals[**] (4-HO-2*E*-alkenals)
6.82	dd	CHO-CH=CH-	
6.31	dddd	CHO-CH=CH-	
4.42	m	-CH-OH	
9.58[b]	d	-C**H**O	4-hydroperoxy-2*E*-alkenals[**] (4-HPO-2*E*-alkenals)
9.30	br,s	-OOH	
6.81	dd	CHO-CH=CH-	
6.29	m	CHO-CH=CH-	
4.63	dd	-CH-OOH	
9.75[b]	t	-C**H**O	n-alkanals[**]
2.40	dt	-CH_2-	
9.77[b]	d	-C**H**O	4-oxo-2*E*-alkenals (4-KO-2*E*-alkenals)
6.87	d	CHO-CH=CH-	
6.78	dd	CHO-CH=CH-	
2.69	t	-C=OC**H**-	

Abbreviations: d: doublet; t: triplet; m: multiplet; br: broad singlet; dd: double doublet.

[*]area of the signals due to the protons in bold, together with the area of the sn-1 and sn-3 signals of TG shown, in Table S1 and in Figure 1, were used for the quantification of each compound, using the [eq. 1] showed in the Materials and Methods.

[**]The assignment of the ^{1}H NMR signals of the protons was made with the aid of standard compounds.

[***]The assignment of the ^{1}H NMR signals of the protons was made with the data taken from the literature:

[a]Data taken from:

Daiboun, T., Elalaoui, M.A., Thaler-Dao, H., Chavis, C., & Maury, G. (1993). *Biocatalysis*, *7*(4), 227-236.

[b]Data taken from:

Guillén, M.D., & Ruiz, A. (2004). *Eur. J. Lipid Sci. Tech. 106*(10), 680-687.

Guillén, M.D., & Ruiz, A. (2005a). *Eur. J. Lipid Sci. Tech. 107*(1), 36-47.

Guillén, M.D., & Ruiz, A. (2005b). *J. Sci. Food Agric. 85*(14), 2413-2420.

Goicoechea, E., & Guillen, M.D. (2010). *J. Agric. Food Chem. 58*(10), 6234-6245.

Table S8. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some sterols oxidation products coming from the oxidation of minor components detected in the corn oil during the oxidation process.

Chemical shift (ppm)	Multiplicity	Functional group	Compounds
Sterols oxidation products			
0.61	s	-C**H**$_3$ (C-18)	5α,6α-epoxysitosterol + campesterol
0.64	s	-C**H**$_3$ (C-18)	5β,6β-epoxysitosterol + campesterol

Abbreviations: s: singlet.

*area of the signals due to the protons in bold, together with the area of the sn-1 and sn-3 signals of TG shown, in Table S1 and in Figure 1, were used for the quantification of each compound, using the [eq. 1] showed in the Materials and Methods.

**The assignment of the ^{1}H NMR signals of the protons was made as in previous studies (Zhang, X., Geoffroy, P., Miesch, M., Julien-David, D., Raul, F., Aoudé-Werner, D., & Marchioni, E. (2005). *Steroids*, *70*(13), 886-895)

Table S9. Some oxidation compounds or structures detected in corn oil submitted to accelerated storage conditions, together with their detection time (day), the moment (day) in which they reach maximum concentration and the maximum concentration reached (mmol/molTG).

Compounds and / or structures	Time (day) of maximum concentration	Maximum concentration reached (mmol/molTG)
Detected from day 4		
mHPO-c(*Z*,*E*)-dEs	13	48.5 ± 2.4
mHPO-c(*E*,*E*)-dEs	13	140.9 ± 3.4
Detected from day 8		
mHO-c(*Z*,E)-dEs*	13	4.0 ± 0.8
Detected from day 9		
dHPO-nc(*E*,*E*)-dEs	13	19.2 ± 0.2
Detected from day 10		
HPO-*E*-EPO-*E*-m*E*s	14	38.7 ± 0.6
Detected from day 11		
m-KO-c(*E*,*E*)-dEs*	15	11.2 ± 0.1
4-HPO-2*E*-alkenals*	14	11.5 ± 1.5
2*E*-alkenals*	16	15.6 ± 0.7
Detected from day 12		
m-KO-c(*Z*,*E*)-dEs*	13	5.0 ± 0.8
dHO-*Z*-mEs* / p-OH	16	6.3 ± 0.3
Formic acid	16	2.2 ± 0.1
2*E*,4*E*-alkadienals*	16	7.6 ± 0.1
n-alkanals*	16	10.0 ± 1.1
Signal at 3.98	16	14.2 ± 0.0
Detected from day 13		
HO-*E*-EPO-*E*-mEs*	14	4.8 ± 1.2
Z-EPO-Z-mEs*	16	27.3 ± 3.1
E-EPO-Z-mEs*	16	17.0 ± 1.6
Poly-formates (pF)*	16	18.6 ± 2.1
5-pentyl-(5H)-furan-2-one	16	8.6 ± 0.5
4,5-EPO-2*E*-alkenals*	16	6.9 ± 0.5
4-HO-2*E*-alkenals*	16	19.8 ± 0.9
4-KO-2*E*-alkenals*	16	1.9 ± 0.1
Signal at 3.62	16	10.2 ± 0.5
Signal at 4.23	16	40.3 ± 0.2
Detected from day 14		
Threo-HO-*E*-EPO-*E*-mEs HO-KO-*Z*-mEs*	16	3.0 ± 0.7
KO-*E*-EPO-*E*-mEs*	16	15.3 ± 2.0
KO-*Z*-EPO-*E*-mEs	16	3.6 ± 0.6
Alkyl-furans*	16	0.7 ± 0.1
Detected from day 15		
2,3-EPO-alkanals	16	0.9 ± 0.0

* Compounds with an asterisk were acquired commercially and used as standards for identification purposes.

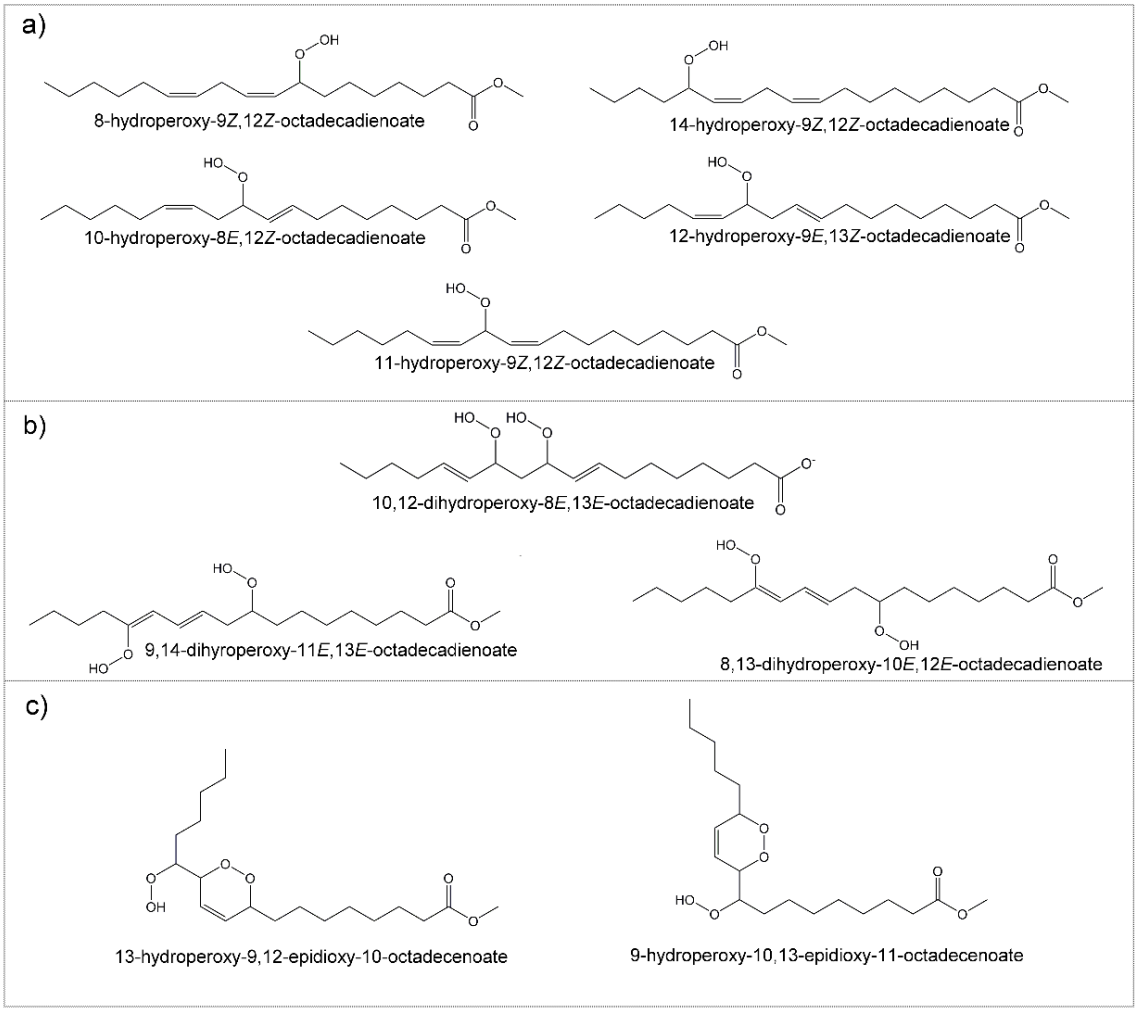

Figure S1. Chemical structures of some oxidation compounds having hydroperoxy groups in their structures, that could be formed during linoleic acyl groups oxidation but that have not been observed in the oxidation of corn oil under the conditions of this study, as commented on. **a)** monohydroperoxy-non conjugated dienes; **b)** dihydroperoxy-non conjugated dienes and dihydroperoxy-conjugated dienes; **c)** hydroperoxy-epidioxy-monoene.

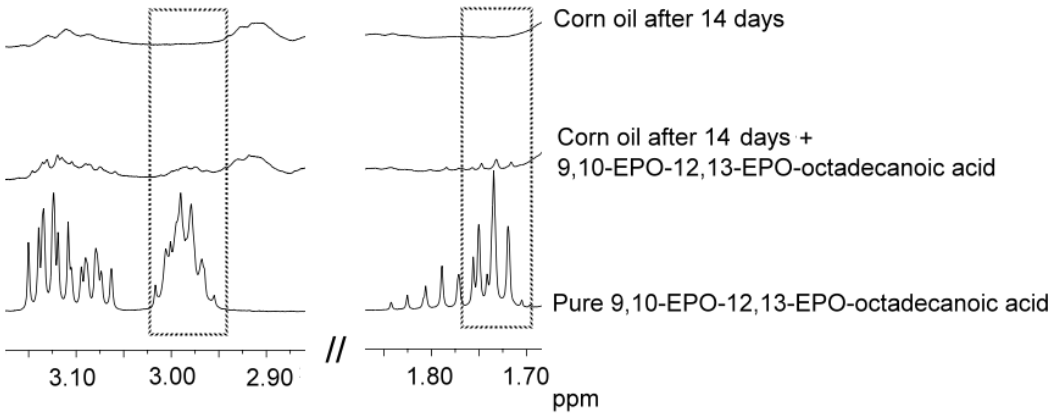

Figure S2. Enlargement of some regions of the ^{1}H NMR spectra of pure 9,10-EPO-12,13-EPO-octadecanoic acid, corn oil after 14 days under oxidative conditions enriched with 9,10-EPO-12,13-EPO-octadecanoic acid and corn oil after 14 days under oxidative conditions.

Article 5

CHANGES PROVOKED BY NIXTAMALIZATION AND TORTILLA MAKING IN THE LIPIDS OF TWO CORN VARIETIES. A STUDY BY ^{1}H NMR

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ABSTRACT

The aim of this study is to analyze in depth, by means of proton nuclear magnetic resonance, ^{1}H NMR, the changes caused by nixtamalization and tortilla making in the lipid composition of two corn varieties. This technique permits the characterization of not only main but also minor lipid components of both corn and tortilla. Ferulates have been found for the first time among the minor components of these lipids. It has been proved that this processing affects the lipids of both corn varieties in a similar way. The total loss of fatty acids occurs as does partial loss of minor components. Furthermore, a slight oxidation is provoked during this processing as well as a small reduction in the unsaturation degree of the lipids. In spite of this a similar distribution of the different kinds of acyl groups has been found in corn and tortilla within each variety.

KEYWORDS: corn lipids; nixtamalization and tortilla-making; proton nuclear magnetic resonance ^{1}H NMR; main and minor components; oxidation.

Chemical compounds studied in this article: 1,2-diglyceride (PubChem CID: 49693474); Phosphatidylcholine (PubChem CID: 5287971); β-Sitosterol (PubChem CID: 222284); Δ7-avenasterol (PubChem CID: 1279536); Sitostanol (PubChem CID: 6743); Campestanol (PubChem CID: 119394); Linoleic acid (PubChem CID: 5280450); Linolenic acid (PubChem CID: 5280934); Oleic acid (PubChem CID: 445639).

1. INTRODUCTION

Corn is a domesticated cereal in Mesoamerica since prehistory. Its cultivation spread throughout all America in ancient times. For centuries it has been the basis of the diet of many American peoples. For thousands of years they have used a processing technique called "nixtamalization". This involves an alkaline thermal treatment of the corn grains with a calcium hydroxide solution, keeping the grains in this solution for a certain period of time, after which they are washed with water and ground to obtain the nixtamalized masa or dough with which tortillas and other culinary preparations are made.

Corn was introduced in Europe in the sixteenth century but not widely consumed by humans until the eighteenth century, coinciding with periods of famine. In Northern Italy and Northern Spain the diet of poor people was based on corn in these periods. In these regions, the corn was processed for culinary preparations like other cereals and people whose diet was almost exclusively based on corn developed the disease named pellagra due to the lack of niacin. However, this health problem did not affect American people whose diet was also based mainly in corn, due to the nixtamalization method used in its processing.

The changes which occur in corn components resulting from this process have been subject of different studies. Thus, it has been proved that it significantly improves corn nutritional characteristics, such as niacin bioavailability (Koetz & Neukom, 1977), for which reason pellagra was not a health problem in America. Likewise, the enrichment of the grains in calcium ions (Serna-Saldivar, Rooney & Greene, 1991) has also been evidenced, this being another improvement in its nutritive value (Serna-Saldivar et al., 1991). Furthermore, modification of the crystalline structure of corn starch (Mondragón, Bello-Perez, Agama, Melo, Betancur-Ancona & Peña, 2004) as well as the variation in phenolic compound content (Del Pozo-Insfran, Brenes, Saldivar & Talcott, 2006) brought about by nixtamalization have also been subject of study, among other aspects.

The effect of this processing on corn lipids has been also addressed. Regarding this issue loss of lipids has been described (Bedolla, Palacios & Rooney, 1983; Pflugfelder, Rooney & Waniska, 1988). Thus Bedolla et al. (1983) found losses of lipid content between raw corn and tortillas of around 28 percent by weight. Pflugfelder et al. (1988), who found losses in lipids of between 11.8 and 18.1 percent by weight, suggested that this loss could be attributed partly to the handling of cooked corn. Regarding this issue it has been pointed out that it could be due to the loss of specific parts of the grain and of compounds soluble in organic solvents like ether, without being fat. Furthermore, no significant changes in the distribution of the different kinds of fatty acids have been reported as consequence of nixtamalization (Bressani, Benavides, Acevedo & Ortiz, 1990). For this reason, it could be thought that this loss could takes place in the same percentage in all corn lipidic components. However, recently it has been reported that in a high-oil corn hybrid rich in linoleic groups nixtamalization and the manufacture of tortillas brings about an important reduction in the percentage of this acyl group. This has been attributed to the saponification provoked by calcium hydroxide and, also to the potential formation of amylose-lipid complexes (Preciado-Ortíz, Vázquez-Carrillo, de Dios Figueroa-Cárdenas, Guzmán-Maldonado, Santiago-Ramos & Topete-Betancourt, 2018). These authors have proved that the loss mainly occurred during nixtamalization, while being almost unappreciable during tortilla making.

Furthermore, the possibility that nixtamalization could provoke other modifications in corn lipids has also been considered. In this sense, studies aimed at evaluating the effect of different calcium hydroxide concentrations on parameters such as iodine value, saponification index and peroxide value have been addressed (Martinez-Flores, Garnica-Romo, Romero & Yahuaca, 2006). It has also been reported that this process can produce corn lipid oxidation, under certain conditions, steeping time being the most influential factor. The parameters used to establish this conclusion were peroxide value, extinction coefficients

at 232 and 270 nm in the UV spectra, and the absorbance of some bands of the infrared spectra (Yahuaca-Juárez, Martínez-Flores, Huerta-Ruelas, Pless, Vazquez-Landaverde & Santillán, 2013a; Yahuaca-Juárez et al., 2013b).

In addition to the above-mentioned changes associated to corn lipids, it has also been suggested that hydrolysis of triglycerides or of other compounds to give di- and mono-glycerides can also occur during nixtamalization (Qamar, 2018). However, until now, we have not found studies in which this fact has been proved.

In this context, this study aims to contribute to a deeper knowledge about the effect of nixtamalization and tortilla making on the lipids of two corn varieties. To do this, both before and after nixtamalization and tortilla making, the corn lipids will be extracted using hexane as solvent and the extracts will be studied by proton nuclear magnetic resonance spectroscopy (^{1}H NMR). The usefulness of this technique, which has not been used for this purpose before, will be tested bearing in mind that it quickly provides from the one spectrum and at the same time information on lipid main and minor components without requiring sample chemical modification. It is to be expected that this information will show the changes occurring during processing to the lipids of both kinds of corn, in terms of losses of main and minor components, changes to the unsaturation degree, and the occurrence of certain reactions, such as oxidation. It must taken into account that the corn varieties subject of study have different colours (white and blue) due to their different content in anthocyanins, other polyphenolic compounds and in carotenoids (Del Pozo-Insfran et al., 2006). For this reason, it could be of interest to analyze if these differences in components with antioxidant ability could influence the changes that nixtamalization and tortilla making provoke on corn lipids.

2. MATERIALS AND METHODS

2.1. Raw samples and extraction of their lipids

The study was carried out with two different varieties of corn (*Zea mays* L.*)* coming from Mexico. One of them is a blue native corn, B, of Tziranza variety, coming from Paracho, Michoacán, and the other is a white corn, W, of Sinaloa variety, coming from Sinaloa. Both varieties of corn have hard endosperm and nixtamalera-tortillera quality. Their grains were ground and mixed with hexane (High Performance Liquid Chromatography (HPLC) grade, 98.5%) in a proportion 1:3 and maintained under vigorous agitation for 24 hours at 25 ºC. After filtration, the solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. The extracted lipids obtained from B and W raw samples were named BR and WR, their yields being 4% and 4.5% by weight respectively.

2.2. Nixtamalization, tortilla manufacture and lipid extraction

Nixtamalization is a technology developed by the Mesoamerican peoples that involves alkaline thermal treatment of the corn grains with calcium hydroxide solution, keeping the grains in this solution for a certain period of time, and cleaning up the grains with water. In this case both corn samples were nixtamalized following the classical methodology. Corn grains were boiled in a solution of calcium hydroxide in a proportion (1/3) (corn/solution). The calcium hydroxide concentration was 1%. The cooking time was established, in agreement with the Mexican normative (NMX-FF-034/1-SCFI-2002), at 30 and 45 minutes for B and W corn respectively. After cooking the grains were steeped in the cooking solution for 12 hours at room temperature. Then the cooked corn grains were washed with distilled water and ground to obtain dough with which tortillas were prepared and cooked on a griddle at 230 ºC (Gomez, Rooney, Waniska & Pflugfelder, 1987; Rooney & Serna-Saldivar, 1987; Serna-Saldivar et al., 1991). The tortillas were dried to 6% moisture and ground to carry out

the extraction of their lipids. This was carried out using hexane under the same conditions mentioned for raw corn samples before. The lipids extracted were named BT and WT, their yields being 2.8% and 3.1% by weight respectively.

2.3. Acquisition of the ^{1}H Nuclear Magnetic Resonance spectra (^{1}H NMR)

The ^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz. Each lipid sample, weighing 0.2 g was mixed with 400 μl of deuterated chloroform and a small proportion of TMS as internal reference; this mixture was introduced into a 5 mm diameter tube. The acquisition conditions were the same as those used in previous studies (Guillén & Ruiz, 2004; Guillén & Uriarte, 2009). The relaxation delays and acquisition times allow the complete relaxation of the protons, the signal areas thus being proportional to the number of protons that generate them, making their use for quantitative purposes possible. Two spectra were acquired from each sample. The ^{1}H NMR spectra were plotted at a fixed value of absolute intensity to be valid for comparative purposes using the MestreNova program (Mestrelab Research, Santiago de Compostela, Spain).

The identification of the compounds was made on the basis of the chemical shifts of their protons for well known compounds. In other cases their identification was confirmed by using standard compounds such as phosphatidylcholine, ethyl and isopropyl ferulate and 1,2-diglyceride, β-sitosterol, Δ5-campesterol, Δ5-avenasterol and sitostanol acquired from Sigma Aldrich (St. Louis, MO, USA), ChemFaces Biochemical Co., LTD (Wuhan, China), Larodan (Malmö, Sweeden). Table 1 shows the signal assignment to the corresponding hydrogen atoms of the several compounds present in the samples subject of study.

Bearing in mind that the area of each ^{1}H NMR spectral signal is proportional to the number of protons that generates it, and that the proportionality constant is the same for all kinds of protons, it is possible, using the area of some spectral signals, to estimate the molar percentage of the several kinds of acyl groups and fatty acids, as well as the concentration of

some minor components. The area of the signals used was determined by using the equipment software and the integrations were made three times to obtain average values.

2.4. Statistical Analysis

The significance of the differences on the several determinations referred to ^{1}H NMR spectral data made among the samples was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS software v.22 (IBM, NY, USA).

Table 1. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of the main protons of glycerides, fatty acids, methyl esters and of phosphatidylcholine present in the samples studied, in agreement with previous studies (Iida et al., 1980; Guillén & Ruiz, 2004; Guillén & Uriarte 2009, 2012; Tariq et al., 2011; Vidal et al., 2012; Bao et al., 2013; Ibargoitia et al., 2014; Ruiz-Aracama et al., 2017). The signal letters agree with those given in Figure 1.

Signal	Chemical shift (ppm)	Multi-plicity	Functional group	
			Type of protons	Compounds
Acyl groups (AG), fatty acids (FA) and other minor components				
Δ7A	0.54	s	-C**H**$_3$ (C-18)	Δ7-avenasterol
STN	0.65	s	-C**H**$_3$ (C-18)	sitostanol
CTN	0.66	s	-C**H**$_3$ (C-18)	campestanol
S+C+Δ5A	0.68	s	-C**H**$_3$ (C-18)	β-sitosterol + campesterol+Δ5-avenasterol
ST	0.70	s	-C**H**$_3$ (C-18)	stigmasterol
A$_1$	0.88	t	-C**H**$_3$	Saturated and monounsaturated **ω-9 AG** and **FA**
A$_1$	0.89	t	-C**H**$_3$	Unsaturated **ω-6 AG** and **FA**
A$_2$	0.97	t	-C**H**$_3$	Unsaturated **ω-3 AG** and **FA**
B	1.19-1.42	m*	-(C**H**$_2$)$_n$-	**AG** and **FA**
C	1.61	m	-OCO-CH$_2$-C**H**$_2$-	**AG** and **FA**
D	1.92-2.15	m**	-C**H**$_2$-CH=CH-	**AG** and **FA**
E	2.26-2.36	dt	-OCO-C**H**$_2$-	**AG** and **FA** in **TG** , except for DHA AG
F$_1$	2.77	t	=HC-C**H**$_2$-CH=	*Bis*-allylic protons in Linoleic **AG** and **FA**
F$_2$	2.77-2.90	m	=HC-C**H**$_2$-CH=	*Bis*-allylic protons in Linolenic **AG** and **FA**
PC	3.30	s	-N(C**H**$_3$)$_3$	Phosphatidylcholine
ME	3.65	s	C**H**$_3$-OCO-CH$_2$	Methyl esters
1,2-DG	3.73	m***	ROCH$_2$-CH(OR')-C**H**$_2$OH	1,2-diglycerides
FE	3.90	s	C**H**$_3$O-	Ferulates
FE	6.18	d	-CH=C**H**-CO- (C-2´)	
FE	6.92	d	-HOC=C**H**-CH- (C-8´)	
FE	7.04	d	CH$_3$OC=C**H**- (C-5´)	
FE	7.08	dd	-HOC=CH-C**H**=C- (C-9´)	
TG	4.22	dd,dd	ROCOC**H**$_2$-CH(OCOR')-C**H**$_2$OCOR''	Glyceryl group in Triglycerides
G	5.22-5.29	m	>C**H**OCOR	Glyceryl groups
H	5.29-5.47	m	-C**H**=C**H**-	Acyl groups

		Oxidation compounds		
		Conjugated dienic systems associated with hydroperoxy-groups		
ZE	6.00	ddtd	-C**H**=C**H**-C**H**=C**H**-	(*Z,E*)-conjugated double bonds
ZE	6.58	dddd		associated with hydroperoxy group (OOH) in AG or FA
		Aldehydes (free or in truncated AG)		
AL	9.74	d	-C**H**O	Alkanals

Abbreviations: t, triplet; m, multiplet; d, doublet; s, singlet; *overlapping of multiplets of methylenic protons in the different acyl groups or in fatty acids either in *beta*-position, or further, in relation to double bonds, or in *gamma*-position, or further, in relation to the carbonyl group;
**overlapping of multiplets of the *alpha*-methylenic protons in relation to a single double bond of the different unsaturated acyl groups or fatty acids;
***this signal shows different multiplicity if the spectrum is acquired from the pure compound or taking part in the mixture.

3. RESULTS AND DISCUSSION

As mentioned the lipid content in B and W corns is of 4 and 4.5 % by weight respectively, whereas in B and W tortillas this amount is reduced to 2.8 and 3.1 %. This evidences that the processing from corn up to tortilla involves the loss of a certain amount of lipids in agreement with previous studies (Bedolla et al., 1983; Pflugfelder et al., 1988). Despite this, little is known about the effect of this processing on the different lipid components of corn (Bressani et al., 1990; Preciado-Ortíz et al., 2018). In order to contribute to shedding light into this issue, corn (BR and WR) and tortillas (BT and WT) hexane extracts were studied by ^{1}H NMR.

3.1. Study of the composition of raw corn lipids by ^{1}H NMR

The proton NMR spectra of the lipids of both raw corn samples contain the typical signals of vegetable oils main components, with some features specific to each one of them. Figure 1a shows the entire spectrum of BR sample as example. These spectra in addition contain signals of some minor lipidic components as Figure 1b shows. From these signals qualitative and quantitative information about the composition of the lipids of both samples can be obtained.

3.1.1. Qualitative characterization of their components. As may be expected, both BR and of WR hexane extracts contain triglycerides (TG) which, as is well known, are the main edible oil components. This is evidenced in their ^{1}H NMR spectra, because these contain the well-known signals of their protons. They are indicated in Table 1 and are shown in Figures 1a and 1b. Thus, signals G and H are due to protons of the triglyceryl backbone and signals A, B, C, D, E and F to the protons of the different kinds of acyl groups (AG) supported on the triglyceryl backbone.

In addition, these spectra show that both BR and WR (Figure 1b) extracts also contain signals of *1,2-diglycerides* (1,2-DG), which are minor components. These give specific ^{1}H NMR signals generated by the protons of the glyceryl backbone, the signal of 1,2-DG being very clear at 3.73 ppm (see Table 1 and Figure 1b), which has similar intensity in the spectra of both extracts, so their concentrations will also be similar. In addition, 1,2-DG give other signals due to the protons of the different acyl groups (AG) supported on the glyceryl backbone; all these latter overlap with that of all other acyl groups, such as the above mentioned triglycerides.

Alkyl esters of fatty acids could also be present in raw corn lipid extracts. The protons of the acyl groups of these compounds give ^{1}H NMR spectral signals that overlap with those of the acyl groups of triglycerides and diglycerides previously mentioned. However, they have in addition signals of the alkyl group, thus allowing their detection and identification. From the two studied samples the presence of *methyl esters* (ME) was only detected in the spectrum of BR by the singlet at 3.654 ppm due to the methyl group. However this signal is absent in the spectrum of WR, indicating that if this kind of compounds are present in the lipids of this corn, their abundance is not enough as to be detected by this technique.

Other minor components which are present in BR and WR samples are *phospholipids*, which in vegetable oils are eliminated during refining. The protons of their two acyl groups

give spectral signals that overlap with the signals A, B, C, D, E and F of triglycerides, diglycerides and alkyl fatty esters. In addition, they give specific signals of the protons of other groups different than acyl groups. In the spectrum of BR no signal attributable to phospholipids is observed, however in that of WR there is a singlet at 3.303 ppm due to the methylic protons of the $-N(CH_3)_3$ group of *phosphatidylcholine* (PC), evidencing in this way its presence in this extract (see Table 1 and Figure 1b).

Furthermore, the ^{1}H NMR spectra of both BR and WR samples indicate that they contain *sterols*. The sterols detected are, as Figure 1b and Table 1 show, *β-sitosterol*, *campesterol*, and *Δ5-avenasterol* (S+C+Δ5A) (signal at 0.685 ppm) as well as *Δ7-avenasterol* (Δ7A) (signal at 0.541 ppm). In addition, these oils also contain *stigmasterol* whose signal at 0.704 ppm overlaps with the side band of the methylic groups. All these signals are due to the methylic protons supported on the C18 of their structure (Iida, Tamura & Matsumoto, 1980; Sopelana, Arizabaleta, Ibargoitia & Guillén, 2013; Ibargoitia, Sopelana & Guillén, 2014; Ruiz-Aracama, Goicoechea & Guillén, 2017).

Likewise, the occurrence of *stanols* has also been observed in the spectra of both samples (see Figure 1b and Table 1). Among these are *sitostanol* (STN) (signal at 0.652 ppm), and *campestanol* (CTN) (signal at 0.661 ppm). These signals of stanols are also due to their methylic protons supported on the C18 of their structure (Iida et al., 1980; Sopelana et al., 2013; Ibargoitia et al., 2014; Sopelana et al., 2016).

The intensity of the signals of sterols and stanols are of a similar order in the spectra of both extracts (see Figure 1b), so their concentrations will also be similar. Their presence in edible oils is well known (Phillips, Ruggio, Toivo, Swank & Simpkins, 2002); however, their direct identification from ^{1}H NMR spectra has been rarely carried out. Both kinds of compounds can be either free or esterified.

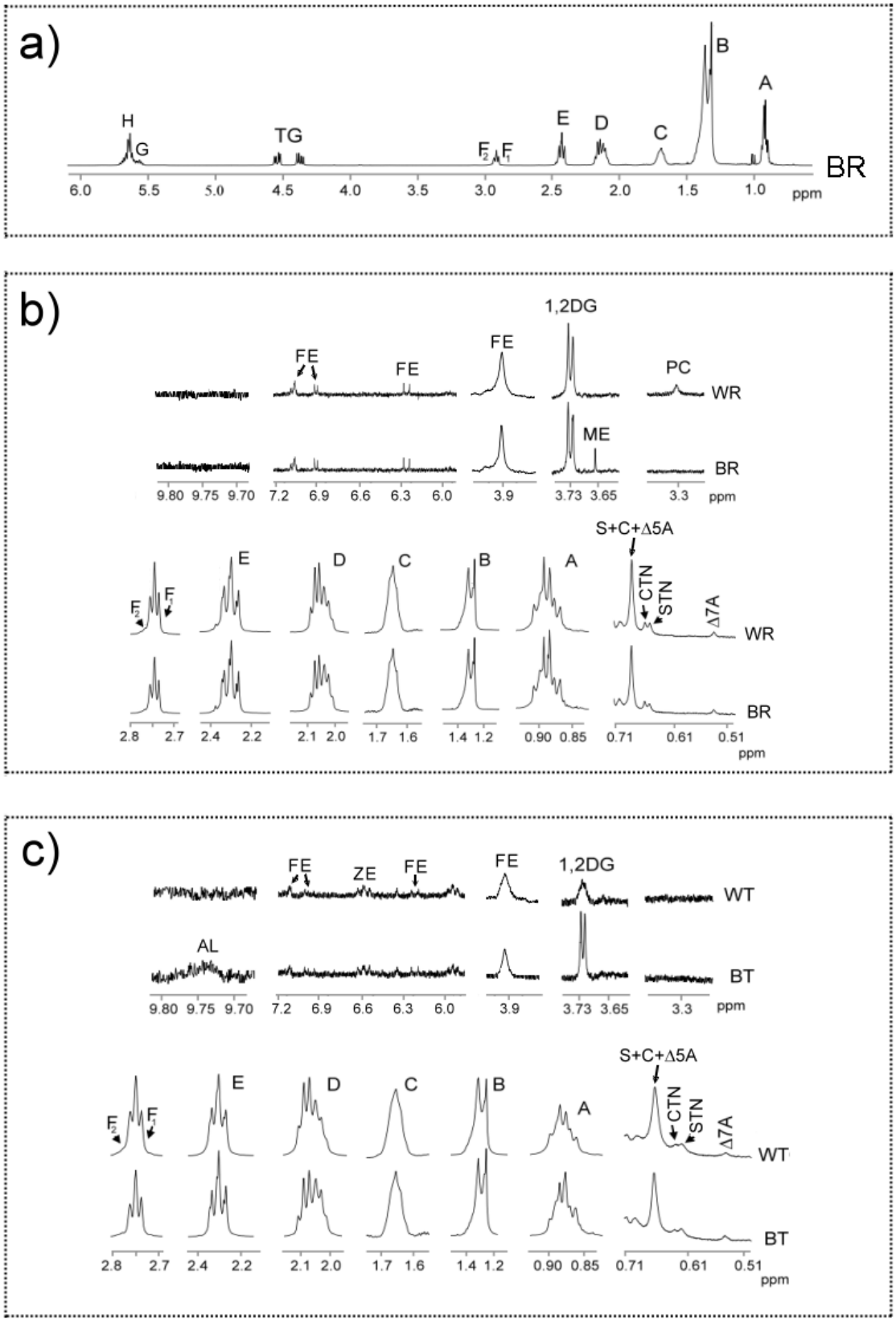

Figure 1. **a)** ^{1}H NMR spectrum of the lipids of B raw corn (BR); **b)** Some spectral regions conveniently enlarged of the lipids of B and W raw corns (WR and BR); **c)** Some spectral regions conveniently enlarged of the lipids of the corresponding tortillas (WT and BT).

In addition to the above-mentioned minor components, the presence of *ferulates* (FE) is evidenced in both BR and WR samples by their ^{1}H NMR spectral signals, as Figure 1b shows. All signals of these compounds (see Table 1) are clearly visible in the spectra and these are in agreement with data given by other authors (Bao, Yanase & Nakatsuka, 2013). The main signal is the singlet at 3.904 ppm due to protons of the methoxy group bonded to the aromatic ring of the ferulate group. It is noteworthy that, although the presence of ferulic acid has been described in corn (Pandey, Singh, Maurya, Singh & Singh, 2013), this compound is absent in these extracts probably because of its low solubility in hexane (Mota, Queimada, Pinho & Macedo, 2008). To the best of our knowledge, this is the first time that the occurrence of ferulates is detected in corn lipids simultaneously with other minor and main lipid components. This finding has been made possible by the technique and methodology used. The occurrence of several steryl and stanyl ferulates in the inner pericarp and aleurone layer of corn kernels has been described previously, and although they have been found in corn bran, they have never been detected in corn lipid extracts (Seitz, 1989; Norton, 1994; Hakala et al., 2002).

Nevertheless, although *γ-tocopherol* is the most abundant tocopherol in corn lipids (Winkler-Moser & Breyer, 2011; Alberdi-Cedeño, Ibargoitia, Cristillo, Sopelana & Guillén, 2017a), it has not been detected by ^{1}H NMR spectroscopy (signal at 6.362 ppm) (Martin-Rubio, Sopelana & Guillén, 2018) either in BR or in WR. This is due to its concentration being much lower than that of the other minor components already mentioned (Winkler-Moser & Breyer, 2011).

Among the minor components detected, there are also, as could be expected, *fatty acids* (FA). Most of the signals of the protons of fatty acids (A, B, C, D, E and F) overlaps totally with that of acyl groups (AG) supported on triglycerides, diglycerides and phospholipids. However, one of these signals shows only a partial overlapping that allows it to be seen that

both corn extracts contain higher proportions of fatty acids than common refined edible oils. This is signal E due to protons supported on the carbon atom in *alpha* position in relation to the carbonyl group in esters and to the carboxyl group in acids (see Figure 1b and Table 1). It is about a triplet centred near 2.35 ppm in fatty acids, and, in the spectra of these extracts, partially overlaps with that of protons bonded to the carbon atom in *alpha* position in relation to the carbonyl groups of AG of TG (2.310 ppm), of 1,2-DG (2.330 ppm) and of other ester groups (Nieva-Echevarría, Goicoechea, Manzanos & Guillén, 2014). This signal is usually not observable in the ^{1}H NMR spectra of refined corn oils (Guillén & Ruiz, 2003) due to their concentration of fatty acids being much lower than in the raw corn hexane extracts here studied.

3.1.2. Quantitative estimation of their minor components. As mentioned, the area of the ^{1}H NMR spectral signals is proportional to the number of protons that generates it, and this proportionality constant is the same for all kind of protons. Due to this, the concentration of the minor components in relation to the number of moles of TG, can be estimated by using the following equations:

$$[\text{1,2-DG}]\ (\text{mol/mol TG}) = (2A_{12DG}/A_{TG}) \quad [\text{eq. 1}]$$

$$[\text{ME}]\ (\text{mol/mol TG}) = (4A_{ME}/3A_{TG}) \quad [\text{eq. 2}]$$

$$[\text{PC}]\ (\text{mol/mol TG}) = (4A_{PC}/9A_{TG}) \quad [\text{eq. 3}]$$

$$[\text{S+C+}\Delta\text{5A}]\ (\text{mol/mol TG}) = (4A_{S+C+\Delta5A}/3A_{TG}) \quad [\text{eq. 4}]$$

$$[\Delta\text{7A}]\ (\text{mol/mol TG}) = (4A_{\Delta7A}/3A_{TG}) \quad [\text{eq. 5}]$$

$$[\text{STN}]\ (\text{mol/mol TG}) = (4A_{STN}/3A_{TG}) \quad [\text{eq. 6}]$$

$$[\text{CTN}]\ (\text{mol/mol TG}) = (4A_{CTN}/3A_{TG}) \quad [\text{eq. 7}]$$

$$[\text{FE}]\ (\text{mol/mol TG}) = (4A_{FE}/3A_{TG}) \quad [\text{eq. 8}]$$

$$[\text{FA}]\ (\text{mol/mol TG}) = (2A_{E}/A_{TG})-(4A_{12DG}/A_{TG})-(4A_{ME}/3A_{TG})-(8A_{PC}/9A_{TG})-3 \quad [\text{eq. 9}]$$

In these equations: A_{12DG}, is the area of the signal at 3.73 ppm; A_{TG}, is the area of the signals of the four protons of sn1 and sn3 positions of the glyceryl backbone of triglycerides TG; A_{ME}, is the area of the signal at 3.65 ppm; A_{PC}, is the area of the signal at 3.30 ppm; $A_{S+C+\Delta5A}$, $A_{\Delta7A}$, A_{STN} and A_{CTN} are the areas of the signals of methylic protons supported on the C18 of sitosterol, campesterol plus Δ5-avenasterol, of Δ7-avenasterol, of sitostanol and of campestanol respectively; A_{FE}, is the area of the signal due to protons of the methoxy group bonded to the aromatic ring of the ferulates; and finally, A_E, is the area of the signal of the methylenic protons supported on the carbon atom in *alpha* position in relation to the carbonyl group in esters and carboxyl group in acids. All of them are shown in Figure 1b and indicated in Table 1. The concentration of stigmaterol cannot be estimated due to the overlapping of the signal of its methylic protons on the C18 carbon atom with the side band of methylic protons of acyl groups and fatty acids.

The concentration of these minor components can also be estimated in relation to the number of moles of fatty acids plus acyl groups (FA+AG) which are the main structures in corn lipids. The equations used for this estimation are given in Supplementary material (see equations S1 to S9). The results obtained using both approaches are given in Table 2.

Among these minor components 1,2-DG and S+C+Δ5A are in similar concentration. Ferulate concentration is around six times lower than that of the minor components mentioned before. Stanols and the rest of sterols are in a lower concentration than that of ferulates. And finally, PC and ME in addition to being in very low concentrations as expected are absent in BR and in WR respectively.

Table 2. Concentrations, given in mmol/mol TG and in mmol/mol (AG+FA), of some minor components in BR and WR hexane extracts of raw corn oils B and W respectively and in hexane extracts of tortillas BT and WT obtained from the same corns after nixtamalization. Different letters within each row indicate a significant difference ($p < 0.05$).

Minor compounds	**BR**	**WR**	**BT**	**WT**
1,2-DG (mmol/mol TG)*	14.43 ± 0.81a	17.38 ± 0.44b	8.27 ± 0.01c	3.48 ± 0.48d
ME (mmol/mol TG)	0.74 ± 0.02	-	-	-
PC (mmol/mol TG)*	-	0.45 ± 0.07	-	-
S+C+Δ5A (mmol/mol TG)*	15.75 ± 1.84ab	19.84 ± 0.34b	13.55 ± 1.08a	14.38 ± 0.44a
Δ7A (mmol/mol TG)	1.02 ± 0.07a	1.18 ± 0.01a	1.01 ± 0.15a	0.91 ± 0.06a
STN (mmol/mol TG)*	1.66 ± 1.46a	2.53 ± 0.07b	1.42 ± 0.17a	1.28 ± 0.06a
CTN (mmol/mol TG)	1.46 ± 0.26a	1.72 ± 0.02a	0.54 ± 0.10b	0.52 ± 0.06b
FE (mmol/mol TG)*	2.55 ± 0.21a	3.27 ± 0.24a	1.21 ± 0.52b	0.98 ± 0.12b
FA (mmol/mol TG)	173.44 ± 0.06	190.29 ± 0.01	-	-
1,2-DG (mmol/mol (AG+FA))*	4.50 ± 0.17a	5.39 ± 0.11b	2.86 ± 0.04c	1.19 ± 0.15d
ME (mmol/mol (AG+FA))	0.23 ± 0.00	-	-	-
PC (mmol/mol (AG+FA))*	-	0.14 ± 0.02	-	-
S+C+Δ5A (mmol/mol (AG+FA))*	4.91 ± 0.48ab	6.15 ± 0.13b	4.70 ± 0.44a	4.94 ± 0.13ab
Δ7A (mmol/mol (AG+FA))	0.32 ± 0.03a	0.37 ± 0.00a	0.35 ± 0.06a	0.31 ± 0.02a
STN (mmol/mol (AG+FA))*	0.52 ± 0.02a	0.79 ± 0.02b	0.49 ± 0.07a	0.44 ± 0.02a
CTN (mmol/mol (AG+FA))	0.45 ± 0.07a	0.53 ± 0.00a	0.19 ± 0.04b	0.18 ± 0.02b
FE (mmol/mol (AG+FA))*	0.79 ± 0.05a	1.01 ± 0.08a	0.42 ± 0.18b	0.34 ± 0.04b
FA (mmol/mol (AG+FA))	53.77 ± 0.02	58.98 ± 0.00	-	-

*Asterisked compounds were acquired commercially and used as standards for identification purposes.
−, no detected.

Fatty acids are by far the most abundant of the minor components, both samples having near 200 mmoles of FA per mol of TG. Moreover, as can be deduced from data referred to AG+FA, given in Table 2, the molar percentage of FA is of 5.4 % and 5.9 % in relation to the sum of AG+FA in BR and WR respectively. This concentration is of a similar order to that found by other authors in crude corn oil obtained by extraction with hexane (Winkler-Moser & Breyer, 2011).

In summary, there are no great differences in relation to the minor components present in BR and WR, although most of the common components are in a slightly higher concentration in WR than in BR (see Table 2). It is worth noting the great concordance between the concentrations referring to TG and those referring to AG+FA, as is to be

expected in accurate and robust approaches; it must be remembered that the ^{1}H NMR signals involved in both approaches are not the same.

3.1.3. Oxidation status. The ^{1}H NMR spectra of the corn hexane extracts can also provide information about the presence of compounds coming from corn lipid oxidation processes, in other words about the oxidation status of the corn hexane extracts. It is well known that oxidation generates firstly hydroperoxides, also named primary oxidation compounds. These compounds can contain either *Z,E*- or/and *E,E*-conjugated dienic structures when the oxidation is produced in either polyunsaturated fatty acids or acyl groups which are the main structures affected by this reaction in these extracts. The protons of these compounds generate well-known signals in the ^{1}H NMR spectra, that allows their quantification if their concentration is sufficient to be detected by this technique. The primary oxidation compounds degrade to form so-called secondary oxidation compounds. These include a great number of compounds of different natures, many of which can also be detected and quantified by ^{1}H NMR (Guillén & Ruiz, 2004; Guillén & Uriarte, 2009; Martínez-Yusta, Goicoechea & Guillén, 2014a; Martin-Rubio et al., 2018). However, in the ^{1}H NMR spectra of BR and WR, (see in Figure 1b, regions between 6.0 and 9.8 ppms) no signals due to protons of oxidation compounds appear**.** This indicates that the hexane extracts of the raw corn oils are unoxidized, as might be expected.

3.1.4. Unsaturation degree. The unsaturation degree of edible oils and of lipidic extracts is a matter of great interest because it is closely related to its oxidative stability, although this latter property is also dependent on their content in fatty acids and in compounds able to exhibit either antioxidant or prooxidant ability. It can be estimated directly in a very simple, accurate and rapid way from ^{1}H NMR spectral data, and can be expressed by different parameters with the same meaning.

One of these parameters is P_1. This is defined as the percentage of olefinic protons, op, in relation to the total protons supported on acyl groups and on fatty acids tp, these latter being the sum of olefinic protons, op, and of protons bonded to saturated carbon atoms, sp, in acyl groups and fatty acids (P1= 100 op/tp = 100 op/(op+sp)). The determination of this parameter P_1, is very easy and simple to carry out, by means of the equation P_1=100 $A_{op}/(A_{op+}A_{sp})$ [eq. 10]. In this equation A_{op} is the area of the signals between near 5.26 ppm and 5.47 ppm corresponding to olefinic protons and A_{sp} is the sum of the areas between 0.80 ppm and 3.0 ppm due to protons bonded to saturated carbon atoms of acyl groups and fatty acids shown in Figure 1b.

Another parameter which is useful for the same aim is P_2, defined as the ratio between olefinic protons and protons supported on saturated carbon atoms in fatty acids and acyl groups, which can be estimated from the equation $P_2 = 100\ A_{op}/A_{sp}$ [eq. 11]. Table 3 gives the values of P_1 and P_2 parameters of BR and WR. Both indicate that the lipids of B corn have a lower unsaturation degree than the lipids of W corn.

Nevertheless, classically the unsaturation degree of edible oils has been expressed by the well-known Iodine Value, IV. For its determination different chemical methods, all of them involving the chemical modification of the sample and the use of different reagents have been described. Among them, the Hanus method (AOAC, 920.158, 1990) is one of the most commonly used. The determination of this parameter can also be carried out from ^{1}H NMR spectral data (Guillén & Ruiz, 2003), by using the equation IV= 10.54+13.39*P1 [eq. 12], where P1 has the same meaning as above. The IVs obtained in this way are given in Table 3 and indicate, unsurprisingly, that WR has a higher unsaturation degree than BR.

Table 3. Several parameters indicators of unsaturation degree (P_1, percentage of olefinic protons in relation to olefinic plus saturated protons; P_2, percentage of olefinic protons in relation to saturated protons; and Iodine Value, IV), as well as molar percentages of the different kinds of acyl groups and fatty acids (AG+FA), obtained from ^{1}H NMR spectral data of hexane extracts of raw native blue and white corns (BR and WR respectively) and of the corresponding tortillas (BT and WT). Different letters within each row indicate a significant difference ($p < 0.05$).

	BR	WR	BT	WT
Several Parameters Indicators of Unsaturation Degree				
P_1	7.99 ± 0.19a	8.75 ± 0.08b	7.80 ± 0.02a	8.56 ± 0.04b
P_2	8.68 ± 0.23a	9.59 ± 0.10b	8.46 ± 0.03a	9.36 ± 0.04b
IV	117.53 ± 2.61a	127.70 ± 1.10b	114.98 ± 0.32a	125.16 ± 0.48b
Molar Percentages of the Several Kinds of Acyl Groups+Fatty Acids				
Linolenic (Ln)	1.05 ± 0.05a	1.29 ± 0.06b	0.92 ± 0.00c	1.26 ± 0.00b
Linoleic (L)	41.88 ± 0.39a	49.16 ± 0.47b	41.20 ± 0.13a	49.06 ± 1.99b
Oleic (O)	40.51 ± 0.19a	34.94 ± 0.01b	41.72 ± 0.01a	35.59 ± 2.30b
Saturated (S)	16.56 ± 0.17a	14.61 ± 0.40b	16.16 ± 0.12a	14.09 ± 0.31b

3.1.5. Molar percentages of the several kinds of acyl groups and fatty acids. As is well known, the main corn lipid components are triglycerides, TG, which support several kinds of acyl groups, AG, having different unsaturation degrees and chain length. As previously commented on, the 1,2-DG, ME, PC detected can also have different kinds of acyl groups, AG; and finally, the detected fatty acids, FA, can also have different unsaturation degrees and chain lengths. All acyl groups and fatty acids (AG+FA) present in both corn lipid samples can be grouped in linolenic (Ln), linoleic (L), oleic (O) and saturated (S) groups; this latter group includes all saturated structures regardless of their length.

Taking into account the assignment of the signals of the ^{1}H NMR spectra, shown in Figures 1a and 1b, to the different protons indicated in Table 1, and their intensities, the distribution of Ln, L, O and S groups in the corn extracts can be estimated using the following equations (Guillén & Ruiz, 2003, Guillén & Uriarte, 2009):

$$\% Ln = 100 (A_{F2}/2A_E) \quad \text{[eq. 13]}$$

$$\% L = 100 (A_{F1}/A_E) \quad \text{[eq. 14]}$$

$$\% O = 100 [(A_D/2\ A_E)-(A_{F1}/A_E)-(A_{F2}/2A_E)] \quad \text{[eq. 15]}$$

$$\% S = 100[1- (A_D/2\ A_E) \quad \text{[eq. 16]}$$

In these equations A_{F2} and A_{F1} are the areas of the signals of *bis*-allylic protons of Ln and L groups respectively, and A_D is the area of the signals of all *mono*-allylic protons present in the samples. The meaning of A_E has been described before.

The results obtained are given in Table 3. It can be observed that both corn lipids are richer in linoleic than in oleic groups, as is common in corn oils (Winkler-Moser & Breyer, 2011; Alberdi-Cedeño, Ibargoitia & Guillén, 2017b). However, in BR corn lipids the difference between the concentrations of these two groups is much smaller than in WR corn lipids. Both corn varieties have a very small concentration of linolenic groups, as expected, and BR is richer in saturated groups than WR.

In summary the ^{1}H NMR spectra of these corn lipids provide a great deal of information concerning their composition. It is noteworthy that all this information is obtained from the same spectrum of the original sample in a very simple and quick way, not requiring any chemical changes.

3.2. Changes undergone by corn lipids during nixtamalization and tortilla making estimated from ^{1}H NMR spectral data.

As mentioned, corn kernels of the two before mentioned varieties were submitted separately to nixtamalization. The nixtamal obtained was separated from the liquid phase, washed to remove the excess calcium hydroxide and the remains of pericarp as is customary, and ground to obtain the corn *masa*, which was submitted to thermal treatment to obtain tortillas BT and WT. The lipids contained in both kinds of tortillas (BT and WT) were

extracted using the same extraction method used for corn lipids and studied by ^{1}H NMR. Figure 1c shows the signals of the ^{1}H NMR spectra of these lipids, due to protons of their minor and main components, from which their concentrations can be estimated.

3.2.1. Changes in lipid minor component concentrations. Figure 1c shows that both BT and WT spectra contain the same signals already described in BR and WR spectra of protons of 1,2-DG, sterols, stanols, and ferulates. However, the concentration of all these compounds in tortilla lipids is lower than in BR and WR, as Table 2 shows. This indicates that nixtamalization and tortilla-making entail a certain loss of these compounds but not their total disappearance. The permanence of certain amounts of sterols, stanols and ferulates in tortillas is of great interest because it has been described that they are able to act as antioxidants and, for this reason, they could contribute to maintaining the tortilla oxidative stability and also positively influence consumer health.

It is noteworthy, as Figures 1b and 1c shows, that signals of ME and of PC, present respectively in BR and WR spectra with a very low intensity, are not visible in BT and WT spectra, which evidences their absence in tortilla lipids.

The most outstanding fact is the absence of the signal of methylenic protons in alpha position in relation to the carboxyl group of fatty acids in the spectra of BT and WT. This shows that fatty acids, which are the principal minor components in the extracts of both kinds of corn, either have been totally eliminated during the processing or are in very low concentrations unable to be detected by ^{1}H NMR. This is very important because of the great tendency of fatty acids to oxidize. This fact, reported here for the first time, shows that this processing entails, from the technological point of view, an important improvement, because it provokes an increase in the oxidative stability of the lipids contained in tortillas in relation to the lipids contained in corn. It could be said that this processing has common points with vegetable oil refining due to the loss of fatty acids and the partial loss of certain minor lipid

components. The loss of fatty acids can be explained by their reaction with calcium hydroxide, giving rise to the formation of salts and their subsequent elimination during washing.

It has also been described that triglycerides are broken down into monoglycerides and diglycerides during nixtamalization of corn (Qamar, 2018). Nevertheless, monoglycerides are not present in tortilla lipids because their signals (Guillén & Uriarte, 2012; Martínez-Yusta, Goicoechea & Guillén, 2014b; Nieva-Echevarría et al., 2014) are absent in the ^{1}H NMR spectra of the lipids of both tortillas. Diglycerides are present in tortilla lipids, as their spectra show, however in smaller concentration than in corn lipids (see Table 2). It is possible that the saponification of triglycerides leads to the formation of diglycerides and of monoglycerides as intermediate compounds, and that these, after a later saponification, are almost totally eliminated by washing. This could be the reason for which, if these compounds are formed during nixtamalization, monoglycerides are not present in tortilla lipids and diglycerides are present even in smaller concentration than in corn lipids.

3.2.2. Changes in lipid oxidation status. Formation of oxidation compounds. In all processes taking place in presence of oxygen, food lipids can be oxidized, even in the presence of compounds able to act as antioxidants. For this reason, oxidation cannot be discarded in this case.

The ^{1}H NMR spectra also contain information about the oxidation status of lipid samples. Figure 1c shows that the spectra of the lipids of both tortillas (BT and WT) have signals (*ZE*) of protons of certain primary oxidation compounds, such as hydroperoxides supported on structures having *Z*,*E*-conjugated diene groups (OOH-*Z*,*E*-CD) (multiplets centered at 6.58 ppms). This indicates that some oxidation has occurred during the nixtamalization and tortilla-making processes. The concentration of these hydroperoxides was estimated by the equation [OOH-*Z*,*E*-CD] = $4A_{ZE}/A_{TG}$ [eq. 17], where A_{ZE} is the area of

signal ZE in Figure 1c. The concentrations of hydroperoxides thus estimated are 5.93±0.85 mmol/mol TG in BT and 5.54 ± 0.22 mmol/mol TG in WT. This shows that the oxidation level reached by the lipids of both tortillas is low, and of a similar order, although somewhat higher in BT.

Regarding secondary oxidation compounds, alkanals (AL) (signal AL in Figure 1c and Table 1) have been detected, but only in BT. Their concentration, estimated by using equation $[AL] = 4A_{AL}/A_{TG}$ [eq. 18], where A_{AL} is the area of signal AL, reaches a value of 1.55 ± 0.13 mmol/mol TG. This result also suggests that BT has a slightly higher oxidation degree than WT.

From these results, it is evident that a certain degree of oxidation takes place during nixtamalization and tortilla-making, affecting mainly polyunsaturated groups, as is indicated by the hydroperoxides formed. For this reason, a slightly lower unsaturation degree and a slightly smaller molar percentage of unsaturated groups should be expected in tortilla than in corn lipids.

3.2.3. Unsaturation degree of tortillas lipids. This characteristic was estimated by the same previously mentioned parameters, P1, P2 and IV. Their values are given in Table 3. It can be observed that the three parameters indicate that during the processing of corn to make tortillas the unsaturation degree of their lipids was slightly reduced. This slight reduction in unsaturation degree could be due to the low oxidation degree occurring specifically in the unsaturated groups. However, in a previous study the preferential loss of linoleic groups was attributed to the greater tendency of these groups towards saponification or forming amylose-lipid complexes than that of the others (Preciado-Ortíz et al., 2018). Furthermore, it is also noteworthy that the evident loss of fatty acids in this processing does not affect to the unsaturation degree of tortilla lipids; this could suggest that the distribution profile of the different kinds of fatty acids and of acyl groups in corn lipids are very similar. For this reason,

the loss of fatty acids during processing do not brought about significant changes in the lipids unsaturation degree.

3.2.4. Distribution of acyl groups in tortillas lipids. The molar percentage of the different kinds of acyl groups of tortilla lipids was estimated from ^{1}H NMR spectra, as previously described, and the results are shown in Table 3. Comparison of the data from lipids of tortilla and of raw corn evidences that the distribution of the percentages of the different acyl groups did not undergo a significant change from corn to tortilla. A small reduction in the molar percentage of linoleic acyl groups is observed, in agreement with all the above mentioned in relation to unsaturation degree, and with the formation of hydroperoxides supported on structures having *Z,E*-conjugated dienic groups derived from linoleic groups. These results are basically in agreement with those of a previous study in which no significant changes, in the distribution of the different kinds of groups were reported in the processing of corn into tortilla (Bressani et al., 1990).

The results obtained here indicate that the loss of acyl groups and of fatty acids during this processing affects all of them to a similar extent regardless of their unsaturation degree, in spite of oxidation specifically affects the unsaturated groups.

4. CONCLUSIONS

^{1}H NMR has been shown to be a very efficient and useful technique to study not only the lipids of the two corn varieties, also in their tortilla form, and as a consequence to evaluate the changes provoked by nixtamalization and tortilla-making in the corn lipids.

It has been proved that the lipids of both corn varieties contain almost the same minor components in a somewhat higher concentration in the white than in the blue corn. Among these the principal are fatty acids, 1,2-diglycerides, sterols, and ferulates also being present. For the first time, the presence of ferulates in corn lipids is described. With regard to main

components, it has been shown that white corn lipids are richer in linoleic groups than blue corn lipids and the opposite is true for oleic groups. As a consequence, white corn lipids have a higher unsaturation degree than blue corn lipids.

It has been shown that nixtamalization and tortilla-making provoke a total loss of fatty acids present in corn lipids, a partial loss of the other minor components, a small reduction in the unsaturation degree of the main lipid components, and a slight oxidation undergone by the unsaturated acyl groups. The total loss of fatty acids increases the oxidative stability of tortilla lipids in relation to corn lipids, but the distribution of the different kinds of acyl groups is fairly similar in corn and tortilla lipids.

AUTHOR CONTRIBUTIONS

M. Molina performed the experimental work referred to nixtamalization, tortilla-making and lipids extraction. B. Yahuaca-Júarez supervised the experimental work referred to nixtamalization, tortilla-making and lipids extraction. J. Alberdi-Cedeño performed the experimental work referred to ^{1}H NMR spectroscopy, contributed to data interpretation and to manuscript preparation. M.L. Ibargoitia supervised the analyses performed by ^{1}H NMR spectroscopy and contributed to data interpretation and to manuscript preparation. M.D. Guillén conceived the work, supervised the whole work and the results obtained, and contributed to data interpretation and to the manuscript preparation.

CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

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Supplementary Material of

Article 5

CHANGES PROVOKED BY NIXTAMALIZATION AND TORTILLA MAKING IN THE LIPIDS OF TWO CORN VARIETIES. A STUDY BY ^{1}H NMR

Jon Alberdi-Cedeño, María L. Ibargoitia, María D. Guillén

Content:

- Equations for the estimation of minor components concentration referring to the number of moles of fatty acids plus acyl groups (FA+ AG).

Estimation from ^{1}H NMR spectral data of the concentration of some minor components in the lipids of B and W raw corns (BR and WR) and of the corresponding tortillas (BT and WT) referring to the number of moles of fatty acids plus acyl groups (FA+AG)

As before mentioned, the area of the ^{1}H NMR signals is proportional to the number of protons that generates it, which are indicated in Table 1, and this proportionality constant is the same for all kind of protons. Due to this, the concentration of minor lipid components of raw corn and tortilla can be estimated. The estimation referring to the number of moles of fatty acids plus acyl groups (FA+ AG) can be made by using the following equations:

1,2-DG (mol/mol (AG+FA)) = (A_{12DG}/A_E) [eq. S1]

ME (mol/mol (AG+FA)) = $(2A_{ME}/3A_E)$ [eq. S2]

PC (mol/mol (AG+FA)) = $(2A_{PC}/9A_E)$ [eq. S3]

S+C+Δ5A (mol/mol (AG+FA)) = $(2A_{S+C+\Delta5A}/3A_E)$ [eq. S4]

Δ7A (mol/mol (AG+FA)) = $(2A_{\Delta7A}/3A_E)$ [eq. S5]

STN (mol/mol (AG+FA)) = $(2A_{STN}/3A_E)$ [eq. S6]

CTN (mol/mol (AG+FA)) = $(2A_{CTN}/3A_E)$ [eq. S7]

FE (mol/mol (AG+FA)) = $(2A_{FE}/3A_E)$ [eq. S8]

FA(mol/mol (AG+FA)) = $1-(3A_{TG}/2A_E)-(4A_{PC}/9A_E)-(2A_{12DG}/A_E)-(2A_{ME}/3A_E)$ [eq. S9]

In these equations: A_{12DG} is the area of the signal at 3.73 ppm; A_E is the area of the signal of all methylene protons supported on carbon atoms in *alpha* position in relation to carbonyl and to carboxyl groups indicated in Table 1 due to acyl groups and to fatty acids; A_{ME} is the area of the signal at 3.65 ppm; A_{PC} is the area of the signal at 3.30 ppm; $A_{S+C+\Delta5A}$, $A_{\Delta7A}$, A_{STN}

and A_{CTN} are the areas of the signals of methylic protons supported on the C18 of sitosterol, campesterol plus Δ5-avenasterol, of Δ7-avenasterol, sitostanol and campestanol respectively; A_{FE} is the area of the protons of the methoxy group of the aromatic ring of the ferulates; and finally, A_{TG} is the area of the signals of the four protons of sn1 and sn3 positions of the glyceryl backbone of triglycerides TG.

Article 6

EFFECT OF THE ENRICHMENT OF CORN OIL WITH *ALPHA*- OR *GAMMA*-TOCOPHEROL ON ITS *IN VITRO* DIGESTION STUDIED BY ^{1}H NMR AND SPME-GC/MS. FORMATION OF HYDROPEROXY-, HYDROXY-, KETO-DIENES AND KETO-*E*-EPOXY-*E*-MONOENES IN THE MORE *ALPHA*-TOCOPHEROL ENRICHED SAMPLES

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ABSTRACT

The aim of this study is the analysis of the *in vitro* digestion of corn oil, and of the effect of its enrichment with three levels of *gamma*- and *alpha*-tocopherol, by using for the first time ^{1}H nuclear magnetic resonance (^{1}H NMR) and solid phase microextraction followed by gas chromatography/mass spectrometry (SPME-GC/MS). The attention is focused on hydrolysis degree, degradation of oil main components, occurrence of oxidation reactions and main compounds formed, as well as on bioaccessibility of oil main components, of compounds formed in the oxidation, and, of *gamma*- and *alpha*-tocopherol. Lypolisis levels reached are high and show a similar pattern in all cases. Oxidation of corn oil components during *in vitro* digestion is proved, as is the action of *gamma*-tocopherol as antioxidant and *alpha*-tocopherol as prooxidant. In the more *alpha*-tocopherol enriched samples hydroperoxy-, hydroxy-, and keto-dienes, as well as keto-epoxy-monoenes and aldehydes are generated. The bioaccessibility of the oil main components is high. The compounds formed in the oxidation process during *in vitro* digestion can also be considered bioaccessible. The bioaccessibility of *alpha*-tocopherol is smaller than that of *gamma*-tocopherol. The concentration of this latter compound remains unchanged during *in vitro* digestion of the more *alpha*-tocopherol enriched oil samples.

KEYWORDS: corn oil, *in vitro* digestion, ^{1}H NMR, SPME-GC/MS, *gamma*- and *alpha*-tocopherols, antioxidant, prooxidant, bioaccessibility.

1. INTRODUCTION

During gastrointestinal digestion several reactions take place, which have important repercussions in food component bioaccessibility. Hydrolysis of proteins, carbohydrates and triglycerides is the main one, giving rise to smaller building blocks which are able to be absorbed. Some evidence suggests that other reactions may also be possible, such as the Maillard reaction, esterification and oxidation [1-3]. Of these, oxidation could be a cause for concern because it is known that certain compounds resulting from lipid oxidation are toxic and have been held responsible for several degenerative diseases [4-6].

Nowadays, it seems to be well established that diets high in vegetable and fruit content are healthy mainly due to these commodities being rich in compounds with antioxidant ability. For this reason, the enrichment of foods with compounds of natural origin with potential antioxidant ability is becoming common in the industry, for both technological and health reasons. In spite of this, knowledge of the effect that this enrichment provokes in foods when they are submitted to different processes is scarce. Recently, it has been shown that some compounds, attributed antioxidant ability, act as prooxidants in some foods, when submitted to certain oxidative conditions [7-9]. Taking into account all the above mentioned, the study of the effect of the enrichment of lipid foods with compounds with potential antioxidant ability on their behavior during digestion, is important for both industry and consumers.

Vegetable oils, widely consumed around the world, are the quintessential lipid foods and their enrichment with natural antioxidants may be considered of interest. The great number of natural compounds with potential antioxidant ability include tocopherols, which may be considered very suitable for enriching oils due to their lipophilicity and because they are also minor components in vegetable oils.

Nevertheless, so far no study has been carried out on the effect of *gamma*-tocopherol enrichment on the digestion of lipid foods. With regard to the effect enrichment with *alpha*-tocopherol, some studies have been published; however, their results are not conclusive. Some of these studies have concluded that this latter enrichment does not cause changes in the digestion of lipid foods [10,11], or that the changes are not clear [12]. Others have found a decrease in the values of certain oxidation parameters, from which it follows that *alpha*-tocopherol acts as antioxidant [2,12-15]. Finally, other ones have reported that *alpha*-tocopherol acts as prooxidant [14,16]. These discrepancies between results may be due to several reasons, which may include: the kind of lipid food involved; the degree of enrichment in *alpha*-tocopherol; the digestion model employed; and the methodology used to evaluate the oxidation level of the digestate.

In this context, the aim of this study is to analyze the effect of enriching corn oil, with either *alpha*-tocopherol or with *gamma*-tocopherol, on its *in vitro* digestion. Corn oil is an edible oil consumed worldwide, and *alpha*- and *gamma*-tocopherol are two natural forms of vitamin E, to which antioxidant ability has been attributed. The study will simultaneously deal with various subjects such as: the lipolysis degree reached and the bioaccessibility of the compounds coming from oil main component hydrolysis; the occurrence of oxidation reactions during digestion and the nature of the main oxidation compounds formed, if any; the elucidation of the effect of enrichment with each tocopherol on the potential oil component oxidation during digestion; and, finally, *gamma*-tocopherol and *alpha*-tocopherol bioaccessibility as well as the influence of the *alpha*-tocopherol added on the bioaccessibility of naturally present *gamma*-tocopherol in corn oil. Furthermore, in order to analyze the influence of tocopherol concentration on all the above mentioned issues the study will be carried out with three different enrichment degrees of each tocopherol; it should be remembered that the current legislation allows

one to add this kind of compounds to refined vegetable oils under the "*quantum satis*" principle. All these matters will be studied using data obtained by ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra of the oil samples and of the lipid extracts of the digestates. Furthermore, solid phase microextraction (SPME) followed by gas chromatography/mass spectrometry (GC/MS) will be used, as a complementary technique, to ensure the occurrence and level of oxidation reached during digestion. This will be elucidated by the presence and abundance of oxidation markers in the headspace of the digestate. Although different digestion models can be used, a classical *in vitro* model which has given satisfactory results for some time [17], will be used in this study; nevertheless, our laboratory is in a process of change in this respect and we plan to use a consensus model in the future.

2. MATERIALS AND METHODS

2.1. Samples subject of study

The study was carried out with refined corn oil C, acquired in a local supermarket. Its composition in molar percentages of linolenic (Ln), linoleic (L), oleic (O) and saturated (S) acyl groups is, 0.6 ± 0.0%, 49.2 ± 0.5%, 34.1 ± 0.3% and 16.1 ± 0.1% respectively. This was determined from ^{1}H NMR spectral data as in previous studies [18, 19]. The tocopherols used were *alpha*-tocopherol (αT) (purity of 98.2%) purchased in Sigma-Aldrich (St. Louis, MO, USA), and *gamma*-tocopherol (γT) (purity of ≥ 90%) provided by Eisai Food & Chemical Co. Ltd. (Tokyo, Japan). Aliquots of the oil were enriched with *alpha*-tocopherol or *gamma*-tocopherol at 0.2, 2 and 5% by weight in each case. The samples submitted to *in vitro* digestion were the original oil C, and all samples enriched in αT (C0.2αT, C2αT and C5αT) and in γT (C0.2γT, C2γT and C5γT).

2.2. Digestion experiments

Aliquots (0.5 g) of the above-mentioned samples were digested following the semi-static *in vitro* gastrointestinal digestion model developed by Versantvoort, *et al*. (2005) [20]. This validated method was optimized, in order to improve the lipids digestion, attempting to reach lipolysis levels of a similar order to *in vivo* digestion [21]. It has three-stages which simulates digestive processes in mouth, stomach, and small intestine, by sequentially adding the corresponding digestive juices (saliva, gastric juice, duodenal juice and bile), whose composition is given in Table S1 (see Supplementary Material). The first stage begins by adding 6 mL of saliva to the sample. After 5 min of incubation, 12 mL of gastric juice are added and the mixture is rotated head-over-heels at 40 rpm for 2 h at 37±2ºC. One hour after the start of the gastric stage, pH is set between 2 and 3 with HCl (37%), simulating the gradual acidification of the chyme occurring *in vivo*. After 2 h of the gastric stage, 2 mL of sodium bicarbonate solution (1 M), 12 mL of duodenal juice and 6 mL of bile juice are added. Subsequently, pH was set between 6 and 7, and the mixture is again rotated at 40 rpm and incubated at 37±2ºC for 4 h. All the reagents and enzymes for the preparation of digestive juices were acquired from Sigma-Aldrich (St. Louis, MO, USA): α-amylase from *Aspergillus oryzae* (10065, ~30 U/mg); pepsin from porcine gastric mucosa (P7125, ≥400 U/mg protein); amano lipase A from *Aspergillus niger* (534781, ≥120,000 U/g); pancreatin from porcine pancreas (P1750); lipase type II crude from porcine pancreas (L3126, 100-500 U/mg protein (using olive oil, 30 min incubation)) and bovine bile extract (B3883). The digested samples were named after the original samples but preceded by D (DC, DC0.2αT, DC2αT, DC5αT, DC0.2γT, DC2γT and DC5γT). Three digestion experiments, each including duplicate samples, were performed. Blank samples corresponding to the mixture of juices submitted to digestive conditions were also taken for further analysis.

2.3. Digestate lipid extraction

Lipids of the digestates were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich) following a methodology that also allows fatty acid extraction as in a previous studies [22]. This methodology involves a three-stage liquid-liquid extraction process with 20 ml of CH_2Cl_2 each. Afterwards, to ensure a complete protonation of fatty acids and/or the dissociation of the potential salts formed, the remaining water phase was acidified to pH 2 with HCl (37%) and a second extraction was carried out in three steps. All the CH_2Cl_2 extracts of each sample were mixed and any solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. The extraction yield was in all cases near 85%. This extracts contain triglycerides, diglycerides and monoglycerides, as well as fatty acids and tocopherols and other minor lipophilic compounds either present in the original oil samples or formed from oil components in the digestion process.

2.4. Study by ^{1}H NMR of oil samples and lipid extracts of digestates

2.4.1. Operating conditions

The ^{1}H NMR spectra of the original oil C, and of the oil samples enriched with each one of the tocopherols at the different concentrations (C0.2αT, C2αT, C5αT; C0.2γT, C2γT and C5γT), and of the lipids extracted from their digestates (DC, DC0.2αT, DC2αT, DC5αT, DC0.2γT, DC2γT and DC5γT), were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. For this purpose, the above-mentioned samples (approximately 0.16 g) were dissolved in 400 µl of deuterated chloroform, which contained tetramethylsilane (TMS), as internal reference (Cortec, Paris, France). The acquisition conditions were the same as those used in previous studies [23]. It must be noted that the relaxation delay and acquisition time allow the complete relaxation of the

protons, the signal areas thus being proportional to the number of protons that generate them, making it possible to use them for quantitative purposes.

2.4.2. Identification of the components

The identification of the components present in the original oil, in the oil samples enriched with tocopherol and in the lipid extracts of their digestates, was carried out on the basis of the assignments of the ^{1}H NMR signals, present in Figures 1 and 2, to the different kinds of hydrogen atoms, and in definitive to the different compounds.

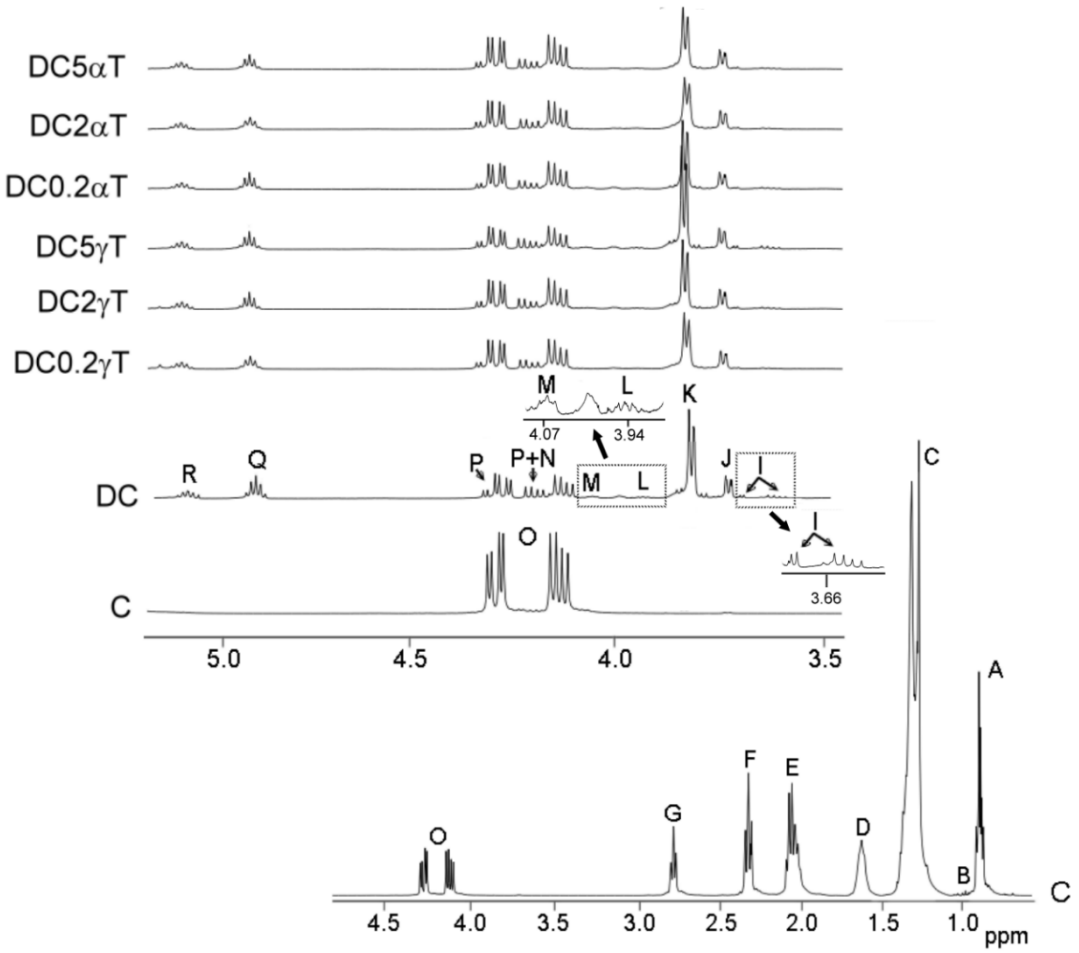

Figure 1. Region comprise between 0.0 and 4.9 ppm, of corn oil C ^{1}H NMR spectrum, and region comprised between 3.5 ppm and 5.10 ppm, conveniently enlarged, of the ^{1}H NMR spectra of the oil C and of the lipids extracted from the several digestates (DC, DC0.2γT, DC2γT, DC5γT and DC0.2αT, DC2αT, DC5αT) in which signals of protons of their main components appear. The signal letters agree with those of Tables S2 and S3.

These signals, their chemical shifts and assignments are given in Tables S2, S3, S4 and S5 (Supplementary Material). Their assignments were made taken into account previous studies as indicated in each table, or on the basis of the signals of standard compounds acquired for this study. Among these later are: *trans*-12,13-epoxy-9-keto-10(*E*)-octadecenoic acid, linolein hydroperoxides, linolein hydroxides, 9-oxo-10*E*,12*Z*-octadecadienoic acid and 13-oxo-9*Z*,11*E*-octadecadienoic acid purchased from Cayman Chemical (Ann Arbor, MI, USA), 9(S)-Hydroxy-10(*E*),12(*E*)-octadecadienoic acid (Dimorphecolic acid) acquired from Larodan (Malmö, Sweeden).

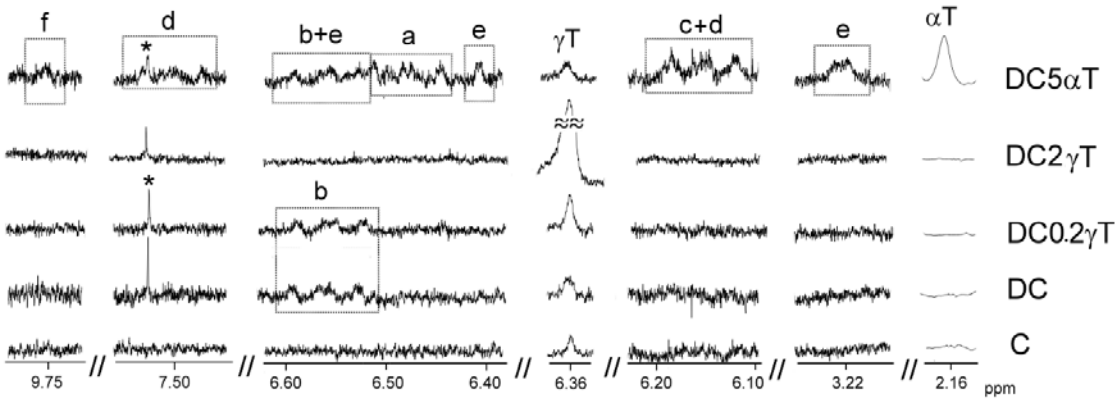

Figure 2. Some regions of the ^{1}H NMR spectra of oil C and of the lipids extracted from the digestates of some enriched in tocopherol samples, conveniently enlarged, in which appear signals of protons belonging to oxidation compounds and to *gamma*- and to *alpha*-tocopherol. The signal letters agree with those of Tables S4 and S5. The singlet marked with * is a satellite peak of chloroform.

Table S2 shows ^{1}H NMR signals of specific protons of the different glyceride structures, such triglycerides, diglycerides and monoglycerides. Table S3 shows ^{1}H NMR signals of protons of linolenic, linoleic, oleic and saturated acyl groups and fatty acids, and the signals of methylenic protons supported on carbons atoms in *alpha* position in relation to carbonyl-carboxyl groups Table S4 shows ^{1}H NMR signals of protons of oxidation compounds coming from main oil components degradation occurred during digestion. Finally, Table S5 gives ^{1}H NMR signals of protons of *alpha*- and *gamma*-

tocopherol. The areas of some of these spectral signals were used to quantify the concentration of the different kinds of above mentioned structures in the corresponding samples, as it will be explained below.

2.4.3. Quantifications made from ^{1}H NMR spectral data

This technique allows the estimation of the concentrations, expressed in different ways, of all identified compounds above mentioned. This is possible because, as has been above explained, the area of the ^{1}H NMR signals is proportional to the number of protons that generate the signal. The quantification of the different kinds of compounds or structures is explained below.

(A) Estimation of the molar percentage of the different kinds of glycerides in the digestates

The estimation of the molar percentage of each kind of glyceride structures can be carried out by using the intensity of some signals indicated in Tables S2 and S3, which can also be observed in Figure 1. Although glycerol is formed during digestion, due to its polar nature, it is not present in the lipid extract of the digestates. However, its concentration can be estimated indirectly. This is possible because the concentration of total fatty acids plus acyl groups, of only acyl groups, and of fatty acids released in the formation of diglycerides and monoglycerides can be determined from ^{1}H NMR data. Thus, the estimation of the molar percentage of triglycerides (TG), 1,2-diglycerides (1,2-DG), 1,3-diglycerides (1,3-DG), 2-monoglycerides (2-MG), 1-monoglycerides (1-MG) and glycerol (Gol) in relation to the total glyceryl structures present in the digestate, was carried out using equations [eq. S1 - eq. S10] given in Supplementary Material. They are based exclusively on the area of ^{1}H NMR spectral signals [24].

(B) Estimation of the percentage of fatty acids plus acyl groups that have linoleic structure in relation to the total of all types of fatty acids and acyl groups in digestates

In refined oils the concentration of fatty acids is very small and unappreciable in comparison with the concentration of acyl groups. However, as is known, hydrolysis during oil digestion provokes the transformation of a certain number of acyl groups into fatty acids. The fatty acids formed maintain the same number of carbon atoms and unsaturation pattern as the starting acyl groups. Acyl groups and fatty acids having the same structure provide NMR spectra signals with a high degree of overlapping, which allows their joint quantification. In this study the molar percentage of the linoleic acyl groups plus linoleic fatty acids in the digestates was estimated in relation to the total number of moles of all kinds of fatty acids plus acyl groups. This estimation was made using the equation [eq. S11], given in Supplementary Material, in which the areas of some signals that are shown in Figure 1 and in Table S3 are involved. This equation is the same employed in previous studies [18,19], but using the signal of methylenic protons supported on carbon atoms in *alpha* position in relation to carbonyl-carboxyl groups, instead of the signal of triglyceride protons used in edible oil studies.

(C) Estimation of the concentration of specific compounds (SC) in oil samples and in the digestates

The concentration of oxidation compounds, and of other ones, such as *gamma*- and *alpha*-tocopherol, either in oils or in digestates, can be estimated by using the general equation [eq. S12] given in Supplementary Material and the intensity of one of their non-overlapped ^{1}H NMR spectral signal , which are shown in Figures 1 and 2, in Figure S1 and in Tables S3, S4 and S5. This equation allows one to estimate the concentration of any compound in oils or in digestates in relation to the concentration of fatty acids plus acyl groups, which are considered the internal reference.

2.5. Study by SPME-GC/MS of the headspace of digestates and of the mixture of the digestive juices submitted to digestion conditions with the corn oil

The extraction of the volatile components constituting the headspace of the several samples (0.5 g in a 10 ml screw-cap vial) was carried out automatically using a CombiPAL autosampler (Agilent Technologies, Santa Clara, CA, USA). The samples studied were the several digestates (DC, DC0.2αT, DC2αT, DC5αT, DC0.2γT, DC2γT and DC5γT) and the mixture CDJ of digestive juices DJ, after submission to digestion conditions, and corn oil C. The comparison of the headspaces of the several samples enables one to deduce differences provoked in them by *in vitro* digestion.

The fiber used for the headspace components extraction was coated with Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm film thickness, 1 cm long; acquired from Supelco (Sigma-Aldrich)). It was inserted into the headspace of the sample and maintained for 55 min at 50 ºC, after a pre-equilibration time of 5 min. The fiber containing the components extracted was desorbed for 10 min in the injection port (splitless mode with 5 min purge time) of a 7890A gas chromatograph equipped with a 5975C inert MSD with Triple Axis Detector (Agilent Technologies) and a computer operating with the ChemStation program. A fused silica capillary column was used (60 m length, 0.25 mm inside diameter, 0.25 μm film thickness; from Agilent Technologies Inc., PaloAlto, CA), coated with a nonpolar stationary phase (HP-5MS, 5%phenyl methyl siloxane). The operation conditions were the following: the injector and interface temperatures were held at 250°C and 305°C respectively, and helium at a constant pressure of 117 kPa (16.9 psi) was used as the carrier gas. The oven temperature was initially held at 50°C for 5 min, increased from 50 to 300°C at a rate of 4°C/min, and then held at 300°C for 30 min. Mass spectra were recorded at an ionization energy of 70 eV, with data acquisition in Scan mode. The temperatures of the ion source and the

quadrupole mass analyzer were kept at 230 and 150°C, respectively. A reference sample of known composition was periodically analyzed in order to verify the sensitivity of the SPME-GC/MS experiments as in previous studies [25].

Identification of the headspace components was performed using several commercial standard compounds, acquired from Sigma-Aldrich (St. Louis, MO, USA). When standard compounds were not available by coincidence higher than 85% of the spectra obtained, with those of commercial libraries (Wiley W9N08, Mass Spectral Database of the National Institute of Standards and Technology), or with those provided by the scientific literature, as in previous studies [25]

Semi-quantification of the compounds was based on the area counts of the base peak (Bp) of the mass spectrum of each compound divided by 10^6. When the Bp of a compound overlapped with some ion peak of the mass spectrum of another compound, an alternative ion peak was selected for the semi-quantification of the former [25]. Although the chromatographic response factor of each compound is different, the area counts thus determined are useful for the comparison of the abundance of each compound in the different samples. The detection limit was established at an abundance of 50000 area counts. Data given in the following tables are average values of duplicate experiments

2.6. Statistical analysis

The significance of the differences in the several kinds of data among samples, was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM, NY, USA).

3. RESULTS AND DISCUSSION

3.1. Extent and pattern of lipolysis produced by the *in vitro* digestion in the several samples

As is known, the main components of edible oils are triglycerides, and when they are submitted to digestion, hydrolysis of their ester bonds occurs, yielding diglycerides and monoglycerides, as well as fatty acids and glycerol. For this reason, in the ^{1}H NMR spectral region shown in Figure 1 of corn oil C, almost the only signal observable, due to glycerides, is signal O of triglyceride protons. Nevertheless, in the same spectral region of the lipid extract of the corn oil digestates (DC, DC0.2γT, DC2γT DC5γT, DC0.2αT, DC2αT and DC5αT), which is also shown in Figure 1, signals J, P and R of 1,2-diglicerydes, signals K and Q of 2-monoglycerides, signal M of 1,3-diglycerides, and signals I, L and N of 1-monoglycerides are clearly observable. In addition, signal O of triglycerides also appears, but in much lower intensity than in the oil C spectrum. This indicates that triglyceride hydrolysis has taken place during this digestion. The extent of the hydrolysis can be inferred from the molar percentages of each kind of glyceride structure in relation to the total. These molar percentages were estimated, in all these samples, by means of the equations [eq. S1-eq. S10], by using the intensity of some signals given in Tables S2 and S3 and shown in Figure 1, as described in the Supplementary Material. The results obtained are given in Table 1.

Table 1. Lipolysis extent. Molar percentages of triglycerides (TG), diglycerides (1,2-DG and 1,3-DG), monoglycerides (2-MG and 1-MG) and glycerol (Gol) in relation to the total glyceride structures, in corn oil C, in the digestates of this oil DC and in those of the samples enriched in *gamma*- and *alpha*-tocopherol (DC0.2γT, DC2γT, DC5γT DC0.2αT, DC2αT and DC5αT). *In vitro* bioaccessibility of oil main components B_{OMC}, defined by the ratio (mol [FA]+[MG])$_D$/mol ([FA]+[AG])$_D$. Different letter within each column indicate statistically significant difference among the samples ($p < 0.05$).

Samples	Lipolysis Extent						B_{OMC}
	TG (%)	1,2-DG (%)	1,3-DG (%)	2-MG (%)	1-MG (%)	Gol (%)	
C	99.8±0.2a	1.1±0.1a	-	-	-	-	-
DC	22.3±5.9b	14.0±1.6b	1.8±1.0a	26.6±5.6a	4.4±1.1a	30.8±1.8a	0.67±0.07a
DC0.2γT	22.2±4.4b	14.7±1.0b	1.7±0.2a	26.0±1.6a	4.1±0.7a	31.3±1.6a	0.67±0.04a
DC2γT	20.7±3.4b	14.1±2.2b	1.4±0.5a	28.2±2.7a	5.3±0.4a	30.3±2.6a	0.69±0.02a
DC5γT	17.0±7.8b	14.3±0.6b	1.7±0.9a	30.2±6.0a	5.1±2.3a	31.7±0.2a	0.72±0.08a
DC0.2αT	20.1±4.3b	13.9±0.7b	1.5±0.5a	28.0±2.9a	5.0±0.5a	31.4±1.9a	0.70±0.05a
DC2αT	21.8±1.8b	14.1±1.1b	1.9±0.2a	28.5±2.9a	5.3±0.4a	28.5±0.6a	0.68±0.02a
DC5αT	24.8±2.0b	14.9±0.9b	1.5±0.7a	24.7±2.9a	4.3±0.2a	29.9±1.1a	0.64± 0.03a

-: not detected

i) Lipolysis extent in corn oil digestate. It may be observed in Table 1 that the lipolysis extent provoked by the *in vitro* digestion in corn oil C is fairly high. About 78% of triglycerides have been hydrolyzed partially or totally. Monoglycerides and glycerol are the main glycerides formed (the yield of each one of these two kinds of compounds is approximately 31%); however, only about 15% of the triglycerides have been transformed into diglycerides. In general terms, this hydrolysis pattern is similar to that previously found in other edible oils submitted to the same digestion model [26].

ii) Lipolysis extent in the enriched in tocopherol corn oil digestates. As data in Table 1 show, the triglyceride hydrolysis pattern of these samples is very similar to that found in the digestate of corn oil: monoglycerides and glycerol are the main hydrolytic products, followed by diglycerides, whose concentration is approximately half that of the other two hydrolytic products. Statistical treatment finds no significant differences between the lipolysis pattern and extent occurred during *in vitro* digestion of the original oil and of the tocopherol enriched samples. Nevertheless, the results suggest that enrichment with tocopherols could have had some influence on the hydrolysis extent. Data in Table 1 could suggest that a slightly higher degree of hydrolysis took place in samples enriched with

gamma-tocopherol than it did in the unenriched oil. However no conclusive results were found in the samples enriched with *alpha*-tocopherol. This potential small effect in the tocopherol-enriched samples could be attributed, among other reasons, to the different interactions that these compounds are able to establish with the components of the complex mixture involved in the digestion. It must be remembered that these two tocopherols have structural differences that translate into differences in polarity and lipophilicity etcetera, and as a consequence in differences in their behavior [27].

3.2. Bioaccessibility of oil main components

The bioaccessibility of a compound or a group of compounds has been defined as the quantity or fraction of this compound or of the group of compounds, which is released from the food matrix into the gastrointestinal tract and becomes available for absorption [28]. As mentioned before, several reactions take place during digestion, and consequently the food components undergo modifications in such a way that the amount of food components absorbable after digestion is different from the amount present in the original food. The methodology used in this study allows one to determine the bioaccessibility of the oil main components.

The only compounds released during digestion as a result of triglyceride hydrolysis which can be absorbed by enterocytes of the intestinal wall are fatty acids and monoglycerides. For this reason, the bioaccessibility of oil main components (B_{OMC}), which is totally dependent on the extent and pattern of the hydrolysis occurring during digestion, is defined by the ratio between the concentration of compounds really absorbable present in the digestate, which are fatty acids plus monoglycerides $([FA]+[MG])_D$ and the concentration of all potentially absorbable compounds before digestion, which coincides with that of fatty acids plus all acyl groups $([FA]+[AG])_D$, as indicated in the following equation: $B_{OMC}=([FA]+[MG])_D/([FA]+[AG])_D$. All these

concentration data can be estimated from the intensities of certain ^{1}H NMR signals of the corresponding samples by using the equations given in the Supplementary Material. The data obtained are given in Table 1.

These data indicate that the bioaccessibility of these compounds is around 0.70mol([FA]+[MG])$_D$/mol([FA]+[AG])$_D$ in the corn oil digestate and reaches very close values to this in the digestates of the oil samples enriched in tocopherols. That is to say, from every hundred original acyl groups supported in the oil triglycerides, nearly seventy are transformed either into fatty acids or into monoglycerides during digestion and for this reason are available for absorption. As in the case of the molar percentages of the different kinds of glyceryl structures the differences found between the bioaccessibility of oil main components in the original oil and in that enriched with tocopherol are not statistically significant. However, the data may suggest that enrichment with *gamma*-tocopherol could slightly favor the bioaccessibility of corn oil triglycerides whereas enrichment with *alpha*-tocopherol could slightly reduce this bioaccessibility.

3.3. Study of the occurrence of oxidation reactions during *in vitro* digestion of corn oil and of corn oil samples enriched in tocopherols

As mentioned in the introduction, the occurrence of oxidation reactions during *in vitro* digestion has been described previously [1,2]. In a lipid system, such as corn oil, it is well accepted that oxidation first provokes the progressive degradation of its unsaturated acyl groups [29]. This degradation yields the so-called primary oxidation compounds, which evolve to give rise to the formation of secondary oxidation compounds of very varied nature and size. The study of the oxidation process of edible oils can be tackled by means of very different methodologies and techniques. Nevertheless, there are two that have great interest, not only by their efficiency, versatility, simplicity and rapidity, but also because they are very environmentally friendly and do not provoke any

chemical modification of the sample, namely ^{1}H NMR spectroscopy and SPME-GC/MS. The first allows detection of the occurrence of oxidation reactions, not only because it is able to quantify the concentration of the different kinds of acyl groups and fatty acids in oil or in lipid extracts, but also because it is able to detect, identify, and quantify the compounds formed in oxidation processes. SPME-GC/MS is much more sensitive than ^{1}H NMR and allows one to identify and semi-quantify only volatile components, among which there may be oxidation markers. For all these reasons, both techniques were used to study the potential occurrence of oxidation reactions during *in vitro* digestion.

3.3.1. Information provided by ^{1}H NMR spectroscopy about the occurrence of oxidation reactions during in vitro digestion

As mentioned, two main facts take place during oxidation, namely the degradation of unsaturated acyl groups and unsaturated fatty acids and the formation of compounds derived from them. This technique is able to evaluate both.

(a) Evaluation of the concentration of acyl groups and fatty acids having linoleic structure in the corn oil, in its digestate and in those of corn oil samples enriched in tocopherols

Linoleic is the main unsaturated acyl group in corn oil. After hydrolysis provoked by *in vitro* digestion, linoleic structures of acyl groups and of fatty acids are also the main unsaturated lipid structures in the digestates. For this reason, analysis of the concentration of this unsaturated structure, both in corn oil and in the lipid extracts of the several digestates, will provide information concerning the occurrence of its degradation during *in vitro* digestion.

As before mentioned, linoleic structures of fatty acids and acyl groups give ^{1}H NMR signals, very or totally overlapped, for which reason their concentration can be

determined jointly. This can be carried out by using equation [eq. S11] given in Supplementary Material. The data obtained, given in molar percentage in relation to the total fatty acids plus acyl groups, are shown in Table 2.

Table 2. Molar percentage of linoleic FA+AG, in relation to the total moles of all kinds of AG and FA, in the corn oil C, in the digestates of this oil DC, and in those of the oil samples enriched in *gamma*- and *alpha*-tocopherol (DC0.2γT, DC2γT, DC5γT, DC0.2αT, DC2αT and DC5αT). Concentration of some oxidation compounds, expressed by mmol per mol of AG+FA in the above samples. Different letter within each column indicate statistically significant difference among the samples ($p < 0.05$).

Samples	**Linoleic FA+AG (%)**	**Concentration of some oxidation compounds (mmol/mol (AG+FA))**					
		HPO-c(*Z*,*E*)dEs	**HO-c(*Z*,*E*)dEs**	**HO-c(*E*,*E*)dEs**	**KO-c(*Z*,*E*)dEs**	**KO-*E*-EPO-*E*-mEs**	**n-alkanals**
C	49.2±0.5a	-	-	-	-	-	-
DC	41.3±0.0b	1.82±0.31a	-	-	-	-	-
DC0.2γT	43.3±0.0c	1.54±0.27a	-	-	-	-	-
DC2γT	43.1±0.2c	-	-	-	-	-	-
DC5γT	44.4±0.3c	-	-	-	-	-	-
DC0.2αT	40.5±0.6b	2.77±0.25b	-	-	-	-	-
DC2αT	40.8±0.0b	2.21±0.31ab	1.22±0.07a	2.27±0.00a	0.53±0.03a	0.71±0.00a	-
DC5αT	40.6±1.2b	2.06±0.38ab	2.78±0.19b	2.43±0.71a	1.09±0.14b	1.12±0.16b	0.27±0.09

-: not detected; HPO-c(*Z*,*E*)dEs: hydroperoxy-conjugated-(*Z*,*E*)-dienes; HO-c(*Z*,*E*)dEs: hydroxy-conjugated-(*Z*,*E*)-dienes; HO-c(*E*,*E*)dEs: hydroxy-conjugated-(*E*,*E*)-dienes; KO-c(*Z*,*E*)dEs: keto-conjugated-(*Z*,*E*)-dienes; KO-*E*-EPO-*E*-mEs: keto-(*E*)-epoxy-(*E*)-monoenes

From these data, it is evident that a significant and clear reduction of the molar percentage of linoleic structures occurs during the corn oil *in vitro* digestion, which evidences their degradation. However, in the samples enriched with *gamma*-tocopherol this reduction is significantly smaller than in the non-enriched oil. It can be inferred from these results that this compound has a protective effect against degradation of linoleic structures. Finally, the loss of linoleic structures in the digestion of the samples enriched with *alpha*-tocopherol is slightly higher than that found in the non-enriched oil, although the differences are not statistically significant. Taking into account that the main cause of degradation of these unsaturated structures is oxidation, it could be thought that *in vitro* digestion of corn oil provokes its oxidation and that while *gamma*-tocopherol reduces this degradative process, *alpha*-tocopherol could have the opposite effect. If lipid oxidation reactions have taken place during *in vitro* digestion, primary or secondary oxidation

compounds, or both, should have been generated and they could be detected and quantified by ^{1}H NMR spectroscopy.

(b) Study of the formation of oxidation compounds during in vitro digestion of corn oil and of corn oil samples enriched in tocopherols

The first oxidation compounds formed in any oil oxidation process are hydroperoxydes, and when they come from linoleic groups, as is the case here, these hydroperoxides are supported on chains having also conjugated dienic systems. These give well known signals in the ^{1}H NMR spectra [29]. The degradation of these intermediate compounds generates secondary oxidation compounds of very varied nature. Among these latter can be cited hydroxy-dienes [30] and keto-dienes [7] with known ^{1}H NMR spectral signals. In addition, other derived compounds having much more complex structures can also be formed such as keto-epoxy-monoenes [8] and other ones formed in the rupture of secondary oxidation compounds [31] such as aldehydes.^{1}H NMR spectral signals of all these compounds are given in Table S4. Their concentration can be estimated from the intensity of some of their signals in the spectrum and using equation [eq. S12] as is indicated in point 2.4.3.3.

i) Oxidation compounds in corn oil digestate. As Figure 2 shows, the ^{1}H NMR spectrum of the corn oil (C) has no signals of protons of oxidation compounds. This indicates that this oil has not been oxidized. However, the spectrum of the lipid extract of its digestate (DC) contains signals of protons of hydroperoxy-conjugated-(*Z,E*)-dienes (HPO-c(*Z,E*)dEs) (see Table S4 and Figure 2). The concentration of these compounds in DC is given in Table 2. This is clear evidence that *in vitro* digestion provokes the oxidation of corn oil generating this specific kind of primary oxidation compounds.

ii) Oxidation compounds in the digestates of corn oil enriched in gamma-tocopherol.

The ^{1}H NMR spectrum of the lipid extract of the digestate of corn oil enriched with *gamma*-tocopherol at the lowest concentration essayed (DC0.2γT) also contain signals of hydroperoxy-conjugated-(Z,*E*)-dienes (HPO-c(*Z*,*E*)dEs) (Figure 2) but, as Table 2 shows, in smaller concentrations than in DC. It is noteworthy that, in the ^{1}H NMR spectra of the extracts of the digestates DC2γT and DC5γT, no signals of hydroperoxy-conjugated-(*Z*,*E*)-dienes, nor those of any other oxidation compound are present (Table 2 and Figure 2). This confirms that *gamma*-tocopherol acts as antioxidant during this *in vitro* digestion, reducing the oxidation of corn oil (at an enrichment of 0.2%) or avoiding it totally (at enrichments of 2% and 5%).

iii) Oxidation compounds in the digestates of corn oil enriched in alpha-tocopherol.

The effect provoked by the enrichment with *alpha*-tocopherol is the opposite of that provoked by the enrichment with *gamma*-tocopherol. The lipid extract of the digestates of all samples enriched with the first compound have a higher oxidation degree than DC. The ^{1}H NMR spectrum of the extract of DC0.2αT only shows signals of hydroperoxy-conjugated-(*Z*,*E*)-dienes(HPO-c(*Z*,*E*)dEs), but as Table 2 shows, at a higher concentration than in the extract of DC. At higher concentrations of enrichment with *alpha*-tocopherol the effect is even clearer. As can be observed in Figure 2, not only are there signals of hydroperoxy-conjugated-(*Z*,*E*)-dienes (HPO-c(*Z*,*E*)dEs) in the spectra of extracts of DC2αT and DC5αT but also those of secondary and further oxidation compounds. These latter include signals of hydroxy-conjugated-(*Z*,*E*)-dienes, (HO-c(*Z*,*E*)dEs), such as those of hydroxy-conjugated-(*E*,*E*)-dienes (HO-c(*E*,*E*)dEs) such as of keto-conjugated-(*Z*,*E*)-dienes (KO-c(*Z*,*E*)dEs) as well as of keto-(*E*)-epoxy-(*E*)-monoenes (KO-*E*-EPO-*E*-mEs) and of saturated aldehydes. The concentration of primary oxidation compounds, in both samplesDC2αT and DC5αT, is only slightly higher than in

DC, but these samples contain other secondary and further oxidation compounds in even higher concentrations than those of the primary oxidation compounds, as shown in Table 2. Figure 3 shows some of the oxidation compounds with the above mentioned structures.

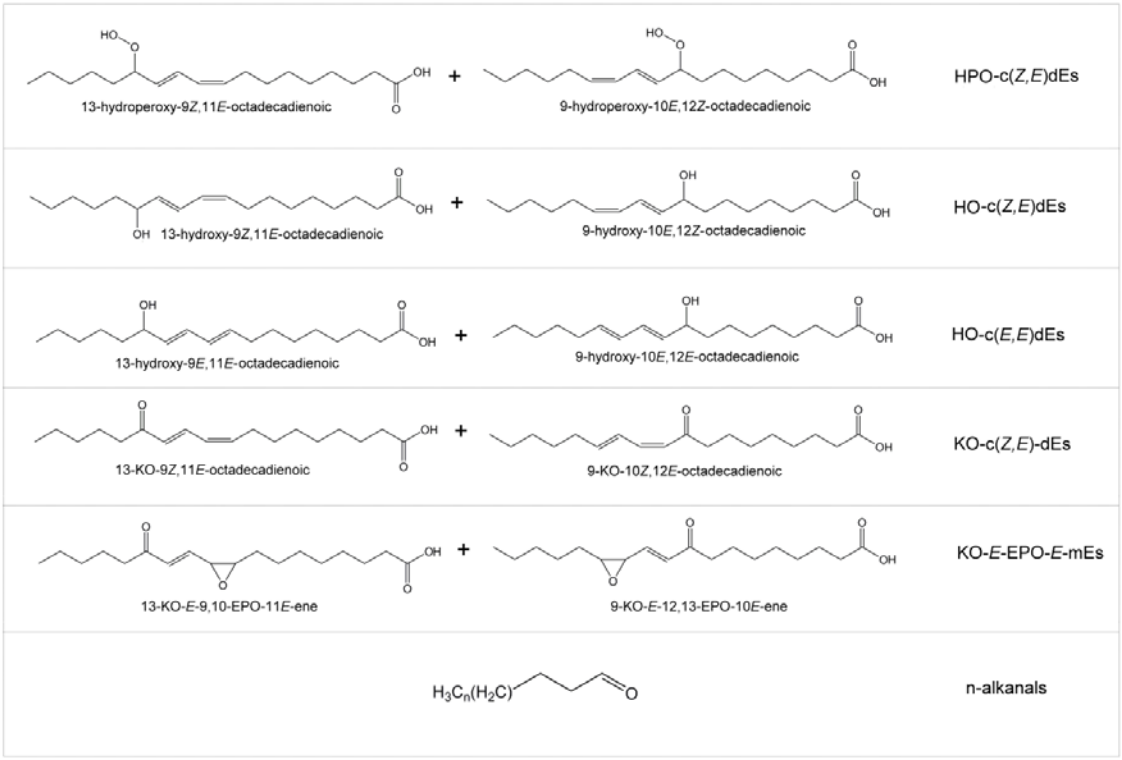

Figure 3. Chemical structures of some potential oxidation compounds present in the digestates of the corn oil samples enriched in *alpha*-tocopherol.

These results may lead to several observations:

a) In spite of *alpha*-tocopherol and *gamma*-tocopherol being known as two forms of Vitamin E, their behaviour is totally different during *in vitro* digestion. The first acts as prooxidant at the three concentrations essayed in agreement with some previous studies [14, 16] whereas the second acts as antioxidant. This difference in behaviour has been also observed in studies of edible oils enriched in these compounds submitted to conditions of accelerated storage. In these studies, *alpha*-tocopherol exhibits a prooxidant effect which increases in line with its concentration, whereas *gamma*-tocopherol only

accelerates oxidation at very early oxidation stages. However, the global effect in the total oxidation process could be considered antioxidant [7-9].

b) Elucidation of the behaviour of a compound as either antioxidant or prooxidant during digestion cannot be made exclusively on the basis of the estimation of a parameter such as peroxide value or other equivalents. In this study the concentration of hydroperoxides is fairly similar in DC and DC5αT, as Table 2 shows, and from these data it could be concluded that *alpha*-tocopherol has no great influence on the oxidation occurred during digestion, against the evidence.

c) In *in vitro* digestion, which takes place at 37ºC, oil oxidation advances very quickly in comparison with experiments on corn oil, or on other oils of similar composition, submitted to accelerated storage conditions at 70ºC. In the *in vitro* digestion of this corn oil, primary and secondary or further oxidation compounds are present in the digestates after 6 hours and in concentrations of a similar order. However, in experiments with corn oil, or with other oils which are rich in linoleic groups, submitted to accelerated storage conditions, hydroperoxides can be detected only after at least 48 hours, or even later, and the appearance of secondary oxidation compounds is sequential with differences of many hours between them, and in very different concentrations [8, 29]. These differences suggest that both oxidation pathways are very different.

d) The fact that fatty acids have smaller oxidative stability than alkyl and glyceryl esters suggest that the structural units formed in the oxidation during *in vitro* digestion, shown in Table 2 and Figure 3, belong to fatty acids instead to acyl groups. The immediate consequence is that these oxidized fatty acids are able to be absorbed. Their concentrations, given in Table 2 in mmol per mol of FA+AG in the digestate, coincide with their bioaccessibility. It should be commented on that the bioaccessibility of these

oxidation compounds is very low, in the range of between 0.3-2.8 mmol per mol of FA+AG in the digestate. The total bioaccessibility of these kinds of compounds ranges from 1.5 mmol (in the sample enriched with the lowest concentration of *gamma*-tocopherol) to 8.9 mmol (in the sample enriched with the highest level of *alpha*-tocopherol. Nevertheless, taking into account that these oxidized fatty acids are toxic, their absorption has direct detrimental effects on human health [6,32-34]. By contrast, these compounds are not present in the digestates of the samples enriched with 2% and 5% of *gamma*-tocopherol, because in the digestion of these samples no oxidation is produced.

e) These results are in agreement with the degradation observed in linoleic structures during digestion in the different samples (see Table 2). They also reinforce the differences (not statistically significant) found in the B_{OMC} values (see Table 1).

f) From all the above mentioned it can be deduced that an in depth analysis, case by case, of the safety of enriching foods with compounds considered antioxidant should be mandatory, and caution should be taken in the indiscriminate intake of certain supplements. It would also be advisable to review the suitability of the European legislation that permits enrichment of refined edible oils with *alpha*-tocopherol under the principle of "*quantum satis*".

3.3.2. Information provided by SPME-GC/MS about the occurrence of oxidation reactions during in vitro digestion

The aim of this section is to analyse if the results obtained from ^{1}H NMR data in previous sections are confirmed by the information provided by SPME-GC/MS. This technique allows us to estimate the abundance of volatile oxidation markers present in the headspace of the samples. Taking into account that the liquid matrix of the samples

subject of study is similar, the abundance of the same volatile component in the different samples is valid for comparative purposes. The most common volatile oxidation markers formed in the oxidation of edible oils are well known. These are alkanals, (*E*)-2-alkenals, (*E,E*)-alkadienals, (*Z,E*)-alkadienals and oxygenated *alpha,beta* unsaturated aldehydes. Likewise, some furanones and furan derivatives are also well-known oil oxidation markers [25].

Table 3 gives the abundances of the most important oxidation markers found in the headspace of the mixture made up of digestive juice, previously submitted the digestion conditions, and corn oil in the same proportions as those employed in the digestion of the oil, this sample being considered as reference sample CDJ. Table 3 also gives the same information referring to the digestates of the corn oil DC, and of the corn oil samples enriched in both kinds of tocopherols, with different enrichment degree (DC0.2γT, DC2γT, DC5γT and DC0.2αT, DC2αT, DC5αT). As an example**,** Figure S2 shows the region between 4-34 min of the total ion chromatogram obtained by SPME-GC/MS of the digestate of corn oil sample enriched in *alpha*-tocopherol DC5αT. In it, the peaks and retention times of some of the volatile oxidation compounds can be observed.

i) *Headspace of the mixture CDJ.* It can be observed in Table 3 that, as expected, the headspace of the sample reference, CDJ, which contains the undigested corn oil, has the lowest number and concentration of oxidation markers of all samples. This is because the oil has not undergone digestion and as a consequence has not been oxidized. For this reason, its headspace only contains a reduced number of aldehydes, mainly alkanals, at a basal concentration, which is common in all non-oxidized edible oils.

Table 3. Abundances of some aldehydes (alkanals, (*E*)-2-alkenals, 2,4-alkadienals and oxygenated *alpha,beta* unsaturated aldehydes), furanones and furan derivatives identified by SPME-GC/MS in the headspace of the mixture of digestive juices and non-enriched corn oil sample (CDJ), in the digestates of the corn oil (DC) and in those of oil samples enriched in *gamma*- and *alpha*-tocopherol (DC0.2γT, DC2γT, DC5γT DC0.2αT, DC2αT and DC5αT). Data are given in area counts of their mass spectra base peak (Bp) multiplied by 10^{-6}, obtained as average of two determinations together with their standard deviations.

Compound (molecular weight)	Bp	CDJ	DC	DC0.2γT	DC2γT	DC5γT	DC0.2αT	DC2αT	DC5αT
Aldehydes									
Alkanals									
Pentanal (86)*	44	17.8 ± 0.9	41.4 ± 1.8	36.0 ± 0.5	48.4 ± 2.9	22.3 ± 1.8	41.4 ± 5.4	52.4 ± 7.3	83.9 ± 6.4
Hexanal (100)*	44	12.9 ± 3.2	76.0 ± 3.1	79.1 ± 11.8	72.4 ± 4.9	45.6 ± 4.9	184.4 ± 12.3	480.6 ± 75.3	604.0 ± 56.0
Heptanal (114)*	70	0.7 ± 0.0	3.0 ± 1.2	4.4 ± 0.7	3.5 ± 0.5	2.4 ± 0.4	6.8 ± 0.1	51.2 ± 11.7	164.3 ± 2.1
Octanal (128)*	41	-	-	-	-	-	-	34.0 ± 6.4	46.4 ± 5.1
Nonanal (142)*	57	3.3 ± 0.2	11.4 ± 0.3	11.3 ± 0.5	17.0 ± 1.7	17.1 ± 1.1	12.4 ± 1.2	42.6 ± 2.4	69.7 ± 11.4
Decanal (156)*	41	-	-	-	-	-	-	-	4.6 ± 0.5
Total		**34.7 ± 2.1**	**131.8 ± 0.1**	**130.8±12.47**	**141.3±10.1**	**87.5 ± 8.3**	**245.1 ± 5.9**	**660.8±103.1**	**972.9 ± 64.4**
(E)-2-alkenals									
(*E*)-2-Butenal (70)*	70	19.5 ± 2.8	20.6 ± 3.4	20.8 ± 5.8	16.1 ± 2.3	15.2 ± 2.6	18.2 ± 3.3	20.7 ± 2.3	23.0± 7.8
(*E*)-2-Pentenal (84)	55	-	-	-	-	-	-	1.3 ± 0.0	5.1 ± 1.1
(*Z*)-4-Heptenal (112)	41	-	-	-	-	-	-	3.9 ± 0.7	5.4 ± 0.5
(*E*)-2-Heptenal (112)*	41	2.3 ± 0.3	47.4 ± 8.1	40.2 ± 6.4	16.5 ± 8.9	8.8 ± 3.0	64.5 ± 8.7	186.3 ± 28.3	275.0 ± 47.7
(*E*)-2-Octenal (126)*	70	-	-	-	-	-	42.3 ± 2.2	186.3 ± 26.2	346.3 ± 58.9
(*E*)-2-Nonenal (140)*	55	-	1.2 ± 0.2	1.1 ± 0.1	1.4 ± 0.1	1.8 ± 0.1	1.3 ± 0.1	2.8 ± 0.8	4.7 ± 0.4
(*E*)-2-Decenal (154)*	70	-	-	-	-	-	4.1 ± 0.6	6.7 ± 1.0	10.8 ± 1.8
(*E*)-2-Undecenal (168)*	70	-	-	-	-	-	0.2 ± 0.0	1.4 ± 0.3	3.0 ± 0.4
Total		**21.8 ± 3.1**	**69.2 ± 4.9**	**62.08±12.28**	**38.9 ± 4.4**	**25.8 ± 5.4**	**130.6 ± 8.3**	**409.3 ± 58.1**	**673.3±102.0**
2,4-Alkadienals									
(*Z*,*E*)-2,4-Heptadienal (110)	81	-	-	-	-	-	3.8 ± 0.1	24.0 ± 4.9	38.6 ± 1.6
(*E*,*E*)-2,4-Heptadienal (110)*	81	-	-	-	-	-	3.5 ± 0.5	24.4 ± 3.0	36.4 ± 10.5
(*Z*,*E*)-2,4-Octadienal (124)	81	-	-	-	-	-	-	-	1.9 ± 1.0

Compound (molecular weight)	Bp	CDJ	DC	DC0.2γT	DC2γT	DC5γT	DC0.2αT	DC2αT	DC5αT
(*E,E*)-2,4-Octadienal (124)	81	-	-	-	-	-	0.8 ± 0.0	3.4 ± 0.1	6.1 ± 0.8
(*E,E*)-2,4-Nonadienal (138)	81	-	1.0 ± 0.1	0.7 ± 0.2	0.7 ± 0.1	0.4 ± 0.1	1.2 ± 0.2	1.9 ± 0.2	2.4 ± 0.5
(*Z,E*)-2,4-Decadienal (152)	81	-	0.6 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	2.6 ± 0.8	19.3 ± 3.1	38.4 ± 10.1
(*E,E*)-2,4-Decadienal (152)*	81	-	0.8 ± 0.1	0.5 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	3.3 ± 0.3	15.8 ± 0.5	29.0 ± 13.2
Total		-	**2.4 ± 0.2**	**1.6 ± 0.3**	**1.3 ± 0.1**	**1.0 ± 0.2**	**15.3 ± 1.6**	**88.7 ± 11.7**	**152.8 ± 37.6**
Oxigenated α,β-unsaturated									
4,5-epoxy-(*E*)-2-heptenal (126)	68	-	-	-	-	-	-	0.9 ± 0.2	3.1 ± 1.0
4,5-epoxy-2-decenal (isomer) (168)	68	-	-	-	-	-	0.4 ± 0.1	2.7 ± 0.2	4.3 ± 2.2
4,5-epoxy-(*E*)-2-decenal (168)*	68	-	-	-	-	-	1.1 ± 0.2	10.9 ± 0.4	31.8 ± 0.0
Total		-	-	-	-	-	**1.5 ± 0.3**	**15.0 ± 0.1**	**41.1 ± 3.2**
Furanones									
5-butyl-5H-furan-2-one (140)	84	-	-	-	-	-	-	0.9 ± 0.0	1.6 ± 0.1
5-pentyl-2(3H)-furanone (154) (or isomer)	98	-	-	-	-	-	-	0.5± 0.0	0.9± 0.2
5-pentyl-2(5H)-furanone (154)	84	-	-	-	-	-	-	2.7 ± 0.3	4.4 ± 0.9
Total		-	-	-	-	-	-	**4.1 ± 0.4**	**6.9 ± 1.2**
Furan derivatives									
Furan, 2-ethyl (96)	81	-	-	-	-	-	0.2 ± 0.1	0.3 ± 0.1	0.6 ± 0.0
Furan, 2-propyl (110)	81	-	-	-	-	-	0.2 ± 0.0	0.6 ± 0.0	0.9 ± 0.1
Furan, 2-butyl (124)	81	-	0.5 ± 0.0	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	2.5 ± 0.5	5.4 ± 0.1
Furan, 2-pentyl (138)*	81	4.7 ± 1.4	21.5 ± 1.5	19.8 ± 0.8	19.4 ± 0.5	17.6 ± 1.1	43.5 ± 2.1	186.8 ± 42.8	308.3 ± 21.4
Furan, 2-heptyl (166)	81	-	-	-	-	-	-	0.7 ± 0.2	8.3 ± 1.7
Total		**4.7 ± 1.4**	**22.4 ± 1.5**	**20.4 ± 0.6**	**20.1 ± 0.4**	**18.1 ± 1.0**	**44.5 ± 2.2**	**190.8 ± 43.5**	**316.0 ± 21.5**

*Asterisked compounds were acquired commercially and used as standards for identification purposes; -: not detected.

ii) *Corn oil digestate headspace.* The headspace of the digestate DC contains a higher concentration of alkanals, of (*E*)-2-alkenals and of furan, 2-pentyl than CDJ and some 2,4-alkadienals which are absent in the headspace of CDJ, all well-known oxidation markers. The presence of these compounds in the headspace of DC proves that the corn oil has undergone oxidation during digestion.

iii) *Gamma-tocopherol enriched corn oil samples headspace.* The antioxidant effect exhibited by *gamma*-tocopherol can also be inferred by comparison of the headspace composition of DC0.2γT, DC2γT and DC5γT samples with that of the headspace of the sample DC. In the headspace of the digestates of the three in *gamma*-tocopherol enriched samples, the same oxidation markers have been found as in DC but in small abundance in all cases. This may be due to these three samples having undergone an oxidation process of a much smaller intensity than DC resulting from the action of the added *gamma*-tocopherol. In agreement with results obtained by ^{1}H NMR, this effect is much more evident in the most *gamma*-tocopherol enriched samples (DC2γT and DC5γT) in which the abundance of (*E*)-2-alkenals is of a similar order to that found in CDJ.

iv) *Alpha-tocopherol enriched corn oil samples headspace.* The comparison of the headspaces composition of the digestates of the oil samples enriched in *alpha*-tocopherol DC0.2αT, DC2αT and DC5αT with that of the headspace of DC evidences, beyond a doubt, the prooxidant behaviour of this compound. The headspace of this digestate contains not only the oxidation compounds detected in DC in much more abundance, but also new oxidation markers, not present in DC, including alkanals, (*E*)-2-alkenals, 2,4-alkadienals and even oxygenated *alpha,beta* unsaturated aldehydes of known toxicity, furanones and a significant number of furan derivatives, some of these in considerable abundance. The great abundance of 2,4-alkadienals and furan, 2-pentyl and the presence of a large number of oxidation

markers which absent in the headspace of DC, evidences the greater prooxidant role of *alpha*-tocopherol in line with its higher concentration in the oil sample. In summary, the results obtained by studying the headspaces of the digestates are in total agreement with those obtained by ^{1}H NMR.

In addition, a consideration can also be made, from the comparison between the ratio of the abundances of some of these oxidation markers generated under *in vitro* digestion conditions and under accelerated storage conditions. It is known that the oxidation of oils rich in linoleic acyl groups, as corn oil, under accelerated storage conditions, forms (*E,E*)-2,4-decadienals in much more abundance than (*Z,E*)-2,4-decadienals (near 4 times higher) [25, 35]. However, under *in vitro* digestion conditions, both alkadienals are formed in similar abundance (see data of DC, DC0.2γT, DC2γT, DC5γT and DC0.2αT in Table 3), and in fact the ratio between the abundances of these two oxidation markers is reversed in the samples enriched with the higher concentrations of *alpha*-tocopherol (DC2αT and DC5αT). Moreover, the ratio between furan, 2-pentyl and 2,4-decadienals abundances is fairly different between oils submitted to accelerated storage conditions and those submitted to *in vitro* digestion. In the first case, this ratio for oils rich in linoleic acyl groups, is always smaller than 1 [25,35]. However in corn oil samples submitted to *in vitro* digestion this ratio is always higher than 1, with the highest values in those samples with the lowest oxidation level (15.3 ± 2.3 in DC, 22.0 ± 0.1 in DC0.2γT, 27.7 ± 1.5 in DC2γT, 29.3 ± 2.7 in DC5γT). In fact, among all digestates, those coming from the samples enriched in *alpha*-tocopherol, which have the highest oxidation levels, exhibit the lowest values of this ratio (7.3 ± 2.3 in DC0.2αT, 5.3 ± 0.7 in DC2αT, and 4.6 ± 1.5 in DC5αT). This shows, in agreement with the previous comments regarding results obtained by ^{1}H NMR, that different mechanisms are involved in these two oxidation processes. It should be taken into account that nothing happens randomly in oxidation processes, but rather that all reactions are governed by the

interactions established between all the components of the sample. Whenever a process is carried out under the same conditions with samples of the same composition, the same results are obtained.

3.4. Bioaccessibility of *gamma*- and *alpha*-tocopherols in the different digestates. Influence of the enrichment with *alpha*-tocopherol on the bioaccessibility of *gamma*–tocopherol naturally present in corn oil

The concentration of these tocopherols in the corn oil or in the digestates can be determined from the intensity of some of their ^{1}H NMR spectral signals, as indicated in the experimental section, by using the equation [eq. S12] shown in Supplementary Material. This is possible because, as Table S5, Figure 2 and Figure S1 show, both tocopherols have some spectral signals (*gamma*-tocopherol a singlet at 6.36 ppm and *alpha*-tocopherol another singlet at 2.16 ppm) not overlapping with either that of the oil component signals nor with those of the oxidation compounds formed during digestion.

In this case, data provided by ^{1}H NMR spectroscopy allows one to estimate the bioaccessibility of these compounds, which can be expressed in two different ways, thus providing very complete information. One way of describing the bioaccessibility of tocopherols could be through the parameter $B_{T,}$ defined by the ratio between the concentration of tocopherol (T) in the digestate (D) and the concentration of fatty acids plus acyl groups in the same sample ($B_T=([T_D]/[FA+AG]_D)$). This parameter informs about the amount of tocopherol (given in mmol) that can be absorbed in relation to the amount of the main lipid components in the sample (given in mol). B_T allows us to contextualize the importance of the bioaccessibility of tocopherols, in quantitative terms, in the whole digestate. In addition, this parameter also allows comparison with the bioaccessibility of the other compounds before

mentioned, which are also present in the digestates such as the bioaccessible main components of the oil and the toxic compounds formed in the oxidation process.

Table 4. Bioaccessibility of *gamma*-tocopherol (γT) and *alpha*-tocopherol (αT) in the digestates of the different samples in which these compounds are present, expressed in two different ways. $B_{T\,=}$ (mmol T_D/mol (AF+GA) $_D$) and $B'_{T\,=}$ (mmol T_D/mmol T_O). Values are the average of two determinations together with their standard deviations.

Samples	$B_{\gamma T}$	$B'_{\gamma T}$	$B_{\alpha T}$	$B'_{\alpha T}$
DC	0.33±0.00	0.67±0.00		
DC0.2γT	0.89±0.18	0.65±0.14		
DC2γT	8.55±0.00	0.70±0.01		
DC5γT	35.65±1.12	0.82±0.03		
DC0.2αT	0.33±0.03	0.67±0.01	-	-
DC2αT	0.46±0.02	0.94±0.00	6.36±0.00	0.58±0.00
DC5αT	0.47±0.04	0.96±0.01	12.91±0.15	0.32±0.00

-: not detected

Another way to express bioaccessibility, B'_T, is through the ratio between the concentration of tocopherol in the digestate, T_D (given in mmol), and the concentration of tocopherol in the oil before digestion T_O (also given in mmol), ($B'_T=([T_D]/[T_O])$). Parameter B'_T gives information about the amount of this compound lost during *in vitro* digestion and indicates the fraction of the original amount of this compound in the oil that after digestion is available to be absorbed. Bioaccessibility data obtained for the different samples, estimated using both approaches mentioned, are given in Table 4.

i) *Regarding the bioaccessibility of gamma-tocopherol naturally present in the corn oil C*. The $B_{\gamma T}$ value of DC digestate (0.33 ± 0.00 mmol/mol (FA+AG)) indicates that a certain amount of this compound remains after *in vitro* digestion without degrading. This amount is very small in comparison with that of the toxic oxidation compounds also present in the same sample (see data in Table 2). Moreover, $B'_{\gamma T}$ is 0.67 ± 0.0 mmol/mmol(γT_O), indicating that up to around 67% of the *gamma*-tocopherol contained in the oil remains in the digestate without degrading and is able to be absorbed. A similar value to this has been found with reference to *gamma*-tocopherol in the *in vitro* digestion of broccoli [36]. Both parameters,

$B_{\gamma T}$ and $B'_{\gamma T,}$ also show that oxidation occurs during this *in vitro* digestion even having *gamma*-tocopherol, at this low concentration, without degrading. This is not surprising because, oil oxidation and *gamma*-tocopherol degradation run in parallel in the oxidation of corn oil, under other conditions than these for a certain period of time, that is to say the presence of *gamma*-tocopherol does not avoid oil oxidation, but only slows down the process [35].

ii) *Regarding the bioaccessibility of gamma-tocopherol in the digestates of the samples enriched with this compound*. As Table 4 shows the $B_{\gamma T}$ values of the digestates of these samples are, as could be expected, higher the higher the enrichment degree of the sample is. These range between 0.89 ± 0.18 and 35.65 ± 1.12 mmol/mol (FA+AG). In sample DC0.2γT, in agreement with what takes place in the non-enriched sample, oxidation of the oil main components has been produced (see Table 2) although a certain amount of undegraded *gamma*-tocopherol remains in the sample. However, in the samples enriched with higher levels of *gamma*-tocopherol (DC2γT and DC5γT), this compound avoids the oxidation of corn oil main components during this *in vitro* digestion. The ratio between the amount of *gamma*-tocopherol that remain undegraded after digestion and the initial, given by $B'_{\gamma T}$, also shows high values (see Table 4), but in all cases a certain amount (between 35-18%) of *gamma*-tocopherol has been lost due to its degradation, in this case avoiding totally or partially the oil component oxidation during *in vitro* digestion.

iii) *Regarding the bioaccessibility of gamma-tocopherol in the digestates of the samples enriched with alpha-tocopherol.* The $B_{\gamma T}$ values of these samples, as expected, are small because this compound is a minor corn oil component. They are given in Table 4. It is noteworthy that in the digestate of the less *alpha*-tocopherol enriched sample DC0.2αT, $B_{\gamma T}$ reaches the same value as in the digestate of the unenriched DC. This indicates that oil

enrichment with this small amount of *alpha*-tocopherol has no effect on *gamma*-tocopherol degradation. Furthermore, in the other two enriched samples DC2αT and DC5αT the $B_{\gamma T}$ value is almost the same for both samples (near 0.46 mmol/mol (FA+AG)). Bearing in mind that the concentration of *gamma*-tocopherol naturally present in corn oil C is 0.49 ± 0.00 mmol/mol (FA+AG), it is evident that this compound has almost undergone no degradation during *in vitro* digestion in the presence of high concentrations of *alpha*-tocopherol. As a result, $B'_{\gamma T}$ has the same value (0.67 ± 0.01) in sample DC0.2αT as in DC, and reaches 0.94-0.96 values in samples DC2αT and DC5αT. It is noteworthy that although *gamma*-tocopherol acts as antioxidant, and *alpha*-tocopherol as prooxidant, the concentration of *gamma*-tocopherol remains unaltered during the *in vitro* digestion in the presence of high concentrations of *alpha*-tocopherol. This could be due to the great difference in concentration between *gamma*- and *alpha*-tocopherol in these two latter samples. This leads one to think that this great difference in concentration significantly reduces the probability of *gamma*-tocopherol molecules being near the oxidation sites of the oil main components, in comparison with that of *alpha*-tocopherol. This could explain *gamma*-tocopherol concentration remaining unaltered during digestion in these samples.

iv) *Regarding the bioaccessibility of alpha-tocopherol in the digestates of the samples enriched with this compound*. The bioaccessibility of *alpha*-tocopherol is null or small depending on the enrichment degree. $B_{\alpha T}$ data given in Table 4 indicate that the added *alpha*-tocopherol degrade during digestion to a greater extent than the added *gamma*-tocopherol. In fact, *alpha*-tocopherol totally degrades in the sample having the smaller enrichment level. For this reason $B_{\alpha T}$ and $B'_{\alpha T}$ values are zero in the less enriched sample. In addition, in the other two samples (DC2αT and DC5αT) $B'_{\alpha T}$ shows small values, especially in the most enriched sample. These results are in agreement with others recently published [37]. The

participation of this compound in the oxidative reactions taking place during *in vitro* digestion could explain their high level of degradation.

4. CONCLUSIONS

The lipolysis extent reached in the *in vitro* digestion of corn oil and of samples enriched in *gamma*- and *alpha*-tocopherol is high and of a similar order in all cases. The effect of the enrichment with these compounds does not provokes significant changes in this process even if the results could suggest a slightly increase in lipolysis extent in the case of enrichment with *gamma*-tocopherol and a slight decrease in the case of *alpha*-tocopherol. These subtle differences could be attributed to the small differences in polarity and in other molecular characteristics of both tocopherols. The lipolysis pattern is also very similar in all samples, glycerol and mono-glycerides being the main glyceride structures present in the digestates, followed by triglycerides. However, diglycerides are the structures whose abundance is the smallest. This hydrolytic pattern could be considered typical of the enzymatic cocktail and digestive juices used. As a consequence of the high lipolysis extent and of its pattern, high bioaccessibility is reached for oil main components, namely fatty acids and monoglycerides. This ranges between 0.64 and 0.72 $mol([FA]+[MG])_D/mol([FA]+[AG])_D$. The subtle differences among samples, regarding the lipolysis extent are also translated to bioaccesibility of oil main components: the highest value is exhibited by the sample most enriched with *gamma*-tocopherol, and the opposite is true for the sample most enriched with *alpha*-tocopherol.

In agreement with several previous studies it is again confirmed that *in vitro* digestion provokes lipid oxidation. This is confirmed by the clear diminution of linoleic structures in the digestate in relation to those existing in the oil, which prove their degradation during *in vitro* digestion. Analysis of the concentration of these linoleic structures in the digestates of

the oil samples enriched in *gamma*- and in *alpha*-tocopherol, indicates that the former tocopherol avoids, to a certain degree, the degradation of linoleic, acting as antioxidant, whereas the opposite is true for the latter compound. It has been proved that enrichment of the oil with *gamma*-tocopherol at a level of 2% in weight or higher avoids oil component oxidation during *in vitro* digestion. However, an enrichment level of 0.2 in weight of *gamma*-tocopherol, barely brings about a reduction in oxidation degree. From these results, it is evident that this compound behaves as antioxidant. However, *alpha*-tocopherol behaves as prooxidant in line with its higher enrichment degree. Oxidation under *in vitro* digestion conditions evolves at a very high rate, generating very different oxidation compounds in concentrations of a similar order, unlike what happens in other oxidation processes. At high enrichment degrees of *alpha*-tocopherol, hydroperoxy-, hydroxy- and keto-conjugated dienes as well as keto-*E*-epoxy-*E*-monoenes and aldehydes have been detected in the digestates, all of them well known oxidation markers and some of them associated to degenerative diseases. The study of the volatile components of the different digestates leads to the same conclusions regarding the behaviour of *gamma*-tocopherol as antioxidant and of *alpha*-tocopherol as prooxidant. Once again, the usefulness of the two techniques employed in this study is proved, as well as the need to use as many oxidation markers as possible, in order to have the most complete picture as possible of the oxidation process in order to avoid erroneous interpretations.

The bioaccessibility of the naturally present *gamma*-tocopherol in the corn oil cannot be considered low, although a certain amount of this compound has been lost during *in vitro* digestion. Nevertheless, this bioaccessibility is smaller than that of the toxic oxidation compounds generated during *in vitro* digestion. *Gamma*-tocopherol bioaccessibility in the samples enriched in this compound is higher the higher the enrichment level is, showing high values, in addition to avoid the oxidation of oil components during *in vitro* digestion.

Bioaccessibility of the naturally present *gamma*-tocopherol in the oil samples enriched in *alpha*-tocopherol is near hundred percent in the most enriched samples, in spite of *alpha*-tocopherol provoking oil component oxidation in line with its higher enrichment degree. By contrast, *alpha*-tocopherol exhibits a low bioaccessibility, being even null in the less enriched sample, probably due to its behaviour as prooxidant. Thus, the safety of the intake of supplements which are rich in *alpha*-tocopherol should be the subject of broader and deeper studies. Likewise, the suitability of European legislation that allows enriching edible oils with this compound under the principle of "*quantum satis*" should be reviewed

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3921/9/3/246/s1, Table S1: Composition and pH values of the juices employed in the *in vitro* digestion model employed in this study; Table S2: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of glycerides. TG: triglycerides; DG: diglycerides; MG: monoglycerides. The signal letters agree with those given in Figure 1; Table S3: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of acyl groups and fatty acids. AG: acyl groups; FA: fatty acids. The signal letters agree with those given in Figure 1; Table S4: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some oxidation compounds detected in the digestates and formed during the *in vitro* digestion. The signal letters agree with those given in Figure 2; Table S5: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of *gamma*- and *alpha*-tocopherol present in the samples before and after *in vitro* digestion. The signal letters agree with those given in Figure 2; Figure S1: Chemical structures of tocopherols involved in this of study, together with some chemical shifts (ppm) of some of their hydrogen atoms. Equations used for the quantification from ^{1}H NMR spectral data of several compounds present in the starting samples and/or in the lipid extracts of the digestates. Figure S2**:** Region between 4-34 min of the total ion chromatogram obtained by SPME-GC/MS of the digestate of corn oil sample enriched in *alpha*-tocopherol DC5αT. Peaks identified: **(1)** pentanal; **(2)** hexanal; **(3)** furan, 2-butyl; **(4)** heptanal; **(5)** (*E*)-2-heptenal; **(6)** furan, 2-pentyl; **(7)** (*Z*,*E*)-2,4-heptadienal; **(8)** (*E*,*E*)-2,4-heptadienal; **(9)** (*E*)-2-octenal; **(10)** nonanal; **(11)** (*E*)-2-nonenal; **(12)** decanal; **(13)** (*E*)-2-decenal; **(14)** (*Z*,*E*)-2,4-decadienal; **(15)** (*E*,*E*)-2,4-decadienal; **(16)** 5-pentyl-2(5H)-furanone; **(17)** 4,5-epoxy-2-decenal (isomer); **(18)** 4,5-epoxy-(*E*)-2-decenal.

AUTHOR CONTRIBUTIONS

J.A-C.; performed the experimental work, contributed to data interpretation and to manuscript preparation. M.L.I.; supervised the analyses performed and contributed to data interpretation and to manuscript preparation. M.D.G.; conceived the work, supervised the whole work and the results obtained, and contributed to data interpretation and to the manuscript preparation.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest, financial or otherwise

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Supplementary Material of

Article 6

EFFECT OF THE ENRICHMENT OF CORN OIL WITH *ALPHA*- OR *GAMMA*-TOCOPHEROL ON ITS *IN VITRO* DIGESTION STUDIED BY ^{1}H NMR AND SPME-GC/MS. FORMATION OF HYDROPEROXY-, HYDROXY-, KETO-DIENES AND KETO-*E*-EPOXY-*E*-MONOENES IN THE MORE *ALPHA*-TOCOPHEROL ENRICHED SAMPLES

Jon Alberdi-Cedeño, María L. Ibargoitia, María D. Guillén

Table S1. Composition and pH values of the juices employed in the *in vitro* digestion model employed in this study.

Components	**Saliva**	**Gastric juice**	**Duodenal juice**	**Bile juice**
KCl (mmol/L)	12.02	11.06	7.57	5.05
NaCl (mmol/L)	5.10	47.09	119.98	89.99
$NaHCO_3$ (mmol/L)	20.17	-	40.33	68.86
NaH_2PO_4 (mmol/L)	7.40	0.22	-	-
NH_4Cl (mmol/L)	-	5.72	-	-
KH_2PO_4 (mmol/L)	-	-	0.59	-
Na_2SO_4 (mmol/L)	4.79	-	-	-
KSCN (mmol/L)	2.06	-	-	-
$MgCl_2$ (mmol/L)	-	-	0.53	-
$CaCl_2*2H_2O$ (mmol/L)	-	2.72	1.36	1.51
HCl (37%) (mL/L)	-	6.50	0.18	0.15
Urea (mmol/L)	3.33	1.42	1.67	4.16
Glucose (mmol/L)	-	3.61	-	-
Glucuronic acid (mmol/L)	-	0.10	-	-
Uric acid (mmol/L)	0.09	-	-	-
Glucoseamine hydrochloride (mmol/L)	-	1.53	-	-
Bovine serum albumin (g/L)	-	1.00	1.00	1.80
Mucin (g/L)	0.025	3.00	-	-
A. oryzae α-amylase (g/L)	0.29	-	-	-
A. niger lipase (U/mL)	-	100	-	-
Pepsin (g/L)	-	2.50	-	-
Pancreatin (g/L)	-	-	9.00	-
Lipase type II from porcine pancreas (g/L)	-	-	1.50	-
Bovine bile extract (g/L)	-	-	-	18.75
pH	6.9±0.0	1.3±0.1	8.1±0.1	8.2±0.1

Table S2. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of glycerides. TG: triglycerides; DG: diglycerides; MG: monoglycerides. The signal letters agree with those given in Figure 1.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Structures
Glycerides structure protons				
I	3.65	ddd	ROCH_2–CHOH–C**H**$_2$OH	glyceryl group in **1-MG**
J	3.73	m*	ROCH_2–CH(O*R'*)–C**H**$_2$OH	glyceryl group in **1,2-DG**
K	3.84	m*	HOC**H**$_2$–CH(O*R*)–C**H**$_2$OH	glyceryl group in **2-MG**
L	3.94	m	ROCH_2–C**H**OH–CH_2OH	glyceryl group in **1-MG**
M	4.05–4.21	m	ROC**H**$_2$–C**H**OH–C**H**$_2$OR'	glyceryl group in **1,3-DG**
N	4.18	ddd	ROC**H**$_2$–CHOH–CH_2OH	glyceryl group in **1-MG**
O	4.22	dd,dd	ROC**H**$_2$–CH(O*R'*)–C**H**$_2$OR''	glyceryl group in **TG**
P	4.28	ddd	ROC**H**$_2$–CH(O*R'*)–CH_2OH	glyceryl group in **1,2-DG**
Q	4.93	m	HOCH_2–C**H**(O*R*)–CH_2OH	glyceryl group in **2-MG**
R	5.08	m	ROCH_2–C**H**(O*R'*)–CH_2OH	glyceryl group in **1,2-DG**

Abbreviations: d: doublet; m: multiplet.

*This signal shows different multiplicity if the spectrum, is acquired from the pure compound or taking part in the mixture.

**The intensity of some of these signals, also shown in Figure 1, together with signal F of Table S3, were used to estimate the molar percentages of different kinds of glyceryl structures using the equations [eq. S1-eq. S10].

***The assignment of the ^{1}H NMR signals of the protons was made as in previous studies (Guillén & Uriarte, 2012; Nieva-Echevarría et al., 2014).

Table S3. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of acyl groups and fatty acids. AG: acyl groups; FA: fatty acids. The signal letters agree with those given in Figure 1.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Structures
Main acyl groups (AG) and fatty acids (FA)				
A	0.88	t	–C**H**$_3$	saturated and monounsaturated **ω-9** in **AG** and **FA**
	0.89	t	–C**H**$_3$	linoleic in **AG** and **FA**
B	0.97	t	–C**H**$_3$	linolenic in **AG** and **FA**
C	1.19–1.42	m**	–(C**H**$_2$)$_n$–	**AG** and **FA**
D	1.61	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **TG**
	1.62	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **1,2-DG**
	1.63	m	–OCO–CH$_2$–C**H**$_2$–, COOH–CH$_2$–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	1.64	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **2-MG**
E	1.92–2.15	m***	–C**H**$_2$–CH=CH–	**AG** and **FA**
F*	2.26–2.36	dt	–OCO–C**H**$_2$–	**AG** in **TG**
	2.33	m	–OCO–C**H**$_2$–	**AG** in **1,2-DG**
	2.35	t	–OCO–C**H**$_2$–, COOH–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	2.38	t	–OCO–C**H**$_2$–	**AG** in **2-MG**
G*	2.77	t	=HC–C**H**$_2$–CH=	linoleic in **AG** and **FA**

Abbreviations: d: doublet; t: triplet; m: multiplet.

*The intensity of these signals, also shown in Figure 1, was used to estimate the molar percentage of linoleic structures by using equation [eq. S11].

**Overlapping of multiplets of methylenic protons in the different acyl groups either in *β*-position, or further, in relation to double bonds, or in *γ*-position, or further, in relation to the carbonyl group.

***Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups.

****The assignment of the ^{1}H NMR signals of the protons was made as in previous studies (Guillén & Ruiz, 2003; Nieva-Echevarría et al., 2014).

Table S4. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some oxidation compounds detected in the digestates and formed during the *in vitro* digestion. The signal letters agree with those given in Figure 2.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Structures
Oxidation Compounds (OC)				
Conjugated dienic systems associated with hydroperoxy groups				
-	5.51	dtm	–C**H**=CH–CH=CH–	(*Z,E*)-conjugated double bonds associated with hydroperoxy group (OOH) in octadecadienoic **AG** and **FA** **HPO-c(*Z,E*)dEs**
-	5.56	ddm		
-	6.00	ddtd		
b	***6.58***	dddd		
Conjugated dienic systems associated with hydroxy groups				
–	5.44	ddd	–C**H**=CH–CH=CH–	(*Z,E*)-conjugated double bonds associated with hydroxy group (OH) in octadecadienoic **AG** and **FA** **HO-c(*Z,E*)dEs**
-	5.66	dd		
-	5.97	t		
a	***6.49***	dd		
-	5.58	dd	–C**H**=CH–CH=CH–	*(E,E)*-conjugated double bonds associated with hydroxy group (OH) in octadecadienoic **AG** and **FA** **HO-c(*E,E*)dEs**
-	5.71	dd		
-	6.03	dd		
c	***6.18***	dd		
Conjugated keto-dienes (KO-c-dEs)				
d	***7.49***	ddd	-CH=C**H**- (C-11)	*(Z,E)*- conjugated double bonds associated with ketodiene of octadeadienoic **AG** and **FA**acyl groups **KO-c(*Z,E*)dEs**
	6.16	d	-CH=CH- (C-10)	
	6.12	m	-CH=CH- (C-12)	
	5.91	dt	-CH=CH- (C-13)	
	2.54	t	-CH_2-CO	
Keto-epoxy-monoenes (KO-EPO-mES)				
e	6.52	dd	-CH=CH-	13-KO-9,10-*E*-EPO-11-*E*-ene 9-KO-12,13-*E*-EPO-10-*E*-ene **KO-*E*-EPO-*E*-mEs**
	6.38	d	-CH=CH-	
	3.22	dd	-HCOC**H**-	
	2.91	td	-HCOCH-	
	2.53	t	-CH_2-	
Aldehydes				
f	***9.75***	t	-C**H**O	**n-alkanals**
	2.40	dt	-CH_2-	

Abbreviations: d: doublet; t: triplet; m: multiplet.

*The intensities of the signals indicated in bold, together with signal F of Table S3, were used to estimate the concentration (mmol/molAG+FA) using the equation [eq. S12].

**The assignment of the ^{1}H NMR signals of the protons was made with the aid of standard compounds and with the data taken from literature (Guillén & Ruiz, 2005; Dufour & Loonis, 2005; Guillén & Goicoechea, 2007; Lin et al., 2007; Ramsden et al., 2017).

Table S5. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some tocopherols detected in the samples before and after *in vitro* digestion. The signal letters agree with those given in Figure 2.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Compounds
		Tocopherols		
αT	2.16**	s	-C**H$_3$** (C-7)	*alpha*-Tocopherol
γT	6.36**	s	-C**H** (C-5)	*gamma*-Tocopherol

Abbreviation: s: singlet.

*The intensity of these signals, together with signal F of Table S3, were used to estimate the concentration (mmol/molAG+FA) using the equation [eq. S12].

**Assignment was made with the aid of standard compounds and with the data taken from the literature (Baker & Mayers, 1991)

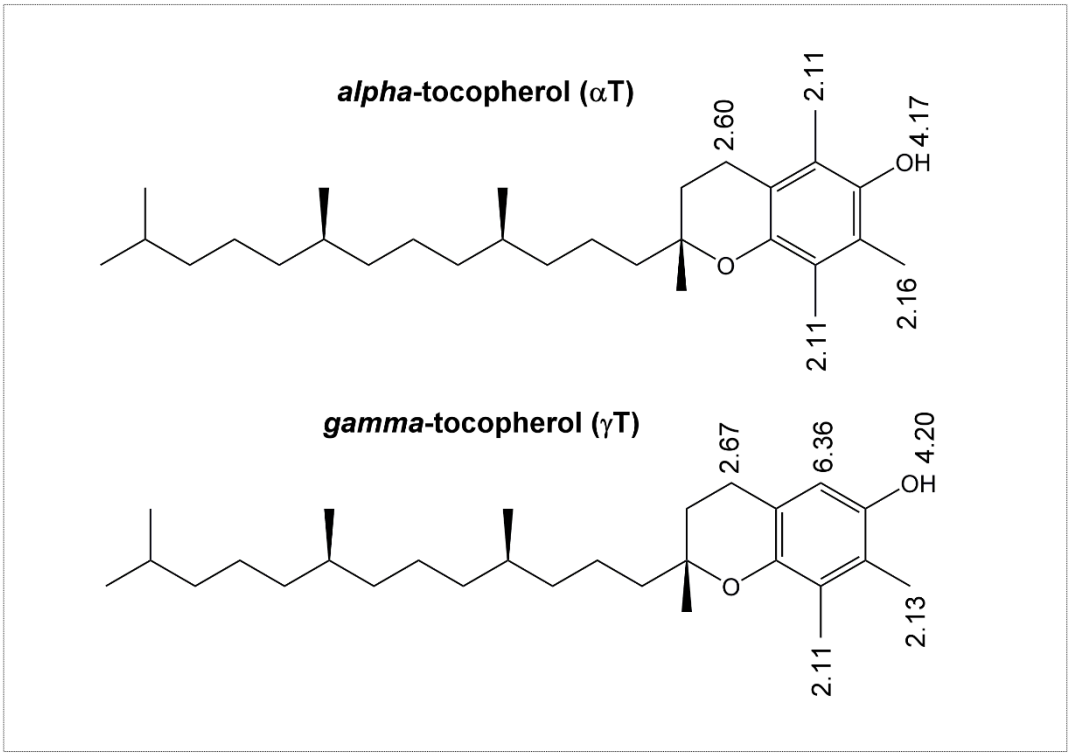

Figure S1. Chemical structures of tocopherols involved in this of study, together with some chemical shifts (ppm) of some of their hydrogen atoms.

Quantification from ^{1}H NMR spectral data of several compounds present in the starting samples and/or in the lipid extracts of the digestates

Bearing in mind that the area of each ^{1}H NMR spectral signal is proportional to the number of protons that generate it, and that the proportionality constant is the same for all kinds of protons, the area of some spectral signals can be employed to quantify a wide variety of compounds, as detailed below.

A. Equations used to estimate the molar percentage (%) of the several glyceride structures present in the lipid extract of digestates and the glycerol.

In these equations the number of moles (N) of fatty acids and all the glycerides were expressed as follows:

$$N_{2\text{-}MG}=Pc*A_K/4 \quad \text{[eq. S1]}$$
$$N_{1\text{-}MG}=Pc*A_L \quad \text{[eq. S2]}$$
$$N_{1,2\text{-}DG}=Pc*(A_{I+J}-2A_L)/2 \quad \text{[eq. S3]}$$
$$N_{TG}=Pc*(2A_{4.26\text{-}4.38}-A_{I+J}+2A_L)/4 \quad \text{[eq. S4]}$$
$$N_{1,3\text{-}DG}=Pc*(A_{4.04\text{-}4.38}-2A_{4.26\text{-}4.38}-2A_L)/5 \quad \text{[eq. S5]}$$
$$N_{FA}=(Pc*A_F-6N_{TG}-4N_{1,2\text{-}DG}-4N_{1,3\text{-}DG}-2N_{1\text{-}MG}-2N_{2\text{-}MG})/2 \quad \text{[eq. S6]}$$
$$N_{Gol}=(N_{FA}-N_{1,2\text{-}DG}-N_{1,3\text{-}DG}-2N_{2\text{-}MG}-2N_{1\text{-}MG})/3 \quad \text{[eq. S7]}$$

where Pc is the proportionality constant existing between the area of the ^{1}H NMR signals and the number of protons that generate them, A_K, A_L, A_{I+J} and A_F are the areas of the corresponding signals indicated in Table S2 and Figure 1, and $A_{4.26\text{-}4.38}$ and $A_{4.04\text{-}4.38}$ represent the areas of the signals between 4.26 and 4.38 ppm, and between 4.04 and 4.38 ppm, respectively.

Using these equations, the molar percentages of the different kinds of glycerides in relation to the total number of moles of glyceryl structures present (NT_{GS}) were determined as follows:

$$NT_{GS}=N_{TG}+N_{1,2\text{-}DG}+N_{1,3\text{-}DG}+N_{2\text{-}MG}+N_{1\text{-}MG}+N_{Gol} \quad \text{[eq. S8]}$$
$$G\%=100N_G/NT_{GS} \quad \text{[eq. S9]}$$

where G is each kind of glyceride (TG, 1,2-DG, 1,3-DG, 2-MG and 1-MG) and N_G the respective number of moles.

$$Gol\%=100N_{Gol}/NT_{GS} \quad \text{[eq. S10]}$$

B. Estimation of the molar percentage of linoleic FA plus AG (FA+AG). The molar percentage of linoleic (L%), AG plus FA, in relation to the total number of moles of AG plus FA (NT_{AG+FA}) present in the starting oils and in the lipid extracts of the corresponding digestates was estimated as follows:

$L\%=100*A_G/ A_F$ [eq. S11]

where A_G and A_F are the areas of signals G and F indicated in Table S3 and Figure 1.

C. Estimation of the concentration of specific compounds in oil samples and in the lipids extract from digestates

The concentration of the several kinds of specific compounds (SC), expressed as millimoles per mole of the sum of AG+FA, was estimated by using the following equations:

$[SC] = [(A_{SC}/n)/(A_F/2)]*1000$ [eq. S12]

where A_{SC}, is the area of the signal selected for the quantification of each specific compound present in the sample, n the number of protons that generate the signals given in Tables S4 , S5 and Figure 2 and A_F is the area of the signal F in Table S3 and in Figure 1.

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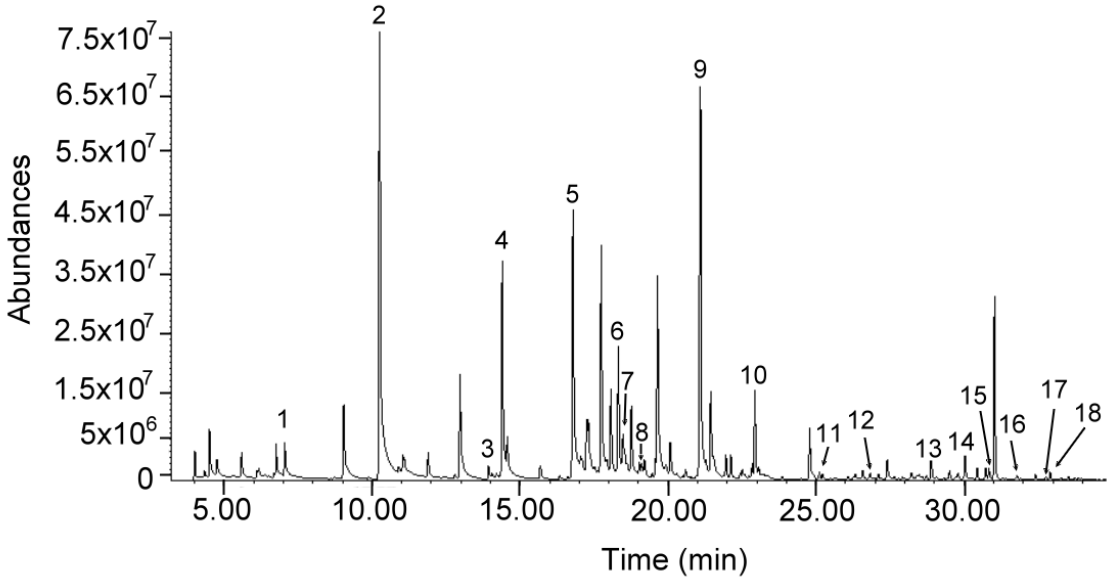

Figure S2. Region between 4-34 min of the total ion chromatogram obtained by SPME-GC/MS of the digestate of corn oil sample enriched in *alpha*-tocopherol DC5αT. Peaks identified: (1) pentanal; (2) hexanal; (3) furan, 2-butyl; (4) heptanal; (5) (*E*)-2-heptenal; (6) furan, 2-pentyl; (7) (*Z*,*E*)-2,4-heptadienal; (8) (*E*,*E*)-2,4-heptadienal; (9) (*E*)-2-octenal; (10) nonanal; (11) (*E*)-2-nonenal; (12) decanal; (13) (*E*)-2-decenal; (14) (*Z*,*E*)-2,4-decadienal; (15) (*E*,*E*)-2,4-decadienal; (16) 5-pentyl-2(5H)-furanone; (17) 4,5-epoxy-2-decenal (isomer); (18) 4,5-epoxy-(*E*)-2-decenal.

Article 7

A GLOBAL STUDY BY ^{1}H NMR SPECTROSCOPY AND SPME-GC/MS OF THE *IN VITRO* DIGESTION OF VIRGIN FLAXSEED OIL ENRICHED OR NOT WITH MONO-, DI- OR TRI-PHENOLIC DERIVATIVES. ANTIOXIDANT EFFICIENCY OF THESE COMPOUNDS

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ABSTRACT

The effect of enriching virgin flaxseed oil with dodecyl gallate, hydroxytyrosol acetate or with *gamma*-tocopherol on its *in vitro* digestion is tackled by means of proton nuclear magnetic resonance and solid phase microextraction followed by gas chromatography/mass spectrometry. The extent and pattern of the lipolysis reached in each sample is analyzed and the bioaccessibility of oil main components. None of the phenolic compounds provokes inhibition of the lipase activity and all of them reduce the lipid oxidation degree caused by the *in vitro* digestion and the bioaccessibility of oxidation compounds. The antioxidant efficiency of the three phenols essayed is in line with the number of phenolic groups in its molecule, and is dose-dependent. The concentration of some minor oil components such as terpenes, sesquiterpenes, cycloartenol and 24-methylenecycloartenol is not modified by *in vitro* digestion. Contrarily *gamma*-tocopherol shows a very low *in vitro* bioaccessibility, probably due to its antioxidant behavior, although increases in line with the oil enrichment in phenolic compounds. Oxidation is produced during *in vitro* digestion even in the presence of important concentrations of *gamma*-tocopherol, which remains bioaccessible after digestion in the enriched samples in this compound.

KEYWORDS: virgin flaxseed oil, *in vitro* digestion, oxidation, *gamma*-tocopherol, hydroxytyrosol acetate, dodecyl gallate, antioxidant efficiency, minor compounds, bioaccessibility.

1. INTRODUCTION

Food lipid oxidation is an issue of great importance from the food technology point of view because it causes food degradation with economic and health repercussions. This can occur during food processing and storage [1,2]. A common strategy to avoid oxidation consists in the incorporation of compounds with antioxidant ability into the food. Many studies refer to the incorporation of antioxidants into lipid foods and evaluate their antioxidant efficiency under different processing conditions [3-5].

In recent years, it has been shown that food lipid oxidation can also occur during food digestion, and some studies have addressed the effect on food digestion provoked by enrichment of lipids with compounds able to act as antioxidants. It should be taken into account that food oxidation during digestion can give rise to the formation of toxic compounds that can be directly absorbed. For this reason, the study of this issue could be considered as even more important than the oxidation of food during processing or storage.

Among compounds having antioxidant ability are phenolic compounds. These are usually secondary metabolites present in fruits and vegetables in very low concentrations; the healthy properties of these foods have been attributed to them. These compounds are considered to be free radical scavengers, and their antioxidant capacity has been related to the number and arrangement of their hydroxyl groups [6]. Due to their potential antioxidant ability and to the beneficial health effects attributed to them [6,7], some studies have focused on the enrichment of lipid foods with different natural or synthetic phenolic compounds in order to prevent oxidation when they are submitted to thermal processes or to gastrointestinal digestion.

In this context the study of the efficiency of different polyphenols such as epicatechin, resveratrol, caffeic and gallic acids in the inhibition of the oxidation of

linoleic acid in authentic fluid from rat small intestine has been tackled [8] by monitoring the oxidation level by the concentration of hexanal. The effect of some hydrophilic and lipophilic phenolic compounds on the oxidation reached by lipids during *in vitro* digestion of high-fat and low-fat beef meat has also been studied by monitoring the concentration of malonaldehyde, 4-hydroxy-nonenal and hexanal [9]. Likewise, the antioxidant effect of 2,6-di-*tert*-butyl-hydroxytoluene (BHT) in the oxidation of cod liver oil during *in vitro* digestion has been proved [10]. Furthermore, several studies have analyzed the effect that enrichment of certain oils with *alpha*-tocopherol has on their oxidation when they are submitted to digestion conditions, with controversial results [9,11-13]. Finally, a comparative study of the effect of the enrichment of corn oil with *alpha*- and *gamma*-tocopherol has shown that the first acts as prooxidant in agreement with some of the above mentioned studies whereas the second acts as antioxidant [14].

However, phenolic compounds, in addition to exhibiting antioxidant or prooxidant ability could also take part in other reactions during digestion because they come in contact both with all food components and with enzyme-containing digestive juices. It should be remembered that phenolic compounds can react with protein, and so with enzymes [15,16], reducing their activity and provoking negative effects on digestion by diminishing the extent of the hydrolytic reactions. In fact, it has been proved that tea polyphenols are able to inhibit the pancreatic lipase activity reducing in this way the gastrointestinal lipolysis [17] and thus absorption of lipids. Likewise, it has been described that alkyl gallates are able to inhibit the activity of amylase, so reducing the absorption of carbohydrates [18,19].

All these studies suggest that the influence of phenolic compounds not only may decrease or increase oxidative reactions that can occur during lipid digestion but also may

in some cases affect the hydrolytic reactions that are the essence of the digestion process, which is to release absorbable building blocks.

Within this framework this study tackles the effect that the enrichment of virgin flaxseed oil with dodecyl gallate, hydroxytyrosol acetate or *gamma*-tocopherol, provokes on its *in vitro* digestion process from a global perspective. This oil has been selected because it can be considered a good model of omega-3 lipids due to its high concentration in linolenic groups, and so has a great tendency towards oxidation. The *in vitro* digestion model used will be a modified version of that developed by Versantvoort, *et al*. (2005) [20] which has given satisfactory results in previous studies [21]. Samples before and after digestion will be studied by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and solid phase microextraction followed by gas chromatography/mass spectrometry (SPME-GC/MS). Subjects such as hydrolysis degree, the occurrence or prevention of lipid oxidation and of formation of oxidation compounds, and *in vitro* bioaccessibility of the main components and of some minor virgin flaxseed oil components will be addressed. This study will shed light on the antioxidant efficiency of the above mentioned tri, di- and mono-phenols under *in vitro* digestion conditions, and clarify if these phenols are able to establish reactions with lipase, in this way having negative effects on the extent of lipolytic reactions.

2. MATERIALS AND METHODS

2.1. Samples subject of study

The study was carried out with virgin flaxseed oil (F), purchased in a local supermarket. Its composition in molar percentages of linolenic (Ln), linoleic (L), oleic (O) and saturated (S) acyl groups is 55.7 ± 0.0%, 14.2 ± 0.3%, 20.5 ± 1.2% and 9.5 ± 0.9% respectively; this was determined from the ^{1}H NMR spectral data as in previous

studies [22,23]. In virgin flaxseed oil there is an important presence of terpenes and of sesquiterpenes, as has been previously shown [24] and the oil used in this study no exception, which will be shown later. Likewise it is also known that the main sterols of this oil are cycloartenol and 24-methylenecyclartenol and the main tocopherol is *gamma*-tocopherol [25, 26]; the concentration of these compounds in this oil will be indicated later. The presence of these minor components is important because they have been assigned various biological activities [27-30].

Aliquots of virgin flaxseed oil containing, a natural concentration of *gamma*-tocopherol of 0.33 mmol/mol (AG+FA), were enriched separately in dodecyl gallate (DG) (purity 98%, from Alfa Aesar., GmbH & Co KG, Germany), in hydroxytyrosol acetate (HTA) (purity of 99.54%, from Seprox Biotech, Madrid, Spain), and in *gamma*-tocopherol (γT) (purity ≥ 90%, Eisai Food & Chemical Co. Ltd., Tokyo, Japan). The samples enriched with dodecyl gallate were named FDG_1 (with an enrichment of 0.14 mmol DG/mol $[FA+AG]_O$) and FDG_2 (with an enrichment of 1.35 mmol DG/mol $[FA+AG]_O$). The samples enriched with hydroxytyrosol acetate, were named $FHTA_1$ (with an enrichment of 0.24 mmol HTA/mol $[FA+AG]_O$) and $FHTA_2$ (with an enrichment of 2.65 mmol HTA/mol $[FA+AG]_O$). Finally, the samples enriched with different additional concentrations of *gamma*-tocopherol, were named $F\gamma T_1$ (with an enrichment of 0.13 mmol γT/mol $[FA+AG]_O$), $F\gamma T_2$ (with an enrichment of 1.30 mmol γT/mol $[FA+AG]_O$), $F\gamma T_3$ (with an enrichment of 14.21 mmol γT/mol $[FA+AG]_O$) and $F\gamma T_4$ (with an enrichment of 32.79 mmol γT/mol $[FA+AG]_O$). These enrichment levels were set in function of the solubility of these compounds in the oil. The above concentrations were obtained trying to reach enrichment degrees near to 0.02 % and 0.2 % in weight for the three phenolic compounds and, in addition, near 2% and 5% in weight in the case of *gamma*-tocopherol due to its high solubility in oils. However, these latter levels of

enrichment were not possible for dodecyl gallate and hydroxytyrosol acetate because of their limited solubility in oils.

2.2. Digestion experiments

Aliquots (0.5 g) of the above-mentioned samples were digested following the semi-static *in vitro* gastrointestinal digestion model developed by Versantvoort, *et al*. (2005) [20]. This validated method was optimized, in order to improve the lipids digestion attempting to reach lipolysis levels of a similar order to in vivo digestion [21]. This method is a three-stage procedure to simulate digestive processes in mouth, stomach, and small intestine, by sequentially adding the corresponding digestive juices (saliva, gastric juice, duodenal juice and bile), whose composition is given in Table S1 (see Supplementary Material). The first stage begins by adding 6 mL of saliva to the sample. After 5 min of incubation, 12 mL of gastric juice are added and the mixture is rotated head-over-heels at 40 rpm for 2 h at 37±2ºC. One hour after the start of the gastric stage, pH is set between 2 and 3 with HCl (37%), simulating the gradual acidification of the chyme occurring *in vivo*. After 2 h of gastric stage, 2 mL of sodium bicarbonate solution (1 M), 12 mL of duodenal juice and 6 mL of bile juice are added. Subsequently, pH was set between 6 and 7, and the mixture is again rotated at 40 rpm and incubated at 37±2ºC for 4 h. All the reagents and enzymes for the preparation of digestive juices were acquired from Sigma-Aldrich (St. Louis, MO, USA): α-amylase from *Aspergillus oryzae* (10065, ~30 U/mg); pepsin from porcine gastric mucosa (P7125, ≥400 U/mg protein); amano lipase A from *Aspergillus niger* (534781, ≥120,000 U/g); pancreatin from porcine pancreas (P1750); lipase type II crude from porcine pancreas (L3126, 100-500 U/mg protein (using olive oil, 30 min incubation) and bovine bile extract (B3883). The digested samples were named as the original samples preceded by D (DF, $DFDG_1$, $DFDG_2$,

DFHTA$_1$, DFHTA$_2$, DFγT$_1$, DFγT$_2$, DFγT$_3$, and DFγT$_4$). Three digestion experiments, each including duplicate samples, were performed. Blank samples corresponding to the mixture of juices submitted to digestive conditions were also taken for further analysis.

2.3. Digestate lipid extraction

Lipids of the digestates were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich, ST. Louis, MO, USA) following a methodology that also allows fatty acids extraction as in a previous studies [10]. This methodology involves a three-stage liquid-liquid extraction process with 20 ml of CH_2Cl_2 each. Afterwards, to ensure a complete protonation of fatty acids and/or the dissociation of the potential salts formed, the remaining water phase was acidified to pH 2 with HCl (37%) and a second extraction was carried out in three steps. All the CH_2Cl_2 extracts of each sample were mixed and solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. The extraction yield was in all cases near 85%. These extracts contain triglycerides, diglycerides and monoglycerides, as well as fatty acids and tocopherols and other minor lipophilic compounds either present in the original oil samples or formed from oil components in the digestion process.

2.4. Study by ^{1}H NMR of oil samples and lipid extracts of digestates

2.4.1. Operating conditions

The ^{1}H NMR spectra of the original oil (F), and of the oil samples enriched with the different compounds at the different concentrations (F, FDG$_1$, FDG$_2$, FHTA$_1$, FHTA$_2$, FγT$_1$, FγT$_2$, FγT$_3$, and FγT$_4$), and of the lipids extracted from their digestates (DF, DFDG$_1$, DFDG$_2$, DFHTA$_1$, DFHTA$_2$, DFγT$_1$, DFγT$_2$, DFγT$_3$, and DFγT$_4$) were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. For this

purpose, the above-mentioned samples (approximately 0.16 g) were dissolved in 400 µl of deuterated chloroform, which contained tetramethylsilane (TMS), as internal reference (Cortec, Paris, France). First, a standard ^{1}HNMR spectrum was acquired and in a second step a NOESYGPPS experiment consisting of a one-dimensional ^{1}H NMR pulse sequence with selective suppression of certain strong signals was carried out. This NOESYGPPS experiment allowed one to obtain a ^{1}H NMR spectrum with higher sensitivity than the standard single pulse ^{1}H NMR in the spectral region ranging from 5.8 to 9.8 ppm [31] at the cost of suppressing some signals in other regions. The relaxation and acquisition time used allow the complete relaxation of the protons, the signal areas thus being proportional to the number of protons that generate them, making it possible to use them for quantitative purposes as in previous studies [32].

2.4.2. Identification of the components

The identification of the components present in the original oil, in the oil samples enriched with phenolic compounds and in the lipid extracts of their digestates, was carried out on the basis of the assignments of the ^{1}H NMR signals to the different kinds of hydrogen atoms, and in short to the different compounds. Figure 1 gives the spectral region comprised between 0.0 and 4.9 ppm, of virgin flaxseed oil F ^{1}H NMR spectrum and the region comprised between 3.5 ppm and 5.10 ppm, conveniently enlarged, of the ^{1}H NMR spectra of the lipids extracted of its digestate (DF), in which signals of protons of their main components appear.

These signals, and other ones due to protons of oxidation compounds and minor components not shown in Figure 1, but present in the spectra of all the samples here studied, their chemical shifts and assignments are given in Tables S2, S3, S4 and S5 (see Supplementary Material). Their assignments were made taken into account previous

studies, as it indicated in each table, or on the basis of the signals of standard compounds acquired for this study. Among these later are: cycloartenol, hexanal and decanal, acquired from Sigma-Aldrich (St. Louis, MO, USA).

Table S2 shows ^{1}H NMR signals of specific protons of the different glyceride structures, such triglycerides, diglycerides and monoglycerides. Table S3 shows ^{1}H NMR signals of protons of linolenic, linoleic, oleic and saturated acyl groups and fatty acids, and the signals of methylenic protons supported on carbons atoms in *alpha* and *beta* position in relation to carbonyl-carboxyl groups. Table S4 shows ^{1}H NMR signals of protons of oxidation compounds coming from main oil components degradation which occurred during digestion. Finally, Table S5 gives ^{1}H NMR signals, present in Figure 2, of some protons of dodecyl gallate, of hydroxytyrosol acetate, and of *gamma* tocopherol and of free and esterified cycloartenol and 24-methylenecycloartenol. The areas of some of these spectral signals were used to quantify the concentration of the different kinds of above mentioned structures in the corresponding samples, as it will be explained below.

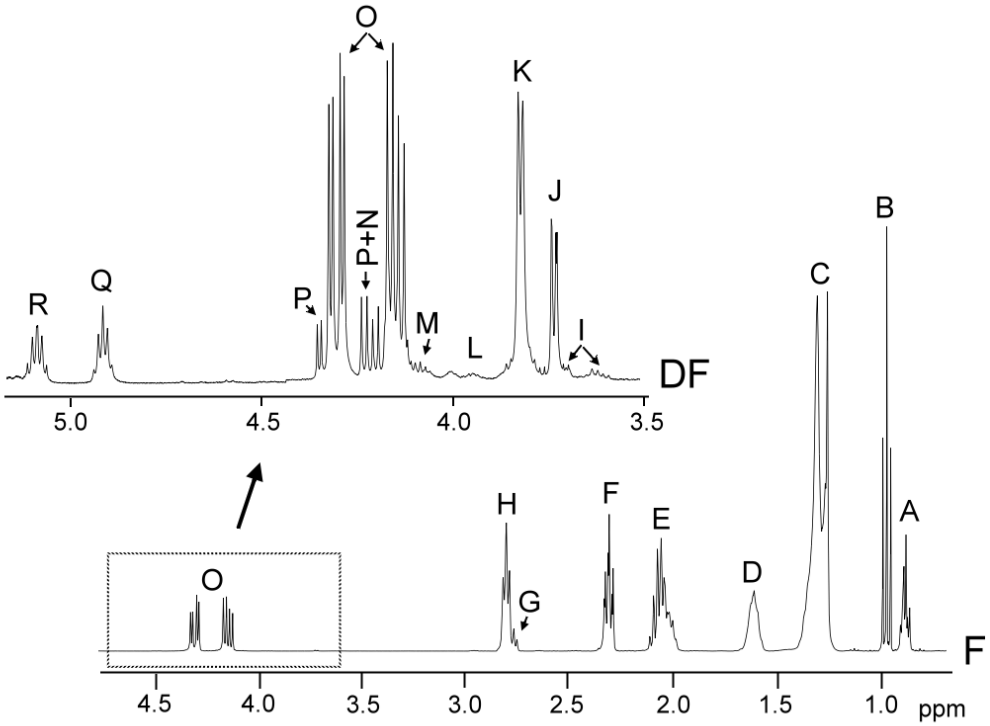

Figure 1. Region comprise between 0.0 and 4.9 ppm, of flaxseed virgin oil, F, ^{1}H NMR spectrum, and enlargement of the region comprised between 3.5 ppm and 5.10 ppm of the ^{1}H NMR of the lipid extracted from the digestate, DF. The signal letters agree with those of Tables S2 and S3 of Supplementary Material.

2.4.3. Quantifications made from ^{1}H NMR spectral data

This technique allows the estimation of the concentrations, expressed in different ways, of all identified compounds already mentioned. This is possible because, as has been above explained, the area of the ^{1}H NMR signals is proportional to the number of protons that generate the signal. The quantification of the different kinds of compounds or structures is explained below.

(A) Estimation of the molar percentage of the different kinds of glycerides in the digestates

Estimation of the molar percentage of each kind of glyceride structures can be carried out by using the intensity of some signals indicated in Tables S2 and S3, which

are also shown in Figure 1. Although glycerol is formed during digestion, due to its polar nature, it is not present in the lipid extract of the digestate, however its concentration can be estimated indirectly. This is possible because the concentration of total fatty acids plus acyl groups, of only acyl groups, and of fatty acids released in the formation of diglycerides and monoglycerides can be determined from ^{1}H NMR data. Thus, the estimation of the molar percentage of triglycerides (TG), 1,2-diglycerides (1,2-DG), 1,3-diglycerides (1,3-DG), 2-monoglycerides (2-MG), 1-monoglycerides (1-MG) and glycerol (Gol) in relation to the total glyceryl structures present in the digestate, was carried out by using equations [eq. S1-eq. S10] given in Supplementary Material. They are based exclusively on the intensity of ^{1}H NMR spectral signals [33].

(B) Estimation of the molar percentage of fatty acids plus acyl groups that have linolenic structure in relation to the total of all types of fatty acids and acyl groups in digestates

In edible oils the concentration of fatty acids is very small and, in many cases, inappreciable in comparison with the concentration of acyl groups. However, as is known, during oils digestion hydrolysis brings about the transformation of a certain number of acyl groups into fatty acids. The fatty acids formed maintain the same number of carbon atoms and unsaturation pattern as the starting acyl groups. Acyl groups and fatty acids having the same structure provide NMR spectra signals with a high degree of overlapping that allow their joint quantification. In this study the molar percentage of the linolenic acyl groups plus linolenic fatty acids in the digestates was estimated in relation to the total number of moles of all kinds of fatty acids plus acyl groups. This estimation was made using the equation [eq. S11], given in Supplementary Material, in which the areas of some signals that are shown in Figure 1 and in Table S3 are involved. This equation is the same employed in previous studies [22,23], but using the signal of methylenic protons

supported on carbons atoms in *alpha* position in relation to carbonyl-carboxyl groups, instead of the signal of triglyceride protons used in edible oils studies.

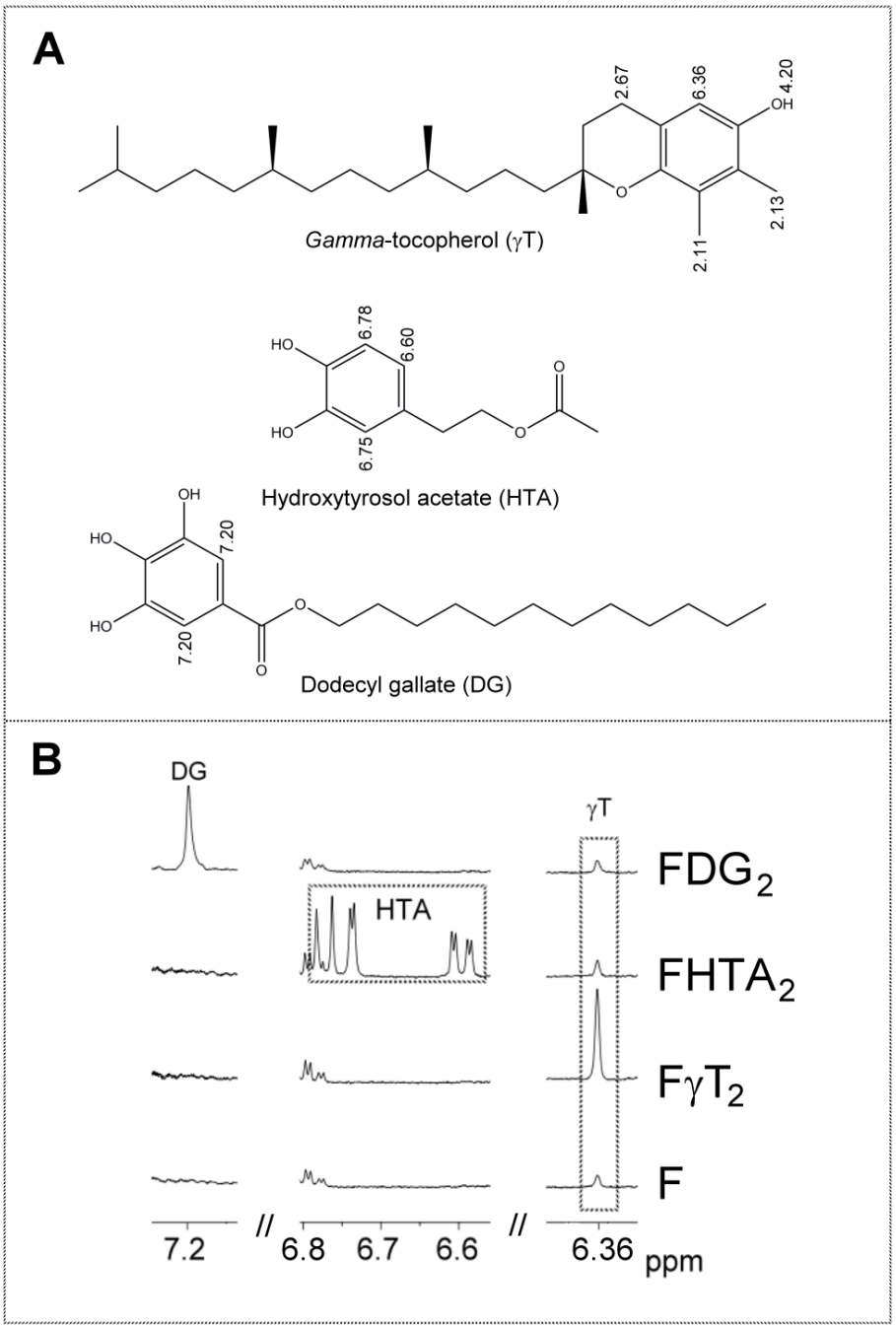

Figure 2. A) Chemical structures of the phenolic compounds involved in this study (*gamma*-tocopherol γT, hydroxytyrosol acetate HTA and dodecyl gallate DG, together with some chemical shifts (ppm) of the ^{1}H NMR signals of some of their hydrogen atoms. B) Enlargement of some regions of the ^{1}H NMR spectra of non-enriched F and some enriched oil samples ($F\gamma T_2$, $FHTA_2$ and FDG_2), in which appears signals of the phenolic compounds above mentioned. The signal letters agree with those of Table S5.

(C) Estimation of the concentration of specific compounds (SC) in oil samples and in the digestates

The concentration of oxidation compounds, and of other ones, such as *gamma*-tocopherol, cycloartenol plus 24-methylenecycloartenol, dodecyl gallate and hydroxytyrosol acetate, either in oils or in digestates can be estimated by using the general equation [eq. S12] given in Supplementary Material using the intensity of one of their non-overlapped ^{1}H NMR spectral signals, which are indicated in Figures 1 and 2, and in Tables S3, S4 and S5. This equation allows one to estimate the concentration of any compound in oils or in digestates in relation to the concentration of fatty acids plus acyl groups, which are considered the internal reference.

2.5. Study by SPME-GC/MS of the headspace of digestates and of the mixture of the digestive juices submitted to digestion conditions with the virgin flaxseed oil

Extraction of the volatile components constituting the headspace of the several samples (0.5 g in a 10 ml screw-cap vial) was accomplished automatically by using a CombiPAL autosampler (Agilent Technologies, Santa Clara, CA, USA). The samples studied were the several digestates (DF, $DFDG_1$, $DFDG_2$, $DFHTA_1$, $DFHTA_2$, $DF\gamma T_1$, $DF\gamma T_2$, $DF\gamma T_3$, and $DF\gamma T_4$) and the mixture FDJ of digestive juices DJ, after undergoing digestion conditions, and virgin flaxseed oil F. The comparison of the headspaces of the several samples enables one to deduce differences provoked in them by *in vitro* digestion.

The fiber used for the headspace components extraction was coated with Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm film thickness, 1 cm long; was acquired from Supelco (Sigma-Aldrich, St. Louis, MO, USA)). It was inserted into the headspace of the sample and maintained for 55 min at 50ºC, after a pre-equilibration time of 5 min. The fiber containing the components extracted was

desorbed for 10 min in the injection port (splitless mode with 5 min purge time) of a 7890A gas chromatograph equipped with a 5975C inert MSD with Triple Axis Detector (Agilent Technologies, Palo Alto, CA, USA) and a computer operating with the ChemStation program. A fused silica capillary column was used (60 m length, 0.25 mm inside diameter, 0.25 μm film thickness; from Agilent Technologies Inc., Palo Alto, CA), coated with a nonpolar stationary phase (HP-5MS, 5%phenyl methyl siloxane). The operation conditions were the following: the injector and interface temperatures were held at 250°C and 305°C respectively, and helium at a constant pressure of 117 kPa (16.9 psi) was used as the carrier gas. The oven temperature was initially held at 50°C for 5 min, increased from 50 to 300°C at a rate of 4°C/min, and then held at 300°C for 30 min. Mass spectra were recorded at an ionization energy of 70 eV, with data acquisition in Scan mode. The temperatures of the ion source and the quadrupole mass analyzer were kept at 230 and 150°C, respectively. A reference sample of known composition was periodically analyzed in order to verify the sensitivity of the SPME-GC/MS experiments as in previous studies [34].

Identification of the headspace components was carried out by using several commercial standard compounds acquired from Sigma-Aldrich (St. Louis, MO, USA). When standard compounds were not available, the identification was made by matching the spectra obtained, higher than 85%, with those of commercial libraries (Wiley W9N08, Mass Spectral Database of the National Institute of Standards and Technology), or with those spectra provided by the scientific literature, as in previous studies [34].

The semi-quantification of the compounds was based on the area counts of the base peak (Bp) of the mass spectrum of each compound divided by 10^6. When the Bp of a compound overlapped with some ion peak of the mass spectrum of another compound, an alternative ion peak was selected for the semi-quantification of the former [34].

Although the chromatographic response factor of each compound is different, the area counts thus determined are useful for the comparison of the abundance of each compound in the different samples. The target compounds of this technique were terpenes and sesquiterpenes, which are characteristics minor volatile components of virgin flaxseed oil, and those volatile oxidation compounds formed in the *in vitro* digestion. Data given in the following tables are average values of triplicate experiments.

2.6. Statistical analysis

The significance of the differences in the several kinds of data among samples, was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM, NY, USA).

3. RESULTS

As mentioned above, the comparison between *in vitro* digestion of virgin flaxseed oil and that of the same enriched oil with different concentrations of dodecyl gallate, hydroxytyrosol acetate or *gamma*-tocopherol is approached as globally as possible, attending to all those aspects, about which, the used techniques provide information.

3.1. Extent and pattern of lipolysis produced by the *in vitro* digestion of virgin flaxseed oil and effect of the enrichment with the different phenolic compounds

In vitro digestion, as expected, provokes the partial hydrolysis of the ester bond of triglycerides, yielding diglycerides, monoglycerides, fatty acids and glycerol. The concentration of the different glyceryl species can be estimated from ^{1}H NMR spectral data of the virgin flaxseed oil, F, and of the lipid extracts of the different digestates, as explained in the experimental section. The results obtained, indicated in Table 1, show that a very important percentage of triglycerides has not undergone hydrolysis and, in

fact, they remain as the main glyceride specie after *in vitro* digestion. Monoglycerides and glycerol are present in the digestates in fairly high concentrations, indicating that the species able to be absorbed by enterocytes of the intestinal wall (monoglycerides and fatty acids) are in important concentrations after this *in vitro* digestion. And finally, diglycerides, which are not able to be absorbed, are in the lower concentration of all glyceride species.

As the statistical treatment shows, the enrichment of the oil with these phenolic compounds, in the concentrations essayed, does not significantly affect to the hydrolysis reached in the digestion. This fact indicates that the added phenolic compounds have not inhibited the lipase activity, or in other words, have not reacted with lipases diminishing their activity, because in this case, the hydrolysis yield should be smaller in the enriched samples than in the not-enriched sample. It should be mentioned, that tea polyphenolic compounds, which can inhibit lipase, are very polar lipophobic compounds, some of them are even polymer polyphenols [17,35]. To the best of our knowledge, this is the first time that it has been shown that dodecyl gallate and hydroxytyrosol acetate are not able to inhibit lipase activity. The inability of *gamma*-tocopherol to inhibit lipase activity has been shown in a previous study and the results here obtained confirm this fact [14].

Contrarily to the before mentioned, and although the differences are not significant, a slightly smaller percentage of triglycerides, and a slightly higher percentage of glycerol in the digestates of the enriched samples than in that of the not-enriched sample may be observed. This could suggest a slightly greater hydrolysis extent as a consequence of the enrichment in these phenolic compounds.

Table 1. Lipolysis extent. Molar percentages of triglycerides (TG), diglycerides (1,2-DG and 1,3-DG), monoglycerides (1-MG and 2-MG) and glycerol (Gol), in relation to the total glyceride structures, in virgin flaxseed oil samples F, in the digestate of this oil DF and in those of the samples enriched in dodecyl gallate, hydroxytyrosol acetate and *gamma*-tocopherol ($DFDG_1$, $DFDG_2$, $DFHTA_1$, $DFHTA_2$ $DF\gamma T_1$, $DF\gamma T_2$, $DF\gamma T_3$ and $DF\gamma T_4$). Bioaccessibility of oil main components after *in vitro* digestion (B_{OMC}), defined by the ratio (mol [FA] + [MG])$_D$ /mol ([FA]+[AG])$_D$. Different letters within each column indicate a statistically significant difference among the samples ($p < 0.05$).

Samples	Lipolysis extent (molar %)						Bioaccessibility
	TG (%)	1,2-DG (%)	2-MG (%)	1,3-DG (%)	1-MG (%)	Gol (%)	(B_{OMC})
Oil							
F	99.4 ± 0.0a	1.2 ± 0.0a	-	-	-	-	-
DF	33.1 ± 2.7b	18.1 ± 2.1b	21.7 ± 0.5a	4.8 ± 1.0a	2.2 ± 0.8a	20.2 ± 4.3a	0.52 ± 0.05a
Oil-dodecyl gallate							
$DFDG_1$	33.6 ± 0.3b	16.8 ± 0.2b	20.3 ± 0.0a	6.0 ± 0.3a	1.6 ± 0.1a	21.9 ± 0.0a	0.51 ± 0.00a
$DFDG_2$	32.7 ± 1.9b	17.3 ± 1.2b	20.6 ± 2.1a	4.7 ± 0.2a	2.6 ± 0.1a	22.1 ± 1.8a	0.53 ± 0.01a
Oil-hydroxytyrosol acetate							
$DFHTA_1$	32.4 ± 0.5b	17.1 ± 0.4b	20.5 ± 1.5a	5.3 ± 0.2a	2.1 ± 0.1a	22.6 ± 2.0a	0.53 ± 0.01a
$DFHTA_2$	31.2 ± 0.5b	16.6 ± 0.4b	22.3 ± 0.5a	4.3 ± 0.4a	2.0 ± 0.2a	23.7 ± 2.0a	0.55 ± 0.01a
Oil-gamma-tocopherol							
$DF\gamma T_1$	32.3 ± 0.9b	17.9 ± 2.5b	22.3 ± 0.7a	3.9 ± 0.9a	1.5 ± 0.1a	22.2 ± 3.3a	0.53 ± 0.01a
$DF\gamma T_2$	32.4 ± 2.0b	16.9 ± 0.1b	20.3 ± 0.5a	5.5 ± 2.9a	1.4 ± 0.1a	23.4 ± 0.5a	0.53 ± 0.00a
$DF\gamma T_3$	30.1 ± 0.7b	17.3 ± 0.0b	20.2 ± 0.6a	7.4 ± 0.4a	2.2 ± 0.1a	22.7 ± 0.9a	0.53 ± 0.00a
$DF\gamma T_4$	32.4 ± 0.0b	17.6 ± 0.9b	20.6 ± 0.6a	4.4 ± 0.5a	2.1 ± 0.0a	22.9 ± 0.9a	0.53 ± 0.01a

-: not detected

3.2. *In vitro* bioaccessibility of oil main components and influence of the enrichment with the different phenolic compounds

The *in vitro* bioaccessibility of virgin flaxseed oil main components depends of both the extent and the pattern of lipolysis. As before mentioned, of all those glyceryl species released during digestion only fatty acids (FA) and monoglycerides (MG) are able to be absorbed by the intestinal wall. For this reason, the *in vitro* bioaccessibility of the oil main components is well described by the ratio between the concentration of these absorbable species and all fatty acids (FA) plus acyl groups (AG) present in the corresponding digestate. This parameter is defined by the equation $B_{OMC} = ([FA]+[MG])_D/([FA]+[AG])_D$ as in previous studies [14]. The data thus obtained are also given in Table 1. As can be observed that, the *in vitro* bioaccessibility of the virgin flaxseed oil main components in the unenriched sample is only around fifty percent. Similar values have been found for the virgin flaxseed oil samples enriched with the different phenolic compounds, without the differences among them being statistically significant. However, although these differences are not significant, in most of the cases, this parameter is slightly higher in the digestates of the oil samples enriched with the phenolic compounds above mentioned than in the digestate of the unenriched oil. This could be related with the antioxidant activity of the compounds added.

3.3. Oxidation reactions during *in vitro* digestion of virgin flaxseed oil and of virgin flaxseed oil samples enriched in dodecyl gallate, hydroxytyrosol acetate and *gamma*-tocopherol

Oxidation reactions in oils lead to the degradation of some components and the formation of others which are new. For this reason, this subject can be tackled by either monitoring the changes in the concentration of the former as consequence of the *in vitro* digestion, or by monitoring the formation of the latter after digestion, or by both.

3.3.1. Changes provoked by in vitro digestion, in the concentration of linolenic structures. Antioxidant efficiency of the added phenolic compounds

In virgin flaxseed oil, linolenic acyl groups are the main ones, as indicated in the experimental section. Furthermore, linolenic acyl group and linolenic fatty acid tend to oxidize more than any other due to their well known low oxidative stability. For this reason, if oxidation reactions take place during digestion, linolenic structures will be clearly affected, diminishing their concentration in the sample. Due to this, they are the subject of this monitoring. ^{1}H NMR spectroscopy allows the determination of the concentration of linolenic structures in relation to the all fatty acids plus acyl groups in the sample subject of study, as described before. Table 2 gives the concentration of these structures in the oil before digestion and in the digestates of each one of the samples. It can be observed that *in vitro* digestion of oil F causes a significant diminution in the concentration of linolenic structures. In other words, this group undergoes degradation which provokes a reduction in their molar percentage in relation to the total moles of all kinds of fatty acids plus acyl groups (AG+FA) from 55.7 % in F to 47.9% in DF.

The addition of different concentrations of the above mentioned phenolic compounds avoids this degradation during digestion to a certain extent, even at the lower enrichment level essayed. This indicates that the three compounds act as antioxidants. Nevertheless, their efficiency at avoiding the degradation of the linolenic structures, or in other words their oxidation, is not the same for the three antioxidants tested. From the comparison of data in Table 2, it is evident that in the range of enrichment levels studied, dodecyl gallate is the most efficient antioxidant of the three essayed, under the *in vitro* digestion conditions employed here. It can be observed that approximately the half of mols of enrichment with dodecyl gallate is required (0.14 or 1.35 mmol/mol (AG+FA)) than with hydroxytyrosol acetate (0.24 or 2.65 mmol/mol (AG+FA)) to reach the same reduction in the degradation of

linolenic groups. Likewise, the higher antioxidant efficiency of dodecyl gallate than that of *gamma*-tocopherol is also evident because with similar enrichment levels of both compounds (see Table 2) the first avoids the degradation of linolenic groups to a greater extent than the second. Finally, also taking data from Table 2, it is evident that hydroxytyrosol acetate is a more efficient antioxidant than *gamma*-tocopherol, because a concentration about five times greater of this latter compound than of hydroxytyrosol acetate is required to avoid a similar degree of oxidation of linolenic structures.

These results demonstrate that, during *in vitro* digestion of virgin flaxseed oil, the antioxidant efficiency of dodecyl gallate is greater than that of hydroxytyrosol acetate, which is in turn greater than that of *gamma*-tocopherol. This is also in agreement with the general idea that a greater number of phenolic groups in the molecule involves a higher antioxidant ability as described by some authors [6].

In the case of the enrichment with *gamma*-tocopherol and due to the availability of enough experimental data it was possible to look for quantitative relationships between enrichment degree in the oil and linolenic structures concentration, expressed in molar percentage in relation to the total (AG+FA) moles in the corresponding sample, which is inversely related with oxidation level reached during *in vitro* digestion. It has been found that the molar percentage of linolenic structures in the digestate [Ln] and enrichment level in *gamma*-tocopherol in the oil sample [γT], given in mmol/mol$(AG+FA)_O$, fit well to the equation [Ln] = 51.06+1.16 *Ln* [γT] , R=0.9931, where *Ln* is the Napierian logarithm. This relationships is represented in Figure S1.

Table 2. Concentration of linolenic structures given in molar percentage in relation to the total moles of AG+FA present in the virgin flaxseed oil F, in the digestate of this oil DF and in those of the samples enriched in hydroxytyrosol acetate, dodecyl gallate and gamma-tocopherol ($DFDG_1$, $DFDG_2$, $DFHTA_1$, $DFHTA_2$, $DF\gamma T_1$, $DF\gamma T_2$, $DF\gamma T_3$ and $DF\gamma T_4$), together with the enrichment level in phenolic compounds of the samples before digestion. Concentration of oxidation compounds such as hydroperoxides and n-alkanals in the same samples determined from ^{1}H NMR spectral data. Different letters within each column indicate a statistically significant difference among the samples ($p < 0.05$).

Oil, Digestates and **Enrichment Level** in phenolic compounds of the oil samples before digestion, given in mmol/mol [AG+FA]	**Linolenic structures** (%)	**Oxidation compounds**	
		HPO-c(*Z*,*E*)-dEs mmol/mol [AG+FA]	**n-alkanals** mmol/mol [AG+FA]
Oil			
F	55.7 ± 0.0a	-	-
DF	47.9 ± 0.8d	0.39 ± 0.04a	0.09 ± 0.00a
Oil-dodecyl gallate			
$DFDG_1$(0.14)	50.9 ± 1.1abc	0.33 ± 0.04ab	0.08 ± 0.01a
$DFDG_2$(1.35)	53.1 ± 1.8ab	0.28 ± 0.02b	0.07 ± 0.01a
Oil-hydroxytyrosol acetate			
$DFHTA_1$ (0.24)	50.9 ± 1.1abc	0.30 ± 0.02b	0.08 ± 0.00a
$DFHTA_2$ (2.65)	53.7 ± 0.3ab	n.d.	0.07 ± 0.00a
Oil-*gamma*-tocopherol			
$DF\gamma T_1$ (0.13)	48.5 ± 3.1d	0.40 ± 0.02a	0.09 ± 0.01a
$DF\gamma T_2$ (1.30)	51.8 ± 1.0abc	0.33 ± 0.02ab	0.07 ± 0.01a
$DF\gamma T_3$ (14.21)	53.8 ± 0.1ab	-	0.07 ± 0.00a
$DF\gamma T_4$ (32.79)	55.2 ± 0.2a	-	0.04 ± 0.00b

-: not detected; n.d.: not determined due to interfering signals; HPO-c(*Z*,*E*)-dEs: hydroperoxy-conjugated (*Z*,*E*)-dienes.

According to this equation the relationship between the molar percentage of linolenic structures, which is higher the higher the antioxidant efficiency, is directly related with *gamma*-tocopherol enrichment through the above logarithmic relation. This means that an increase in the enrichment level of *gamma*-tocopherol when the concentration of this compound in the sample is low provokes a higher antioxidant effect than the same increase

in the enrichment level when the concentration of this compound in the oil sample is high. In other words, increased enrichment in *gamma*-tocopherol appreciably improves the antioxidant effect to a certain degree of enrichment after which additional enrichments are much less efficient at improving this antioxidant effect (see Figure S1). According to this equation, to reach total prevent of the linolenic oxidation during *in vitro* digestion requires an enrichment level of *gamma*-tocopherol near 54.59 mmol/mol$(AG+FA)_O$. This result indicates that the higher enrichment level essayed does not totally avoid oil oxidation during this *in vitro* digestion, as will be shown later.

The approach above described shows a methodology which may be used with any other antioxidant whenever enough experimental data are available. The generalization of its use will provide very interesting data, not only to estimate the antioxidant efficiency of different compounds, but also to predict, in a fairly accurate way, the enrichment degree required of a compound to avoid lipid oxidation during *in vitro* digestion.

3.3.2. Formation of oxidation compounds derived from virgin flaxseed oil main components during in vitro digestion

As mentioned, the degradation of the oil main components during *in vitro* digestion gives rise to the formation of oxidation compounds, most of which should be present in the lipid extracts of the corresponding digestates. The detection and quantification of these can be carried out by means of ^{1}H Nuclear Magnetic Resonance spectroscopy using both the standard and NOESYGPPS experiments, and by means of Solid Phase Microextraction (SPME) followed by Gas Chromatography Mass Spectrometry (GC/MS). The first technique views the sample as a whole and allows one to detect and quantify compounds of the same family having protons with similar electronic environment in a global way, without the previous steps of sample separation and without provoking chemical changes in it. The

second technique extracts the headspace components of the sample, among which, if the sample has undergone oxidation, there are volatile oxidation markers.

(a) Oxidation compounds detected in the different digestates by ^{1}H NMR. Effect of the enrichment in phenolic compounds

Two kinds of oxidation compounds have been detected by this technique in the lipid extracts of the digestates, evidencing that oxidation has taken place during *in vitro* digestion. These are hydroperoxides supported on chains having *Z,E* conjugated dienic systems (HPO-c(*Z,E*)-dEs) derived from octadecatrienoic groups, which are primary oxidation compounds (multiplet signal at 6.58 ppm in the ^{1}H NMR spectrum indicated in Table S4), and n-alkanals, which are secondary oxidation compounds (singlet at 9.75 ppm signal in the ^{1}H NMR spectrum indicated also in Table S4). The concentrations of both kinds of compounds were determined in the manner that has been described before. They are given in Table 2 and are in agreement with the previous discussion relating to the degradation of linolenic structures. In general, the higher the degradation of linolenic structures the higher the concentration of both kinds of oxidation compounds. Likewise, the greater the enrichment in phenolic compounds in the sample is, the lower the concentration of oxidation compounds in the digestates. Finally, it is again demonstrated that, under the *in vitro* digestion conditions, dodecyl gallate shows higher antioxidant efficiency than hydroxytyrosol acetate and *gamma*-tocopherol. To reduce the same oxidation level during *in vitro* digestion, measured by the concentration of hydroperoxydes and n-alkanals, needs a level of enrichment with hydroxytyrosol acetate (or with *gamma*-tocopherol) approximately two times (or ten times) higher than with dodecyl gallate. Likewise, to reduce the same level of oxidation, measured by the above mentioned markers, requires a much higher concentration of *gamma*-tocopherol than of hydroxytyrosol acetate, which indicates that the efficiency of the second one as antioxidant is higher than that of the first. Although hydroperoxides cannot be detected in the

digestates of the samples more enriched in *gamma*-tocopherol, the presence of n-alkanals indicates that oxidation has taken place in agreement with data of linolenic degradation and with data of volatile markers that will be commented on later.

As the rate of oxidation of fatty acids is much higher than that of the corresponding acyl groups it could be thought that the first are the lipid compounds that have been oxidized, incorporating in their molecules hydroperoxy groups and conjugated dienic systems and also giving rise to the formation of aldehydes, as mentioned before. This suggests that the new formed compounds could also be bioaccessible in the concentrations indicated in Table 2. That is to say, bioaccessibility of HPO-c(*Z,E*)-dEs (or of n-alkanals) will range between zero (or between 0.04mmol/mol(FA+AG)) in the digestates of the samples which are more enriched in *gamma*-tocopherol, to near 0.4 (or to near 0.09 mmol/mol(FA+AG)) in the unenriched sample or in the sample having the lowest enrichment in *gamma*-tocopherol. Furthermore, the smaller concentration of oxidation compounds in the enriched samples indicates that a lower degradation of fatty acids has taken place in them during digestion. This could be the reason why the samples enriched with phenolic compounds have slightly higher (although not statistically significant) bioaccessibility of the main components of virgin flaxseed oil than the unenriched sample.

(b) Oxidation markers detected by SPME-GC/MS in the different digestates. Effect of the enrichment in phenolic compounds

The samples subject of this study were, in addition to the digestate of the virgin flaxseed oil DF and that of the oil samples enriched with the different phenols ($DFDG_1$, $DFDG_2$, $DFHTA_1$, $DFHTA_2$, $DF\gamma T_1$, $DF\gamma T_2$, $DF\gamma T_3$, and $DF\gamma T_4$), the mixture constituted by the juices submitted to *in vitro* digestion conditions and the undigested oil FDJ. This latter sample was

taken as reference because it contains the oil not submitted to digestion, and for this reason not to oxidation, but in a similar matrix to the digestates.

Among the main volatile compounds coming from lipid oxidation are aldehydes, furan derivatives and ketones and these are the target of this study. As an example, Figure S2 shows the region between 4–30 min of the total ion chromatogram obtained by SPME-GC/MS of the FDJ sample and of the DF digestate. In it, the increase in the intensity of some peaks or the appearance of new peaks related to the main volatile oxidation compounds formed during *in vitro* digestion can be observed. The abundances of the different compounds are directly related with the oxidation degree reached by the sample during digestion. Their values were estimated as indicated in the experimental section, and are given in Table 3, together with the enrichment levels of the different samples in the different phenols. Both sets of data allow one to evaluate the antioxidant efficiency of each one of these phenolic compounds in the oxidation undergone by the virgin flaxseed oil during *in vitro* digestion.

It can be observed in Table 3 that the headspace of the sample FDJ, formed by the mixture of the undigested oil and the juices submitted to *in vitro* digestion conditions, contains a basal concentration of some of these oxidation markers, common in unoxidized oils. As already shown, the *in vitro* digestion provokes the oxidation of virgin flaxseed oil main components, generating volatile oxidation compounds derived mainly from the linolenic structures. For this reason, the volatile oxidation compounds are in much higher abundance in the headspace of DF sample than in that of FDJ mixture (see Table 3). To be found among them are typical compounds coming from the oxidation of linolenic structures, such as 2,4-heptadienals, 2,3-pentanedione and 2,3-octanedione as well as 3,5-octadien-2-one. However, enrichment of the oil with phenolic compounds has as consequence a reduction in the oxidation level reached during *in vitro* digestion. This is proved because, as can be observed in Table 3, the concentration of these volatile oxidation compounds in the

headspace of the digestates of the samples enriched with phenolic compounds is smaller than that found in the headspace of the digestate of the unenriched sample. That is to say, data in Table 3 confirm the same facts inferred from data coming from ^{1}H NMR, not only about the oxidation level reached by the different samples but also about the antioxidant efficiency of each phenolic compound.

In summary, under these *in vitro* digestion conditions dodecyl gallate shows greater antioxidant efficiency than has hydroxytyrosol acetate, which in turn has greater antioxidant efficiency than *gamma*-tocopherol. Nevertheless, it should be pointed out that the antioxidant efficiency order is not the same if it concerns the number of moles of the compound required to avoid or reduce oxidation or if it refers to the amount (expressed in weight or in percentage in weight) required of the compound to reach the same endpoint. If antioxidant efficiency referred to the amount of compound, the hydroxytyrosol acetate would have higher antioxidant efficiency than dodecyl gallate, due to the great difference in the molecular weight of these compounds. For this reason, it is very important to indicate the accurate units to which the antioxidant efficiency is referred measured. Perhaps, the lack of clarity of some studies in this respect could be the cause of the divergences, between some studies, regarding the antioxidant efficiency order of some compounds.

Table 3. Abundances of some volatile oxidation markers extracted by SPME from the headspace of the mixture of digestive juices and virgin flaxseed oil FDJ, from the digestate of this oil DF, and from the digestates of the samples enriched in dodecyl gallate, hydroxytyrosol acetate and *gamma*-tocopherol (DF, $DFDG_1$, $DFDG_2$, $DFHTA_1$, $DFHTA_2$, $DF\gamma T_1$, $DF\gamma T_2$, $DF\gamma T_3$, and $DF\gamma T_4$), separated, identified and semiquantified by GC/MS, together with the enrichment level of each phenol in each oil sample before digestion given in mmol/mol $(AG+FA)_O$. Data are expressed as area counts of the mass spectra base peak (Bp) of each compound multiplied by 10^{-6}, obtained as average of two determinations together with their standard deviations.

Compound (molecular weight)	**Bp**	**FDJ (0.0)**	**DF (0.0)**	**$DFDG_1$ (0.14)**	**$DFDG_2$ (1.35)**	**$DFHTA_1$ (0.24)**	**$DFHTA_2$ (2.65)**	**$DF\gamma T_1$ (0.13)**	**$DF\gamma T_2$ (1.30)**	**$DF\gamma T_3$ (14.21)**	**$DF\gamma T_4$ (32.79)**
Aldehydes											
Alkanals											
Pentanal (86)*	44	10.60±3.78	65.99±5.57	38.23±4.68	35.09±1.29	54.45±0.84	35.19±0.68	77.53±7.47	55.66±3.44	39.70±4.33	31.03±2.10
Hexanal (100)*	44	7.85±2.16	63.22±9.92	56.89±7.03	52.86±1.25	66.69±6.45	51.03±5.29	63.71±2.84	55.52±3.62	45.84±2.07	39.02±0.55
Heptanal (114)*	70	0.49±0.05	4.10±0.03	3.30±.98	2.16±0.23	3.61±0.35	3.70±0.05	4.67±0.07	4.17±0.18	3.14±0.01	2.80±0.05
Octanal (128)*	41	-	7.71±0.51	-	-	-	-	8.36±0.20	-	-	-
Nonanal (142)*	57	2.97±0.80	12.05±1.17	15.73±4.14	10.02±0.57	11.21±3.46	10.75±0.32	14.98±4.65	11.79±0.55	9.55±0.67	8.70±0.12
Total		**21.91±6.78**	**153.07±17.52**	**114.14±16.83**	**100.14±3.34**	**135.96±11.10**	**100.67±6.34**	**169.25±15.23**	**127.14±7.79**	**98.23±7.09**	**81.55±2.82**
(E)-2-Alkenals											
(*E*)-2-Pentenal (84)	55	2.23±0.29	10.67±0.34	5.24±0.44	5.64±0.13	6.70±1.15	5.12±1.15	8.25±0.82	5.41±0.55	5.96±1.04	5.46±0.29
(*E*)-2-Hexenal (98)*	41	0.39±0.07	1.38±0.03	0.77±0.07	0.43±0.15	0.79±0.03	0.60±0.01	1.03±0.03	1.14±0.16	0.95±0.03	0.53±0.03
(*Z*)-4-Heptenal (112)	41	1.53±0.06	6.09±1.40	4.27±0.49	4.55±0.44	4.78±0.06	3.74±0.42	6.18±1.82	4.50±0.38	3.61±0.44	2.87±0.14
(*E*)-2-Nonenal (140)*	55	-	0.32±0.01	-	-	-	-	2.9±0.1	-	-	-
Total		**4.15±0.41**	**18.46±1.77**	**10.21±1.00**	**10.63±0.73**	**12.26±1.24**	**10.02±1.82**	**15.76±2.69**	**11.05±1.09**	**10.52±1.58**	**8.86±0.46**
2,4-Alkadienals											
(*E,E*)-2,4-Hexadienal (96)*	81	-	2.86±0.21	1.19±0.11	1.14±1.8	1.60±0.16	1.18±0.34	1.64±0.04	1.87±0.04	1.25±0.14	1.06±0.00
(*Z,E*)-2,4-Heptadienal (110)	81	3.31±0.93	19.66±0.62	9.35±0.15	7.97±0.37	14.02±0.50	8.07±1.48	14.05±0.46	12.48±0.08	11.54±1.35	8.35±0.78
(*E,E*)-2,4-Heptadienal (110)*	81	2.24±0.39	21.30±0.66	17.38±0.66	10.49±0.19	19.02±0.08	11.62±0.66	19.79±3.60	13.34±0.29	12.77±1.19	11.21±0.44
Total		**5.55±1.32**	**43.81±1.48**	**27.92±0.92**	**19.60±0.74**	**34.64±0.73**	**20.87±2.49**	**35.48±4.12**	**27.69±0.41**	**25.57±2.68**	**20.63±1.23**
Aromatic aldehydes											
Benzaldehyde (106)*	106	3.85±1.71	8.15±1.06	4.50±0.30	5.11±0.31	6.79±0.59	6.71±0.89	7.08±1.01	6.99±1.03	6.32±0.51	5.25±0.13
Furan derivatives											
Furan, 2-ethyl (96)*	81	1.88±0.03	6.29±2.64	4.51±0.64	3.59±0.22	4.64±0.38	3.12±0.43	6.91±2.0	3.30±0.72	3.34±0.89	2.81±0.77
Furan, 2-butyl (124)	81	0.29±0.04	0.46±0.08	-	-	-	-	0.70±0.28	0.70±0.01	-	-

Compound (molecular weight)	**Bp**	**FDJ (0.0)**	**DF (0.0)**	**DFDG$_1$ (0.14)**	**DFDG$_2$ (1.35)**	**DFHTA$_1$ (0.24)**	**DFHTA$_2$ (2.65)**	**DFγT$_1$ (0.13)**	**DFγT$_2$ (1.30)**	**DFγT$_3$ (14.21)**	**DFγT$_4$ (32.79)**
Furan, 2-pentyl (138)*	81	4.20±1.11	11.69±0.71	9.83±0.43	8.50±0.42	11.20±0.37	10.13±0.13	11.12±0.50	11.53±0.47	11.28±0.09	9.25±0.18
Total		**6.37±1.18**	**18.43±3.42**	**13.94±1.04**	**12.09±0.64**	**15.84±0.75**	**13.25±0.56**	**18.72±0.98**	**15.52±1.20**	**14.62±0.98**	**12.06±0.95**
Ketones											
2,3-Pentanedione (100)*	43	1.64±0.12	26.39±8.58	24.25±2.21	9.78±0.30	19.24±0.77	16.58±0.43	25.22±5.44	14.00±1.73	4.54±0.05	3.32±0.41
2-Hexanone (100)	43	0.58±0.02	2.46±0.10	1.20±0.28	1.55±0.43	1.83±0.04	1.02±0.03	1.94±0.15	1.38±0.06	1.59±0.00	1.18±0.21
2-Heptanone (114)*	43	5.63±0.12	9.27±0.56	9.58±1.01	6.75±0.14	8.68±1.65	7.40±0.12	9.79±1.37	8.24±0.54	7.31±0.83	6.79±0.42
2,3-Octanedione (142)	43	1.49±0.29	6.92±1.25	6.09±0.13	4.38±0.00	6.68±0.49	5.50±0.31	6.50±0.46	6.31±0.34	4.55±0.14	2.21±0.01
2-Octanone (128)*	43	2.14±0.01	3.84±0.57	3.71±0.70	3.26±0.07	3.76±0.08	2.56±0.34	4.24±0.47	3.99±0.24	3.67±0.14	2.47±0.05
3-Octen-2-one (126)	55	1.15±0.12	1.91±0.37	1.52±0.04	1.21±0.21	2.00±0.11	0.96±0.05	1.85±0.34	1.95±0.29	1.05±0.48	0.96±0.32
3*E*,5*Z*-Octadien-2-one (124)*	95	0.78±0.08	9.40±0.18	5.40±0.11	2.17±0.01	7.98±0.05	2.70±0.19	6.13±0.16	3.23±0.11	2.15±0.07	1.97±0.13
Total		**13.41±0.69**	**60.18±11.62**	**49.94±4.49**	**29.09±1.16**	**50.16±3.20**	**36.72±1.41**	**55.67±8.39**	**39.11±3.32**	**24.85±1.72**	**18.90±1.56**
Alcohols											
1-Hexanol (102)*	56	10.53±1.15	12.55±1.51	13.15±0.16	9.02±0.45	9.38±0.52	8.61±0.02	12.09±0.09	9.73±0.85	8.02±0.73	5.26±0.19
1-Octen-3-ol (128)*	57	-	8.62±0.97	11.09±0.19	6.72±0.56	10.74±0.87	6.10±0.53	9.63±0.28	6.24±0.57	5.51±0.35	2.53±0.11
Total		**10.53±1.15**	**21.17±2.48**	**24.24±0.35**	**15.74±1.01**	**20.12±1.38**	**14.72±0.55**	**21.72±0.37**	**15.97±1.43**	**13.53±1.09**	**7.79±0.29**

*Asterisked compounds were acquired commercially and used as standards for identification purposes; -: not detected.

3.4. *In vitro* bioaccessibility of some minor components of virgin flaxseed oil and specifically of *gamma*-tocopherol in the different digestates

This virgin flaxseed oil contains, as is common in vegetable oils, tocopherols and sterols. Furthermore, this oil also contains terpenes and sesquiterpenes as has been shown in previous studies [24,26]. All these compounds has been attributed important biological activities, and in some cases antioxidant ability. Some of these minor oil components give signals in the ^{1}H NMR spectra, which do not overlap with any other, for which reason their concentration in the sample before and after *in vitro* digestion can be estimated. These are, (i) of the tocopherols, *gamma*-tocopherol singlet signal at 6.36 ppm due to proton aromatic proton C-5 as is indicated in Table S5 and shown in Figure 2); and (ii) of the sterols, cycloartenol and 24-methylenecycloartenol free or esterified (two doublet signals overlapping due to the methylene protons on carbon atom C-19 yielding a triplet joint signal centred at 0.33 ppm as is indicated in Table S5, which permit their joint quantification) [36]. They are respectively the main components of the tocopherols and of the sterols in this oil [26]. Other minor virgin flaxseed oil components such as terpenes and sesquiterpenes, can, due to their volatility, be extracted from the headspace of the samples by means of solid phase microextraction (SPME) and can be separated, identified and semiquantified by means of gas chromatography/mass spectrometry (GC/MS). Due to this, by using these techniques the *in vitro* bioaccessibility of these compounds can be estimated.

As mentioned, the *gamma*-tocopherol concentration can be estimated in the different samples before and after *in vitro* digestion. The *in vitro* bioaccessibility of this compound can be expressed by the concentration of this compound in the digestate $[\gamma T]_D$, given in mmoles, in relation to the concentration of the main components also in the digestate $[FA+AG]_D$, given in moles, by the equation $B_{\gamma T}=[\gamma T]_D/[FA+AG]_D$. This *in vitro* bioaccessibility definition gives information from a global quantitative point of view. The

values of the thus defined *in vitro* bioaccessibility of *gamma*-tocopherol are given in Table 4 for the different samples. It can be observed that during the *in vitro* digestion of the sample not enriched in phenols almost the totality of the *gamma*-tocopherol present in the oil (0.33 mmol/mol(FA+AG)) is degraded, only a small amount remaining undegraded. In spite of this, oxidation of the sample took place. In the samples enriched in dodecyl gallate and hydroxytyrosol acetate a larger amount of *gamma*-tocopherol remained undegraded than it did in the unenriched sample. It could be interpreted that these added di- or tri-phenolic compounds have some protective effect on *gamma*-tocopherol. It is noteworthy that these samples, in spite of having *gamma*-tocopherol present, were also oxidized though to a lesser degree than the not enriched sample. Finally, the amount of this compound that remains undegraded in the samples enriched in *gamma*-tocopherol after digestion increases in line with the enrichment level, as could be expected. However, it is noticeable that even in the more *gamma*-tocopherol enriched samples, having important concentrations of this compound that did not degrade, a small oxidation degree took place, as has been shown before.

The *in vitro* bioaccessibility of *gamma*-tocopherol can also be expressed as the ratio between the concentration of the compound in the digestate $[\gamma T]_D$ and the concentration in the sample before digestion, $[\gamma T]_O$, as indicated by the equation $B'_{\gamma T}=[\gamma T]_D/[\gamma T]_O$. This parameter informs one directly about the degradation level undergone by the *gamma*-tocopherol during the *in vitro* digestion. The values of the *in vitro* bioaccessibility thus defined are also given in Table 4. It can be observed in this table that in the samples enriched in dodecyl gallate and in hydroxytyrosol acetate this parameter reaches values around four times higher than in the not enriched sample, suggesting again a potential protective effect of these phenolic compounds on *gamma*-tocopherol. Furthermore, in the samples enriched with the latter compound, its bioaccessibility increases as does the enrichment level, and an

important amount of the *gamma*-tocopherol remains after digestion which can be absorbed, in agreement with BγT. This fact is important due to the biological activities attributed to this compound [27-29].

Table 4. *In vitro* bioaccessibility of *gamma*-tocopherol in the different samples, defined by $B_{\gamma T}$ = mmol $(\gamma T)_D$/mol $(AF+GA)_D$ and by $B'_{\gamma T}$ = mmol$(\gamma T)_D$/mmol $(\gamma T)_O$ Values are the average of two determinations together with their standard deviations.

Samples	$B_{\gamma T}$	$B'_{\gamma T}$
Oil		
DF	0.04 ± 0.01	0.12 ± 0.00
Oil-dodecyl gallate		
$DFDG_1$	0.14 ± 0.00	0.42± 0.01
$DFDG_2$	0.16 ± 0.01	0.48 ± 0.03
Oil-hydroxytyrosol acetate		
$DFHTA_1$	0.13 ± 0.00	0.39 ± 0.02
$DFHTA_2$	0.16 ± 0.01	0.48 ± 0.01
Oil-gamma-tocopherol		
$DF\gamma T_1$	0.09 ± 0.01	0.19 ± 0.05
$DF\gamma T_2$	0.95 ± 0.03	0.58 ± 0.02
$DF\gamma T_3$	11.41 ± 0.29	0.78 ± 0.02
$DF\gamma T_4$	28.73 ± 0.27	0.86 ± 0.01

The concentration of cycloartenol and 24-methylenecycloartenol free or esterified, as above mentioned, can be estimated jointly in samples before and after digestion. It is noteworthy that its concentration remains unchanged during *in vitro* digestion, being of 0.6 mmol/mol(FA+AG) in both undigested and digested samples. Their *in vitro* bioaccessibility defined as the ratio between its concentrations in the undigested and digested samples is the unity. This is an important fact because to these compounds have been attributed beneficial biological activities [30, 37].

Likewise, an important group of terpenes and sesquiterpenes are present in the virgin flaxseed oil and their abundances before and after digestion, which reflect their

concentrations in these samples, can be estimated by using SPME-GC/MS as mentioned before. Analysis of the results obtained indicates that the abundances of these compounds in the headspaces of the digestates of the samples enriched with different concentrations of each phenolic compound have very similar values, for which reason they are given in Table 5 as an average value. Furthermore, it can also be observed in Table 5 that abundance values of terpenes and sesquiterpenes in all digestates are very similar in both the unenriched sample and in the samples enriched in phenolic compounds; in fact these abundances are even higher in the headspace of the digestates than in that of FDJ mixture, probably due to the matrix effect. These results indicate that the concentration of these compounds is not affected by either the digestion process or by the oxidation reactions that also take place during this process. The preserving of these compounds during digestion, and for this reason, their great *in vitro* bioaccessibility to be absorbed is of great interest, due to the biological activities attributed to them [38].

Finally, dodecyl gallate and hydroxytyrosol acetate have unoverlapped signals in the ^{1}H NMR spectrum (see Table S5 and Figure 2) for which reason if they are present in the lipid extract of the digestates their spectra will show these signals. However, only very small signals of both compounds are visible in the spectra of the samples most enriched with these phenols, but they are not enough to be quantified, due to their low intensity. This low concentration of these phenols in the digestates could be due to either their being degraded almost totally during *in vitro* digestion, transforming into other compounds by their action as antioxidant, or by other reactions among which their hydrolysis could be cited. In fact there are some previous studies concerning hydroxytyrosol alkyl esters that describe their partial hydrolysis under digestion conditions [39,40]. The hydrolysis of these compounds yield very polar compounds that will remain in the aqueous phase, so they are not detected in the lipid extract of the digestates. Although hydrolysis of alkyl gallates in digestion has not been

reported, to the best of our knowledge, it could not be discarded. Furthermore, reactions between phenolic compounds and reactive aldehydes formed in the lipid oxidation during digestion could also take place [41] contributing to the disappearance of the phenolic compounds in the digestates.

Table 5. Terpenes and sesquiterpenes of virgin flaxseed oil, detected by SPME-GC/MS in the headspaces of the mixture of digestive juices submitted to digestive conditions and virgin flaxseed oil FDJ, of the digestate of this oil DF and of the digestates of the samples enriched with different levels of dodecyl gallate, hydroxytyrosol acetate, and *gamma*-tocopherol (DFDG, DFHTA, DFγT). Data are average abundances expressed as area counts of the mass spectra base peak Bp of each compound multiplied by 10^{-6}, together with their standard deviations. For samples enriched with phenolic compounds data given are average values of the abundances of the headspace of digestates coming from samples having different enrichment levels of phenolic compounds. Different letters within each row indicate a statistically significant difference among the samples ($p < 0.05$).

Terpenes and sesquiterpenes	**Bp**	**FDJ**	**DF**	**DFDG$_{average}$**	**DFHTA$_{average}$**	**DFγT$_{average}$**
alpha-thujene	93	24.33±2.42a	41.87±5.39b	38.49±6.62b	38.30±1.85b	44.45±3.34b
alpha-pinene*	93	18.81±2.07a	35.15±8.52b	31.32±4.24b	30.79±1.48b	33.11±4.63b
Sabinene	93	2.10±0.13a	3.27±0.01b	2.44±0.42a	2.49±0.08a	2.80±0.41ab
beta-pinene*	93	4.70±0.25a	10.90±0.74b	8.88±1.34bc	8.15±0.31c	8.86±1.09bc
l-phellandrene*	93	0.28±0.07a	0.45±0.08b	0.50±0.07b	0.39±0.06ab	0.49±0.06b
delta-3-carene	93	1.04±0.15a	2.79±0.07b	2.40±0.11bc	1.92±0.09c	2.30±0.28c
alpha-terpinene*	93	0.56±0.28a	1.70±0.02b	1.22±0.18cd	1.14±0.15d	1.56±0.15bc
Cymene*	119	189.10±0.54a	240.24±24.37b	243.91±15.58b	268.78±12.34b	248.03±18.38b
Limonene*	68	18.32±1.29a	35.81±0.95b	37.24±1.54b	32.21±1.88b	33.96±3.74b
gamma-terpinene*	93	2.45±0.31a	4.95±0.58b	4.10±0.78b	4.27±0.12b	4.65±0.49b
alpha-terpinolene*	93	0.25±0.01a	0.35±0.07ab	0.45±0.06b	0.40±0.06ab	0.43±0.08b
4-Terpineol*	71	0.22±0.03a	2.49±0.04b	2.15±0.52bc	1.77±0.05c	2.55±0.19b
alpha-terpineol*	59	0.28±0.03a	1.61±0.13b	1.27±0.22b	1.21±0.08b	1.59±0.18b
Carvone*	82	1.63±0.30a	4.36±0.46bc	4.54±0.40c	3.38±0.25b	4.75±0.62c
alpha-copaene	119	0.21±0.03a	0.54±0.02bc	0.46±0.15bc	0.40±0.02b	0.63±0.04c
beta-elemene	93	0.10±0.02a	0.26±0.0b	0.25±0.04b	0.20±0.01b	0.26±0.02b
Calarene	161	0.30±0.01a	0.80±0.04bc	0.74±0.23bc	0.65±0.04b	0.97±0.11c
Cadinene	161	0.10±0.03a	0.32±0.04bc	0.27±0.07bc	0.24±0.02b	0.35±0.04c

*Asterisked compounds were acquired commercially and used as standards for identification purposes.

4. CONCLUSIONS

Enrichment of virgin flaxseed oil with different concentrations of dodecyl gallate, hydroxytyrosol acetate and *gamma*-tocopherol, does not appreciably modify either the lipolysis degree reached during *in vitro* digestion or its lipolysis pattern in comparison with the not enriched virgin flaxseed oil. These results show that the phenolic compounds involved in this study, under the *in vitro* digestion conditions essayed, do not inhibit the activity of lipases, or in other words do not react with them affecting the hydrolytic reactions. By contrast, and although no significant differences were observed, the digestates of the virgin flaxseed oil enriched in the above mentioned phenols, shows in general a slightly smaller percentage of triglycerides, and a slightly higher percentage of glycerol than the digestate of the not enriched sample. These results are also reflected in the bioaccessibility of the oil main components. *In vitro* digestion provokes a small oxidation degree clearly affecting to the linolenic structures whose concentration diminish, forming hydroperoxydes supporting conjugated *Z,E* dienic systems, which are primary oxidation compounds, and also volatile secondary oxidation compounds, well known oxidation markers. Enrichment with the different phenols, in the levels essayed, reduces the oxidation degree reached during the virgin flaxseed oil digestion and, for this reason, the bioaccessibility of oxidation compounds, but not totally. Dodecyl gallate shows the higher antioxidant efficiency followed by hydroxytyrosol acetate and *gamma*-tocopherol successively. In the case of enrichment with *gamma*-tocopherol it has been observed that its antioxidant efficiency is related with the *gamma*-tocopherol concentration through a logarithmic relation. The concentrations of some minor components of the virgin flaxseed oil involved in this study, such as cycloartenol, 24-methylenecycloartenol, terpenes and sesquiterpenes are not modified by *in vitro* digestion showing an *in vitro* bioaccessibility near the unity. However, the *in vitro* bioaccessibility of the *gamma*-tocopherol contained in virgin flaxseed oil is very small, but increases in line

with the enrichment in phenolic compounds. It has been shown that oxidation is produced during *in vitro* digestion even in the presence of important concentrations of *gamma*-tocopherol, which remain bioaccessible after digestion in the enriched samples in this compound. Finally, it should be remarking the importance of indicating the accurate units to which the antioxidant efficiency is referred, since the lack of clarity in this respect could be the cause of the divergences between studies, regarding the antioxidant efficiency order of some compounds.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3921/9/4/312/s1, Table S1: Composition and pH values of the juices employed in the *in vitro* digestion model employed in this study; Table S2: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of glycerides; Table S3: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of acyl groups and fatty acids; Table S4: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some oxidation compounds detected in the digestates and formed during the *in vitro* digestion; Table S5: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of cycloartenol and methylencycloartenol, esters of cycloartenol and methylencycloartenol, *gamma*-tocopherols, hydroxytyrosol acetate and dodecyl gallate detected in the samples before and after *in vitro* digestion; Equations used for the quantification from ^{1}H NMR spectral data of several compounds present in the starting samples and / or in the lipid extract of the digestates. Figure S1. Graphical representation of linolenic structures concentration in the digestates of the different virgin flaxseed oil samples enriched in *gamma*-tocopherol given in molar percentage of [Ln] referred to the total moles of $[AG+FA]_D$ *versus* enrichment level of *gamma*-tocopherol in the corresponding oil samples, given in mmol γT/mol$[AG+FA]_O$. Figure S2. Region between 4-30 min of the total ion chromatogram obtained by SPME-GC/MS of the FDJ sample and of the digestate of the virgin flaxseed oil DF.

AUTHOR CONTRIBUTIONS

J.A-C.; performed the experimental work, contributed to data interpretation and to manuscript preparation. M.L.I.; supervised the analyses performed and contributed to data

interpretation and to manuscript preparation. M.D.G.; conceived the work, supervised the whole work and the results obtained, and contributed to data interpretation and to the manuscript preparation.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Supplementary Material of

Article 7

A GLOBAL STUDY BY ^{1}H NMR SPECTROSCOPY AND SPME-GC/MS OF THE *IN VITRO* DIGESTION OF VIRGIN FLAXSEED OIL ENRICHED OR NOT WITH MONO-, DI- OR TRI-PHENOLIC DERIVATIVES. ANTIOXIDANT EFFICIENCY OF THESE COMPOUNDS

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Table S1. Composition and pH values of the juices employed in the *in vitro* digestion model employed in this study.

Components	**Saliva**	**Gastric juice**	**Duodenal juice**	**Bile juice**
KCl (mmol/L)	12.02	11.06	7.57	5.05
NaCl (mmol/L)	5.10	47.09	119.98	89.99
$NaHCO_3$ (mmol/L)	20.17	-	40.33	68.86
NaH_2PO_4 (mmol/L)	7.40	0.22	-	-
NH_4Cl (mmol/L)	-	5.72	-	-
KH_2PO_4 (mmol/L)	-	-	0.59	-
Na_2SO_4 (mmol/L)	4.79	-	-	-
KSCN (mmol/L)	2.06	-	-	-
$MgCl_2$ (mmol/L)	-	-	0.53	-
$CaCl_2*2H_2O$ (mmol/L)	-	2.72	1.36	1.51
HCl (37%) (mL/L)	-	6.50	0.18	0.15
Urea (mmol/L)	3.33	1.42	1.67	4.16
Glucose (mmol/L)	-	3.61	-	-
Glucuronic acid (mmol/L)	-	0.10	-	-
Uric acid (mmol/L)	0.09	-	-	-
Glucoseamine hydrochloride (mmol/L)	-	1.53	-	-
Bovine serum albumin (g/L)	-	1.00	1.00	1.80
Mucin (g/L)	0.025	3.00	-	-
A. oryzae α-amylase (g/L)	0.29	-	-	-
A. niger lipase (U/mL)	-	100	-	-
Pepsin (g/L)	-	2.50	-	-
Pancreatin (g/L)	-	-	9.00	-
Lipase type II from porcine pancreas (g/L)	-	-	1.50	-
Bovine bile extract (g/L)	-	-	-	18.75
pH	6.8±0.0	1.4±0.1	8.06±0.0	8.1±0.1

Table S2. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of glycerides. TG: triglycerides; DG: diglycerides; MG: monoglycerides. The signal letters agree with those given in Figure 1.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Structures
			Glycerides structure protons	
I	3.65	ddd	ROCH$_2$–CHOH–C**H$_2$**OH	glyceryl group in **1-MG**
J	3.73	m*	ROCH$_2$–CH(O*R'*)–C**H$_2$**OH	glyceryl group in **1,2-DG**
K	3.84	m*	HOC**H$_2$**–CH(O*R*)–C**H$_2$**OH	glyceryl group in **2-MG**
L	3.94	m	ROCH$_2$–C**H**OH–CH$_2$OH	glyceryl group in **1-MG**
M	4.05-4.21	m	ROC**H$_2$**–C**H**OH–C**H$_2$**OR'	glyceryl group in **1,3-DG**
N	4.18	ddd	ROC**H$_2$**–CHOH–CH$_2$OH	glyceryl group in **1-MG**
O	4.22	dd,dd	ROC**H$_2$**–CH(O*R'*)–C**H$_2$**OR''	glyceryl group in **TG**
P	4.28	ddd	ROC**H$_2$**–CH(O*R'*)–CH$_2$OH	glyceryl group in **1,2-DG**
Q	4.93	m	HOCH$_2$–C**H**(O*R*)–CH$_2$OH	glyceryl group in **2-MG**
R	5.08	m	ROCH$_2$–C**H**(O*R'*)–CH$_2$OH	glyceryl group in **1,2-DG**

Abbreviations: d: doublet; m: multiplet.

*This signal shows different multiplicity if the spectrum, is acquired from the pure compound or taking part in the mixture.

**The intensity of some of these signals, also shown in Figure 1, together with signal F of Table S3, were used to estimate the molar percentages of different kinds of glyceryl structures using the equations [eq. S1-eq. S10].

***The assignment of the ^{1}H NMR signals of the protons was made as in previous studies (Guillén & Uriarte, 2012; Nieva-Echevarría et al., 2014).

Table S3. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of acyl groups and fatty acids. AG: acyl groups; FA: fatty acids. The signal letters agree with those given in Figure 1.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Structures
			Main acyl groups (AG) and fatty acids (FA)	
A	0.88	t	–C**H**$_3$	saturated and monounsaturated **ω-9** in **AG** and **FA**
	0.89	t	–C**H**$_3$	linoleic in **AG** and **FA**
B	0.97	t	–C**H**$_3$	linolenic in **AG** and **FA**
C	1.19–1.42	m**	–(C**H**$_2$)$_n$–	**AG** and **FA**
D	1.61	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **TG**
	1.62	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **1,2-DG**
	1.63	m	–OCO–CH$_2$–C**H**$_2$–, COOH–CH$_2$–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	1.64	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **2-MG**
E	1.92–2.15	m***	–C**H**$_2$–CH=CH–	**AG** and **FA**
F*	2.26–2.36	dt	–OCO–C**H**$_2$–	**AG** in **TG**
	2.33	m	–OCO–C**H**$_2$–	**AG** in **1,2-DG**
	2.35	t	–OCO–C**H**$_2$–, COOH–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	2.38	t	–OCO–C**H**$_2$–	**AG** in **2-MG**
G	2.77	t	=HC–C**H**$_2$–CH=	linoleic in **AG** and **FA**
H*	2.80	t	=HC–C**H**$_2$–CH=	linolenic in **AG** and **FA**

Abbreviations: d: doublet; t: triplet; m: multiplet.

*The intensity of these signals, also shown in Figure 1, was used to estimate the molar percentage of linolenic acyl groups plus fatty acids by using equations [eq. S11].

**Overlapping of multiplets of methylenic protons in the different acyl groups either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group.

***Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups.

****The assignment of the ^{1}H NMR signals of the protons was made as in previous studies (Guillén & Ruiz, 2003; Nieva-Echevarría et al., 2014).

Table S4. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some oxidation compounds detected in the digestates and formed during the *in vitro* digestion.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Structures
Oxidation Compounds (OC)				
Conjugated dienic systems associated with hydroperoxy groups				
-	5.51	dtm	–C**H**=CH–CH=CH–	(*Z*,*E*)-conjugated double bonds
-	5.56	ddm		associated with hydroperoxy group
-	6.00	ddtd		(OOH) in octadecatrienoic
b	***6.58***	dddd		**AG** and **FA**
				HPO-c(*Z*,*E*)-dEs
Aldehydes				
f	***9.75***	t	-C**H**O	**n-alkanals**
	2.40	dt	-CH_2-	

Abbreviations: d: doublet; t: triplet; m: multiplet.

*The intensities of the signals indicated in bold, together with signal D of Table S3, were used to estimate the concentration (mmol/molAG+FA) using the equation [eq. S12].

**The assignment of the ^{1}H NMR signals of the protons was made with the aid of standard compounds and with the data taken from literature (Guillén & Ruiz, 2005).

Table S5. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of cycloartenol and methylencycloartenol, esters of cycloartenol and methylencycloartenol, *gamma*-tocopherols, hydroxytyrosol acetate and dodecyl gallate detected in the samples before and after *in vitro* digestion. Some of the signal letters agree with those given in Figure 2.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Compounds
		Sterols		
4,4´-DiMe-St.	***0.33*****	d	-C**H**$_2$- (exo, C-19)	Cycloartenol/ 24-Methylenecycloartenol Esters of Cycloartenol/ 24-Methylenecycloartenol
4,4´-DiMe-St´.	***0.34*****	d	-C**H**$_2$- (exo, C-19)	
		***gamma*-tocopherol**		
γT	***6.36*****	s	-C**H** (C-5)	*gamma*-tocopherol
		Hydroxytyrosol acetate		
HTA	6.60	dd	Ar**H** (C-8)	Hydroxytyrosol acetate
	6.75**	d	Ar**H** (C-4)	
	6.78	d	Ar**H** (C-7)	
		Dodecyl gallate		
DG	***7.20***	s	Ar**H**(C-3; C-7)	Dodecyl gallate

Abbreviation: s: singlet; d: doublet.

*The intensity of these signals, together with signal D of Table S3, were used to estimate the concentration (mmol/molAG+FA) using the equation [eq. S12].

**Assignment was made with the aid of standard compounds and with the data taken from the literature (Baker & Mayers, 1991; Kubo et al., 2002; Kawai et al., 2007).

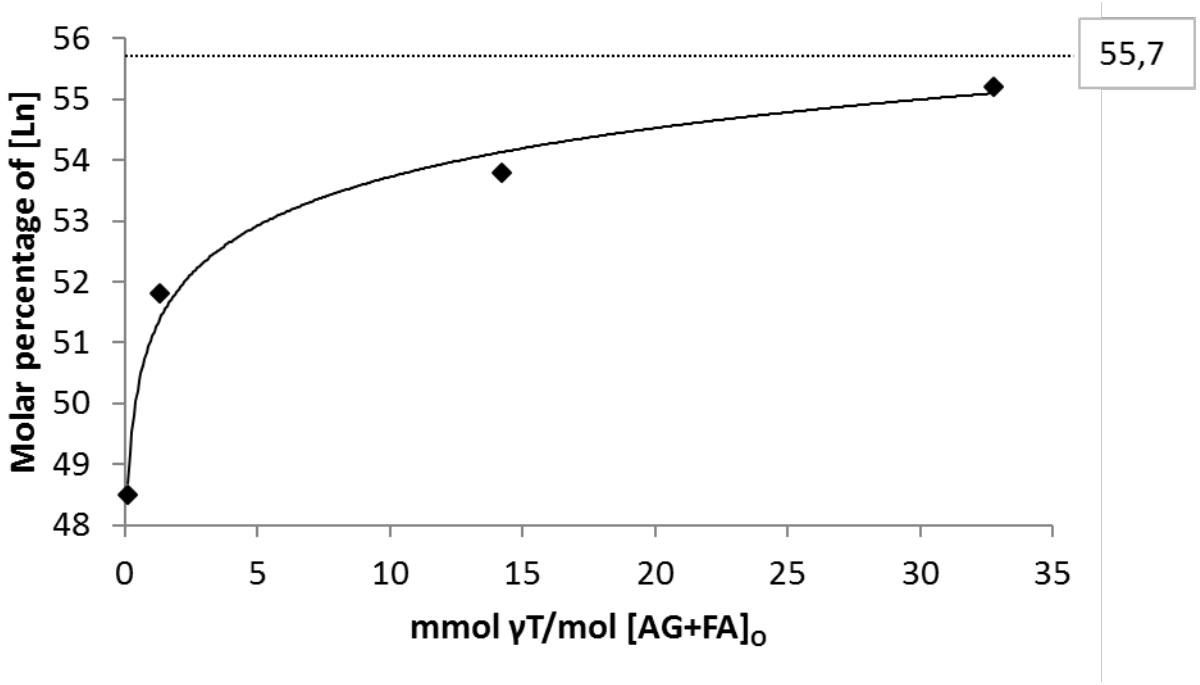

Figure S1. Graphical representation of linolenic structures concentration in the digestates of the different virgin flaxseed oil samples enriched in *gamma*-tocopherol given in molar percentage of [Ln] referred to the total moles of $[AG+FA]_D$ *versus* enrichment level of *gamma*-tocopherol in the corresponding oil samples, given in mmol γT/mol$[AG+FA]_O$.

Quantification from ^{1}H NMR spectral data of several compounds present in the starting samples and/or in the lipid extracts of the digestates.

Bearing in mind that the area of each ^{1}H NMR spectral signal is proportional to the number of protons that generate it, and that the proportionality constant is the same for all kinds of protons, the area of some spectral signals can be employed to quantify a wide variety of compounds, as detailed below.

A. Equations used to estimate the molar percentage (%) of the several glyceride structures present in the lipid extract of digestates and the glycerol.

In these equations, the number of moles (N) of fatty acids and all the glycerides were expressed as follows:

$$N_{2\text{-}MG}=Pc*A_K/4 \quad \text{[eq. S1]}$$

$$N_{1\text{-}MG}=Pc*A_L \quad \text{[eq. S2]}$$

$$N_{1,2\text{-}DG}=Pc*(A_{I+J}-2A_L)/2 \quad \text{[eq. S3]}$$

$$N_{TG}=Pc*(2A_{4.26\text{-}4.38}-A_{I+J}+2A_L)/4 \quad \text{[eq. S4]}$$

$$N_{1,3\text{-}DG}=Pc*(A_{4.04\text{-}4.38}-2A_{4.26\text{-}4.38}-2A_L)/5 \quad \text{[eq. S5]}$$

$$N_{FA}=(Pc*A_F-6N_{TG}-4N_{1,2\text{-}DG}-4N_{1,3\text{-}DG}-2N_{1\text{-}MG}-2N_{2\text{-}MG})/2 \quad \text{[eq. S6]}$$

$$N_{Gol}=(N_{FA}-N_{1,2\text{-}DG}-N_{1,3\text{-}DG}-2N_{2\text{-}MG}-2N_{1\text{-}MG})/3 \quad \text{[eq. S7]}$$

where Pc is the proportionality constant existing between the area of the ^{1}H NMR signals and the number of protons that generate them, A_K, A_L, A_{I+J} and A_F are the areas of the corresponding signals indicated in Table S2, and $A_{4.26\text{-}4.38}$ and $A_{4.04\text{-}4.38}$ represent the areas of the signals between 4.26 and 4.38 ppm, and between 4.04 and 4.38 ppm, respectively.

Using these equations, the molar percentages of the different kinds of glycerides in relation to the total number of moles of glyceryl structures present (NT_{GS}) were determined as follows:

$$NT_{GS}=N_{TG}+N_{1,2\text{-}DG}+N_{1,3\text{-}DG}+N_{2\text{-}MG}+N_{1\text{-}MG}+N_{Gol} \quad \text{[eq. S8]}$$

$$G\%=100N_G/NT_{GS} \quad \text{[eq. S9]}$$

where G is each kind of glyceride (TG, 1,2-DG, 1,3-DG, 2-MG and 1-MG) and N_G the respective number of moles.

$$Gol\%=100N_{Gol}/NT_{GS} \quad \text{[eq. S10]}$$

B. Estimation of the molar percentages of linolenic fatty acids (FA) plus acyl groups (AG) (FA+AG). The molar percentages of linolenic (Ln%) FA plus AG, in relation to the total number of moles of AG plus FA (NT_{AG+FA}) present in the starting oils and in the lipid extracts of the corresponding digestates were estimated as follows:

$$Ln\%=100*(A_H/2*A_F) \quad \text{[eq. S11]}$$

where A_H and A_F are the areas of signals H and F indicated in Table S3.

C. Estimation of the concentration of specific compounds in oil samples and in the lipids extract from digestates.

The concentration of the several kinds of specific compounds (SC), expressed as micromoles per mole of the sum of AG+FA present, was estimated by using the following equations:

$$[SC] = [(A_{SC}/n)/(A_D/2)]*1000 \quad \text{[eq. S12]}$$

where A_{SC}, is the areas of the signals selected for the quantification of each specific compound (SC), present in the oil samples and in the lipid extract from digestates and n the number of protons that generate each signal given in Tables S4 and S5 and Figure 1 and A_D is the area of the signal D in Table S3.

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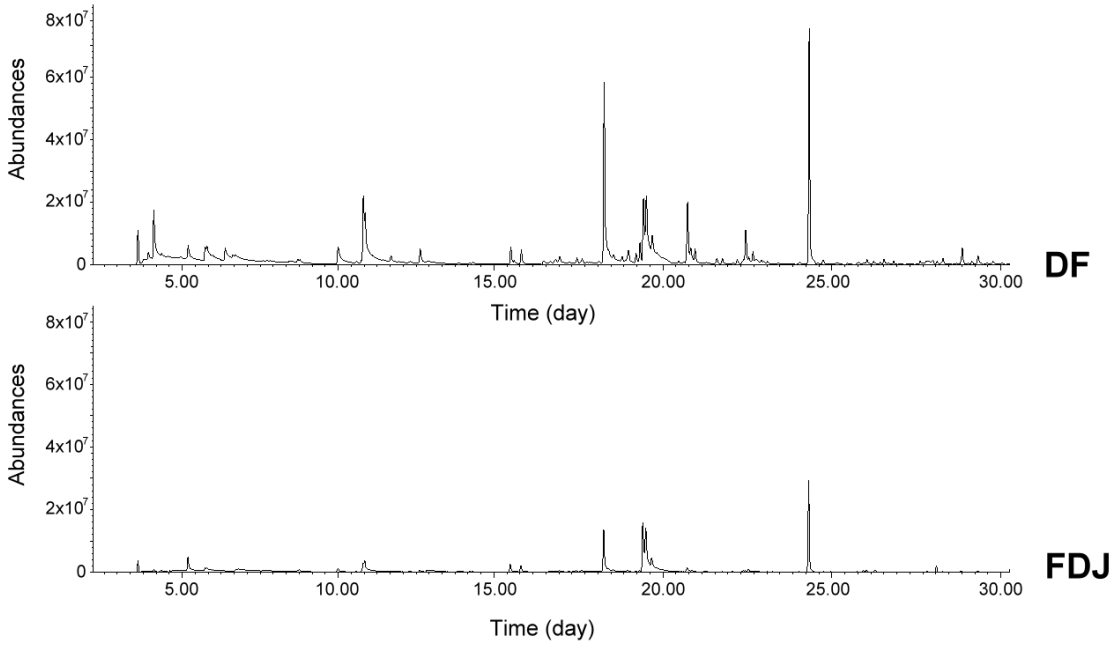

Figure S2. Region between 4-30 min of the total ion chromatogram obtained by SPME-GC/MS of the FDJ sample and of the digestate of the virgin flaxseed oil DF.

Manuscript 8

STUDY OF THE *IN VITRO* DIGESTION OF OLIVE OIL ENRICHED OR NOT WITH ANTIOXIDANT PHENOLIC COMPOUNDS. RELATIONSHIPS BETWEEN BIOACCESSIBILITY OF MAIN COMPONENTS OF DIFFERENT OILS AND THEIR COMPOSITION

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ABSTRACT

The changes provoked by the *in vitro* digestion in the lipids of olive oil enriched or not with different phenolic compounds, were studied by proton nuclear magnetic resonance (^{1}H NMR) and solid phase microextraction followed by gas chromatography/mass spectrometry (SPME-GC/MS). These were compared with those provoked in the lipids of corn oil and of virgin flaxseed oil submitted to the same digestive conditions. Lipolysis and oxidation were the two reactions under consideration. The bioaccessibility of main and minor components of olive oil, of phenolic compounds added and of compounds formed as consequence of the oxidation, if any, were matters of attention. Enrichment of olive oil with antioxidant phenolic compounds does not affect the extent of lipolysis, but reduces the oxidation degree to minimum values or avoids it almost entirely. The *in vitro* bioaccessibility of nutritional and bioactive compounds was greater in the olive oil digestate than in those of other oils, whereas that of compounds formed in oxidation was minimal, if any. Very close quantitative relationships were found between the composition of the oils in main components and their *in vitro* bioaccessibility. These relationships, some of which have predictive value, can help to design lipid diets for different nutritional purposes.

KEYWORDS: lipolysis; oxidation phenolic compounds; antioxidant efficiency; *gamma*-tocopherol; hydroxytyrosol acetate; dodecyl gallate; olive oil minor components; corn oil; virgin flaxseed oil.

1. INTRODUCTION

Digestion is a very complex process in which the main reactions provoke hydrolysis of proteins, carbohydrates and lipids to yield smaller building blocks, which may be absorbed through the intestinal wall. Furthermore, other secondary reactions, such as oxidation, Maillard reaction and even esterification, among others, can also be produced during digestion [1-3]. All of them make up this process, which is essential to cover human nutritional needs.

Lipids are an important group of macronutrients which include many different compounds. Triglycerides are their main components, and edible oils are the principal food lipid. During oil digestion triglycerides are hydrolyzed to give smaller molecules, of which only fatty acids and monoglycerides can be absorbed. Lipolysis extent determines the yield of molecules derived from oil main components that are able to be absorbed. Knowledge of the factors that influence the lipolytic process is a subject of great importance in monitoring the digestive process and designing lipids and mixtures of lipids with other components to cover different nutritional needs [4]. In this context, it has been proved that tea polyphenols are able to inhibit pancreatic lipase activity, reducing gastrointestinal lipolysis [5] and the absorption of lipids, thus diminishing the nutritional value of the lipids ingested. Likewise, it has been reported that the lipolysis degree reached during *in vitro* digestion of some oils is related to the oil composition [6-8]. This means that both main and minor oil components influence lipolysis yield, as could be expected. If the above mentioned relations between oil composition and lipolysis degree were known in depth they could be used to design lipids which are able to bring about specific degrees of lipolysis and suitable bioaccessibility of oil main components for different nutritional needs.

In addition to lipolysis, lipids oxidation can also take place during digestion [9-14] leading to the formation of toxic compounds with detrimental effects on health. It may be

expected that the extent of this reaction will not be the same for all kinds of oils and that it will be determined by the oxidative stability of the lipids involved as well as by the presence or absence of minor compounds capable of acting as antioxidants or of prooxidants during digestion. This subject is also of great importance and should be taken into account in digestion studies because, in addition to generating undesirable toxic bioaccessible compounds, the most reactive oxidation compounds could also influence or interfere with the digestion process in turn.

When edible oils are submitted to digestion, the above mentioned reactions can also affect minor oil components, and the nature and properties of these can also, in turn, influence the digestive process [11]. As is known, edible oils are vehicles for vitamins and bioactive compounds, and it is desirable that the bioaccessibility of these minor components will be as high as possible, for their potential health effects.

In summary, in order to advance in the understanding of the *in vitro* digestion of edible oils and to achieve a broad view, both of the evolution of the process and of the bioaccessibility of the compounds involved, as many influential factors as possible should be taken into account.

In this context, the *in vitro* digestion of olive oil is tackled. This oil is made up of a mixture of olive refined oil and of olive extra virgin oil, and as consequence is much poorer in antioxidant components than the latter. To the best of our knowledge, the behaviour of this oil under *in vitro* digestion conditions has not been previously studied. The study will pay attention firstly to lipolysis extent and to the pattern produced as well as to the bioaccessibility of the oil main components estimated using ^{1}H NMR spectroscopic data of the lipid extracts of the digestates. In order to have a complete view of this lipolytic process the results will be analyzed jointly with those of other oils such as corn oil and virgin flaxseed oil, these latter from previous studies [13,14]. Relationships between the composition of these

oils in their main components and *in vitro* bioaccessibility will be studied in order to find quantitative models to explain these relationships if any. The interest of these potential quantitative relationships is considerable because if sound they could be used as tools to design mixtures of oils for specific nutritional needs. Likewise, there will be an analysis of the effect of the enrichment of olive oil with various concentrations of dodecyl gallate, hydroxytyrosol acetate and *gamma*-tocopherol on the bioaccessibility of the oil main components in order to evaluate if these phenolic compounds are able to inhibit lipase activity. Furthermore, oxidation extent, if any, during *in vitro* digestion of olive oil enriched, or not, with phenolic compounds will be evaluated and compared with that undergone by corn oil and virgin flaxseed oil. Monitoring of oxidation extent, if any, will be tackled by using two different techniques. First of all, ^{1}H NMR will be used to evaluate differences in the concentration of unsaturated fatty acids and acyl groups, in oil and in the lipid extract of the digestate, due to oxidation reactions, and secondly to quantify potential oxidation compounds in the lipid extract of the digestates. In addition, the abundance of volatile oxidation markers will also be estimated by means of SPME-GC/MS to clarify and/or reinforce the oxidation extent results obtained by the first technique. Finally, the bioaccessibility of all minor compounds involved in the *in vitro* digestion of olive oil enriched or not with phenolic compounds will also be determined. These compounds include natural olive oil minor components, phenolic added compounds and potentially compounds formed by oxidation if any.

2. MATERIALS AND METHODS

2.1. Samples subject of study

The study was carried out with two different olive oils O_1 and O_2, of the same brand, acquired in a local supermarket. As already mentioned, olive oil is made up of a mixture of

extra virgin olive oil and of refined olive oil. The composition of both oils in molar percentages of linolenic (Ln), linoleic (L), oleic (O) and saturated (S) acyl groups, is very similar (O_1: Ln%= 0.6 ± 0.1, L%= 8.0 ± 0.4, O%= 75.5 ± 0.6, and S%= 15.8 ± 0.2; O_2: Ln%= 0.7 ± 0.1, L%= 8.0 ± 0.1, O%= 75.1 ± 0.6, and S%= 16.2 ± 0.5). This was determined from ^{1}H NMR spectral data as in previous studies [15,16]. Both olive oils also contain a small concentration of alkanals. These are the aldehydes with the lowest reactivity of all, which could have been produced by a lipoxygenase mediated oxidation of unsaturated acyl groups during the crushing and malaxation steps of olive oil production, contributing, in low concentrations, to the green odour of olive oils [17]. Nevertheless, as is well known, these compounds may be formed, in the absence of these enzymes, under very varied oxidative conditions. These olive oils also contain squalene and sterols, detectable by ^{1}H NMR spectroscopy, and a certain number of terpenes and sesquiterpenes, detectable by SPME-GS/MS. Nevertheless, both abundance and number of these compounds are much smaller in olive oil than in extra virgin olive oils [18,19]. Likewise, the content of polyphenols is very small in these olive oils and they are not detectable by ^{1}H NMR spectroscopy either in the standard proton spectrum or in the spectrum acquired by using the NOESYGPPS experiment, which will be explained later [18].

Aliquots of olive oil O_1 were enriched with two different concentrations either of dodecyl gallate DG (purity 98%, from Alfa Aesar., GmbH & Co KG, Germany) or of hydroxytyrosol acetate HTA (purity of 99.54%, from Seprox Biotech, Madrid, Spain). Likewise, aliquots of the olive oil O_2 were enriched with different concentrations of *gamma*-tocopherol (γT) (purity ≥ 90%, Eisai Food & Chemical Co. Ltd., Tokyo, Japan). These compounds were chosen due to their differing number of phenolic groups, which may influence on their activity. The samples enriched with dodecyl gallate were named, O_1DG_1 (with an enrichment of 0.12 mmol DG/mol $[FA+AG]_O$) and O_1DG_2 (with an enrichment of 1.36 mmol DG/mol

$[FA+AG]_O$). The samples enriched with hydroxytyrosol acetate, were named O_1HTA_1 (with an enrichment of 0.28 mmol HTA/mol $[FA+AG]_O$) and O_1HTA_2 (with an enrichment of 2.53 mmol HTA/mol $[FA+AG]_O$). Finally, the oil O_2 samples enriched with different concentrations of *gamma*-tocopherol were named $O_2\gamma T_1$ (with an enrichment of 0.11 mmol γT/mol $[FA+AG]_O$), $O_2\gamma T_2$ (with an enrichment of 1.17 mmol γT/mol $[FA+AG]_O$) and $O_2\gamma T_3$ (with an enrichment of 12.58 mmol γT/mol $[FA+AG]_O$). These enrichment levels were set in function of the solubility of these compounds in the oil. Thus, the above concentrations were obtained in order to reach enrichment degrees near to 0.02% and 0.2% in weight for the three phenolic compounds and, in addition, near 2% in weight in the case of *gamma*-tocopherol due to its high solubility in oils. However, this latter level of enrichment was not possible for dodecyl gallate and hydroxytyrosol acetate because of their limited solubility in oils. All these samples were submitted to *in vitro* digestion.

2.2. *Digestion experiments*

Aliquots (0.5 g) of the above-mentioned samples were digested by using a semi-static *in vitro* gastrointestinal digestion model developed by Versantvoort *et al*. (2005) [20]. This validated method was optimized, in order both to improve lipid digestion and to reach lipolysis levels of a similar order to *in vivo* digestion [21]. It has three stages, which simulate digestive processes in mouth, stomach, and small intestine, by sequentially adding the corresponding digestive juices (saliva, gastric juice, duodenal juice and bile), whose composition is given in Table S1 (see Supplementary Material). The digestive juices were prepared in the following way: the electrolyte solutions of the digestate juices were prepared the day before the *in vitro* digestion experiment and the enzymes were added just before starting the *in vitro* digestion. Once the digestive juices were prepared, they were heated to 37±2 °C to start de digestion experiment. The first stage begins by adding 6 mL of saliva to

the sample. After 5 min of incubation, 12 mL of gastric juice are added and the mixture is rotated head-over-heels at 40 rpm for 2 h at 37±2 °C. One hour after the start of the gastric stage, pH is set between 2 and 3 with HCl (37%), simulating the gradual acidification of the chyme occurring *in vivo*. After 2 h of the gastric stage, 2 mL of sodium bicarbonate solution (1 M), 12 mL of duodenal juice and 6 mL of bile juice are added. Subsequently, pH is set between 6 and 7, and the mixture is again rotated at 40 rpm and incubated at 37±2 °C for 4 h. All the reagents and enzymes for the preparation of digestive juices were acquired from Sigma-Aldrich (St. Louis, MO, USA): α-amylase from *Aspergillus oryzae* (10065, ~30 U/mg); pepsin from porcine gastric mucosa (P7125, ≥400 U/mg protein); amano lipase A from *Aspergillus niger* (534781, ≥120,000 U/g); pancreatin from porcine pancreas (P1750); lipase type II crude from porcine pancreas (L3126, 100-500 U/mg protein (using olive oil, 30 min incubation); and bovine bile extract (B3883). The digested samples were named like the original samples preceded by D (DO_1, DO_1DG_1, DO_1DG_2, DO_1HTA_1, DO_1HTA_2, DO_2, $DO_2\gamma T_1$, $DO_2\gamma T_2$ and $DO_2\gamma T_3$). Two digestion experiments, each including duplicate samples, were performed. Blank samples corresponding to the mixture of juices submitted to digestive conditions were also taken for further analysis.

2.3. Digestate lipid extraction

Lipids from the digestates were extracted using dichloromethane as solvent, added directly to the digestates without any previous step, (CH_2Cl_2, HPLC grade, Sigma-Aldrich) following a methodology that also allows fatty acid extraction as in previous studies [9]. This methodology involves a three-stage liquid-liquid extraction process with 20 mL of CH_2Cl_2 each. Afterwards, to ensure a complete protonation of fatty acids and/or the dissociation of the potential salts formed, the remaining water phase was acidified to pH 2 with HCl (37%) and a second extraction was carried out in three steps using again 20 mL of CH_2Cl_2. For this

purpose, a Sigma 3K30 centrifugal machine (Sigma Laboratory Centrifuges, Germany) working at 2724 g was used, each extraction step lasting 6.50 min. All the CH_2Cl_2 extracts of each sample were mixed and the solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. The extraction yield was in all cases near 85%. These extracts contain triglycerides, diglycerides and monoglycerides, as well as fatty acids and minor lipophilic compounds either present in the original samples or formed in the digestion process.

2.4. Study by ^{1}H NMR of oil samples and lipid extracts of digestates

2.4.1. Samples subject of study and operating conditions

The ^{1}H NMR spectra of the original oils **O_1 and O_2**, and of the oil samples enriched with each one of the **phenolic compounds above mentioned** at the different concentrations (O_1DG_1, O_1DG_2, O_1HTA_1, O_1HTA_2, $O_2\gamma T_1$, $O_2\gamma T_2$ and $O_2\gamma T_3$), and of the lipids extracted from their digestates (DO_1, DO_1DG_1, DO_1DG_2, DO_1HTA_1, DO_1HTA_2, DO_2, $DO_2\gamma T_1$, $DO_2\gamma T_2$ and $DO_2\gamma T_3$), were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. As in previous studies [18,22] standard ^{1}H NMR and multisuppressed spectra were acquired, these latter by using NOESYGPPS experiments. Details about operating conditions are given in Supplementary Material.

2.4.2. Identification of the components from ^{1}H NMR spectral data

The identification of the components present in the original oils, in the oil samples enriched with phenolic compounds and in the lipid extracts of their digestates, was carried out on the basis of the assignments of the ^{1}H NMR signals to the different kinds of hydrogen atoms, and in short to the different compounds. Figure 1 gives the spectral regions comprised between 0.0 and 4.9 ppm, of olive oil O_1 ^{1}H NMR spectrum, and between 3.5 ppm and 5.10

ppm, conveniently enlarged, of the ^{1}H NMR spectra of the lipids extracted from the several digestates (DO_1, DO_1DG_2, DO_1HTA_2, DO_2, and $DO_2\gamma T_2$), in which signals of protons of their main components appear.

These above mentioned signals, and others due to protons of minor components not shown in Figure 1, but present in the spectra of the above mentioned samples, their chemical shifts and assignments are given in Tables S2, S3, S4 and S5 (see Supplementary Material). These assignments were made taken into account previous studies as is indicated in each table, or were based on the signals of standard compounds acquired for this study, which include cycloartenol, squalene, hexanal and decanal, acquired from Sigma-Aldrich (St. Louis, MO, USA) and linolein hydroperoxides purchased from Cayman Chemical (Ann Arbor, MI, USA).

Table S2 shows ^{1}H NMR signals of specific protons of the different glyceride structures, such triglycerides, diglycerides and monoglycerides. Table S3 shows ^{1}H NMR signals of protons of linolenic, linoleic, oleic and saturated acyl groups and fatty acids, and the signals of methylenic protons supported on carbons atoms in *alpha* and *beta* position in relation to carbonyl-carboxyl groups. Table S4 shows ^{1}H NMR signals of protons of oxidation compounds coming from main oil component degradation, which occurred during digestion. Finally, Table S5 gives ^{1}H NMR signals of some protons of dodecyl gallate, hydroxytyrosol acetate, *gamma*-tocopherol, of free and esterified cycloartenol plus 24-methylenecycloartenol and of squalene. The areas of some of these spectral signals were used to quantify the concentration of the different kinds of above-mentioned structures in the corresponding samples, as will be explained below.

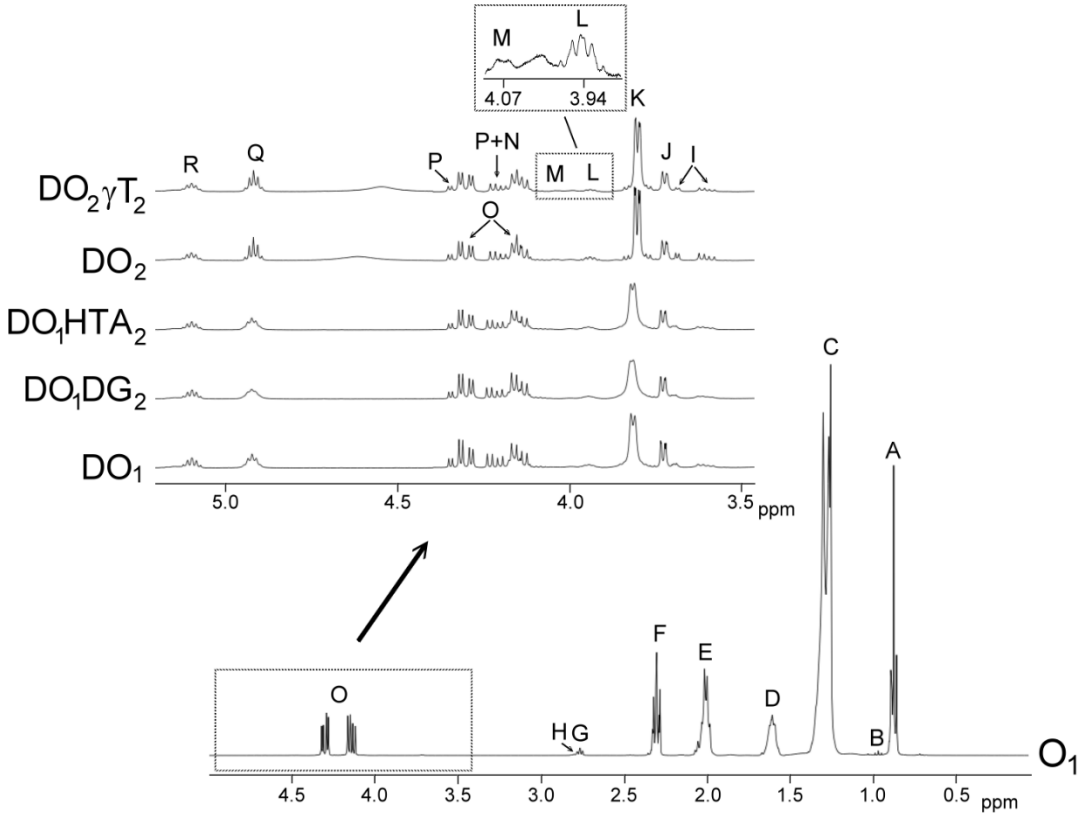

Figure 1. Region comprised between 0.0 and 4.9 ppm, of olive oil O_1 ^{1}H NMR spectrum, and region comprised between 3.5 ppm and 5.10 ppm, conveniently enlarged, of the ^{1}H NMR spectra of the lipids extracted from the several digestates (DO_1, DO_1DG_2, DO_1HTA_2, DO_2 and $DO_2\gamma T_2$), in which signals of protons of their main components appear. The signal letters agree with those of Tables S2 and S3 of Supplementary Material.

2.4.3. Quantifications made from ^{1}H NMR spectral data

This technique allows the estimation of the concentrations, expressed in different ways, of all identified compounds if they do not have overlapped signals in the corresponding spectra. This is possible because, as has been explained above, the area of the ^{1}H NMR signals is proportional to the number of protons that generate the signal. The quantification of the different kinds of compounds or structures is explained below.

(A) Estimation of the molar percentage of the different kinds of glycerides in the digestates

This estimation can be carried out by using the intensity of some signals indicated in Tables S2 and S3, which are also shown in Figure 1. Although glycerol is formed during

digestion, due to its polar nature it is not present in the lipid extract of the digestate. However, its concentration can be estimated indirectly. This is possible because the concentration of total fatty acids plus acyl groups, of only acyl groups, and of fatty acids released in the formation of diglycerides and monoglycerides can be determined from ^{1}H NMR data. Thus, the estimation of the molar percentage of triglycerides (TG), 1,2-diglycerides (1,2-DG), 1,3-diglycerides (1,3-DG), 2-monoglycerides (2-MG), 1-monoglycerides (1-MG) and glycerol (Gol) in relation to the total glyceryl structures present in the digestate, was carried out by using equations [eq. S1- eq. S10] given in Supplementary Material and the areas of signals included in Tables S2 and S3 . They are based exclusively on the intensity of ^{1}H NMR spectral signals [23].

(B) Estimation of the molar percentage of fatty acids plus acyl groups that have linolenic, linoleic, oleic and saturated structures in relation to the total fatty acids and acyl groups in digestates

In edible oils the concentration of fatty acids is very low and, in many cases, inappreciable in comparison with the concentration of acyl groups. However, as is known, during oils digestion hydrolysis provokes the transformation of a certain number of acyl groups into fatty acids. The fatty acids formed maintain the same number of carbon atoms and unsaturation pattern as the starting acyl groups. Acyl groups and fatty acids having the same structure provide NMR spectra signals with a high degree of overlapping that allow their joint quantification. In this study, the molar percentage of *linolenic, linoleic, oleic and saturated structures* found in acyl groups and fatty acids in the digestates was estimated in relation to the total number of moles of fatty acids plus acyl groups. This estimation was made using the equation [eq. S11- eq. S14], given in Supplementary Material, in which the areas of some signals that are shown in Figure 1 and in Table S3 are involved. These

equations are the same as those employed in previous studies [15,16], but using the signal of methylenic protons supported on carbons atoms in *alpha* position in relation to carbonyl-carboxyl groups, instead of the signal of triglyceride protons used in edible oils studies.

(C) Estimation of the concentration of specific compounds (X) in oil samples and in the digestates

The concentration of oxidation compounds, and of others such as squalene, cycloartenol plus 24-methylenecycloartenol, dodecyl gallate, hydroxytyrosol acetate and *gamma*-tocopherol either in oils or in digestates can be estimated by using the general equation [eq. S15] given in Supplementary Material and the intensity of one of their non-overlapped ^{1}H NMR spectral signals, which are indicated in Figure 1, and in Tables S3, S4 and S5. This equation allows one to estimate the concentration of any compound in oils or in digestates in relation to the concentration of fatty acids plus acyl groups, which are considered the internal reference.

(D) Estimation of in vitro bioaccessibility

The *in vitro* bioaccessibility of a compound can be defined as the concentration of the compound that remains absorbable after *in vitro* digestion. This concentration may refer either to an internal reference or to the initial concentration of the compound in the sample before digestion. The first approach is much more general because it can be used for compounds formed during digestion and absent in the sample before digestion. In this study the internal reference can be the concentration of oil main components expressed by the sum of the concentration of fatty acids plus acyl groups in the digestate $([FA]+[AG])_D$. I*n vitro* bioaccessibility, thus defined, of the oil main components, can be estimated by using the equation $B_{OMC} = ([FA]+[MG])_D /([FA]+[AG])_D$, because the only absorbable compounds coming from oil main components are fatty acids, FA, and monoglycerides, MG. For any

other compound X present in the oil sample before digestion or not, the equation to be used to determine the bioaccessibility in this approach is $B_x = [X]_D/([FA]+[AG])_D$, where $[X]_D$ is the concentration of the compound X in the lipid extract of the digestate.

In the second approach, bioaccessibility B´ can be estimated by the ratio between the concentration of the compound in the lipid extract of the digestate $[X]_D$ and the concentration in the oil before digestion, $[X]_O$, as indicated in the equation $B'_x = [X]_D/[X]_O$. This definition gives information about the loss of the compounds during *in vitro* digestion, or about the fraction of the compounds released during digestion that are really absorbable as in the case of oil main components. For oil main components B_{OMC} and B'_{OMC} are very similar because the reference is barely modified during digestion.

2.5. Study by SPME-GC/MS of the headspace of the digestates and of the mixture of the digestive juices submitted to digestion conditions with olive oil

Extraction of the volatile components constituting the headspace of the several samples (0.5 g in a 10 mL screw-cap vial) was accomplished automatically by using a CombiPAL autosampler (Agilent Technologies, Santa Clara, CA, USA). The samples studied were the several digestates (DO_1, DO_1DG_1, DO_1DG_2, DO_1HTA_1, DO_1HTA_2, DO_2, $DO_2\gamma T_1$, $DO_2\gamma T_2$ and $DO_2\gamma T_3$) and the mixtures O_1DJ and O_2DJ of digestive juices DJ, after undergoing digestion conditions, and olive oils O_1 and O_2. The comparison of the headspaces of the several samples enables one to deduce differences provoked by *in vitro* digestion. The operating conditions were the same as those used in previous studies [24] and are explained in Supplementary Material.

Identification of the headspace components was carried out by using several commercial standard compounds acquired from Sigma-Aldrich (St. Louis, MO, USA). When standard compounds were not available, identification was made by matching the spectra obtained,

higher than 85%, with those of commercial libraries (Wiley W9N08, Mass Spectral Database of the National Institute of Standards and Technology), or with those spectra provided by the scientific literature, as in previous studies [24].

The semi-quantification of the compounds was based on the area counts of the base peak (Bp) of the mass spectrum of each compound divided by 10^6. When the Bp of a compound overlapped with some ion peak of the mass spectrum of another compound, an alternative ion peak was selected for the semi-quantification of the former [24]. Although the chromatographic response factor of each compound is different, the area counts thus determined are useful for the comparison of the abundance of each compound in the different samples. The target compounds of this technique were the volatile oxidation compounds formed in *in vitro* digestion, and terpenes and sesquiterpenes, which are characteristic minor volatile components of olive oil. Data given in the corresponding tables are average values of duplicate experiments.

2.6. Statistical analysis

The significance of the differences among samples in the several kinds of data, was determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p < 0.05$, using SPSS Statistics 24 software (IBM, NY, USA).

3. RESULTS

The main reaction that takes place during digestion is the hydrolysis of large molecules, such as proteins, triglycerides and carbohydrates, to release molecules of a small size capable of being absorbed through the intestinal wall. The extent and pattern of this reaction determines the bioaccessibility of these main components. Nevertheless, hydrolysis also could affect smaller molecules whenever they have hydrolyzable bonds. Furthermore, other reactions such as oxidation reactions could also be produced affecting both main and minor

components, either present in or added to the food. These latter reactions could also give rise to the formation of derived compounds, some of which could also be absorbed, affecting the bioaccessibility of the different kinds of compounds. In this context the *in vitro* digestion of olive oil, enriched or not with phenolic compounds, will be addressed and compared with that of other oils of very different composition, such as, corn and virgin linseed oils.

3.1. Lipolysis extent and in vitro bioaccessibility of oil main components of olive oil, comparison with those of corn and virgin flaxseed oil, and effect of olive oil enrichment with phenolic compounds.

3.1.1. Lipolysis and in vitro bioaccessibility of olive oil main components

Lipolysis provokes the release of fatty acids (FA) by breaking the ester bonds of triglycerides, yielding also diglycerides (DG), monoglycerides (MG) and glycerol (Gol). Table 1 gives the molar percentages of each of the glyceride structures present in the digestates formed during the digestion of two different olive oils, estimated as described in the experimental section. It can be observed that the main glyceryl lipolytic products formed in the digestion of both oils O_1 and O_2 are monoglycerides (near 44% and 42% in DO_1 and DO_2 respectively) and glycerol (near 28% and 26% in DO_1 and DO_2 respectively). This is very important because both fatty acids and monoglycerides are able to be absorbed through the intestinal wall. By contrast, the concentration of triglycerides in the digestates of both oils is low, reduced to near 13%, and that of diglycerides reaches near 15% and near 18% in DO_1 and DO_2 respectively.

A parameter that represents both the extent and pattern of the lipolysis reached during *in vitro* digestion in a global way is the *in vitro* bioaccessibility of the oil main components, B_{OMC}, defined as the ratio between the real absorbable molecules after digestion and all absorbable potential molecules before digestion. The really absorbable molecules after

digestion are the released fatty acids (FA) and monoglycerides (MG), $(FA+MG)_{D,}$ present in the digestate. The potential absorbable molecules are fatty acids plus all acyl groups, $(FA+AG)_D$. In agreement with the molar percentage of the different kinds of glyceryl structures, given in Table 1, B_{OMC} (determined as indicated in the experimental section) of DO_1 is slightly higher than DO_2. This parameter is very important because it not only summarizes in a single value the level of lipolysis reached during the *in vitro* digestion but also because of its nutritional meaning.

3.1.2. Comparison between lipolysis yield of olive oil and in vitro bioaccessibility of its main components and those of corn and virgin flaxseed oils submitted to the same digestive conditions

The data of lipolysis yields of other edible oils such as corn oil C and virgin flaxseed oil F, obtained in previous studies [13,14] under the same digestive conditions as in this study, are given in Table 1. It can be observed that the extent and pattern of lipolysis is very different to that of olive oils O_1 and O_2. The concentration of monoglycerides in the digestates of corn oil DC and of virgin flaxseed oil DF reaches values near 31% and 24% respectively, somewhat lower than that of the olive oils. These results are in agreement with previous studies in which important differences in the extent of the lipolysis reached during *in vitro* digestion of edible oils of different compositions were also found [6-8]. As expected, there is a clear difference between the bioaccessibility of oil main components in DF and in DC, DO_1 or DO_2, as Table 1 shows. Although the difference between the bioaccessibility of the main components of the oil in DC and in DO_1 or DO_2 is not statistically significant, the bioaccesibility in DC tends to be smaller than in DO_1 and DO_2. This is in line with the lower extent of the lipolysis undergone by C and F oils during *in vitro* digestion compared with that

of olive oils, which is also reflected in the molar percentages of different glyceryl species of the corresponding digestates.

There may be many factors that influence the *in vitro* digestion lipolytic process. However, under the same digestive conditions, the minor and main oil components present can be considered the main ones. To the best of our knowledge, there are few studies regarding the influence of oil minor components. As one example, no significant differences have been found in the distribution of the glycerides in the digestates of refined and virgin soybean oils with different content in minor components [11]. The minor components of the three oils considered here are very different not only in their nature but also in their concentrations, which is why no conclusion could be drawn in this regard. It could only be mentioned that the three oils, as Table 1 shows, contain a small concentration of 1,2-diglycerides which can act from the beginning of the *in vitro* digestion process as emulsifiers, favouring contact between enzymes and lipid active sites to facilitate the lipolytic reactions. However, as the difference in the initial concentration of 1,2-diglycerides in olive, corn and virgin flaxseed oils is very small it is to be expected that this factor has no influence on the lipolysis extent produced in these oils during digestion.

As already mentioned, main oil components can also be determinant factors of the lipolysis extent during *in vitro* digestion. There are some studies on this issue [8,25-29]. The oil main components are triglycerides, which support different kinds of acyl groups, with varied number of carbon atoms and unsaturation degrees. Furthermore, the acyl groups can occupy different positions in the backbone of the glyceryl group, forming in this way different kinds of triglycerides. All these variables can influence the extent and pattern of lipolysis during *in vitro* digestion.

Table 1. Lipolysis extent. Molar percentages of triglycerides (TG), diglycerides (1,2-DG and 1,3-DG), monoglycerides (1-MG and 2-MG) and glycerol (Gol) in relation to the total glyceride structures, in olive oils (O_1 and O_2), in corn oil (C) and in virgin flaxseed oil (F), in the digestates of these oils (DO_1, DO_2,, DC and DF) and in those of the samples enriched with dodecyl gallate, hydroxytyrosol acetate and *gamma*-tocopherol (DO_1DG_1, DO_1DG_2, DO_1HTA_1, DO_1HTA_2, $DO_2\gamma T_1$, $DO_2\gamma T_2$,and $DO_2\gamma T_3$) whose level of enrichment in phenolic compounds is given in brackets in mmol/mol $(AG+FA)_O$. Bioaccessibility of oil main components after *in vitro* digestion (B_{OMC}), defined by the ratio (mol $[FA]+[MG])_D$/mol $([FA]+[AG])_D$), where FA means fatty acid and AG acyl groups. Different letters within each column indicate statistically significant differences among the samples ($p < 0.05$). Data of corn and virgin flaxseed oils and of their digestates were taken from previous studies [13,14].

Samples	**Lipolysis extent (molar %)**						**Bioaccessibility**
	TG (%)	**1,2-DG (%)**	**1,3-DG (%)**	**2-MG (%)**	**1-MG (%)**	**Gol (%)**	**(B_{OMC})**
Oils							
O_1	98.1 ± 0.2a	1.5 ± 0.0a	-	-	-	-	
DO_1	13.0 ± 0.6b	13.4 ± 1.7bc	1.9 ± 0.0ab	29.9 ± 2.2ab	13.7 ± 1.8a	28.1 ± 1.7abc	0.77 ± 0.02a
O_2	98.3 ± 1.4a	1.4 ± 0.0a	-	-	-	-	
DO_2	13.3 ± 2.0b	16.0 ± 1.0bc	2.5 ± 0.6b	33.5 ± 2.0a	8.9 ± 0.9b	25.8 ± 1.5abc	0.74 ± 0.01a
C	99.8 ± 0.2a	1.1 ± 0.1a	-	-	-	-	
DC	22.3 ± 5.9bc	14.0 ± 1.6bc	1.8 ± 1.0ab	26.6 ± 5.6ab	4.4 ± 1.1cd	30.8 ± 1.8ab	0.67 ± 0.07a
F	99.4 ± 0.0a	1.2 ± 0.0a	-	-	-	-	
DF	33.1 ± 2.7c	18.1 ± 2.1c	4.8 ± 1.0c	21.7 ± 0.5b	2.2 ± 0.8df	20.2 ± 4.3c	0.52 ± 0.05b
Olive oil-dodecyl gallate							
DO_1DG_1 (0.12)	12.9 ± 3.8b	11.6 ± 3.7b	1.8 ± 0.6ab	28.7 ± 0.2ab	12.1 ± 1.1a	32.9 ± 6.9a	0.78 ± 0.07a
DO_1DG_2 (1.36)	14.9 ± 1.1b	13.8 ± 1.3bc	1.4 ± 0.6ab	28.8 ± 0.6ab	13.9 ± 1.9a	27.2 ± 1.8abc	0.75 ± 0.00a
Olive oil-hydroxytyrosol acetate							
DO_1HTA_1 (0.28)	12.0 ± 1.5b	12.6 ± 0.2b	1.7 ± 0.1ab	31.2 ± 0.7a	13.0 ± 0.4a	29.5 ± 0.5ab	0.79 ± 0.01a
DO_1HTA_2 (2.53)	12.2 ± 0.3b	12.9 ± 0.1bc	2.0 ± 0.2b	31.4 ± 0.3a	12.0 ± 0.4a	29.4 ± 0.7ab	0.78 ± 0.01a
Olive oil-gamma-tocopherol							
$DO_2\gamma T_1$ (0.11)	14.7 ± 0.4b	15.2 ± 2.2bc	1.8 ± 0.2ab	35.4 ± 0.9a	7.1 ± 1.7bc	25.8 ± 0.1abc	0.74 ± 0.02a
$DO_2\gamma T_2$ (1.17)	15.6 ± 1.6b	15.4 ± 0.3bc	1.8 ± 0.4ab	35.9 ± 4.3a	7.8 ± 0.3b	23.6 ± 2.4bc	0.73 ± 0.02a
$DO_2\gamma T_3$ (12.58)	15.5 ± 1.3b	16.7 ± 0.6bc	2.2 ± 0.1b	35.1 ± 4.1a	7.9 ± 0.3b	22.5 ± 1.8bc	0.72 ± 0.02a

-: not detected

a) Influence of the length and unsaturation degree of acyl groups present in the oil

The acyl groups of the oils here considered differ in length and, as Table 2 shows, have important differences in the unsaturation degree. Furthermore, it should be mentioned that, in olive and corn oils, which have similar molar percentages of saturated acyl groups S, the distribution of these between palmitic and stearic groups is of a similar order in both oils, the second group in a much smaller percentage than the first, as is well known [30]. However, in virgin flaxseed oil the molar percentage of saturated acyl groups S is smaller than in the other two oils, having only a slightly smaller percentage of stearic than of palmitic groups [31]. Taking into account all the compositional data and lipolysis extent of each oil shown in Table 1, it seems evident that the unsaturation degree (or the saturation degree) of the oils greatly influences lipolysis extent reached during their digestion, in contrast to the reported in some previous studies [28,32]. As Tables 1 and 2 show, the most unsaturated oil (virgin flaxseed oil), reaches the lowest lipolysis extent during digestion and the opposite is true for olive oils. Likewise, from data of these tables it is evident that oleic acyl group has a slightly greater tendency to be hydrolysed during digestion than linoleic acyl group. This is evident because olive and corn oils have similar molar percentages of saturated acyl groups, but olive oils, which are richer in oleic acyl groups, reach a lipolysis extent during digestion which is slightly higher than the second oil, which is richer in linoleic groups. This fact is in disagreement with the similar tendency of oleic, linoleic and even of linolenic acyl groups to hydrolyze reported by some authors [27]. Furthermore, it has also been described that ester bonds of saturated acyl groups, such as palmitic and stearic groups, are hydrolyzed more easily or faster by pancreatic lipase than unsaturated acyl groups such as oleic, linoleic and linolenic acyl groups [27-29]. In addition, it has also been reported that hydrolysis is more efficient the smaller the number of carbon atoms of the acyl groups [6,27,29]. For this reason, the tendency of palmitic group to hydrolyze should be greater than that of stearic group,

although some authors find no difference between them [32-34]. Compositional data of the oils involved in this study given in Table 2 do not permit an analysis of some of the above-cited considerations.

b) Influence of the distribution of the different kinds of acyl groups in the backbone of the triglycerides in each oil

Some authors have also pointed out that, in addition to the above mentioned concerning different tendencies of fatty acyl groups to be hydrolyzed in function of their unsaturation degree and length, the positions which they occupy in the backbone of triglyceride could also influence the hydrolysis extent reached during their *in vitro* digestion. The importance of the distribution of the different acyl groups in triglyceride is due to the ester hydrolysis which takes place mainly in the sn-1 and sn-3 positions of the triglyceride when pancreatic lipase and *A. niger* lipase are used [8,25,29]. Due to this, the distribution of the different kinds of acyl groups in the backbone of the triglycerides of oils obtained by a similar processing and same vegetable origin as those involved in this study was analyzed using data from the literature [35,36]. Published data about triglyceride profiles of these three kinds of oils evidence that none of these oils have triglycerides with both sn-1 and sn-3 positions occupied simultaneously by saturated acyl group, which is considered the group most likely to be hydrolyzed [27-29]. Saturated acyl groups occupy, almost exclusively, the sn-1 position in the triglycerides of these three oils and the abundance of this class of triglycerides in each oil depends on the molar percentage of this type of acyl groups in the oil. As Table 2 shows, the molar percentage of saturated acyl groups is very similar in olive and corn oils and slightly less in virgin flaxseed oil. For this reason, the formation of 1,2-diglycerides as consequence of the hydrolysis of the ester group of saturated acyl groups should be of the same order in

olive and corn and lower in virgin flaxseed oil. However, this does not explain the differences found in the total lipolysis extent undergone by these oils during *in vitro* digestion.

Analysis of the profile of the triglycerides of these oils evidences that those acyl groups, which are in greater concentrations in the oil, are those that more frequently occupy the sn-1 and sn-3 positions in the triglyceride. From this, it seems clear that the differences in the lipolysis extent of these oils, under same digestive conditions, depend mainly on the different tendency of each acyl group to hydrolyze and on its concentration in the oil, because the frequency of their presence in sn-1 and sn-3 positions of the backbone of the triglyceride is a function of the concentration of each acyl group in the oil.

Table 2. Molar percentage of the main acyl groups plus fatty acids (AG + FA), in relation to the total moles of all kinds of AG and FA, in olive oils (O_1 and O_2), in corn oil (C) and in virgin flaxseed oil (F), in the digestates of these oils (DO_1, DO_2,, DC and DF) and in those of the samples enriched with dodecyl gallate, hydroxytyrosol acetate and *gamma*-tocopherol (DO_1DG_1, DO_1DG_2, DO_1HTA_1, DO_1HTA_2, $DO_2\gamma T_1$, $DO_2\gamma T_2$,and $DO_2\gamma T_3$) whose level of enrichment in phenolic compounds is given in brackets in mmol/mol $(AG+FA)_O$.. Concentration of some oxidation compounds, expressed by mmol per mol of AG+FA in the above samples. Different letters within each column indicate statistically significant differences among the samples ($p < 0.05$). Data of corn and virgin flaxseed oils and of their digestates were taken from previous studies [13,14].

Samples	**Molar (%) of total acyl groups + fatty acids**				**Oxidation compounds (mmol/mol [AG+FA])**	
	Linolenic	**Linoleic**	**Oleic**	**Saturated**	**HPO-c(*Z*,*E*)-dEs**	**n-alkanals**
Oil						
O_1	0.6 ± 0.1a	8.0 ± 0.4a	75.5 ± 0.6a	15.8 ± 0.2a	-	0.12 ± 0.00a
DO_1	0.7 ± 0.0a	7.7 ± 0.1a	75.4 ± 0.0a	16.3 ± 0.1a	-	0.08 ± 0.00a
O_2	0.7 ± 0.1a	8.0 ± 0.1a	75.1 ± 0.6a	16.2 ± 0.5a	-	0.10 ± 0.00a
DO_2	0.7 ± 0.0a	8.0 ± 0.1a	74.4 ± 0.4a	16.9 ± 0.6a	0.26 ± 0.04ac	0.08 ± 0.02a
C	0.6 ± 0.0a	49.2 ± 0.5b	34.1 ± 0.3b	16.1 ± 0.1a	-	-
DC	0.6 ± 0.1a	41.3 ± 0.0c	42.6 ± 0.2c	15.5 ± 0.0a	1.82 ± 0.31b	-
F	55.7 ± 0.0b	14.2 ± 0.3d	20.5 ± 1.2d	9.5 ± 0.9b	-	-
DF	47.9 ± 0.8c	14.1 ± 0.7d	25.7 ± 3.9e	12.3 ±3.5b	0.39 ± 0.04c	0.09 ± 0.00a
Olive oil-dodecyl gallate					-	
DO_1DG_1 (0.12)	0.9 ± 0.2a	8.0 ± 0.9a	74.8 ± 0.6a	16.3 ± 0.0a	-	0.11 ± 0.03a
DO_1DG_2 (1.36)	0.7 ± 0.1a	8.2 ± 0.1a	75.2 ± 0.2a	15.9 ± 0.2a	-	0.10 ± 0.04a
Olive oil-hydroxytyrosol acetate					-	
DO_1HTA_1 (0.28)	0.8 ± 0.1a	7.4 ± 0.4a	75.2 ± 0.2a	16.6 ± 0.2a	-	0.08 ± 0.02a
DO_1HTA_2 (2.53)	0.8 ± 0.1a	7.9 ± 0.7a	75.1 ± 0.4a	16.3 ± 0.3a	-	0.08 ± 0.02a
Olive oil-gamma-tocopherol						
$DO_2\gamma T_1$ (0.11)	0.7 ± 0.0a	7.8 ± 0.2a	74.2 ± 0.2a	17.4 ± 0.0a	0.27 ± 0.01ac	0.08 ± 0.00a
$DO_2\gamma T_2$ (1.17)	0.6 ± 0.1a	7.6 ± 0.1a	74.1 ± 0.2a	17.7 ± 0.0a	-	0.07 ± 0.00a
$DO_2\gamma T_3$ (12.58)	0.8 ± 0.1a	7.8 ± 0.1a	74.2 ± 0.2a	17.2 ± 0.2a	-	0.09 ± 0.00a

: not detected

c) Quantitative relationships between lipolysis extent reached and concentrations of the different kinds of acyl groups in the oil

In order to go into this matter in more depth, potential quantitative relationships between lipolysis yield, expressed by the *in vitro* bioaccessibility of the oil main components B_{OMC} in the corresponding digestates, and concentration of oil main components in the oils submitted to *in vitro* digestion, expressed by the molar percentages of the different kinds of acyl groups, were tested. Both kinds of data are given in Tables 1 and 2 respectively. As before mentioned, data of corn and virgin flaxseed oils were taken from previous studies [13,14]. Regarding data in Table 2 of the molar percentages the different kinds of acyl groups in each oil, it should be mentioned that that although there are four compositional data (%S, %O, %L, %Ln), only three are independent variables. This is corroborated by the correlation matrix of the molar percentages of the different kinds of acyl groups in the four oils showed in Table S6. This table shows that molar percentages of linolenic (%Ln) and of saturated (%S) acyl groups are closely related in an inverse way (R = - 0.9986), which indicates that they provide similar information. The molar percentage of the other two acyl groups (%L) and (%O) are not as closely related with any other.

In a first approach simple linear relationship between *in vitro* bioaccessibility of oil main components B_{OMC} in the digestates and molar percentage of the different kinds of acyl groups in the oil were tested. The results evidenced that there is a close relationship with molar percentage of saturated (%S) acyl groups and also, as expected, with the molar percentage of linolenic (%Ln) acyl groups in the oil. The equations that describe these relations are B_{OMC} = 0.226 + 0.031 (%S) with a correlation coefficient R= 0.9148 and B_{OMC} = 0.729 - 0.004 (%Ln) with a correlation coefficient R= 0.9265. The relationship of B_{OCM} with the molar percentage of oleic (%O) acyl groups is also close (B_{OMC} = 0.489 + 0.004 (%O), R= 0.9208).

However, a very slight, almost non existent relationship between *in vitro* bioaccessibility and molar percentage of linoleic (%L) groups was observed.

The above equations can lead to some considerations being made. The first is that the molar percentage of saturated, linolenic and of oleic acyl groups have an important influence on the lipolysis extent reached during *in vitro* digestion of these oils. Secondly that the molar percentages of saturated and also of oleic acyl groups are related to $B_{OMC,}$ or to lipolysis extent, in a direct way, which is to say that the higher the concentration of saturated and of oleic acyl groups the higher B_{OMC}. However, the opposite is true for the molar percentage of linolenic acyl groups: the higher the molar percentage of linolenic acyl groups the smaller the lipolysis extent and the lower the B_{OMC} values. This result shows the important negative influence of a high concentration of linolenic groups, or of a high unsaturation degree in the oil, on its lipolytic process during its *in vitro* digestion, in agreement with some previous studies [8,26,27]. Furthermore, these results reaffirm previous findings on the direct relationship between concentration of saturated acyl groups and lipolysis extent [28,29]. Finally, they evidence that the concentration of oleic acyl groups is also positively related with lipolysis extent, which has been proved, for the first time, in this study.

In order to deepen the study of the influence of the oil composition on the lipolysis extent reached during its *in vitro* digestion, equations involving two variables were tested in an attempt to find relationships, closer than the above, between *in vitro* bioaccessibility and concentrations of the different acyl groups in the oil. The equations obtained are indicated in Table 3. It can be observed that in the five equations shown there is a very close relationship between *in vitro* bioaccessibility and the molar percentage of two kinds of acyl groups. These equations demonstrate once again the direct relationships between lipolysis extent and concentration of saturated and oleic acyl groups, the weight of the molar percentage of saturated acyl groups being around ten times higher than that of the oleic groups. Likewise,

it can again be observed that lipolysis extent is inversely related with the molar percentage of linolenic and linoleic acyl groups, the weight of the first being double than that of the second. Furthermore, equations involving saturated and linoleic acyl groups (or linolenic and oleic acyl groups) also have high correlation coefficients, as can be expected due to the very close relationship between %S and %Ln. In these two latter equations it is again shown that the greater the concentration of saturated or of oleic acyl groups the higher the *in vitro* bioaccessibility, and the opposite is true for linolenic and linoleic groups. These results suggest that the trend of saturated groups to be hydrolyzed is high, and this decreases progressively as the unsaturation degree of the acyl group increases, reaching the least tendency in linolenic groups, whereas oleic and linoleic groups show an intermediate tendency. Finally, the equation that involves these latter acyl groups has also a very high correlation coefficient, the weight of the molar percentage of oleic group being double than that of linoleic group.

Table 3. Coefficients of the equations $B_{OMC} = a + b\ X_1 + c\ X_2$ that relate the *in vitro* bioaccessibility of the oil main components (B_{OMC}) and the molar percentage of certain acyl groups saturated (%S), oleic (%O), linoleic (%L) or linolenic (%Ln) in the oil before digestion, together with their correlation coefficients R.

Equation Number	**Variables**	**Equation Coefficients**			**Correlation Coefficient**
	X_1, X_2	**a**	**b**	**c**	**R**
1	%S, %O	0.307	0.018	0.002	0.9911
2	%S, %L	0.230	0.034	- 0.002	0.9872
3	%Ln, %O	0.601	- 0.002	0.002	0.9944
4	%Ln, %L	0.774	- 0.004	- 0.002	0.9941
5	%O, %L	0.390	0.004	0.002	0.9947

Finally, the predictive values of equations given in Table 3 were analyzed with data coming from a previous study concerning virgin and refined soybean oils [11]. The introduction of the molar percentages of the different kinds of acyl groups of these oils (virgin

soybean oil: %Ln=5.5, %L=44.7, %O=32.5, %S=17.3, B_{OMC}=0.65; refined soybean oil: %Ln=4.9, %L=47.6, %O=32.1, %S=16.3, B_{OMC}=0.66) in the different equations given in Table 3 allows one to evaluate their predictive value. It has been proved that equations 1, 3 and 4 of Table 3 can predict the *in vitro* bioaccessibility of these soybean oils with a high level of approximation, which evidences their soundness. This could be carried out over a greater number of edible oils, all of them submitted to the same *in vitro* digestive conditions, to obtain much more general equations, which can be of interest to design mixtures of edible oils with a specific bioaccessibility in order to prepare diets for special needs with different nutritional purposes.

3.1.3. Effect of the enrichment of olive oil with different phenolic compounds on lipolysis extent and oil main component bioaccessibility reached during in vitro digestion

It has been reported that certain polyphenolic compounds, polymeric or not, are able to inhibit lipase activity and reduce the lipolysis extent reached during lipid digestion [5,38]. However, this ability has not been observed in the phenolic compounds involved in this study [14]. Table 1 shows the molar percentages of the different glyceryl species found in the digestates of olive oil enriched with various concentrations of dodecyl gallate (DG), hydroxytyrosol acetate (HTA) and *gamma*-tocopherol (γT). As this table shows, no significant differences have been found either in the molar percentage of any of the glyceryl species or in the bioaccessibility of oil main components between the digestates of the not enriched olive oils and those of the olive oils enriched with phenolic compounds. This reinforces previous results that showed no inhibitory capacity of these phenolic compounds on pancreatic lipase [14].

3.2. Occurrence of oxidation reactions during in vitro digestion of olive oil, comparison with that of corn and virgin flaxseed oil, and effect of olive oil enrichment with phenolic compounds on this issue

In previous studies on the *in vitro* digestion of edible oils of different unsaturation degree it was proved that lipid oxidation takes place during this process [9-14]. This is a subject of great importance due to the toxicity of some of the oxidation compounds that can be formed, because they can be absorbed through the intestinal wall. The occurrence of lipid oxidation reactions during digestion can be evaluated either by the degradation of fatty acids and acyl groups or by the formation of oxidation compounds.

Due to esters having greater oxidative stability than fatty acids, it may be supposed that the fatty acids released in the lipolytic process will be the candidates for oxidization if it takes place. Both, acyl groups and fatty acids can be estimated jointly by ^{1}H NMR spectroscopy as indicated in the experimental section and in previous studies [13,14]. The differences between the concentration of acyl groups plus fatty acids in the oil and in the corresponding digestate will inform about the degradation of some of them during digestion due to oxidation [9-14].

Likewise, the formation of oxidation compounds during digestion can be evaluated by both ^{1}H NMR spectroscopy and by solid phase microextraction followed by gas chromatography-mass spectrometry (SPME-GC/MS) as indicated in the experimental section [18,24]. The first technique permits one, whenever the concentration of oxidation compounds in the extract of the digestate is high enough, to detect and quantify oxidation compounds contained in it [9-14]. The second technique allows measurement of the abundance of secondary oxidation compounds volatile present in the headspace of the digestate, as indicated in the experimental section and in previous studies [9-14].

3.2.1. Analysis of potential changes in the concentration of unsaturated fatty acids-acyl groups during in vitro digestion

a) In olive oils

The molar percentages of the different kinds of fatty acids and acyl groups determined jointly in the olive oils subject of study and in their corresponding digestates are given in Table 2. It can be observed that no significant differences have been found between the molar percentages of the different kinds of acyl groups and fatty acids in both olive oils and their digestates. The lack of differences indicates that, if oxidation has taken place, this has not produced variations in the concentration of the different kinds of acyl groups+fatty acids, at a level detectable by means of ^{1}H NMR spectroscopy.

b) Comparison between changes occurred in olive oil with those of corn and virgin flaxseed oils submitted to the same digestive conditions

Table 2 also gives the same data of corn and virgin flaxseed oils and of their digestates, obtained after submission of these oils to *in vitro* digestion under the same conditions as in this study. These data have been taken from previous studies [13,14]. The comparison of the molar percentages of the main unsaturated acyl groups and fatty acids in these two oils and in their corresponding digestates evidence that in both oils oxidation took place during their *in vitro* digestion processes [13,14]. This was evident because the molar percentages of their main unsaturated fatty acids and acyl groups, linoleic in corn oil and linolenic in virgin flaxseed oil, have smaller values in the digestates than in the oils.

Given these results, an important consideration can be made: these olive oils have greater oxidative stability than the corn and virgin flaxseed oils previously studied. If oxidation has taken place during olive oil *in vitro* digestion it has occurred at such a low extent that it does not provokes measurable changes by ^{1}H NMR spectroscopy in their main unsaturated acyl

groups and fatty acids. The higher oxidative stability of the olive oil than that of other oils, including corn and virgin flaxseed oil, has been proved before under accelerated storage conditions [39-41] and it is also confirmed here under *in vitro* digestion conditions, reinforcing the higher quality of these olive oils from this point of view.

c) Effect of olive oil enrichment with phenolic compounds

Taking into account that no degradation of olive oil acyl groups and fatty acids has been detected by ^{1}H NMR spectroscopy, during *in vitro* digestion, or in other words that no oxidation measurable by this technique has been observed, it is to be expected that enrichment of these oils with phenolic compounds will not cause changes in this regard. Data in Table 2 of the molar percentages of the different kinds of fatty acids and acyl groups in oils enriched in the before mentioned phenolic compounds and in their digestates evidence this fact. The lack of oxidation during *in vitro* digestion of these olive oils, measurable by the absence of degradation of the main unsaturated fatty acids and acyl groups, will have as its consequence greater *in vitro* bioaccessibility of the added phenolic compounds than in other oils in which oxidation can take place during *in vitro* digestion. In other words, olive oil enriched in these bioactive compounds could have more beneficial effects on health than oils in which a higher oxidation extent can take place during *in vitro* digestion.

3.2.2. Analysis by ^{1}H NMR of the formation of oxidation compounds during in vitro digestion

In lipid oxidation processes the degradation of fatty acids and acyl groups leads to the formation of primary and of secondary oxidation compounds which can be identified and quantified by ^{1}H NMR, if they are present in enough concentration as to be detected by this technique. It is well known that the first compounds formed in oxidation processes have groups containing hydroperoxy conjugated *Z,E*- or *E,E*-dienes (HPO-c(*Z,E*)-dEs or HPO-c(*E,E*)-dEs) supported on either fatty acids or on acyl groups chains. These primary oxidation

compounds are intermediate compounds in the oxidation process and can evolve to form secondary oxidation compounds. The nature of these secondary oxidation compounds is very varied [39-42], among which there are aldehydes.

a) In olive oils

As Table 2 shows, O_1 and O_2 olive oils before being submitted to *in vitro* digestion do not contain primary oxidation compounds, however both have a small concentration of saturated aldehydes. This does not means that they are oxidized at all. Some olive oils, as is been mentioned in the experimental section, contain a small concentration of these compounds that could have been formed in their production process by action of the lipoxygenases on the oil unsaturated acyl groups [17]. Nevertheless, as is well known, these compounds can also be formed, in absence of these enzymes, in oxidation processes under very varied conditions. The *in vitro* digestion of O_1 oil does not provoke the formation either of primary or of secondary oxidation compounds measurable by ^{1}H NMR spectroscopy. As Table 2 shows, DO_1 does not contain primary oxidation compounds and only n-alkanals are detected in a concentration similar to that found in the oil before digestion. Data in Table 2 indicate that during the *in vitro* digestion of O_2 very little oxidation has taken place due to the digestate DO_2 containing a very low concentration of hydroperoxy conjugated *Z,E*-dienes (HPO-c(*Z,E*)-dEs), which were not present in the oil before digestion. DO_2 also contains n-alkanals but in similar concentrations to those in the original oil O_2. For this reason, their formation could not be attributed to oxidation reactions during *in vitro* digestion. These results are in agreement with the absence of changes in the molar percentage of the main unsaturated fatty acids and acyl groups during digestion of these oils studied by this technique.

b) Comparison between the oxidation compounds formed in olive oil and in corn and virgin flaxseed oils submitted to the same digestive conditions

Table 2 gives data, taken from previous studies [13,14], regarding oxidation compounds, detected by ^{1}H NMR spectroscopy, in corn C and virgin flaxseed F oils and in their digestates, obtained under the same *in vitro* digestion conditions as in this study. It can be observed that both oils C and F are free of oxidation compounds in concentrations detectable by ^{1}H NMR spectroscopy. However their digestates contain primary (HPO-c(*Z,E*)-dEs) and also secondary oxidation compounds (n-alkanals) which proves that during the *in vitro* digestion of these oils oxidation took place. It is worth noting the important concentration of HPO-c(*Z,E*)-dEs in the digestate of corn oil DC. Likewise it is worth highlighting the presence of HPO-c(*Z,E*)-dEs in the digestate of virgin flaxseed oil DF, although in much smaller concentration than in the digestate of corn oil DC and that of alkanals in a similar concentration as in the digestates of both olive oils. Taking into account the much higher unsaturation degree of virgin flaxseed oil than of refined corn oil it could be expected that during the *in vitro* digestion the lipids of the first could reach a higher oxidation degree than the lipids of the second. However, the results indicate the opposite. This higher oxidative stability shown by the virgin flaxseed oil during *in vitro* digestion might be due to a higher content in natural antioxidants than refined corn oil. Again, both olive oils O_1 and O_2 show higher oxidative stability during *in vitro* digestion than the other two oils before mentioned, and this fact is of great importance from the health point of view. It must be remembered that hydroperoxydes can potentially give rise to several disorders, as has been described either in *in vitro* or *in vivo* studies [43,44]. In addition, these oxidation compounds, taking into account the lower oxidative stability of fatty acids than that of acyl groups, presumably are formed on fatty acids, which are absorbable structures. Moreover, they are precursors of a great number of secondary oxidation compounds many of which are considered responsible for

different degenerative diseases [45,46]. Due to the above mentioned, the behaviour of olive oil is again better than that of the other more unsaturated oils and this reinforces the quality attributes of this oil from the health point of view.

c) Effect of olive oil enrichment with phenolic compounds on the formation of oxidation compounds during in vitro digestion

As commented on before the main components of olive oil either do not undergo oxidation during digestion or do so at a very low degree. For this reason, the enrichment of this kind of oil with phenolic compounds, which are able to exhibit antioxidant activity will only be significant from this point of view in those olive oils able to undergo some slight oxidation. Data in Table 2 confirm the above considerations. Olive oil O_1, which is free of hydroperoxides and only contains a basal concentration of aldehydes, when submitted to digestion does not undergo oxidation or if it does, it is so low that it is undetectable by ^{1}H NMR spectroscopy. The main components of olive oil O_2 during digestion undergo a slightly oxidation generating a small concentration of hydroperoxides, able to be detected by ^{1}H NMR spectroscopy in the lipid extract of digestate DO_2. This oxidation is not avoided with the lowest level of enrichment in *gamma*-tocopherol tested (sample $O_2\gamma T_1$), whereas with higher levels, as in samples $O_2\gamma T_2$ and $O_2\gamma T_3$, this is totally avoided. These results are in line with those obtained in previous studies [13,14]. The inhibition of the formation of hydroperoxides during digestion avoids the formation of secondary oxidation compounds of which, as mentioned, some are toxic compounds [45,46]. Bearing all the above mentioned in mind, enrichment with either dodecyl gallate, hydroxytyrosol acetate or *gamma*-tocopherols could turn olive oil into what could be called a functional food, due to the great bioaccessibility of the phenolic added compounds given the almost total absence of oxidation during *in vitro* digestion. At this point, it should be pointed out that different biological activities have been

attributed to these phenolic compounds, such as anticancer, anti-tumoral, anti-inflammatory, inhibition of platelet aggregation, antioxidant, among others [46-52].

3.2.3. Analysis by SPME-GC/MS of the abundance of volatile oxidation markers in the headspace of the digestates

In order to confirm the previous results about the occurrence or not of oxidation during digestion of the olive oils and of its extent, SPME-GC/MS was used. This technique is highly sensitive and can detect volatile compounds in very low abundances. As mentioned before, all edible oils contain a basal concentration of the most known volatile oxidation markers, and their abundance increases if oxidation takes place. For this reason, this methodology is an excellent tool for the objective pursued.

a) In the headspace of olive oil digestates

Table 4 shows the abundances of a large number of oxidation markers of the digestates DO_1 and DO_2 and of the mixtures constituted by the digestive juices submitted to digestion conditions and the corresponding olive oils named O_1DJ and O_2DJ. A comparison between the abundances of volatile oxidation markers in the headspaces of these samples provides information about the occurrence of oxidation during digestion whenever the abundance in the headspace of DO_1 (DO_2) sample is higher than in the headspace of O_1DJ (O_2DJ). As Table 4 shows, oil O_2 has a slightly higher oxidation level than oil O_1, as shown by the higher abundance in all oxidation markers in the headspace of the mixture O_2DJ than in that of the mixture O_1DJ. This subtle difference in oxidation level, which has not been detected by ^1H NMR in the starting oils, is also reflected in the oxidation reached by these oils during digestion. It can be observed that the headspace of DO_2 sample has higher abundances of volatile oxidation markers than that of DO_1. In addition, these results also indicate that during digestion of both olive oils a very slight oxidation has taken place, as Table 4 shows.

b) Comparison between the oxidation markers abundances in the headspace of olive oil digestates and in those of corn and virgin flaxseed oils

In previous studies in which corn and virgin flaxseed oils were submitted to *in vitro* digestion, the headspaces of their digestates DC and DF and of their corresponding mixtures CDJ and FDJ (these latter have the same meaning as O_1DJ and O_2DJ above described), were analyzed to evaluate their abundance in volatile oxidation markers [13,14]. These data [13,14] are also given in Table 4. Although, as previously shown, both abundance and nature of the volatile oxidation markers formed in the oxidation of each kind of oil are closely dependent on oil composition [22], some considerations in this regard can be made from data in Table 4. The headspace of the unoxidized corn (virgin flaxseed) oil mixed with the digestive juices submitted to digestion conditions, which can be considered the sample reference CDJ (FDJ), has a low basal content of oxidation markers in terms of number of compounds and abundance, which is somewhat higher (or of a similar order) than that of O_1DJ, and lower than that of O_2DJ. However, the headspace of its digestate DC (DF) is richer in oxidation compounds than that of both DO_1 and DO_2 digestates, showing that oxidation occurring during olive oil *in vitro* digestion is less than that during corn (virgin flaxseed) oil *in vitro* digestion. This evidences again the higher oxidative stability of olive oils than of corn and virgin flaxseed oils, under the same *in vitro* digestive conditions, which may be considered very relevant from the health point of view due to the toxicity of oxidation compounds.

c) Effect of the enrichment of olive oils with phenolic compounds on the oxidation marker abundances of their digestates

With data from ^{1}H NMR spectroscopy, as Table 2 shows, no effect of the enrichment in phenolic compounds of olive oil O_1 on the potential oxidation of their main components

produced during *in vitro* digestion, was observed. This was due to both the low oxidation provoked during digestion and because this technique is not sensitive enough to detect compounds in very low concentrations. However, SPME-GC/MS is highly sensitive and is able to detect differences in the headspace of the digestates coming from olive oil samples both unenriched and enriched in phenolic compounds. As Table 5 shows, the low oxidation degree provoked during O_1 and O_2 olive oil digestion is clearly diminished with the enrichment in phenolic compounds, the greater the higher the concentration of the phenolic compound. Furthermore, differences between the antioxidant efficiency of the three phenolic compounds added are also evidenced by the abundances of the oxidation markers in the corresponding samples. Again, and in agreement with a previous study [14], data in Table 5 show that dodecyl gallate has higher antioxidant efficiency than hydroxytyrosol acetate, and the lowest antioxidant efficiency, under these *in vitro* digestion conditions, is that of *gamma*-tocopherol.

Table 4. Abundances of some oxidation markers extracted and identified by SPME-GC/MS in the headspace of mixture of digestive juices and olive oils, corn oil and virgin flaxseed oil (O_1DJ, O_2DJ, CDJ and FDJ) and in the digestate of these oils (DO_1, DO_2, DC and DF). Data are expressed as area counts of the mass spectra base peak (Bp) of each compound multiplied by 10^{-6}, and obtained as average of two determinations together with their standard deviations. Data of corn and virgin flaxseed oils were taken from previous studies [13,14].

Compound (molecular weight)	Bp	**O_1DJ**	**DO_1**	**O_2DJ**	**DO_2**	**CDJ**	**DC**	**FDJ**	**DF**
Aldehydes									
Alkanals									
Pentanal (86)*	44	11.2±0.2	41.2±11.8	27.0±3.3	71.7±4.9	17.8±0.9	41.4±1.8	10.6±3.8	66.0±5.6
Hexanal (100)*	44	3.7±0.2	14.2±0.1	18.9±1.9	26.6±0.6	12.9±3.2	76.0±3.1	7.9±2.2	63.2±9.9
Heptanal (114)*	70	0.6±0.1	2.9±0.3	1.7±0.4	2.7±0.2	0.7±0.0	3.0 ±1.2	0.5±0.1	4.1±0.0
Octanal (128)*	41	1.9±0.4	9.9±0.7	7.0±2.1	8.4±0.0	-	-	-	7.7±0.5
Nonanal (142)*	57	2.9±0.2	16.3±3.1	11.9±3.8	12.8±1.0	3.3±0.2	11.4±0.3	3.0±0.8	12.1±1.2
Total		**20.3±1.1**	**84.5±16.0**	**66.5±11.5**	**122.2±6.7**	**34.7±4.3**	**131.8±6.4**	**19.3±6.9**	**152.9±17.2**
(E)-2-Alkenals									
(*E*)-2-Butenal (70)*	70	-	-	-	-	19.5±2.8	20.6±3.4	-	-
(*E*)-2-Butenal-2-methyl (84)	55	1.2±0.0	2.0±0.3	1.4±0.2	1.9±0.2	-	-	-	-
(*E*)-2-Pentenal (84)	41	-	-	-	-	-	-	2.2±0.3	10.7±0.3
(*E*)-2-Hexenal (98)*	41	0.7±0.1	2.2±0.3	2.2±0.1	3.4±0.2	-	-	0.4±0.1	1.4±0.0
(*Z*)-4-Heptenal (112)	41	-	-	-	-	-	-	1.5±0.1	6.1±1.4
(*E*)-2-Heptenal (112)	41	0.8±0.1	6.8±0.9	2.3±0.0	9.4±2.1	2.3±0.3	47.4±8.1	-	-
(*E*)-2-Nonenal (140)*	55	0.4±0.0	1.4±0.1	0.5±0.0	1.9±0.4	-	1.2±0.2	-	0.3±0.0
Total		**3.1±0.2**	**12.4±1.6**	**6.4±0.3**	**16.6±2.9**	**21.8±3.1**	**69.2±11.7**	**4.1±0.5**	**18.5±1.7**
2,4-Alkadienals									
(*E,E*)-2,4-Hexadienal (96)*	81	0.5±0.0	1.6±0.4	3.1±0.4	8.3±0.7	-	-	-	2.9±0.2
(*Z,E*)-2,4-Heptadienal (110)	81	0.6±0.2	2.6±0.1	0.3±0.0	2.3±0.3	-	-	3.3±0.9	19.7±0.6

Compound (molecular weight)	Bp	**O_1DJ**	**DO_1**	**O_2DJ**	**DO_2**	**CDJ**	**DC**	**FDJ**	**DF**
(*E*,*E*)-2,4-Heptadienal (110)*	81	-	1.3±0.1	-	1.0±0.0	-	-	2.2±0.4	21.3±0.7
(*E*,*E*)-2,4-Nonadienal (138)	81	-	-	-	-	-	1.0±0.1	-	-
(*Z*,*E*)-2,4-Decadienal (152)	81	-	-	-	-	-	0.6±0.0	-	-
(*E*,*E*)-2,4-Decadienal (152)*	81	-	-	-	-	-	0.8±0.1	-	-
Total		**1.1±0.2**	**5.5±0.6**	**3.4±0.4**	**11.6±1.0**	**-**	**2.4±0.2**	**5.5±1.3**	**43.9±1.5**
Furan derivatives									
Furan, 2-ethyl (96)*	81	-	-	0.2±0.0	0.6±0.0	-	-	1.9±0.0	6.3±2.6
Furan, 2-butyl (124)	81	-	-	-	-	-	0.5±0.0	0.3±0.0	0.5±0.1
Furan, 2-pentyl (138)*	81	1.7±0.4	14.5±0.9	2.8±0.8	13.2±0.2	4.7±1.4	21.5±1.5	4.2±1.1	11.7±0.7
Total		**1.7±0.4**	**14.5±0.9**	**3.0±0.8**	**13.8±0.2**	**4.7±1.4**	**22.0±1.5**	**6.4±1.1**	**18.5±3.4**

*Asterisked compounds were acquired commercially and used as standards for identification purposes; -: not detected.

Table 5. Abundances of some oxidation markers extracted and identified by SPME-GC/MS in the headspace of digestates of non-enriched olive oils (DO_1 and DO_2) and in those of the samples enriched with dodecyl gallate, hydroxytyrosol acetate and *gamma*-tocopherol (DO_1DG_1, DO_1DG_2, DO_1HTA_1, DO_1HTA_2, $DO_2\gamma T_1$, $DO_2\gamma T_2$ and $DO_2\gamma T_3$) whose level of enrichment in phenolic compounds is given in brackets in mmol/mol $(AG+FA)_O$.. Data are expressed as area counts of the mass spectra base peak (Bp) of each compound multiplied by 10^{-6}, and obtained as average of two determinations together with their standard deviations.

Compound (molecular weight)	**Bp**	**DO_1**	**DO_1DG_1 (0.12)**	**DO_1DG_2 (1.36)**	**DO_1HTA_1 (0.28)**	**DO_1HTA_2 (2.53)**	**DO_2**	**$DO_2\gamma T_1$ (0.11)**	**$DO_2\gamma T_2$ (1.17)**	**$DO_2\gamma T_3$ (12.58)**
Aldehydes										
Alkanals										
Pentanal (86)*	44	41.2±11.8	14.9±4.0	15.6±0.1	32.7±5.0	22.2±1.9	71.7±4.9	73.1±15.0	50.0±0.2	34.7±0.6
Hexanal (100)*	44	14.2±0.1	5.7±0.3	5.6±1.5	11.3±1.3	8.4±0.3	26.6±0.6	23.0±1.1	19.6±1.0	16.8±0.2
Heptanal (114)*	70	2.9±0.3	1.1±0.2	1.1±0.0	1.0±0.0	1.7±0.3	2.7±0.2	2.6±0.2	2.0±0.2	1.5±0.2
Octanal (128)*	41	9.9±0.7	5.2±0.1	3.7±0.1	3.7±0.1	6.1±0.6	8.4±0.0	10.4±0.5	8.0±0.3	-
Nonanal (142)*	57	16.3±3.1	6.1±0.5	6.3±0.3	6.0±0.4	10.2±1.6	12.8±1.0	11.3±1.0	9.2±0.4	7.9±0.0
Total		**84.5±16.0**	**33.0±5.1**	**32.3±2.0**	**54.7±6.8**	**48.6±4.7**	**122.2±6.7**	**120.4±17.8**	**88.8±2.1**	**60.9±1.0**
(E)-2-Alkenals										
(*E*)-2-Butenal-2-methyl (84)	55	2.0±0.3	1.3±0.3	2.4±0.3	2.1±0.2	1.9±0.5	1.9±0.2	2.4±0.4	2.0±0.2	1.3±0.5
(*E*)-2-Hexenal (98)*	41	2.2±0.3	0.9±0.2	0.8±0.2	1.5±0.2	0.8±0.1	3.4±0.2	3.2±0.1	2.6±0.2	2.1±0.4
(*E*)-2-Heptenal (112)	41	6.8±0.9	2.3±0.2	2.2±0.1	2.2±0.2	2.7±0.6	9.4±2.1	8.8±0.1	5.8±0.4	3.8±0.4
(*E*)-2-Nonenal (140)*	55	1.4±0.1	0.6±0.1	0.8±0.1	1.0±0.1	0.9±0.0	1.9±0.4	2.0±0.2	1.0±0.0	1.0±0.0
Total		**12.4±1.6**	**5.1±0.8**	**6.2±0.7**	**6.8±0.7**	**6.3±1.2**	**16.6±2.9**	**16.4±0.8**	**11.4±0.8**	**8.2±1.3**
2,4-Alkadienals										
(*E,E*)-2,4-Hexadienal (96)*	81	1.6±0.4	1.1±0.2	0.6±0.2	1.1±0.2	0.4±0.1	8.3±0.7	8.9±0.1	7.0±1.8	5.4±0.7
(*Z,E*)-2,4-Heptadienal (110)	81	2.6±0.1	1.3±0.3	0.9±0.0	2.0±0.2	1.5±0.4	2.3±0.3	2.4±0.1	1.8±0.3	1.0±0.0
(*E,E*)-2,4-Heptadienal (110)*	81	1.3±0.1	0.5±0.1	-	0.5±0.0	0.4±0.0	1.0±0.0	1.2±0.1	0.9±0.2	0.6±0.2
Total		**5.5±0.6**	**2.9±0.6**	**1.5±0.2**	**3.6±0.4**	**2.3±0.5**	**11.6±1.0**	**12.5±0.3**	**9.7±2.3**	**7.0±0.9**

Compound (molecular weight)	**Bp**	**DO_1**	**DO_1DG_1 (0.12)**	**DO_1DG_2 (1.36)**	**DO_1HTA_1 (0.28)**	**DO_1HTA_2 (2.53)**	**DO_2**	**$DO_2\gamma T_1$ (0.11)**	**$DO_2\gamma T_2$ (1.17)**	**$DO_2\gamma T_3$ (12.58)**
Furan derivatives										
Furan, 2-ethyl (96)*	81	-	-	-	-	-	0.6±0.0	0.5±0.1	0.2±0.1	-
Furan, 2-pentyl (138)*	81	14.5±0.9	5.4±1.3	5.3±0.5	8.7±1.2	6.1±0.1	13.2±0.2	11.9±1.0	11.4±1.2	11.2±0.7
Total		**14.5±0.9**	**5.4±1.3**	**5.3±0.5**	**8.7±1.2**	**6.1±0.1**	**13.8±0.2**	**12.4±1.1**	**11.6±1.3**	**11.2±0.7**

*Asterisked compounds were acquired commercially and used as standards for identification purposes; -: not detected.

3.3. In vitro bioaccessibility of minor compounds involved in the vitro digestion of unenriched or enriched olive oil in phenolic compounds

Not only is the bioaccessibility of oil main components a very important indicator from the nutritional point of view but so is that of the oil minor components because edible oils are vehicles of vitamins and of other bioactive compounds some of which have very interesting properties from the health point of view. Likewise, it is very important to know the fate during *in vitro* digestion of the added polyphenols and that of compounds formed in this process. For these reasons, the *in vitro* bioaccessibility of different kinds of compounds before mentioned was estimated when it was possible with the techniques used in this study.

3.3.1. In vitro bioaccessibility of olive oils minor components

Olive oils contain squalene, sterols such as cycloartenol and 24-methylencycloartenol. Both kinds of compounds can be detected and quantified by ^{1}H NMR spectroscopy, in olive oils and in their digestates. Furthermore, olive oils also contain, among other minor components, a certain number of terpenes and sesquiterpenes, which can be identified, and semiquantified by SPME-GS/MS in the headspace of both olive oils and in that of their digestates. With these data the *in vitro* bioaccessibility of all these compounds can be estimated.

Using ^{1}H NMR, the quantification of squalene, in both olive oils enriched or not in phenolic compounds and in their corresponding digestates, was carried out by using the area of the singlet at 1.67 ppm due to its methylenic protons in carbon atoms C-1 and C-24, indicated in Table S5. The results prove that its concentration (near 4.76 mmol/mol [AG+FA])) is the same in olive oils and in their digestates, which means that 100 % of squalene remains bioaccessible after *in vitro* digestion. That is to say, B = 4.76 mmol/mol [AG+FA] and B´= 1. This is of great importance since antioxidant, cardioprotective and anti-carcinogenic activities have been attributed to this compound [53].

Likewise, the joint quantification of sterols such as cycloartenol and 24-methylencycloartenol, either free or esterified, was also carried out in both olive oils, enriched or not with phenolic compounds, and in their corresponding digestates, by means of ^{1}H NMR spectral data. For this purpose, the area of the triplet centered at 0.33 ppm, due to the overlapping of the doublets of these compounds shown in Table S5, was used. The concentration of these compounds in olive oil is near 0.30 mmol/mol [AG+FA] and their concentration remains unchanged after the *in vitro* digestion, being 100% bioaccessible afterwards. That is to say, B = 0.30 mmol/mol [AG+FA] and B´= 1. These results are in line with those observed in a previous study on the *in vitro* digestion of virgin flaxseed oil [14]. This is an important fact, since to these compounds have been attributed with, among other beneficial biological activities, anti-cancer, anti-obesity and anti-inflammatory activities [54-56].

As aforementioned some terpenes and sesquiterpenes are also present in olive oils. The bioaccessibility of these compounds can be estimated, using SPME-GC/MS, by the ratio between their abundances in the headspaces of the digestates and of the reference samples. Analysis of the results obtained indicates that the abundances of these compounds in the headspaces of the digestates of the olive oils enriched with different concentrations of each phenolic compound have very similar values, for which reason they are given in Table 6 as average values. As Table 6 shows, the abundances of these compounds are significantly higher in the headspace of all digestates than in O_1DJ or O_2DJ mixtures (which are the reference samples), probably due to the matrix effect. Furthermore, no statistically significant differences were found between the abundances in the headspace of the digestates of the oil samples both unenriched and enriched in phenolic compounds, which means that the abundances of terpenes and sesquiterpenes are not affected by the several reactions that take place during *in vitro* digestion. For this reason, their B´= 1. The behaviour of this kind of

compounds on this occasion agrees with that observed during the *in vitro* digestion of virgin flaxseed oil [14]. The great *in vitro* bioaccessibility of terpenes and of sesquiterpenes is of great importance because some health beneficial properties have been attributed to them [57].

Table 6. Terpenes and sesquiterpenes of olive oil, detected by SPME-GC/MS in the headspaces of the mixture of digestive juices submitted to digestive conditions and olive oils (O_1DJ and O_2DJ), of the digestate of these oils (DO_1 and DO_2) and of the samples enriched with different levels of dodecyl gallate, hydroxytyrosol acetate, and *gamma*-tocopherol (DO_1DG, DO_1HTA , $DO_2\gamma$T). Data are average abundances expressed as area counts of the mass spectra base peak (Bp) of each compound multiplied by 10^{-6}, together with their standard deviations. For samples enriched with phenolic compounds data given are average values of the abundances of the headspace of digestates coming from samples having different enrichment levels of phenolic compounds. Different letters within each row of each oil indicate statistically significant differences among the samples ($p < 0.05$).

Terpenes and sesquiterpenes	**Bp**	**O_1DJ**	**DO_1**	**$DO_1DG_{average}$**	**$DO_1HTA_{average}$**	**O_2DJ**	**DO_2**	**$DO_2\gamma T_{average}$**
alpha-pinene*	93	0.1±0.0a	0.2±0.1b	0.2±0.0b	0.2±0.0b	0.1±0.0a	0.3±0.0b	0.3±0.0b
Limonene*	68	0.4±0.0a	2.0±0.3b	2.2±0.3b	2.1±0.2b	0.6±0.0a	3.5±0.2b	3.5±0.4b
beta-ocimene*	93	0.2±0.0a	0.3±0.0b	0.3±0.0b	0.4±0.1b	0.3±0.1a	0.6±0.1b	0.6±0.1b
alpha-gurjunene	93	0.1±0.0a	0.2±0.0b	0.2±0.0b	0.2±0.0b	0.1±0.0a	0.2±0.0b	0.2±0.0b
alpha-copaene	119	0.1±0.0a	0.4±0.0b	0.4±0.0b	0.4±0.0b	0.2±0.0a	0.5±0.0b	0.5±0.0b
alpha-guaiene	93	0.1±0.0a	0.2±0.0b	0.2±0.0b	0.3±0.0b	0.1±0.0a	0.3±0.0b	0.3±0.0b
alpha-farnesane	161	0.2±0.0a	0.5±0.0b	0.5±0.1b	0.5±0.1b	0.2±0.0a	0.6±0.0b	0.5±0.0b

*Asterisked compounds were acquired commercially and used as standards for identification purposes.

3.3.2. In vitro bioaccessibility of the phenolic added compounds

The concentration of dodecyl gallate and hydroxytyrosol acetate added to olive oil O_1, can be determined, in both olive oils and in the lipid extracts of their digestates if they are present, by ^{1}H NMR, by using the area of non-overlapped signals given in Table S5. Furthermore, due to the very low extent of oxidation undergone by oil O_1 during digestion, it should be expected that the concentration of both phenolic compounds would remain almost unaffected by the *in vitro* digestion. However, no signals of any of the two phenolic compounds were found in the spectra of the lipid extracts of the digestates DO_1DG_1, DO_1DG_2 and DO_1HTA_2 and only signals of hydroxytyrosol acetate were detected in the spectrum of the lipid extract of the digestate of the more enriched oil in this compound, that is to say in DO_1HTA_2. Their absence in the lipid extracts of the above mentioned digestates could not be attributed to reactions between these phenolic compounds with digestive enzymes because the lipolysis reached, in presence or in absence of these phenolic compounds, is of a similar order. Furthermore, although some reactions between phenolic compounds and aldehydes have been described, they are not very common reactions [58]. For these reasons, the absence of these two phenolic compounds in DO_1DG_1, DO_1DG_2 and DO_1HTA_2 digestates could be attributed to their hydrolysis because both are esters. In fact, some previous studies on hydroxytyrosol alkyl esters have described their partial hydrolysis under digestion conditions [59,60], and although, to the best of our knowledge, the hydrolysis of alkyl gallates in digestion has not been reported, this cannot be discarded. Hydrolysis of these phenolic esters yields very polar compounds that remain in the aqueous fraction of the digestates, for which reason they could not be detected in the lipid extract of the digestates, as in a previous study [14]. It only remains to add that the hydrolyzed derived compounds from these phenolic esters (gallic acid and hydroxytyrosol) are also bioactively healthy compounds.

However, as mentioned before, hydroxytyrosol acetate was detected in the lipid extract of the digestate of the most enriched olive oil sample, DO_1HTA_2, probably due to its partial hydrolysis, and *gamma*-tocopherol was also detected in the lipid extracts $DO_2\gamma T_1$, $DO_2\gamma T_2$ and $DO_2\gamma T_3$, due to its liposolubility. Their concentrations were estimated from the area of the ^{1}H NMR signals indicated in Table S5, and with these data the *in vitro* bioaccessibility of these phenolic compounds was determined. As explained in the experimental section, *in vitro* bioaccessibility can be defined by the ratio between the concentration of the phenolic compound, PC, in the digestate $[PC]_D$, given in mmoles, and the concentration of the main components also in the digestate $[FA+AG]_D$, given in moles, by the equation $B_{PC}=[PC]_D/[FA+AG]_D$. This definition gives direct information about the concentration of these bioactive compounds in the digestate, and so is comparable with that of the minor and main oil components. Table 7 gives the bioaccessibilities B_{PC} thus defined for hydroxytyrosol acetate in DO_1HTA_2 and for *gamma*-tocopherol in $DO_2\gamma T_1$, $DO_2\gamma T_2$ and $DO_2\gamma T_3$. As expected, these values are higher the higher the enrichment degree in the oils before digestion. Likewise, *in vitro* bioaccessibility can also be defined by the ratio between the concentration of the compound in the lipid extract of the digestate $[PC]_D$ and the concentration in the oil before digestion, $[PC]_O$, both in the same units, as indicated in the equation $B'_{PC}= [PC]_D/[PC]_O$. This definition gives information about the loss of the compound during *in vitro* digestion, or about the fraction of the phenolic compound added to the oil that remain in the digestate. B'_{PC} data of the same compounds above mentioned are given in Table 7. It is noteworthy that in all cases the loss of these phenolic compounds during *in vitro* digestion of olive oils is smaller than during *in vitro* digestion of corn and virgin flaxseed oils enriched with similar concentrations of these phenolic compounds [13,14]. These results are in agreement with the lower oxidation extent occurring during *in vitro* digestion of olive oil than in that of corn and virgin flaxseed oil mentioned in previous

sections. The higher *in vitro* bioaccessibility of hydroxytyrosol acetate and of *gamma*-tocopherol in olive oil than in the other oils that have higher unsaturation degree is an important fact. It must be remembered that hydroxytyrosol acetate has been attributed beneficial health effects, such as, anti-inflammatory, antioxidant and ability to inhibit of platelet aggregation [48,61]. Likewise, many health beneficial biological activities have been attributed to *gamma*-tocopherol [46,51,52]. For all these reasons, it seems evident that olive oil is a more suitable matrix or carrier than other oils when designing functional foods enriched in liposoluble beneficial health bioactive compounds due their greater preservation e during *in vitro* digestion.

Table 7. Bioaccessibility of hydroxytyrosol acetate (HTA) and *gamma*-tocopherol (γT) in the digestates of the different samples in which these compounds are present, expressed in two different ways. $B_{=}$ (mmol PC_D/mol $(AF+GA)_D$) and $B'_{=}$ (mmol PC_D/mmol PC_O). Values are the average of two determinations together with their standard deviations.

Samples	B_{HTA}	B'_{HTA}	$B_{\gamma T}$	$B'_{\gamma T}$
DO_1HTA_2	1.63±0.03	0.64±0.01	n.a.	n.a.
$DO_2\gamma T_1$	n.a.	n.a.	0.07±0.00	0.63±0.01
$DO_2\gamma T_2$	n.a.	n.a.	0.85±0.02	0.72±0.02
$DO_2\gamma T_3$	n.a.	n.a.	10.57±0.11	0.84±0.01

n.a.: not applicable

3.3.3. In vitro bioaccessibility of compounds formed in secondary reactions during in vitro digestion and potential consequences

In this study and in others previous [9-14] oxidation has been considered as the main secondary reaction that takes place during *in vitro* digestion. It has been proved in previous sections that the oxidation extent occurring during the *in vitro* digestion of both olive oils subject of study is much smaller than that occurring during digestion of other more unsaturated oils such as corn and virgin flaxseed oils. As Table 2 shows, either no hydroperoxydes (primary oxidation compounds) have been detected in the olive oil digestate

DO_1 or they have been detected in DO_2 digestate, in a much smaller concentration than in the digestates of the other oils such as DC and DF. This concentration represents the bioaccessibility B of hydroperoxydes in each digestate. Furthermore, as already mentioned, these hydroperoxydes could be expected to be absorbable, with the consequent negative effects on human health, because presumably oxidation during digestion will be produced in fatty acids rather than in acyl groups, due to the lower oxidative stability of the former.

In addition, as Table 4 shows, a higher concentration of aldehydes are present in the headspace of virgin flaxseed and corn oil digestates than in that of olive oils. All these aldehydes, although in low concentration in the case of digestates of olive oil, are also bioaccessible and susceptible to reacting either with nitrogenated compounds through Maillard-type reactions or with other structures through typical aldehyde group reactions. Finally, one consideration can be made regarding the formation of aldehydes as consequence of the oxidation during digestion. As above mentioned the higher the unsaturation of the oil the greater the formation of aldehydes during *in vitro* digestion and so a smaller lipolysis extent is produced. This suggest that perhaps the ability of aldehydes to react with nitrogenated compounds such as proteins, and for this reason with enzymes, could affect lipase activity negatively. The immediate consequence could be the diminution in the lipolysis extent during digestion, in those oils which tend to be oxidized more, or in other words have lower oxidative stability and a greater facility to generate aldehydes during digestion.

4. CONCLUSIONS

The lipolysis extent produced during the *in vitro* digestion of olive oil is high, yielding an important release of monoglycerides and fatty acids, and as consequence, the *in vitro* bioaccessibility of the olive oil main components is also high, and both are greater than of

those edible oils having higher unsaturation degrees, such as corn oil and virgin flaxseed oil. For the first time very, close quantitative relationships have been found between composition of the oil, expressed in molar percentages of the different kinds of acyl groups, and its *in vitro* bioaccessibility. It has been proved that the *in vitro* bioaccessibility of the oil main components is directly related with the concentration in the oil of saturated and of oleic acyl groups, the weight of the first being almost ten times higher than that of the second. Likewise, it has been proved that *in vitro* bioaccessibility of the oil main components is inversely related with the concentration of the linolenic and of linoleic acyl groups in the oil, the weight of the former being double than that of the latter. Some of the obtained equations have predictive ability and are able to predict with a high level of approximation the *in vitro* bioaccessibility of the main components of oils not involved in the development of these equations. It has also been demonstrated that the intake of a similar amount of edible oils of different composition in acyl groups, can have very different nutritional effects, caused by, among other reasons, the different lipolysis extent reached during digestion. These differences in lipolysis extent have as their consequence differences in the bioaccessibility of the oil main components that could be absorbed through the intestinal wall. The models developed that relate *in vitro* bioaccessibility of oil main components and oil composition provide a very valuable tool for designing diets for very different purposes and for special needs in which high or low lipid bioaccessibility may be required. Enrichment of olive oil with different concentrations of phenolic bioactive compounds, such as dodecyl gallate, hydroxytyrosol acetate or *gamma*-tocopherol, does not modify either the lipolysis extent reached during *in vitro* digestion or the *in vitro* bioaccessibility of oil main components. It has been demonstrated that, unlike other polyphenolic compounds, these phenolic compounds do not inhibit lipase activity.

Furthermore, the oxidation extent reached during the *in vitro* digestion of olive oil is very small, and in some cases is undetectable by ^{1}H NMR spectroscopy but only by the abundance of volatile oxidation markers analyzed by the very sensitive SPME-GC/MS technique. In all cases, the oxidation extent reached during *in vitro* digestion of olive oils is much smaller than that reached during the digestion of other edible oils such as corn and virgin flaxseed oil, with the consequent repercussions on health due to the toxicity of oxidation compounds. Enrichment of olive oils with phenolic compounds either reduces the oxidation extent produced during *in vitro* digestion to minimum values or prevents it almost totally. It has been demonstrated again that the antioxidant efficiency of these compounds is in line with the number of phenolic groups in its molecule, which means that dodecyl gallate has higher antioxidant efficiency than hydroxytyrosol acetate, and the lowest antioxidant efficiency, under these *in vitro* digestion conditions, is that of *gamma*-tocopherol.

It has been shown that the concentration of minor olive oil components such as squalene, some sterols, as well as terpenes and sesquiterpenes is not modified during the *in vitro* digestion, these being totally bioaccessible after digestion. This is of great importance due to the different beneficial bioactive capabilities attributed to most of them. The *in vitro* bioaccessibility of the added phenolic compounds, which are very interesting bioactive compounds, is higher in the digestate of olive oil than in those of corn and virgin flaxseed oil. This could be related to the lower oxidation extent produced during digestion of olive oil than during digestion of corn or virgin flaxseed oil. Nevertheless, estimation of the *in vitro* bioaccessibility of dodecyl gallate and of hydroxytyrosol acetate, this latter at the lower concentration essayed, was not possible, probably due to their hydrolysis during digestion which yields very bioactive polar compounds that remain in the aqueous fraction of the digestate. Finally, due to the very low oxidation level reached during *in vitro* digestion of olive oil, the bioaccessibility of the oxidation compounds formed, if any, is much more

smaller in the digestate of olive oil than in those of corn and virgin flaxseed oil with the consequent repercussions on health.

Among these oxidation compounds there are aldehydes that are generated in much more abundance in the oils having higher unsaturation degree. The great ability of aldehydes to react with nitrogenated compounds such as proteins, by reactions of a Maillard type, and for this reason with enzymes including lipases, could be underlined as a potential cause of the smaller lipolysis extent produced during the *in vitro* digestion of the most unsaturated oils.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Composition and pH values of the juices employed in the *in vitro* digestion model employed in this study; Table S2: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of glycerides; Table S3: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of acyl groups and fatty acids; Table S4: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some oxidation compounds detected in the digestates and formed during the *in vitro* digestion; Table S5: Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of cycloartenol and methylencycloartenol, esters of cycloartenol and methylencycloartenol, squalene, *gamma*-tocopherols, hydroxytyrosol acetate and dodecyl gallate detected in the samples before and after *in vitro* digestion; Table S6: Correlation matrix between the molar percentages of the different kinds of acyl groups present in the four oils involved in this study; Operating conditions for the acquisition of the ^{1}H NMR Spectra; Equations used for the quantification from ^{1}H NMR spectral data of several compounds present in the starting samples and/or in the lipid extract of the digestates; Operating conditions for the SPME-GC/MS Experiments.

AUTHOR CONTRIBUTIONS

J.A-C.; performed the experimental work, contributed to data interpretation and to manuscript preparation. M.L.I.; supervised the analyses performed and contributed to data interpretation and to manuscript preparation. M.D.G.; conceived the work, supervised the

whole work and the results obtained, and contributed to data interpretation and to the manuscript preparation. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Supplementary Material of

Manuscript 8

STUDY OF THE *IN VITRO* DIGESTION OF OLIVE OIL ENRICHED OR NOT WITH ANTIOXIDANT PHENOLIC COMPOUNDS. RELATIONSHIPS BETWEEN BIOACCESSIBILITY OF MAIN COMPONENTS OF DIFFERENT OILS AND THEIR COMPOSITION

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Table S1. Composition and pH values of the juices employed in the *in vitro* digestion model employed in this study.

Components	**Saliva**	**Gastric juice**	**Duodenal juice**	**Bile juice**
KCl (mmol/L)	12.02	11.06 47.09	7.57	5.05
NaCl (mmol/L)	5.10	-	119.98	89.99
$NaHCO_3$ (mmol/L)	20.17	0.22	40.33	68.86
NaH_2PO_4 (mmol/L)	7.40	5.72	-	-
NH_4Cl (mmol/L)	-	-	-	-
KH_2PO_4 (mmol/L)	-	-	0.59	-
Na_2SO_4 (mmol/L)	4.79	-	-	-
KSCN (mmol/L)	2.06	-	-	-
$MgCl_2$ (mmol/L)	-	2.72	0.53	-
$CaCl_2*2H_2O$ (mmol/L)	-	6.50	1.36	1.51
HCl (37%) (mL/L)	-	1.42	0.18	0.15
Urea (mmol/L)	3.33	3.61	1.67	4.16
Glucose (mmol/L)	-	0.10	-	-
Glucuronic acid (mmol/L)	-	-	-	-
Uric acid (mmol/L)	0.09	1.53	-	-
Glucoseamine hydrochloride (mmol/L)	-	1.00	1.00	1.80
Bovine serum albumin (g/L)	-	3.00	-	-
Mucin (g/L)	0.025	-	-	-
A. oryzae α-amylase (g/L)	0.29	100	-	-
A. niger lipase (U/mL)	-	2.50	-	-
Pepsin (g/L)	-	-	9.00	-
Pancreatin (g/L)	-	-	1.50	18.75
Lipase type II from porcine pancreas (g/L)	-	-	-	
Bovine bile extract (g/L)	-	-		
pH	6.9±0.0	1.4±0.1	8.1±0.0	8.2±0.1

Table S2. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of glycerides. TG: triglycerides; DG: diglycerides; MG: monoglycerides.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Structures
			Glycerides structure protons	
I	3.65	ddd	ROCH$_2$–CHOH–C**H$_2$**OH	glyceryl group in **1-MG**
J	3.73	m*	ROCH$_2$–CH(O*R*')–C**H$_2$**OH	glyceryl group in **1,2-DG**
K	3.84	m*	HOC**H$_2$**–CH(O*R*)–C**H$_2$**OH	glyceryl group in **2-MG**
L	3.94	m	ROCH$_2$–C**H**OH–CH$_2$OH	glyceryl group in **1-MG**
M	4.05–4.21	m	ROC**H$_2$**–C**H**OH–C**H$_2$**OR'	glyceryl group in **1,3-DG**
N	4.18	ddd	ROC**H$_2$**–CHOH–CH$_2$OH	glyceryl group in **1-MG**
O	4.22	dd,dd	ROC**H$_2$**–CH(O*R*')–C**H$_2$**OR''	glyceryl group in **TG**
P	4.28	ddd	ROC**H$_2$**–CH(O*R*')–CH$_2$OH	glyceryl group in **1,2-DG**
Q	4.93	m	HOCH$_2$–C**H**(O*R*)–CH$_2$OH	glyceryl group in **2-MG**
R	5.08	m	ROCH$_2$–C**H**(O*R*')–CH$_2$OH	glyceryl group in **1,2-DG**

Abbreviations: d: doublet; m: multiplet.

*This signal shows different multiplicity if the spectrum, is acquired from the pure compound or taking part in the mixture.

**The intensity of some of these signals, together with signal F of Table S3, were used to estimate the molar percentages of different kinds of glyceryl structures using the equations [eq. S1-eq. S10].

***The assignment of the ^{1}H NMR signals of the protons was made as in previous studies (Guillén & Uriarte, 2012a; Nieva-Echevarría et al., 2014).

Table S3. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of acyl groups and fatty acids. AG: acyl groups; FA: fatty acids.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Structures
Main acyl groups (AG) and fatty acids (FA)				
A	0.88	t	–C**H**$_3$	saturated and monounsaturated **ω-9** in **AG** and **FA**
	0.89	t	–C**H**$_3$	linoleic in **AG** and **FA**
B	0.97	t	–C**H**$_3$	linolenic in **AG** and **FA**
C	1.19–1.42	m**	–(C**H**$_2$)$_n$–	**AG** and **FA**
D	1.61	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **TG**
	1.62	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **1,2-DG**
	1.63	m	–OCO–CH$_2$–C**H**$_2$–, COOH–CH$_2$–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	1.64	m	–OCO–CH$_2$–C**H**$_2$–	**AG** in **2-MG**
E*	1.92–2.15	m***	–C**H**$_2$–CH=CH–	**AG** and **FA**
F*	2.26–2.36	dt	–OCO–C**H**$_2$–	**AG** in **TG**
	2.33	m	–OCO–C**H**$_2$–	**AG** in **1,2-DG**
	2.35	t	–OCO–C**H**$_2$–, COOH–C**H**$_2$–	**AG** in **1,3-DG**, **1-MG** and **FA**
	2.38	t	–OCO–C**H**$_2$–	**AG** in **2-MG**
G*	2.77	t	=HC–C**H**$_2$–CH=	linoleic in **AG** and **FA**
H*	2.80	t	=HC–C**H**$_2$–CH=	linolenic in **AG** and **FA**

Abbreviations: d: doublet; t: triplet; m: multiplet.

*The intensity of these signals was used to estimate the molar percentage of the main acyl groups plus fatty acids by using equations [eq. S11-eq. S14].

**Overlapping of multiplets of methylenic protons in the different acyl groups either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group.

***Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups.

****The assignment of the ^{1}H NMR signals of the protons was made as in previous studies (Guillén & Ruiz, 2003; Nieva-Echevarría et al., 2014).

Table S4. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of some oxidation compounds detected in the digestates and formed during the *in vitro* digestion.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Structures
Oxidation Compounds (OC)				
Conjugated dienic systems associated with hydroperoxy groups				
-	5.51	dtm	–C**H**=CH–CH=CH–	(*Z,E*)-conjugated double bonds associated with hydroperoxy group (OOH) in octadecadienoic **AG** and **FA** **HPO-c(*Z,E*)-dEs**
-	5.56	ddm		
-	6.00	ddtd		
b	***6.58***	dddd		
Aldehydes				
f	***9.75***	t	-C**H**O	**n-alkanals**
	2.40	dt	-CH_2-	

Abbreviations: d: doublet; t: triplet; m: multiplet.

*The intensities of the signals indicated in bold, together with signal D of Table S3, were used to estimate the concentration (mmol/molAG+FA) using the equation [eq. S15].

**The assignment of the ^{1}H NMR signals of the protons was made with the aid of standard compounds and with the data taken from literature (Guillén & Ruiz, 2005).

Table S5. Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of protons of cycloartenol and methylencycloartenol, esters of cycloartenol and methylencycloartenol, *gamma*-tocopherols, hydroxytyrosol acetate and dodecyl gallate detected in the samples before and after *in vitro* digestion.

Signal	Chemical shift (ppm)	Multiplicity	Type of protons	Compounds
Sterols				
4,4´-DiMe-St.	***0.33*********	d	-C**H**$_2$- (exo, C-19)	Cycloartenol/ 24-Methylenecycloartenol
4,4´-DiMe-St´.	***0.34*********	d	C**H**$_2$- (exo, C-19)	Esters of Cycloartenol/ 24-Methylenecycloartenol
Squalene				
SQ	***1.67*********	s	-C**H**$_3$ (C-1; C-24)	Squalene
***gamma*-Tocopherol**				
γT	***6.36*********	s	-C**H** (C-5)	*gamma*-tocopherol
Hydroxytyrosol acetate				
HTA	6.60	dd	Ar**H** (C-8)	Hydroxytyrosol acetate
	6.75******	d	Ar**H** (C-4)	
	6.78	d	Ar**H** (C-7)	
Dodecyl gallate				
DG	***7.20*********	s	Ar**H** (C-3; C-7)	Dodecyl gallate

Abbreviations: s: singlet; d: doublet.

*The intensity of these signals, together with signal D of Table S3, were used to estimate the concentration (mmol/molAG+FA) using the equation [eq. S15].

**Assignment was made with the aid of standard compounds and with the data taken from the literature (Baker & Mayers, 1991; Pogliani et al., 1994; Kubo et al., 2002; Kawai et al., 2007).

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Table S6. Correlation matrix between the molar percentages of the different kinds of acyl groups, linoleic (%L), linolenic (%Ln), oleic (%O) and saturated (%S), present in the four oils involved in this study.

	%L	**%Ln**	**%O**	**%S**
%L	1.0000	0.1913	0.5363	0.2042
%Ln	0.1913	1.0000	0.7259	- 0.9986
%O	0.5363	0.7259	1.0000	0.7153
%S	0.2042	- 0.9986	0.7153	1.0000

Operating Conditions for the Acquisition of the ^{1}H NMR Spectra

The ^{1}H NMR spectra were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz. For this purpose, the samples (approximately 0.16 g) were dissolved in 400 μL of deuterated chloroform, which contained tetramethylsilane (TMS), as internal reference (Cortec, Paris, France). First, a standard ^{1}H NMR spectrum was acquired and in a second step, a NOESYGPPS experiment consisting of a one-dimensional ^{1}H NMR pulse sequence with selective suppression of certain strong signals was carried out. This NOESYGPPS experiment allows one to obtain a ^{1}H NMR spectrum with a greater sensitivity than that of the standard single pulse ^{1}H NMR experiment (Ruiz-Aracama et al., 2017) in the spectral region from 5.8 to 9.8 ppm, at the cost of suppressing some signals in other regions. The relaxation and acquisition times used allow the complete relaxation of the protons, the signal areas thus being proportional to the number of protons that generate them, except in the suppressed signals, making it possible to use them for quantitative purposes as in previous studies (Guillén & Uriarte, 2012).

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Quantification from ^{1}H NMR spectral data of several compounds present in the starting samples and/or in the lipid extracts of the digestates.

A. Equations used to estimate the molar percentage (%) of the several glyceride structures present in the lipid extract of digestates and the glycerol. In these equations the number of moles (N) of fatty acids and all the glycerides were expressed as follows:

$$N_{2\text{-}MG}=Pc*A_K/4 \quad \text{[eq. S1]}$$

$$N_{1\text{-}MG}=Pc*A_L \quad \text{[eq. S2]}$$

$$N_{1,2\text{-}DG}=Pc*(A_{I+J}-2A_L)/2 \quad \text{[eq. S3]}$$

$$N_{TG}=Pc*(2A_{4.26\text{-}4.38}-A_{I+J}+2A_L)/4 \quad \text{[eq. S4]}$$

$$N_{1,3\text{-}DG}=Pc*(A_{4.04\text{-}4.38}-2A_{4.26\text{-}4.38}-2A_L)/5 \quad \text{[eq. S5]}$$

$$N_{FA}=(Pc*A_F-6N_{TG}-4N_{1,2\text{-}DG}-4N_{1,3\text{-}DG}-2N_{1\text{-}MG}-2N_{2\text{-}MG})/2 \quad \text{[eq. S6]}$$

$$N_{Gol}=(N_{FA}-N_{1,2\text{-}DG}-N_{1,3\text{-}DG}-2N_{2\text{-}MG}-2N_{1\text{-}MG})/3 \quad \text{[eq. S7]}$$

where Pc is the proportionality constant existing between the area of the ^{1}H NMR signals and the number of protons that generate them, A_K, A_L, A_{I+J} and A_F are the areas of the corresponding signals indicated in Table S2, and $A_{4.26\text{-}4.38}$ and $A_{4.04\text{-}4.38}$ represent the areas of the signals between 4.26 and 4.38 ppm, and between 4.04 and 4.38 ppm, respectively.

Using these equations, the molar percentages of the different kinds of glycerides in relation to the total number of moles of glyceryl structures present (NT_{GS}) were determined as follows:

$$NT_{GS}=N_{TG}+N_{1,2\text{-}DG}+N_{1,3\text{-}DG}+N_{2\text{-}MG}+N_{1\text{-}MG}+N_{Gol} \quad \text{[eq. S8]}$$

$$G\%=100N_G/NT_{GS} \quad \text{[eq. S9]}$$

where G is each kind of glyceride (TG, 1,2-DG, 1,3-DG, 2-MG and 1-MG) and N_G the respective number of moles.

$$Gol\%=100N_{Gol}/NT_{GS} \quad \text{[eq. S10]}$$

B. Estimation of the molar percentages of the main fatty acids (FA) plus acyl groups (AG) (FA+AG). The molar percentages of linolenic (Ln%), linoleic (L%), oleic (O%) and saturated (S%) AG or FA, in relation to the total number of moles of AG plus FA (NT_{AG+FA}) present in the starting oils and in the lipid extracts of the corresponding digestates were estimated as follows:

$Ln\% = 100*(A_H/2*A_F)$ [eq. S11]

$L\% = 100*(A_G/A_F)$ [eq. S12]

$O\% = U\% – L\% – Ln\%$ [eq. S13]

$S\% = 100 – U\%$ [eq. S14]

where A_H, A_G A_F are the areas of signals H, G and F indicated in Table S3.

C. Estimation of the concentration of specific compounds in oil samples and in the lipids extract from digestates.

The concentration of the several kinds of specific compounds (X), expressed as micromoles per mole of the sum of AG+FA present, was estimated by using the following equations:

$[X] = [(A_x/n)/(A_D/2)]*1000$ [eq. S15]

where A_x, is the areas of the signals selected for the quantification of each specific compound (X), present in the oil samples and in the lipid extract from digestates and n the number of protons that generate each signal given in Tables S4 and S5 and A_D is the area of the signal D in Table S3.

Operating Conditions for the SPME-GC/MS Experiments

The fiber used for the headspace components extraction was coated with Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm film thickness, 1 cm long, acquired from Supelco (Sigma-Aldrich, St. Louis, MO, USA)). It was inserted into the headspace of the sample and maintained for 55 min at 50 ºC, after a pre-equilibration time of 5 min. The fiber containing the components extracted was desorbed for 10 min in the injection port (splitless mode with 5 min purge time) of a 7890A gas chromatograph equipped with a 5975C inert MSD with Triple Axis Detector (Agilent Technologies, Palo Alto, CA, USA) and a computer operating with the ChemStation program. A fused silica capillary column was used (60 m length, 0.25 mm inside diameter, 0.25 μm film thickness; from Agilent Technologies Inc., Palo Alto, CA), coated with a nonpolar stationary phase (HP-5MS, 5% phenyl methyl siloxane). The operation conditions were the following: the injector and interface temperatures were held at 250 °C and 305 °C respectively, and helium at a constant pressure of 117 kPa (16.9 psi) was used as the carrier gas. The oven temperature was initially held at 50°C for 5 min, increased from 50 to 300 °C at a rate of 4 °C/min, and then held at 300 °C for 30 min. Mass spectra were recorded at an ionization energy of 70 eV, with data acquisition in Scan mode. The temperatures of the ion source and the quadrupole mass analyzer were kept at 230 and 150 °C, respectively. A reference sample of known composition was periodically analyzed in order to verify the sensitivity of the SPME-GC/MS experiments as in previous studies (Guillén et al., 2005).

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SUMMARY OF RESULTS

BLOCK 1

Methodological development and its application on the advancement of the knowledge about edible oil composition and about the processes they are involved in, such as nixtamalization and oxidation. The Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography coupled to Mass Spectrometry (DI-SPME-GC/MS) and the Proton Nuclear Magnetic Resonance (^{1}H NMR).

(Articles 1, 2, 3, 5 and **Manuscript 4)**

Aim 1.1. Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography coupled to Mass Spectrometry (DI-SPME-GC/MS) as a very useful new tool. **(Articles 1-3)**

Aim 1.2. Proton Nuclear Magnetic Resonance (^{1}H NMR) as a powerful tool in the study of the edible oil oxidation processes. Advancement in the oxidation process knowledge. **(Manuscript 4 and Article 5)**

Aim 1.1. Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography coupled to Mass Spectrometry (DI-SPME-GC/MS) as a very useful new tool. **(Articles 1-3)**

Objective 1.1.1. Development and application of a new methodology based on DI-SPME-GC/MS, for the characterization of volatile and less volatile edible oils components.

(Article 1)

The study was carried out using five commercial edible oils of different vegetable origin among, which are one sunflower oil, SF, one corn oil, C, two soybean oils (one refined, RSB and the other virgin, VSB) and one virgin linseed oil, VL. This new methodology, based on Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography coupled to Mass Spectrometry (DI-SPME-GC/MS), as can be observed bellow, allows in a single run, simultaneous determination of the relative abundances of the oil minor components belonging to several families of compounds.

- Sterols and derivatives: sterols form an important group of minor components of edible oils, to which several healthy properties have been attributed. In the case of the five edible oils here studied, the method used detected more than 25 five sterols and related compounds without the need of either previous saponification or modification of the samples, or even fractionation steps. Regarding the group of *desmethylsterols*, among which are campesterol, *β*-sitosterol, and stigmasterol, RSB was the richest in these compounds, followed by SF and C, which have higher abundances than the VSB and VL. In the group of *4-methylsterols*, cylolaudenol and citrostadienol were the main ones in SF, the richest oil in these

types of components, whereas they were absent in VSB and VL. Finally, *4,4´-dimethylsterols, oxo-sterols* and *sterol esters* were also found in all the oils studied, although in small number.

- Tocols: the method proposed here is able to detect the three kinds of tocols, namely tocopherols, tocotrienols and tocomonoenols. In total, nine tocols have been found, γ-tocopherol being the main one in all the oils except in SF where the main one is α-tocopherol. C and RSB were the richest oils in tocopherols. Both soybean oils also contained δ-, α- and β-tocopherols and γ-, δ-, and α-tocomonoenols in decreasing abundances. C, in addition to γ-tocopherol, contained in decreasing abundances α-, δ-, and β-tocopherol, as well as γ-, and α-tocotrienol and γ- and α-tocomonoenol. In SF, in addition to α-, β- and γ-tocopherols, δ-tocopherol, α- and γ-tocotrienol and α-tocomonoenol have been found. Finally, it should be noted that among the oils here studied, VL was the poorest in tocols.

- Hydrocarbons: Several kinds of hydrocarbons were present in these oils, among which are alkanes, alkenes, aromatic hydrocarbons, polycyclic aromatic hydrocarbons, terpenic hydrocarbons and steroidal hydrocarbons. Of the hydrocarbons studied, squalene was the most abundant in all the oils here studied, C being the richest in this component, followed by SF, VSB, RSB and VL.

- Acids: Other well known compounds present in edible oils which can be detected with this method are *fatty acids.* In these oils, acids from two to eighteen carbon atoms have been detected. The quantification of those having up to sixteen carbon atoms (palmitic acid) can be made individually in an accurate way. However, the

quantification of those of eighteen carbon atoms, namely, oleic, linoleic, linolenic and stearic acids, can only be made in an approximate way if overlapping of signals occurs. In addition to aliphatic acids, an *aromatic acid*, benzoic acid was also detected in all the studied oils.

- Esters: The alkyl esters found in the oils here studied are derived not only from the main acids in edible oils (palmitic, oleic, linoleic, linolenic and stearic acids), but also from fatty acids having either lower or higher numbers of carbon atoms (up to twenty six carbon atoms) than the above mentioned. Among the oils here studied, VL was the richest in number and abundance of alkyl esters. In addition, some *aromatic esters* were found in all the oils.

- Lactones: These oils contained lactones not only of small, but also of high molecular weight. Among those of high molecular weight there were *gamma*- and *delta*-lactones derived from the main fatty acids present in the oils.

- Monoglycerides: These are an important group of compounds also associated with oil quality that can also be detected and quantified with this methodology. Like fatty acids, monoglycerides having the same number of carbon atoms elute very close to each other and, depending on their concentration, their peaks overlap. Thus, among the oils here studied the RSB was the richest in these kinds of compounds.

- Fatty amides: This methodology is also able to detect the occurrence in these oils of some amides derived from fatty acids, such as palmitamide, linoleamide, oleamide and stearamide. The C is the richest in these compounds.

- Aldehydes, ketones, alcohols, epoxides, furan and pyran derivatives, and some terpenic oxygenated derivatives: With regard to *aldehydes,* a large number of *alkanals, alkenals, alkadienals,* certain aromatic aldehydes and some *oxygenated alfa, beta unsaturated aldehydes* have been detected. The highest abundances of these compounds have been found in VL. Furthermore, *aliphatic* and a reduced number of *aromatic ketones*, *alcohols* and *terpenic* derivatives having alcohol, aldehyde, ketone or epoxy groups were also detected.

Objective 1.1.2. New corn oil components detected for the first time by means of DI-SPME-GC/MS

(Article 2)

In this study, the minor components of eleven edible oils (extra virgin olive oil, virgin olive oil, olive oil, refined soybean oil, virgin soybean oil, sunflower oil, virgin linseed oil and four refined corn oils, C1, C2, C3 and C4) were studied looking for nitrogenated compounds. Among the oils studied, only in corn oils were detected cyclic dipeptides and other nitrogenated compounds.

Cyclic dipeptides or 2,5-diketopiperazines (DKPs). Their presence in edible oils has not been described before. However, with the methodology used in this study, twenty-five compounds of this nature were detected only in corn oil samples. The DKPs here found are derived mainly from proline (Pro), leucine/isoleucine (Leu/Ile), phenylalanine (Phe) and other amino acids.

- DKPs detected: Among them there are: one derived from proline-glycine (Pro-Gly); two isomers derived from proline-alanine (Pro-Ala); one derived from proline-proline (Pro-Pro); two isomers derived from proline-valine (Pro-Val); one derived from proline-pyroglutamic (Pro-PyroGlu); two isomers derived from proline-leucine/isoleucine (Pro-Leu/Ile); and two isomers derived from proline-phenylalanine (Pro-Phe); three isomers derived from leucine/isoleucine-valine (Leu/Ile-Val); four isomers derived from leucine/isoleucine-leucine/isoleucine (Leu/Ile-Leu/Ile); and four isomers derived from leucine/isoleucine-phenylalanine (Leu/Ile-Phe); one derived from phenylalanine-glycine (Phe-Gly); and two isomers derived from phenylalanine-valine (Phe-Val).

- Occurrence of these DKPs in other foods and potential origin: Some of the DKPs detected have also been recently found in some foods such as bread, chicken essence, Japanese sake, beer, beef, roasted coffee and cocoa. However, the cyclic dipeptide Pro-PyroGlu has not been found previously in foods. The origin and presence of DKPs in the above-mentioned food has been attributed to their formation in: fermentation processes, in thermal or hydrothermal treatments of proteins, in baking or roasting, or in Maillard reactions. Their presence in corn oil could be related to several sources such as: their occurrence in corn (in fact some cyclic dipeptides have been found in corn steep water), their formation during oil processing because of thermal treatment, or even because the presence of some fungus, yeasts or bacteria are able to synthesize DKPs.

- Importance of the occurrence of DKPs in corn oil: The importance of the presence of DKPs in corn oil is due to their activities. Among them can be cited sensory attributes and bioactive abilities. Regarding sensory attributes, their taste has been broadly described as bitter, metallic, astringent, salty and grainy. With respect to the bioactive abilities, it has been described that some DKPs exhibit a significant antibacterial, antifungal and antilarval activities, as well as anticancer and anti-mutagenic activity and antioxidant effect. Due to their antioxidant ability, the presence of DKPs in corn oil contributes to the oxidative of this oil. In fact, it has been proved that corn oil has higher oxidative stability than sunflower oil in spite of both oils having similar unsaturation degrees, and the DKPs contained in corn oil could contribute to this higher stability.

- DKPs as potential corn oil markers and tool to differentiate between corn oils: It is noteworthy that among the oils here studied only the corn oils (C1, C2, C3 and

C4) contained DKPs. This fact suggests that these compounds could be considered as corn oil markers as far as present-day knowledge can tell, making this a valuable tool to authenticate corn oil and to differentiate from other oils with similar molar percentages of the different kinds of acyl groups. However, differences between corn oils are also found due not only to the concentration of the common DKPs but also to the absence or presence of some of them. Thus, the C1 and C2 oils contain practically the same DKPs although in very different concentrations, the C1 oil being the richest in these components. However, the C4 oil contains, in addition to the DKPs present in the aforementioned oils, other derivatives from proline and glycine, valine and alanine that are absent in C1 and C2. Finally, note that the C3 oil is the poorest in DKPs both in relation to their number and their abundance. From these results it is evident that the corn oils can also be differentiated and classified on the basis of their main DKPs.

- Contribution to current knowledge of peptide presence in edible oils: The results of this study open a new perspective in relation to knowledge of corn oil composition because, to the best of our knowledge, the presence of DKPs in corn oils is shown for the first time in this study. Furthermore, the bioactivity and molecular characteristics of the DKPs increase the added value of this oil and its interest from the technological, nutritional and healthy points of view.

Other nitrogenated compounds. In addition to the above-mentioned DKPs, other nitrogenated compounds, some of them with anticancer and antimicrobial activities, have also been found in corn oils but not in the other oils studied.

- Fatty oxazoniles and pyrrolidines: The *oxazolines* found in the corn oils here studied are 4,4-dimethyl-2-(1-hydroxy-heptadec-8-enyl)-2-oxazoline, the homologous derived from 1-hydroxy-octadecen-8,11-oic acid and that of derived from 1-hydroxyhexadecenoic acid. The fatty *pyrrolidine*s found are those derived from palmitic, linoleic and oleic acids. Both kinds of compounds were found in the four corn oils studied though in somewhat higher abundances in C4 oil.

- Other pyrrol derivatives: Other compounds found in these corn oils containing the pyrrolic ring in their structure are N-furfuryl-2-formylpyrrole, 1-(2-phenylethyl)-2-formylpyrrol, and N-(2-phenylethenyl)-pyrrolidin-2-one. The first has only been detected in C1 and the other two in C1 and C2, although in somewhat higher concentration in C1.

- Indole derivatives: Compounds belonging to this family have also been found in some of the corn oils studied (C1, C2 and C4). These are, 1H-indole-3-carboxaldehyde, acetyl-beta-carboline, 1H-indole-3-acetic acid ethyl ester, and 3-phenylindol, or isomers of these four compounds. All of these compounds were detected in C1 oil. However, in C2 and C4 oils, only the presence of 1H-indole-3-carboxaldehyde and 3-phenylindol has been detected.

- Miscellaneous compounds: This group includes the detected compounds having different functional groups such as 3,5-diphenylpyrazole, alatamide and 3,5-diphenylpyridine. In addition, a group of unknown compounds, supposedly nitrogenated derivatives with well defined mass spectra, have been also found.

Objective 1.1.3. To address by means of DI-SPME-GC/MS the effect of accelerate storage process (70 ºC) on corn oil, throughout the evolution of some of its minor components. Detection of new oil oxidation markers.

(Article 3)

Minor compounds present in the original corn oil. The characterization of the original corn oil minor components was the starting point of this study. It provided information about the identity and abundance of a broad range of compounds, some of which can have antioxidant ability, others which come from oil oxidation and provide information about its oxidation status, and others which are of varied origin and nature.

***Compounds with antioxidant ability.** These include tocols, sterols, squalene and some cyclic dipeptides or DKPs (2,5-diketopiperazines).

- Tocols: The main tocol is γ-tocopherol (γ-T) followed by α- (α-T) and δ-tocopherols (δ-T) and in much smaller abundances β-tocopherol (β-T), as well as some tocomonoenols and tocotrienols.

- Sterols: Corn oil contains mainly β-sitosterol, followed by campesterol and in much smaller abundance stigmasterol, Δ5-avenasterol, as well as one stanol, sitostanol.

- Terpenic hydrocarbon: Squalene [Sq] is the main hydrocarbon in corn oil.

- DKPs: This oil also contains in much more small abundance cyclic dipeptides. These derive from phenylalanine and other amino acids such as valine, leucine, isoleucine and proline.

***Compounds coming from oil oxidation.** This oil also contains a basal concentration of some compounds whose origin could be in the oxidation of main corn oil components. Among them, there are *aldehydes*, small abundance of *2-pentylfuran*, as well as a reduced number of *alcohols*, *ketones*, some *furanones* and *lactones*.

***Other minor compounds.** Apart from the aforementioned compounds, there are a reduced number of *glycidyl fatty acid esters.* The abundance of these compounds is, as expected, very low.

Evolution under oxidative conditions of the antioxidant compounds present initially in the corn oil. When oil is subjected to oxidative conditions, it is to be expected that the antioxidants, initially present, undergo degradation, thus decreasing in concentration. The rate of this degradation depends on the oxidative stability of each one of these compounds under these conditions.

***Degradation kinetic of tocols**

- Tocopherols: They remained practically constant during an initial period of time (approximately up to day 3 under degradative conditions). However, from day 3 until day 9, they underwent a rapid degradation. The abundance of each one of these tocopherols [**T**] and time, **t**, under degradative conditions (considering day 3 the starting point (time = 0) and day 9 the end point (time = 6)) fitted, with a high correlation coefficient, to the general equation [**T**] = **m t** + **b**, being **m** and **b** specific of each tocopherol. The degradation rate **m** of each tocopherol, derived from these equations, is defined by $\mathbf{m} = d\mathbf{[T]}/d\mathbf{t}$. From the data obtained by these equations, the highest degradation rate was shown by γ-T, followed by α-T, δ-T and β-T. Likewise, a first-order kinetic model $Ln[\mathbf{T}] = Ln[\mathbf{T}]_0 + \mathbf{k\ t}$ was tested;

however, the correlation coefficients were somewhat worse than those obtained with the zero-order kinetic model. In summary, it can be said that the degradation of these four tocopherols contained in corn oil fits better with a kinetic model of zero-order, under the oxidative conditions of this study.

- Tocomonoenols and tocotrienols: Their abundances followed a similar path as tocopherols under the oxidative conditions of this study, and after the ninth day, all of them disappear from the corn oil.

***Degradation kinetic of sterols.** The abundance of these compounds remained, like that of tocols, practically unchanged until day 3. Abundance and time, from day 3 (**t**=0) to day 12 (**t**=9) under oxidative conditions, fitted with a high correlation coefficient to linear equations, which is to say that also in this case, the degradation of sterols fits well to a zero-order kinetic model, being the degradation rate of β-sitosterol higher than that of campesterol. The equations obtained considering a first-order kinetic model also have high correlation coefficients although somewhat smaller than those of the zero-order kinetic model.

***Degradation kinetic of squalene.** The abundance of this compound decreases very slowly from day 0 to day 9 and then very sharply from day 9 to day 12. In the first stage (days 0- 9), the abundance [Sq] and time, t, fit, with a high correlation coefficient, to either linear equation [Sq] = -11.1 t + 527.9 or to the equation Ln [Sq] = -0.02 t + 6.3. This indicates that squalene degradation is fairly well described by both either a zero-order or a first-order kinetic model.

***Evolution of DKPs.** The degradation of these compounds appears to begin from day 0, and on day 9, they are totally degraded. Due to their low abundance, the equations relating

their abundance and time under degradative conditions are more affected by experimental errors than those of the components in higher abundance mentioned above, for which reason no kinetic data are given.

In summary, it is evident that under oxidative conditions, from day 3 to day 9, the abundance of tocols has been reduced nearly ten times, whereas that of sterols has been reduced about a half and that of squalene by a factor of around 0.8. From day 9 onwards the degradation rate of all of these oil components increases considerably, and on day 12 the abundance of many of them is small and that of others null.

Formation of compounds derived from the antioxidant components of corn oil and evolution of their abundance. The degradation of each one of the above-mentioned minor corn oil components can lead to the formation of several derived compounds which were not present in the original oil or were present in very low abundance.

***Compounds coming from tocopherols degradation.** In this study six compounds derived from either the four tocopherols or especially from α-tocopherol were detected. The compounds derived from the four tocopherols comprises prist-1-ene, 6,10,14-trimethylpentadecan-2-one, 3,7,11-trimethyl-3-dodecanol and 4,8,12,16-tetramethylheptadecan-4-olide. The abundance [**DT**] of each one of these four compounds and time **t** under oxidative conditions fit well to equations like [**DT**] = **a** e^{kt}, where **a** is a coefficient specific of each compound, and **k** the formation rate constant. From the results obtained it is evident that 4,8,12,16-tetramethylheptadecan-4-olide has the highest formation rate among these four tocopherol derivatives. On the other hand, the compounds coming exclusively from α-T are α-tocopherylquinone-2,3-epoxide and α-tocopherylquinone-5,6-epoxide. The evolution of the abundance of these two

compounds follows a different path than that of the above-mentioned tocopherol derivatives and their presence in the oil also indicates that an oxidation process has taken place. In summary, all of these tocopherol derivatives can be considered corn oil oxidation markers, and this can be extrapolated to all edible oils containing tocopherols.

***Compounds coming from sterols degradation.** Of the various sterols oxidation derivatives that could be formed, only 7-ketositosterol, which is derived from β-sitosterol (the main sterol in this oil), has been detected, but in very small abundance. This compound was detected after 12 days under oxidative conditions, coinciding with an important degree of β-sitosterol degradation

***Compounds coming from squalene degradation.** In total nine squalene derived compounds were found, among which are: four oxygenated derivatives of farnesene; one unsaturated branched hydrocarbon of 25 carbon atoms; and four oxygenated derivatives of squalene, among which there are two epoxides, one ketone and one aldehyde. Some of them were detected on day 9 and others on day 12.

***Compounds coming from cyclic dipeptides degradation.** None of these compounds could be detected.

Evolution with time of compounds coming from corn oil main components oxidation

***Aldehydes.** These compounds are the most numerous and abundant secondary oxidation compounds of those detected here. Since linoleic is the main acyl group in corn oil, its derived aldehydes are the most abundant. The abundance of aldehydes increases as time under oxidative conditions increases. This increase follows a general path of two stages. In the first stage (from day 0 to day 6), the formation rate is much slower than in the second stage (from day 6 onwards). It is noteworthy that the point between these two

stages occurs after six days under oxidative conditions, that is to say, when tocopherols have been degraded to a great extent.

***Other secondary oil oxidation compounds.** In addition to aldehydes, some furan derivatives, alcohols, ketones, as well as furanones and lactones were also detected in the corn oil after its subjection to accelerated storage conditions. The evolution of their abundance, like that of aldehydes, shows two stages of different growth rate.

***Evolution of glycidyl fatty acid esters.** Their abundance seems to increase with time under oxidative conditions until day nine, after which they begin to decline until their total disappearance on day twelve.

Comparison of the performance of this methodology in the study of oil oxidation with that of other methodologies that do not require chemical changes in the sample either

***Performance of HS-SPME-GC/MS.** This methodology provides information about the oil oxidation status through the abundance of volatile secondary oxidation compounds. As described above with DI-SPME-GC/MS, the same volatile compounds as in that technique are also detected, but even earlier due to the lower abundances needed because of the direct extraction of them from the oil matrix. In addition, it should be remembered that DI-SPME-GC/MS, besides the above-mentioned secondary oxidation compounds, also provides information about the oil antioxidant components and of some of their derived compounds

***Performance of ^{1}H NMR spectroscopy.** Of all those compounds which may be studied by ^{1}H NMR spectroscopy, DI-SPME-GC/MS is only able to provide information of some secondary oxidation compounds. However, as mentioned above, this latter technique

provides information of the oil antioxidant components and of some of their derived compounds, which cannot be obtained directly from ^{1}H NMR. In addition, the sensitivity of ^{1}H NMR is lower than that of DI-SPME-GC/MS, for which reason the compounds are only detected from a certain abundance level onwards.

Aim 1.2. Proton Nuclear Magnetic Resonance (^{1}H NMR) as a powerful tool in the study of the edible oil oxidation processes. Advancement in the knowledge of the oxidation process. **(Manuscript 4 and Article 5)**

Objective 1.2.1. Detection and quantification of compounds previously not described during edible oil oxidation processes by means of ^{1}H NMR. A global analysis of the oxidation process.

(Manuscript 4)

^{1}H NMR methodology allows the monitoring throughout all the oxidation process, of the degradation rate of original main and minor corn oil components and simultaneously, permits the detection and quantification of new compounds formed as consequence of the degradation of the firsts.

Evolution of corn oil main components. In corn oil the main acyl group is *linoleic* as it has been indicated before. For this reason and due to its unsaturation degree, this acyl group can be considered an appropriate representative for the study of the degradation rate of corn oil main components. The degradation of linoleic acyl groups shows in general terms, two different rates throughout the oxidative process. After 16 days under degradative conditions, the corn oil has reached a very high polymerization degree, and the linoleic group is in a very low concentration.

Evolution of corn oil minor components. Among the different kinds of minor components of corn oil, *tocopherols* and *sterols-stanols* are important because to them has been attributed antioxidant activity and also because their concentrations are higher than those of other minor corn oil components.

*__Evolution of tocopherols__. Among the tocopherols, only the evolution of *γ-tocopherol*, the main tocopherol of corn oil, was studied. The degradation of *γ-tocopherol* throughout this oxidative process has two stages and at day 11, it presences can not be detected by ^{1}H NMR, reason for which it could be said that it has practically disappeared from the oil.

*__Evolution of sterols-stanols.__ It is well known that corn oil can contain a great number of sterols-stanols, however, only *sitostanol* (STN) and Δ*7-avenasterol* (Δ7A) have, at least, a ^{1}H NMR signal that does not overlaps with any other and are in enough concentration to be detected by ^{1}H NMR. These compounds, which are in much lower concentration than γ-tocopherol, have a higher oxidative stability than this, and as consequence they show a very small degradation rate.

Formation of new compounds. Simultaneously with the degradation of original oil main and minor components, new derived compounds are formed.

*__Compounds derived from corn oil main components.__ A huge number of oxylipins, some of them well known and other news, have been detected. These findings are very important because most of these oxylipins have been found also in cells and have been related to several diseases. Furthermore, for the first time, has also been shown in the oil oxidation processes, the formation of other functional groups such as poly-formates, poly-hydroxy and poly-ether groups which are responsible of the viscosity increase of the oil as consequence of the oxidation and of its polymerization.

a) Monohydroperoxides (mHPO). The firsts structures detected as new (from day 4) in this corn oil submitted to oxidative conditions, are monohydroperoxy-conjugated dienes, mHPO-c-dEs, derived from linoleic group; the well known 9- and 13-hydroperoxy-*Z,E*-

conjugated dienes (mHPO-c(*Z,E*)-dEs) as well as 9- and 13-hydroperoxy-*E,E*-conjugated dienes (mHPO-c(*E,E*)-dEs). Both isomers reach their maximum concentration the same day and after this maximum the concentration of these compounds decreased very sharply.

b) Dihydroperoxides (dHPO). Dihydroperoxy-non conjugated dienes dHPO-nc-dEs, such as 9,12-dihydroperoxy-10*E*,13*E*-octadecadienoate (9,12-dHPO-10*E*,13*E*-dE) and 10,13-dihydroperoxy-8*E*,11*E*-octadecadienoate (10,13-dHPO-8*E*,11*E*-dE) have been detected from day 9 onwards due to double doublet near 4.82 ppm. They reach their maximum concentration at day 13, like mHPO-c-dEs after which their intensity decreases indicating their role as intermediate compounds.

c) Hydroperoxy-epoxy-monoenes (HPO-EPO-mEs). Other compounds able to be formed as consequence of the oxidation of mHPO-c-dE can be hydroperoxy-epoxy-monoenes (HPO-EPO-mEs). Thus, signals (2.84, 3.11 and 5.85 ppms) of 9-HPO-12,13-*E*-EPO-10*E*-octadecenoate are present in the spectra of the corn oil here studied from day 10 onward. This compound reaches the highest concentration near day 14 with a very small decreasing on day 16 up.

d) Monohydroxy-conjugated dienes (mHO-c-dEs). From day 8 to day 13 signals of monohydroxy-conjugated-*Z,E*-dienes, centered at 6.48 ppms, with very low intensity are observed in the spectra of the corn oil.

e) Hydroxy-epoxy-monoenes (HO-EPO-mEs). In the oxidation process of this corn oil signals assignable to two different groups of these compounds appear after 13 days under oxidative conditions. In the first group are included those HO-EPO-mEs having the double bond between the epoxy and hydroxy groups and in the second group are those

having vicinals hydroxy and epoxy groups. The concentration of the HO-EPO-mEs belonging to the first group was estimated jointly using the area of the signal near 5.95-5.94 ppm and regarding the second group of HO-EPO-mEs, due to the overlapping of signals, only the concentration of the *threo* isomers was estimated using the area of the signal near 3.96 ppm.

f) Hydroxy-keto-monoenes (HO-KO-mEs). This kind of structures are also formed after 14 days under degradative conditions. Those found belong to two different groups. In one group, both oxygenated groups are on vicinal carbon atoms and the other groups constituted by structures that do not have the hydroxy and keto groups in vicinal carbon atoms. The concentration of all these compounds was estimated jointly, using the area of the signal at 3.24 ppm due to the methylenic protons in *alpha* position regarding either the keto group and the double bond, or regarding both oxygenated groups.

g) Monoketo-conjugated dienes (mKO-c-dEs). In this corn oil submitted to degradative conditions have been detected and quantified monoketo-conjugated dienes, which are well known oxylipins. The monoketo conjugated-*E,E*-dienes (mKO-c(*E,E*)-dEs) appear in the spectra from day 11 onwards and the *Z,E*-isomers (mKO-c(*Z,E*)-dEs) from day 12 onwards. Likewise, their quantification has been made using the area of signals centered near 7.49 ppm in the case of *Z,E*-isomers and centered near 7.13 ppm in the case of *E,E*-isomers. The concentration of the *E,E* isomers is more than double than that the *Z,E* isomers and both are in line with that of their corresponding precursors mHPO-c(*E,E*)-dES and mHPO-c(*Z,E*)-dES at this time, but much smaller.

h) Keto-epoxy-monoenes (KO-EPO-mEs). Having into account the spectral signals, two groups of possible isomers are present from day 14 onwards. One group of KO-EPO-mEs has the double bond between the keto and epoxy groups, among which are structures as

KO-*E*-EPO-*E*-mEs, signals at 2.53 (t), 2.91 (td), 3.20 (dd), 6.38 (d) and 6.52 (dd) ppm and KO-*Z*-EPO-*E*-mEs, signals at 2.55 (t), 3.20 (dd), 3.52 (dd), 6.40 (d) and 6.66 (dd) ppm. Again the concentration of *E,E* isomers is higher than that of *Z,E* isomers. The second group of KO-EPO-mEs has the keto and epoxy groups in vicinal carbons, with both, the double bond and epoxy group in *trans* configuration, KO-*E*-EPO-*E*-mEs. Signals attributable to this kind of compounds at 3.04-2.98 (ddd) ppm, 3.34-3.28 (d) ppm, 6.23-6.16 (dt) ppm and 7.02 (dt) ppm are also present, with very low intensity in the corn oil here studied.

i) Epoxy-keto-hydroxy derivatives (EPO-KO-HOs). From day 12 onwards under oxidative conditions, in the ^{1}H NMR spectra of this oil appear signals near 2.42 (dd), 2.51 (dd), 3.02-3.08 (ddd), 3.16 (d) ppm and 3.98-4.04 ppm which are overlapped with other ones. For this reason, it could be thought that structures with these three oxygenated functional groups could also have been be formed in this oil.

j) Monoepoxy-monoenes (mEPO-mEs) and diepoxy (dEPO) structures. The appearance in the oil spectra from day 13, of signals at 2.98-2.88 ppm and 2.73-2.66 ppm together with other ones suggests the formation of mono unsaturated epoxy structures (mEPO-mEs). The first signals (2.98-2.88 ppm) are associated with *Z*-EPO-*Z*-mEs and the second signals (2.73-2.66 ppm) are related to *E*-EPO-*Z*-mEs. It should be noted that *Z*-EPO-*Z*-mE isomers are formed in greater concentration than *E*-EPO-Z-mEs isomers, maybe because in the first case a previous isomerization is not required. Regarding diepoxy structures (dEPO), their presence can be ruled out due to the absence of spectral signals of the standard, 9,10-EPO-12,13-EPO-octadecanoic acid.

k) Dihydroxy (dHO) and/or polyhydroxy (pHO) structures. In this study, one broad signal centered at 3.42 ppm, attributable to the methine carbinol protons of 9,10-dHO-12*Z*-

octadecanoate (leukotoxin diol) and/or 12,13-dHO-9*Z*-octadecanoate (isoleukotoxin diol) appears in the spectra from day 12 onwards. Furthermore, the formation of pHO can also occur since the methine carbinol proton of pHO could also contribute to the signal at 3.42 ppm.

l) Acids and formic acid. In the oxidation process of this corn oil the formation of formic acid has been observed from day 12 onwards due to the appearance in the ^{1}H NMR spectra of the singlet signal at 8.01 ppm, and its concentration grows up to day 16.

m) Poly-formate (pF), poly-ester (pEST) and poly-hydroxy (pHO) structures. Formic acid is able to open the epoxydic rings yielding one hydroxy group and one formate group. The existence of this reaction in the oxidation process of this oil under the conditions of this study is confirmed by the appearance of signals between 8.03 and 8.17 ppm of the proton of formate groups and at 5.18 ppm of methine protons of the ester group. The appearance of formate signals in the spectrum occurs on day 13 and the concentration of these groups increases progressively up to the end of the polymerization process. Besides formic acid, other acids present in the oil could also open the oxirane rings in triglycerides forming poly-hydroxy and poly-ester groups.

n) Poly-ether (pET) and poly-hydroxy (pHO) structures. It is known that not only acids are able to open the oxirane rings but also both primary and secondary alcohols can do it. The oxirane ring opening provoked by alcohols yields, one hydroxy group on the one side and on the other side one ether group, which incorporates to the molecule the structure that support the alcohol group. Furthermore, if this reaction takes place between epoxides of one triglyceride and the secondary hydroxy groups of other triglyceride, polymerization is produced by the formation of C-O-C bridges between fatty chains In addition, reactions between hydroxyl groups of different structures are also possible to

generate again ether groups contributing also to generate ether groups and polymerization of the sample. From days 12-13 onwards appears signals at near 3.62, 3.98 and 4.23 ppm, with increasing intensity to the end of the experiment, which can be assignable to methine protons of alcohols or ether groups

o) Structures supporting furan ring (Frs). Among these can be distinguish two types, alkyl-furans and furanones. The first gives signal in the ^{1}H NMR spectra at 7.27 ppm from day 14 onward with growing concentration up to the end of the experiment and the second appear from day 13 onwards with increasing intensity up to the end of the process. Among these there are a characteristic double doublet signal of their unsaturated protons centered near 7.47 ppm. The increasing concentration of these structures up to day 16 suggests that they are oxidation end products.

p) Aldehydes (A). In the oxidation process underwent by this corn oil the first aldehydes formed are 2*E*-alkenals and 4-hydroperoxy-2*E*-alkenals which are detected from day 11 onwards. However, n-alkanals and, 2*E*,4*E*-alkadienals appear in the spectra from day 12, whereas 4-hydroxy-2*E*-alkenals, 4-oxo-2*E*-alkenals and 4,5-epoxy-2*E*-alkenals appear on day 13. Finally, from day 15 the presence of 2,3-epoxyalkanals is observed.

***Compounds derived from corn oil minor components.** In this study the only detected oxidation compounds derived from corn oil minor components are those coming from the main sterols present in this oil, which are sitosterol + campesterol. They are 5α,6α-epoxysitosterol+campesterol and 5β,6β-epoxysitosterol+campesterol and appear the last days of the oxidative process at very low concentracion. It should be pointed out that this is the first time that the presence of some sterols oxidation products have been detected directly in oxidized edible oil by ^{1}H NMR technique.

View of the evolution over time of the oxidation process. The process begins with the degradation of the oil main and minor components. The evolution of degradation of oil main components is well represented by that of linoleic groups, which degradation is divided in four different stages. Along this time the concentration of *gamma*-tocopherol, decrease at almost constant rate from day 2 onwards up to day 11, being totally degraded on day 12 just when the degradation of linoleic groups reach the highest rate. As linoleic groups and *gamma*-tocopherol are degraded the structures aforementioned are being formed. The firsts formed are mHPO-c-dEs which can be detected and quantified from day 4 onwards. After mHPO-c-dEs, the following formed compounds detected by ^{1}H NMR are mHO-c-(*Z*,*E*)-dEs (day 8), dHPO-nc-*E*,*E*-dEs (day 9) and HPO-*E*-EPO-*E*-mEs (day 10). On day 11 appear in the sample in addition to m-KO-c(*E*,*E*)-dEs, two kinds of aldehydes whose formation involve the breaks of the acyl group chain, they are 4-HPO-2*E*-alkenals and 2*E*-alkenals. Likewise, on day 12 in the spectra appear signals of m-KO-c(*Z*,*E*)-dEs, and of methine carbinol protons of secondary alcohols being possible to be vicinal or not, as well of n-alkanals, 2*E*,4*E*-alkadienals and formic acid. A numerous group of different structures appear on day 13. These are *Z*-EPO-*Z*-mEs, *E*-EPO-*Z*-mEs, pF, 4-HO-2*E*-alkenals, 5-pentyl-(5H)-furan-2-one, 4,5-EPO-2*E*-alkenals, and 4-KO-2*E*-alkenals. In addition to these, HO-*E*-EPO-*E*-mEs, HO-*Z*-EPO-*E*-mEs and *E*-EPO-KO-HOs, also appear on day 13, and also signals at 3.62 and 4.24 ppm associated to methine carbinol protons. On day 14 also appear new oxidation compounds such as HO-*E*-EPO-*E*-mEs, HO-KO-*Z*-mEs, HO-KO-*E*-mEs, KO-*E*-EPO-*E*-mEs, KO-*Z*-EPO-*E*-mEs and alkyl-furans and the last detected compounds were 2,3-EPO-alkanals on day 15.

Objective 1.2.2. Study by means of ^{1}H NMR the changes caused by Nixtamalization and Tortilla-making processes in the lipid composition of two corn varieties. **(Article 5)**

In order to contribute to a deeper knowledge about the effect of nixtamalization and tortilla-making processes in the lipids of two corn varieties (blue B and white W), its lipids will be extracted and studied by proton nuclear magnetic resonance (^{1}H NMR), both before (BR and WR) and after (BT and WT) nixtamalization and tortilla-making processes. It is to be expected that the information provided by this technique will show the changes occurring during processing to the lipids of both kinds of corn, in terms of losses of main and minor components, changes to the unsaturation degree, and the occurrence of certain reactions, such as oxidation.

Study of the composition of raw corn lipids (BR and WR) by ^{1}H NMR

***Qualitative characterization of their components**. As may be expected, both BR and of WR hexane extracts contained both triglycerides (TG), which, as is well known, are the main edible oil components, and 1,2-diglycerides (1,2-DG), which are minor components. In addition, the presence of methyl esters (ME) and phosphatidylcholine (PC) was also detected in BR, the first component, and in WR, the second one. Other minor components which were present in both BR and WR samples are sterols, among which are, β-sitosterol, campesterol and Δ5-avenasterol (S+C+Δ5A) and Δ7-avenasterol (Δ7A); ferulates (FE), as well as fatty acids (FA).

***Quantitative estimation of their minor components.** The area of the ^{1}H NMR spectral signals is proportional to the number of protons that generates it, and this proportionality constant is the same for all kind of protons. Due to this, the concentration of the minor components in relation to the number of moles of TG, as well as in relation to the number

of moles of fatty acids plus acyl groups (FA+AG), can be estimated. There are no great differences in relation to the minor components present in BR and WR, although most of the common components are in a slightly higher concentration in WR than in BR. Among these minor components, 1,2-DG and S+C+Δ5A are in similar concentration. Ferulate concentration is around six times lower than that of the minor components mentioned before. Stanols and the rest of sterols are in a lower concentration than that of ferulates, and PC and ME, in addition to being in very low concentrations as expected, are absent in BR and in WR respectively. And finally, FA, which are the most abundant of the minor components in both samples.

***Oxidation status.** The ^{1}H NMR spectra of the corn hexane extracts can also provide information about the presence of compounds coming from corn lipid oxidation processes, in other words about their oxidation status. However, in the ^{1}H NMR spectra of BR and WR, no signals due to protons of oxidation compounds appear. This indicates that, as might be expected, the hexane extracts of the raw corn oils are unoxidized.

***Unsaturation degree.** This can be estimated directly in a very simple, accurate and rapid way from ^{1}H NMR spectral data, and can be expressed by different parameters with the same meaning. One of these parameters is P_1. This is defined as the percentage of olefinic protons (op) in relation to the total protons supported on acyl groups and on fatty acids (tp). Another parameter which is useful for the same aim is P_2, defined as the ratio between olefinic protons and protons supported on saturated carbon atoms in fatty acids and acyl groups. And finally, another parameter classically used to determine the unsaturation degree is Iodine Value, IV. Al these parameters indicate that WR has a higher unsaturation degree than BR.

***Molar percentages of the several kinds of acyl groups and fatty acids.** As is common in corn oils, both corn lipids are richer in linoleic than in oleic groups. However, in BR corn lipids, the difference between the concentrations of these two groups is much smaller than in WR corn lipids. Both corn varieties have a very small concentration of linolenic groups, as expected, and BR is richer in saturated groups than WR.

Changes undergone by corn lipids during nixtamalization and tortilla making (BT and WT) estimated from ^{1}H NMR spectral data.

***Changes in lipid minor component concentrations.** The concentration of the above-mentioned minor components in tortilla lipids is lower than in BR and WR. This indicates that nixtamalization and tortilla-making entail a certain loss of some these compounds and the total disappearance of other ones. It is noteworthy that signals of ME and of PC, present respectively in BR and WR spectra with a very low intensity, and the signal of methylenic protons in *alpha* position in relation to the carboxyl group of FA, are not visible in BT and WT spectra, which evidences their absence in tortilla lipids.

***Changes in lipid oxidation status. Formation of oxidation compounds.** The lipids of both tortillas (BT and WT) have signals (*ZE*) of protons of certain primary oxidation compounds, such as hydroperoxides supported on structures having *Z,E*-conjugated diene groups (OOH-*Z,E*-CD). Moreover, secondary oxidation compounds, alkanals (AL), have been detected, but only in BT. These results suggest that some oxidation has occurred during the nixtamalization and tortilla-making processes and that BT has a slightly higher oxidation degree than WT.

***Unsaturation degree of tortillas lipids**. This characteristic was estimated by the same previously mentioned parameters, P1, P2 and IV. These three parameters indicate that

during the processing of corn to make tortillas, the unsaturation degree of their lipids was slightly reduced.

***Distribution of acyl groups in tortillas lipids.** Comparison of the data from lipids of tortilla and of raw corn evidences that the distribution of the percentages of the different acyl groups did not undergo a significant change from corn to tortilla. A small reduction in the molar percentage of linoleic acyl groups is observed, in agreement with all the above mentioned in relation to unsaturation degree, and with the formation of hydroperoxides supported on structures having *Z,E*-conjugated dienic groups derived from linoleic groups.

BLOCK 2

Advances in the knowledge of the edible oil *in vitro* digestion process. Antioxidant effectiveness of mono- or poly-phenolic compounds. Bioaccessibility of main and minor oil components.

(Articles 6-7 and Manuscript 8)

Aim 2.1. To investigate by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the effect of the addition of *alpha*-tocopherol (α-T) and *gamma*-tocopherol (γ-T) on the lipolysis extent, advance of the oxidation reactions and bioaccessibility of main and minor compounds, during *in vitro* digestion of corn oil. **(Article 6)**

Aim 2.2. To address by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the antioxidant effectiveness of mono-, di- and tri-phenolic compounds (*gamma*-tocopherol (γ-T), hydroxytyrosol acetate (HTA) and dodecyl gallate (DG)) during the *in vitro* digestion of virgin flaxseed oil. **(Article 7)**

Aim 2.3. To study by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the behaviour of olive oil during *in vitro* digestion. Study of the lipolysis extent and pattern and oxidation reactions, as well as of the bioaccessibility of main and minor compounds. Comparison with corn oil and virgin flaxseed oil submitted to the same digestive conditions. **(Manuscript 8)**

Aim 2.1. To investigate by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the effect of the addition of *alpha*-tocopherol (α-T) and *gamma*-tocopherol (γ-T) on the lipolysis extent, advance of the oxidation reactions and bioaccessibility of main and minor compounds, during *in vitro* digestion of corn oil.

(Article 6)

The *in vitro* digestion of corn oil, as well as the effect of its enrichment with three different levels of *gamma* and *alpha*-tocopherol on this process, is analysed for the first time. The study was carried out by using ^{1}H nuclear magnetic resonance (^{1}H NMR) and solid phase microextraction followed by gas chromatography/mass spectrometry (SPME-GC/MS). Attention focused on subjects such as hydrolysis degree, degradation of oil main components, occurrence of oxidation reactions and main compounds formed, as well as on bioaccessibility of oil main components, of compounds formed in the oxidation, and of *gamma*-tocopherol and *alpha*-tocopherol.

Extent and pattern of lipolysis produced by the *in vitro* digestion in the several samples. As is known, the main components of edible oils are triglycerides, and when they are submitted to digestion, hydrolysis of their ester bonds occurs, yielding diglycerides and monoglycerides, as well as fatty acids and glycerol.

***Lipolysis extent in corn oil digestates.** About 78% of triglycerides have been hydrolyzed partially or totally. Monoglycerides and glycerol are the main glycerides formed (approximately 31%).

***Lipolysis extent in the enriched in tocopherol corn oil digestates.** The triglyceride hydrolysis pattern of these samples is very similar to that found in the digestate of corn

oil: monoglycerides and glycerol are the main hydrolytic products, followed by diglycerides, whose concentration is approximately half that of the other two hydrolytic products. Nevertheless, the results suggest that enrichment with tocopherols could have had some influence on the hydrolysis extent.

Bioaccessibility of oil main components (B_{OMC}). The only compounds released during digestion as a result of triglyceride hydrolysis which can be absorbed by enterocytes of the intestinal wall are fatty acids and monoglycerides. The bioaccessibility of these compounds is around 70% in the corn oil digestate and reaches very close values to this in the digestates of the oil samples enriched in tocopherols. That is to say, from every hundred original acyl groups supported in the oil triglycerides, nearly seventy are transformed either into fatty acids or into monoglycerides during digestion and for this reason are available for absorption.

Study of the occurrence of oxidation reactions during *in vitro* digestion of corn oil and of corn oil samples enriched in tocopherols. The study of the occurrence of oxidation reactions was carried out by means of ^{1}H NMR spectroscopy and SPME-GC/MS.

***Information provided by ^{1}H NMR spectroscopy about the occurrence of oxidation reactions during *in vitro* digestion**

(a) Evaluation of the concentration of acyl groups and fatty acids having linoleic structure in the corn oil, in its digestate and in those of corn oil samples enriched in tocopherols. A significant and clear reduction of the molar percentage of linoleic structures occurs during the corn oil *in vitro* digestion, which evidences their degradation. However, in the samples enriched with *gamma*-tocopherol this reduction is significantly

smaller than in the non-enriched oil. Likewise, the loss of linoleic structures in the digestion of the samples enriched with *alpha*-tocopherol is slightly higher than that found in the non-enriched oil, although the differences are not statistically significant.

(b) Study of the formation of oxidation compounds during in vitro digestion of corn oil and of corn oil samples enriched in tocopherols.

- Oxidation compounds in corn oil digestates: *In vitro* digestion provokes oxidation reactions in corn oil generating specific kinds of primary oxidation compounds, such as, hydroperoxy-conjugated-(*Z,E*)-dienes (HPO-c(*Z,E*)-dEs).
- Oxidation compounds in the digestates of corn oil enriched in *gamma*-tocopherol: The digestate of corn oil enriched with *gamma*-tocopherol at the lowest concentration essayed (DC0.2γT) also contain signals of (HPO-c(*Z,E*)-dEs) but in smaller concentrations than in DC, whereas in the digestates of DC2γT and DC5γT, no signals of oxidation compound are detected, showing the clear antioxidant activity of this compound.
- Oxidation compounds in the digestates of corn oil enriched in *alpha*-tocopherol: The effect provoked by the enrichment with *alpha*-tocopherol is the opposite of that provoked by the enrichment with *gamma*-tocopherol. While at the lowest concentration essayed (DC0.2αT), only signals of (HPO-c(*Z,E*)-dEs) are detected, although in higher concentration than in DC, as the concentration of *alpha*-tocopherol increases, further oxidation compounds are generated. These latter include, hydroxy-conjugated-(*Z,E*)-dienes, (HO-c(*Z,E*)dEs), such as those of hydroxy-conjugated-(*E,E*)-dienes (HO-c(*E,E*)dEs), such as of keto-conjugated-(*Z,E*)-dienes (KO-c(*Z,E*)dEs), as well as of keto-(*E*)-epoxy-(E)-monoenes (KO-*E*-EPO-*E*-mEs) and of saturated aldehydes.

***Information provided by SPME-GC/MS about the occurrence of oxidation reactions during *in vitro* digestion**

- Headspace of the mixture CDJ: The headspace of the sample reference, CDJ, which contains the undigested corn oil, has a reduced number of aldehydes, mainly alkanals, at a basal concentration, which is common in all non-oxidized edible oils.
- Corn oil digestate headspace: The headspace of the digestate DC contains a higher concentration of alkanals, of (*E*)-2-alkenals and of furan 2-pentyl than CDJ, and some 2,4-alkadienals absent in the headspace of CDJ, which proves that the corn oil has undergone oxidation during digestion.
- *Gamma*-tocopherol enriched corn oil samples headspace: In the headspace of the digestates of the three in *gamma*-tocopherol enriched samples, the same oxidation markers have been found as in DC, but in small abundance in all cases. In agreement with results obtained by ^{1}H NMR, this effect is much more evident in the most *gamma*-tocopherol enriched samples.
- *Alpha*-tocopherol enriched corn oil samples headspace: The headspace of this digestate contains not only the oxidation compounds detected in DC in much more abundance, but also new oxidation markers not present in DC, including alkanals, (*E*)-2-alkenals, 2,4-alkadienals and even oxygenated *alpha,beta* unsaturated aldehydes of known toxicity, furanones and a significant number of furan derivatives, some of these in considerable abundance. This evidences the greater prooxidant role of *alpha*-tocopherol in line with its higher concentration in the oil sample.

Bioaccessibility of *gamma*- and *alpha*-tocopherol in the different digestates. Influence of the enrichment with *alpha*-tocopherol on the bioaccessibility of *gamma*-tocopherol naturally present in corn oil. The ^{1}H NMR spectroscopy allows one to estimate the bioaccessibility of these compounds, which can be expressed in two different ways. One way of describing the bioaccessibility of tocopherols could be through the parameter B_T, ($B_T=([T_D]/[FA+AG]_D)$), which informs about the amount of tocopherol (given in mmol) that can be absorbed in relation to the amount of the main lipid components in the sample (given in mol). Another way to express bioaccessibility could be through the parameter $B´_T$, ($B´_T=([T_D]/[T_O])$). This parameter gives information about the amount of this compound lost during *in vitro* digestion and indicates the fraction of the original amount of this compound in the oil that after digestion is available to be absorbed.

- Regarding the bioaccessibility of *gamma*-tocopherol naturally present in the corn oil C. The $B_{\gamma T}$ value of DC digestate (0.33±0.00 mmol/mol (FA+AG)) indicates that a certain amount of this compound remains after *in vitro* digestion without degrading. Moreover, $B´_{\gamma T}$ is 0.67 ± 0.0 mmol/mmol (γT_O), indicating that up to around 67% of the *gamma*-tocopherol contained in the oil remains in the digestate without degrading and is able to be absorbed.
- Regarding the bioaccessibility of *gamma*-tocopherol in the digestates of the samples enriched with this compound. $B_{\gamma T}$ and $B´_{\gamma T}$ values of the digestates of these samples are, as could be expected, higher, the higher the enrichment degree of the sample is. These range between 0.89 ± 0.18 and 35.65 ± 1.12mmol/mol (FA+AG) or the same as from 0.65 ± 0.14 to 0.82 ± 0.03mmol/mol (γT_O).

- Regarding the bioaccessibility of *gamma*-tocopherol in the digestates of the samples enriched with *alpha*-tocopherol. In the digestate of the less *alpha*-tocopherol enriched sample DC0.2αT, $B_{\gamma T}$ reaches the same value as in the digestate of the unenriched DC. However, in the other two enriched samples DC2αTand DC5αT the $B_{\gamma T}$ value is almost the same for both samples (near 0.46 mmol/mol (FA+AG)). Bearing in mind that the concentration of *gamma*-tocopherol naturally present in corn oil C is 0.49 ± 0.00 mmol/mol (FA+AG), it is evident that this compound has almost undergone no degradation during *in vitro* digestion in the presence of high concentrations of *alpha*-tocopherol. This could be due to the great difference in concentration between *gamma*- and *alpha*-tocopherol in these two latter samples reducing the probability of *gamma*-tocopherol molecules being near the oxidation sites of the oil main components in comparison with that of *alpha*-tocopherol.
- Regarding the bioaccessibility of *alpha*-tocopherol in the digestates of the samples enriched with this compound. The bioaccesibility of *alpha*-tocopherol is null or small depending on the enrichment degree, and *alpha*-tocopherol is totally degraded in the sample having the smaller enrichment level. In addition, the other two samples (DC2αT and DC5αT) show small values, especially in the most enriched sample.

Aim 2.2. To address by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the antioxidant effectiveness of mono-, di- and tri-phenolic compounds (*gamma*-tocopherol (γ-T), hydroxytyrosol acetate (HTA) and dodecyl gallate (DG)) during the *in vitro* digestion of virgin flaxseed oil.

(Article 7)

The comparison between *in vitro* digestion of virgin flaxseed oil and that of the same enriched oil with different concentrations of dodecyl gallate, hydroxytyrosol acetate or *gamma*-tocopherol is approached as globally as possible, attending different aspects, such as extent and pattern of the lipolysis reached, bioaccessibility of main and minor compounds, as well as the occurrence of oxidation reactions and the bioaccessibility of oxidation compounds.

Extent and pattern of lipolysis produced by the *in vitro* digestion of flaxseed oil and effect of the enrichment with the different phenolic compounds. The results obtained show that a very important percentage of triglycerides have not undergone hydrolysis and, in fact, remain as the main glyceride specie after *in vitro* digestion. Monoglycerides and glycerol are present in the digestates in fairly high concentration, and indicates that the species able to be absorbed by enterocytes of the intestinal wall (monoglycerides and fatty acids) are in not very low concentrations after this *in vitro* digestion. And finally, diglycerides, which are not able to be absorbed, are in the lower concentration of all glyceride species. Moreover, the enrichment of the oil with these phenolic compounds, in the concentrations essayed, does not affect significantly the hydrolysis reached in the

digestion. This fact indicates that the added phenolic compounds have not inhibited the lipase activity. To the best of our knowledge, this is the first time that it has been shown that dodecyl gallate and hydroxytyrosol acetate are not able to inhibit lipase activity.

***In vitro* bioaccessibility of oil main components and influence of the enrichment with the different phenolic compounds.** The *in vitro* bioaccessibility of the flaxseed oil main components in the unenriched sample is only around fifty percent. Similar values have been found for the flaxseed oil samples enriched with the different phenolic compounds, being the differences among them not statistically significant.

Oxidation reactions during *in vitro* digestion of flaxseed oil and of flaxseed oil samples enriched in dodecyl gallate, hydroxytyrosol acetate and *gamma*-tocopherol. As it is well known, the oxidation reactions in oils provoke the degradation of some of their components and the formation of others that are new. For this reason, this subject can be tackled either monitoring the changes that occurred in the concentration of the former as consequence of the *in vitro* digestion, or monitoring the formation of the latter after digestion or by both.

***Changes provoked by the *in vitro* digestion, in the concentration of linolenic structures. Antioxidant efficiency of the added phenolic compounds.** The *in vitro* digestion of flaxseed oil (F) causes a significant diminution in the concentration of linolenic structures. In other words, this group undergoes degradation which provokes a reduction in the molar percentage in relation to the total moles of all kinds of fatty acids plus acyl groups (AG+FA) from 55.7 mol/mol (AG+FA) in F to 47.9 mol/mol (AG+FA) in DF.

The addition of different concentrations of the above-mentioned phenolic compounds avoids this degradation during digestion to a certain extent, even at the lower enrichment

level essayed. This indicates that the three compounds act as antioxidants. Nevertheless, their efficiency at avoiding the degradation of the linolenic structures is not the same for the three antioxidants tested. The results demonstrate that in *in vitro* digestion of flaxseed oil, the antioxidant efficiency of dodecyl gallate is greater than that of hydroxytyrosol acetate, which is in turn greater than that of *gamma*-tocopherol.

In the case of the enrichment with *gamma*-tocopherol and due to the availability of enough experimental data, it was possible to look for quantitative relationships between enrichment degree in the oil and linolenic concentration in the corresponding digestates, which is inversely related with oxidation level reached during *in vitro* digestion. This relationship fits well to a logarithmic equation ([Ln] = 51.06 + 1.16 *Ln* [γT] , R=0.9931). According to this equation, the relationship between the concentration of linolenic structures, which is higher, the higher the antioxidant efficiency, is directly related with *gamma*-tocopherol enrichment through the above logarithmic relation. Moreover, to reach a level to totally prevent the linolenic oxidation during *in vitro* digestion, requires an enrichment level of *gamma*-tocopherol near 54.59 mmol/mol $(AG+FA)_O$. This result indicates that the higher enrichment level essayed does not totally avoid oil oxidation during this *in vitro* digestion, as will be shown later. This approach above described shows a methodology which may be used with any other antioxidant whenever enough experimental data are available to estimate its antioxidant efficiency, and also to predict, in a fairly accurate way, the enrichment degree required of a compound to avoid lipid oxidation during *in vitro* digestion.

***Formation of oxidation compounds derived from flaxseed oil main components during *in vitro* digestion.** The degradation of the oil main components during *in vitro* digestion gives rise to the formation of oxidation compounds, most of which should be present in the lipid extracts of the corresponding digestates. The detection and

quantification of these can be carried out by means of ^{1}H Nuclear Magnetic Resonance spectroscopy using both the standard and NOESYGPPS experiments, and by means of Solid Phase Microextraction (SPME) followed by Gas Chromatography Mass Spectrometry (GC/MS).

a) ***Oxidation compounds detected in the different digestates by ^{1}H NMR. Effect of the enrichment in phenolic compounds.*** Two kinds of oxidation compounds have been detected by this technique in the lipid extracts of the digestates, evidencing that oxidation has taken place during *in vitro* digestion. These are hydroperoxides supported on chains having *Z,E* conjugated dienic systems (HPO-c(*Z,E*)-dEs) derived from octadecatrienoic groups, which are primary oxidation compounds, and n-alkanals, which are secondary oxidation compounds. In general, the higher the degradation of linolenic structures the higher the concentration of both kinds of oxidation compounds. Likewise, the greater the enrichment in phenolic compounds in the sample, the lower the concentration of oxidation compounds in the digestates. Finally, it is again demonstrated that, under the *in vitro* digestion conditions, dodecyl gallate shows higher antioxidant efficiency than hydroxytyrosol acetate and *gamma*-tocopherol. Likewise, hydroxytyrosol acetate shows greater antioxidant activity than that of *gamma*-tocopherol. Although hydroperoxides cannot be detected in the digestates of the samples more enriched in *gamma*-tocopherol, the presence of n-alkanals indicates that oxidation has taken place, in agreement with above-mentioned data. On the other hand, it should be pointed out that the new formed compounds could also be bioaccessible.

b) ***Oxidation markers detected by SPME-GC/MS in the different digestates. Effect of the enrichment in phenolic compounds.*** As already shown, the *in vitro* digestion provokes the oxidation of flaxseed oil main components, generating

volatile oxidation compounds derived mainly from the linolenic structures. Among the main volatile compounds coming from lipid oxidation are aldehydes, furan derivatives and ketones and these are the target of this study. The results obtained show that the enrichment of the oil with phenolic compounds has, as a consequence, a reduction in the oxidation level reached during *in vitro* digestion. This is proved because the concentration of these volatile oxidation compounds in the headspace of the digestates of the samples enriched with phenolic compounds is smaller than that found in the headspace of the digestate of the unenriched sample, confirming the same facts inferred from data coming from ^{1}H NMR.

***In vitro* bioaccessibility of some minor components of virgin flaxseed oil and specifically of *gamma*-tocopherol in the different digestates.** This virgin flaxseed oil contains, as is common in vegetable oils, tocopherols and sterols. Furthermore, this oil also contains a great number of terpenes and sesquiterpenes.

- *In vitro bioaccessibility of gamma-tocopherol.* The *in vitro* bioaccessibility of this compound can be expressed either by the concentration of this compound in the digestate $[\gamma T]_D$, given in mmoles, in relation to the concentration of the main components also in the digestate $[FA+AG]_D$, given in moles, by the equation $B_{\gamma T}= [\gamma T]_D/[FA+AG]_D$, or by the ratio between the concentration of the compound in the digestate $[\gamma T]_D$ and the concentration in the sample before digestion, $[\gamma T]_O$, as indicated by the equation $B'_{\gamma T}= [\gamma T]_D/[\gamma T]_O$. The results obtained using these equations show that during the *in vitro* digestion of the sample not enriched in phenols, almost the totality of the *gamma*-tocopherol present in the oil is degraded with only a small undegraded amount remaining. In the samples enriched in

dodecyl gallate and hydroxytyrosol acetate, a larger amount of *gamma*-tocopherol remained undegraded than it did in the unenriched sample. It could be interpreted that these added di- or tri-phenolic compounds have some protective effect on *gamma*-tocopherol. Furthermore, in the samples enriched with this latter compound, its bioaccessibility increases as does the enrichment level, with an important amount of the *gamma*-tocopherol remaining after digestion capable of being be absorbed, in agreement with BγT.

- *In vitro bioaccessibility of sterols.* The concentration of cycloartenol and 24-methylenecycloartenol, free or esterified, can be estimated jointly in samples before and after digestion. It is noteworthy that its concentration remains unchanged during *in vitro* digestion.
- *In vitro biaccessibility of terpenes and sesquiterpenes.* An important group of these compounds are present in the virgin flaxseed oil and their abundances before and after digestion, which reflect their concentrations in these samples and can be estimated by using SPME-GC/MS. The results indicate that the concentration of these compounds is not affected by either the digestion process or by the oxidation reactions that also take place during this process. In fact, abundance values of terpenes and sesquiterpenes in all digestates are very similar in both the unenriched sample and in the samples enriched in phenolic compounds.
- *In vitro biaccessibility of dodecyl gallate and hydroxytyrosol acetate.* Although these compounds have unoverlapped signals in the ^{1}H NMR spectrum, only small signals of both compounds are visible in the spectra of the samples most enriched with these phenols. However, they are not enough to be quantified, due to their low intensity. This low concentration of these phenols in the digestates could be due to either their being degraded almost totally during in vitro digestion,

transforming into other compounds by their action as antioxidant, or by other reactions among which their hydrolysis could be cited.

Aim 2.3. To study by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the behaviour of olive oil during *in vitro* digestion. Study of the lipolysis extent and pattern and oxidation reactions, as well as of the bioaccessibility of main and minor compounds. Comparison with corn oil and virgin flaxseed oil submitted to the same digestive conditions.

(Manuscript 8)

The *in vitro* digestion of olive oil enriched or not with phenolic compounds, will be addressed and compared with that of other oils of very different composition, such as corn and virgin linseed oils.

Extent and pattern of lipolysis produced during in vitro digestion of not enriched or enriched olive oil in phenolic compounds. In vitro bioaccessibility of oil main components. The lipolysis provokes the release of fatty acids (FA) due to the break of the ester bonds of triglycerides, yielding also diglycerides (DG), monoglycerides (MG) and glycerol (Gol). During *in vitro* digestion of both olive oils O_1 and O_2, the main formed lipolytic products are monoglycerides, around 43% and glycerol, around 27%. A parameter that represents in a global way both the extent and pattern of the lipolysis reached during *in vitro* digestion is the *in vitro* bioaccessibility of the oil main components B_{OMC} defined as the ratio between the real absorbable molecules after digestion and all absorbable potential molecules before digestion. The really absorbable molecules after digestion are the released fatty acids (FA) and monoglycerides (MG), $(FA+MG)_D$, present in the digestate. The potential absorbable molecules are fatty acids plus all acyl groups, $(FA+AG)_D$. Taking the aforementioned into account, the obtained results showed that B_{OMC} of DO_1 is slightly higher than DO_2.

* ***Comparison with the lipolysis yield and in vitro bioaccessibility of oil main components of corn and virgin flaxseed oils submitted to the same digestive conditions*.** The extent and pattern of lipolysis is very different among the oils. The concentration of monoglycerides in the digestates of corn oil DC and of virgin flaxseed oil DF reaches values near 31% and 24% respectively, which are smaller than in that of the olive oils. Therefore, as expected, the *in vitro* bioaccessibilities of oil main components in DC and DF, especially in DF, are smaller than in DO_1 and DO_2. This is in line with the lower extent of the lipolysis undergone by C and F oils during *in vitro* digestion compared with that of olive oils, which is also reflected in the molar percentages of different glyceryl species of the corresponding digestates. There may be many factors that influence the *in vitro* digestion lipolytic process. However, under the same digestive conditions the oil minor and main components present can be considered the main. The minor components of the three oils considered here are very different, not only with regard to their nature but also with regard to their concentrations, which is why no conclusion could be drawn in this regard. The oils main components are triglycerides, which support different kinds of acyl groups, with varied number of carbon atoms and unsaturation degrees. Furthermore, the acyl groups can occupy different positions in the backbone of the glyceryl group, forming in this way different kinds of triglycerides. All these variables can influence the extent and pattern of the lipolysis during *in vitro* digestion.

a) *Influence of the length and unsaturation degree of acyl groups present in the oil.* The oils here considered have important differences in the unsaturation degrees of their acyl groups. Olive oil is the richest in oleic acyl groups while corn and virgin flaxseed oils are rich in linoleic and linolenic acyl groups, respectively. In addition, they also contain acyl groups of different length. Thus, in olive and corn oils that have similar

molar percentages of saturated acyl groups S, the distribution of these between palmitic and stearic groups is of a similar order in both oils, the second group being much smaller in percentage than the first. However, in virgin flaxseed oil the molar percentage of saturated acyl groups S is smaller than in the other two oils, having only a slightly smaller percentage of stearic than of palmitic groups. Taking into account all this above mentioned compositional data and lipolysis extent of each oil, it seems evident that the unsaturation degree (or the saturation degree) of the oils influences greatly on the lipolysis extent reached during their digestion. The results obtained showed that the most unsaturated oil (virgin flaxseed oil) reaches the lowest lipolysis extent during digestion and the opposite is true for olive oils. Likewise, it is evident that the oleic acyl group has a slightly higher trend to be hydrolysed during digestion than linoleic acyl group, since olive oils reaches during digestion a lipolysis extent somewhat higher than corn oil.

b) *Influence of the distribution of the different kinds of acyl groups in the backbone of the triglycerides in each oil.* The importance of the distribution of the different acyl groups in the triglyceride is due to the ester hydrolysis that takes place mainly in the sn-1 and sn-3 positions of the triglyceride when pancreatic lipase and *A. niger* lipase are used. Published data about triglyceride profiles of these three kinds of oils evidence that none of these oils have triglycerides with both sn-1 and sn-3 positions occupied simultaneously by saturated acyl group, which is considered the group having the highest trend to be hydrolyzed. The analysis of the profile of the triglycerides of these oils evidences that those acyl groups in higher concentration in the oil are those that more frequently occupy the sn-1 and sn-3 positions in the triglyceride. From this, it seems to be clear that the differences in the lipolysis extent of these oils, under same digestive conditions, depend mainly on the different trend of each one of the acyl

groups to be hydrolyzed and on the concentration of each one of them in the oil, because the frequency of their presence in sn-1 and sn-3 positions of the backbone of the triglyceride is a function of the concentration of each acyl group in the oil.

c) *Quantitative relationships between lipolysis extent reached and concentration of the different kinds of acyl groups in the oil.* Potential quantitative relationships between lipolysis yield, expressed by the *in vitro* bioaccessibility of the oil main components B_{OMC} in the corresponding digestates, and concentration of oil main components in the oils (olive, corn and flaxseed) submitted to *in vitro* digestion, expressed by the molar percentages of the different kinds of acyl groups, were tested. Thus, the molar percentage of saturated, linolenic and of oleic acyl groups have an important influence in the lipolysis extent reached during *in vitro* digestion of these oils. Likewise, the molar percentages of saturated and also of oleic acyl groups are related to $B_{OMC,}$ or to the lipolysis extent, in a direct way; that is to say, the higher the concentration of saturated and of oleic acyl groups, the higher $B_{OMC,}$ and the opposite is true for the molar percentage of linolenic acyl groups. These results show the important negative influence of a high concentration of linolenic groups, or a high unsaturation degree in the oil, on its lipolytic process during its *in vitro* digestion, and also evidences that the concentration of oleic acyl groups is also positively related with the lipolysis extent, which has been proved for the first time in this study.

In order to go further in the study of the influence of the oil composition on the lipolysis extent reached during its *in vitro* digestion, equations involving two variables were tested trying to find a closer relationship than the above between *in vitro* bioaccessibility and concentrations of the different acyl groups in the oil. In the equations obtained, again the direct relationships between lipolysis extent and concentration of saturated and oleic acyl groups is evidenced, while it is observed that

the lipolysis extent is inversely related with the molar percentage of linolenic and linoleic acyl groups. The results suggest that the trend to be hydrolyzed of the saturated groups is high, and this decreases progressively as the unsaturation degree of the acyl group increases, reaching the lower trend in linolenic groups, whereas oleic and linoleic groups show an intermediated trend

Influence of the enrichment of olive oil with different phenolic compounds on lipolysis extent and oil main component bioaccessibility. The results show that no significant differences are found either in the molar percentage of any of the glyceryl species, or in the bioaccessibility of oil main components between the digestates of the not enriched olive oils and those of the olive oils enriched in phenolic compounds.

Evaluation of the occurrence of oxidation reactions during in vitro digestion of olive oil not enriched or enriched in phenolic compounds. The occurrence of lipid oxidation reactions during digestion can be evaluated either by the degradation of fatty acids and acyl groups by means of ^{1}H NMR spectroscopy or by the formation of oxidation compounds by both ^{1}H NMR spectroscopy and by solid phase microextraction followed by gas chromatography-mass spectrometry (SPME-GC/MS).

Analysis of potential changes in the concentration of unsaturated fatty acids-acyl groups during in vitro digestion of olive oils. No significant differences have been found between the molar percentages of the different kinds of acyl groups and fatty acids in both olive oils and their digestates.

a) *Comparison with corn and virgin flaxseed oils*. The comparison of the molar percentages of the main unsaturated acyl groups and fatty acids in these two oils and in their corresponding digestates evidence that, in both oils, oxidation took place

during their *in vitro* digestion processes. Therefore, the studied olive oils have higher oxidative stability than the corn and virgin flaxseed oils previously studied.

b) *Effect of the enrichment in phenolic compounds*. Taking into account that no degradation of olive oil acyl groups and fatty acids has been detected by ^{1}H NMR spectroscopy during *in vitro* digestion, or in other words, no oxidation measurable by this technique has been observed, the same trend is observed for the oils enriched in phenolic compounds. These will have, as consequence, a higher *in vitro* bioaccessibility of the added phenolic compounds than in other oils in which oxidation can take place during *in vitro* digestion.

Analysis of the formation of oxidation compounds during in vitro digestion of olive oils able to be detected by ^{1}H NMR. O_1 and O_2 olive oils, before being s subjected to *in vitro* digestion, do not contain primary oxidation compounds; however, both have a small concentration of saturated aldehydes. The *in vitro* digestion of O_1 oil does not provoke the formation either of primary or of secondary oxidation compounds able to be measurable by ^{1}H NMR spectroscopy. However, during the *in vitro* digestion of O_2, a very slight oxidation has taken place due to the digestate DO_2 containing a very low concentration of hydroperoxy conjugated *Z,E*-dienes (HPO-c(*Z,E*)-dEs).

a) *Comparison with corn and virgin flaxseed oils.* Both oils C and F are free of oxidation compounds in concentrations detectable by ^{1}H NMR spectroscopy. However, their digestates contain primary (HPO-c(*Z,E*)-dEs) and also secondary oxidation compounds (n-alkanals) which proves that, during the *in vitro* digestion of these oils, oxidation took place. The results show again that, both olive oils O_1 and O_2 present higher oxidative stability during *in vitro* digestion than the other two oils before mentioned, and this fact is of great importance from the health point of view.

b) *Effect of the enrichment in phenolic compounds.* The olive oil O_1 is free of hydroperoxides and only contain a basal concentration of aldehydes and when it is submitted to digestion, or not undergone oxidation, or it is as low that it is not possible to be detected by ^{1}H NMR spectroscopy. In this sense, no effect of enriching O_1 with dodecyl gallate and hydroxytyrosol was observed in the digestates. In the olive oil O_2 enriched with *gamma*-tocopherol, the oxidation is not avoided with the lowest level of enrichment, whereas with higher levels this is totally avoided. Taking into account all the above mentioned, the enrichment with either dodecyl gallate, hydroxytyrosol acetate or *gamma*-tocopherols could turns the olive oil into what could be called a functional food.

Analysis of the abundance of volatile oxidation markers in the headspace of the digestates estimated by using SPME-GC/MS. This technique has a great sensitivity, being able to detect volatile compounds in very low abundance. The increase in the abundances of volatile oxidation markers in the headspace of the olive oil digestates DO_1 and DO_2 with respect to the control, indicate that during digestion of both olive oils, a very slight oxidation has taken place. While it is also true that the headspace of DO_2 sample has higher abundances of volatile oxidation markers than that of DO_1.

a) *Comparison with the corn and virgin flaxseed oils*. The headspace of the corn and virgin flaxseed oils digestates, DC and DF, is richer in oxidation compounds than that of both DO_1 and DO_2 digestates, showing that the oxidation that occurred during olive oils *in vitro* digestion is smaller than that which occurred during corn (virgin flaxseed) oil *in vitro* digestion. This evidences again the higher oxidative stability of olive oils than that of corn and virgin flaxseed oils under the same *in vitro* digestive conditions,

a fact that can be considered very relevant from the health point of view due to the toxicity of oxidation compounds.

b) *Effect of the enrichment in phenolic compounds*. The small oxidation degree provoked during O_1 and O_2 olive oils digestion is clearly diminished with the enrichment in phenolic compounds, and is in higher extent, the higher the concentration of the phenolic compound is. The results evidenced that dodecyl gallate has higher antioxidant efficiency than hydroxytyrosol acetate, and the lowest antioxidant efficiency under these *in vitro* digestion conditions is that of *gamma*-tocopherol.

In vitro bioaccessibility of minor compounds involved in the in vitro digestion of olive oil not enriched or enriched in phenolic compounds. The *in vitro* bioaccessibility of both minor component and added phenolic compounds was estimated, since they are a very important indicator from the nutritional point of view.

****In vitro bioaccessibility of olive oils minor components.*** Olive oils and their digestates contain squalene, sterols such as cycloartenol and 24-methylencycloartenol detected and quantified by ^{1}H NMR spectroscopy and a certain number of terpenes and sesquiterpenes, which can be identified, and semiquantified by SPME-GS/MS. With these data, the *in vitro* bioaccessibility of all these compounds can be estimated. The presence of all these compounds in the digestates is of great importance because some beneficial health properties have been attributed to them.

- Squalene: The results prove that its concentration (near 4.76 mmol/mol [AG+FA]), is the same as in olive oils and in their digestates, which means that 100 % of squalene remains bioaccessible after *in vitro* digestion.

- Cycloartenol and 24-methylencycloartenol: The concentration of these compounds in olive oil is near 0.30 mmol/mol [AG+FA], and their concentration remains unchanged after the *in vitro* digestion, being 100% bioaccessible after digestion.
- Terpenes and sesquiterpenes: The abundances of these compounds are not affected by the several reactions that take place during the *in vitro* digestion, showing a great *in vitro* bioaccessebility.

In vitro bioaccessibility of the phenolic added compounds. The concentration of gamma-tocopherol, dodecyl gallate and hydroxytyrosol acetate added to olive oils can be determined, in both olive oils and in the lipid extracts of their digestates if they are present, by ^{1}H NMR, by using the area of non-overlapped signals. However, only hydroxytyrosol acetate was detected in the lipid extract of the digestate of the most enriched olive oil sample, DO_1HTA_2, and *gamma*-tocopherol was also detected in the lipid extracts $DO_2\gamma T_1$, $DO_2\gamma T_2$ and $DO_2\gamma T_3$ and therefore their *in vitro* bioaccessibility was estimated. It must be remembered that to these compounds have been attributed several beneficial effects. The bioaccessibility values are higher, the higher the enrichment degree in the oils before digestion was. It is noteworthy that in all cases the loss of these phenolic compounds during *in vitro* digestion of olive oils is smaller than during *in vitro* digestion of corn and virgin flaxseed oils enriched with similar concentrations of these phenolic compounds. These results are in agreement with the lower oxidation extent which occurred during *in vitro* digestion of olive oil than in that of corn and virgin flaxseed oil.

In vitro bioaccessibility of compounds formed in secondary reactions during in vitro digestion and potential consequences. The concentration of both primary oxidation

compounds, hydroperoxides, and secondary oxidation compounds, mainly aldehydes, is higher in corn and virgin flaxseed oils than in olive oils. This concentration represents the bioaccessibility of these compounds in each digestate. This fact can be considered very relevant form the health point of view due to the toxicity attributed to some of these oxidation compounds.

CONCLUSIONS

BLOCK 1

Methodological development and its application on the advancement of the knowledge about edible oils composition and the processes they are involved in, such as nixtamalization and oxidation. The Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography coupled to Mass Spectrometry (DI-SPME-GC/MS) and the Proton Nuclear Magnetic Resonance (^{1}H NMR).

(Articles 1, 2, 3, 5 and **Manuscript 4)**

Aim 1.1. Direct Immersion-Solid Phase Microextraction followed by Gas Chromatography coupled to Mass Spectrometry (DI-SPME-GC/MS) as a very useful new tool. **(Article 1-3)**

1) It has been proved that **DI-SPME** of the liquid matrix of edible oils and the study of the extracted compounds by **GC/MS** constitutes a **powerful tool** able to provide qualitative and quantitative data on minor compounds naturally present in edible oils, as well as compounds coming from degradation of both main and minor components of the oils.

2) The **presence** of a **great number** of **minor oil components** belonging to a great variety of chemical families, including sterols, tocols, hydrocarbons, lactones, monoglycerides and fatty amides, among others, have been determined in **different types** of **vegetable edible oil**. **This allows**:

    - To obtain a **great deal** of **information** on diverse aspects of the oils, such as nutritional value, oxidative stability, quality, safety and even fraudulent practices.
    - To **discriminate** in a simple way **between different** types of **oils**, even those from the same vegetable origin.

3) Likewise, the **occurrence only in corn oil**, among different vegetable oils studied, of a great number of minor **nitrogenated components,** to which **sensory attributes** and **bioactive abilities** are attributed, has been demonstrated for the first time. Among those, there are:

    - **Cyclic dipeptides**, derived mainly from proline, leucine/isoleucine and phenylalanine and of other amino acids.

- **Oxazolines, pyrrolidines** and other **pyrrol derivatives**, **indol derivatives** as well as **pyrazol, pyridine** and ***β*-phenylethylamine derivatives**.

4) This **methodology also provides information** at the same time about:
    - The **degradation rate** of some of those above-mentioned **minor compounds** and about the identity and **formation rate** of **compounds coming from** their **degradation** during corn oil accelerate storage process. **Some** of these degradation compounds can be considered a very **good** corn oil **oxidation marker**.
    - The **formation** and **evolution** of **secondary oxidation volatile compounds** derived from main corn oil components. The abundance of these becomes noticeable coinciding with those above-mentioned minor compounds having antioxidant ability.

5) The **usefulness** of **this technique** for the determination of different kinds of compounds, either those naturally present in the oils or even those generated during oxidation processes, **is shown for the first time**, providing new information which would be difficult, if not impossible, to obtain by other methods.

6) Moreover, the **potential** of this technique **is reinforced** by the fact that it completely avoids the use of any type of solvent or reagent, and that all the information is obtained in the same chromatographic run and without sample modification, representing a considerable saving in time and cost.

Aim 1.2. Proton Nuclear Magnetic Resonance (^{1}H NMR) as a powerful tool in the study of the edible oil oxidation processes. Advancement in the knowledge of the oxidation process. **(Manuscript 4 and Article 5)**

1) The **usefulness** and **versatility** of **^{1}H NMR** to study the changes that take place during edible oil processing has been **evidenced** and **reinforced** again. This technique **allows** the **study** and **detection** of the **compounds present** in the **oils** if their spectra have at least one signal that does not overlap with any other and if their concentrations in the oil are high enough.

2) During corn oil **accelerate storage process,** degradation of both main and minor compounds and formation of new ones coming from those takes place.

    - The **degradation** of **linoleic acyl group**, which is the main acyl group in corn oil, shows, in general terms, two different rates throughout the process.
    - Likewise, ***gamma*-tocopherol**, the main tocol in corn oil, is also degraded in two stages. It must be pointed that although its presence in the oil **does not avoid linoleic** acyl groups **degradation**, it is true that it **delays** the oil degradation.
    - It has been observed that, **Δ7-avenasterol** and **sitostanol**, which are in much lower concentration than *gamma*-tocopherol, have a greater stability and lower degradation rate than this. Sitostanol shows higher oxidative stability than Δ7-avenasterol, possibly attributed to the higher unsaturation degree of this latter.
    - As is well known, the **degradation** of original **oil main components** gives rise to formation of so called **primary oxidation compounds,** and, due to

the evolution of these later the formation of **secondary oxidation compounds** takes place. Among them there are:

i. **Well known oxyplipins** such as hydroperoxy- hydroxy- and keto-conjugated-dienes, as well as all kinds of oxygenate-*alfa,beta*-unsaturated aldehydes.

ii. An important number of **new oxylipins** detected **for the first time in edible** oil subjected to degradative conditions such as, dihydroperoxy-non-conjugated-dienes, hydroperoxy-epoxy-monoenes, hydroxy-epoxy-monoenes, keto-epoxy-monoenes, hydroxy-keto-monoenes, keto-hydroxy-epoxy-structures, and epoxy-monoenes. These findings are very important because most of these oxylipins have been found also in cells and have been **related to several diseases**.

iii. Certain concentration of formic acid and for the first time, it has been proved the subsequent formation in the triglycerides of poly-formates (and probably of other poly-esters), and also of poly-hydroxides and poly-ethers provoking the increase of the oil viscosity and their polymerization degree.

- From the **degradation** of original **oil minor components,** the presence of some sterols oxidation products, such as 5α,6α-epoxysitosterol+campesterol and 5β,6β-epoxysitosterol+campesterol is observed. They are only detectable in advanced oxidation level of the sample and in low concentrations.
- To the best of our knowledge, this is the first time that a global oxidation study in great detail has been made by means of ^{1}H NMR.

3) ^{1}H NMR has also been shown to be a very efficient and useful technique to study not only the lipids of **two corn varieties** (blue and white), but also of the tortillas derived, and consequently, helpful in the evaluation of the changes provoked by **nixtamalization** and **tortilla-making** in the corn lipids.

   - White corn lipids are richer in linoleic groups than blue corn lipids and the opposite is true for oleic groups. Therefore, **white corn lipids** show **higher unsaturation** degree.

   - **Both corn varieties** contain almost the **same** minor components, among which are **1,2-diglycerides**, **sterols**, **fatty acids** and **ferulates**, although in somewhat **higher levels in** the **white** than in the blue **corn**. For the first time the presence of ferulates in corn lipids is described.
   - During **nixtamalization** and **tortilla-making,** a total **loss** of **fatty acids**, a **partial** loss of **other minor components**, a small **reduction** in the **unsaturation degree** of the main lipid components, and **slight oxidation,** and consequently, **formation,** of some primary (**hydroperoxides**) and secondary (**alkanals**) oxidation compounds occur.

BLOCK 2

Advances in the knowledge of the edible oil *in vitro* digestion process. Antioxidant effectiveness of mono- or poly-phenolic compounds. Bioaccessibility of main and minor oil components.

(Articles 6-7 and **Manuscript 8)**

Aim 2.1. To investigate by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the effect of the addition of *alpha*-tocopherol (α-T) and *gamma*-tocopherol (γ-T) on the lipolysis extent, advance of the oxidation reactions and bioaccessibility of main and minor compounds, during *in vitro* digestion of corn oil. **(Article 6)**

1) The **lipolysis extent** reached in ***in vitro* digestion** of **corn oil** and of **samples enriched** in ***gamma*** and ***alpha*-tocopherol** is **high** and of a similar order in all cases. As consequence of the high lipolysis extent and of its pattern, **high bioaccessibility** of **oil main components** is reached.

2) The results obtained confirmed that ***in vitro* digestion** provokes corn oil **lipids oxidation** by the clear **diminution** of **linoleic structure**s in the digestate in relation to those existing in the oil, and by the **presence** of some **oxidation compounds**.

3) The presence of ***gamma*-** and ***alpha*-tocopherols** affect in **different ways** the **oxidative stability** of **corn oil** during the *in vitro* digestion.
    - ***Gamma*-tocopherol** clearly acts as **antioxidant**, avoiding to a certain degree the degradation of linoleic structures, as well as the formation of non-volatiles and volatiles oxidation compounds. This effect is clearly dose-dependent.
    - ***Alpha*-tocopherol** behaves as a **prooxidant**, in line with its higher enrichment degree. At high enrichment degree (2% and 5% in weight), hydroperoxy-, hydroxy- and keto-conjugated dienes keto-*E*-epoxy-*E*-monoenes and aldehydes, as well as a great number of volatile oxidation compounds have been detected in the digestates. All of them are well-

known oxidation markers and some of them are associated to degenerative diseases.

4) The results obtained evidence that the oxidation advances in corn oil ***in vitro*** **digestion** process, which takes place at 37 ºC during 6 hours, follows **different oxidation pathways** than that of oils of similar composition submitted to **accelerate storage conditions** (70 ºC).

5) Once again, the **usefulness** of the two techniques employed in this study, **^{1}H NMR** and **SPME-GC/MS**, is proved, as well as the need to use as many oxidation markers as possible in order to get a **global view** about the **oxidation process** and thus **avoid erroneous interpretation**.

6) Regarding the **bioaccessibility** of the **naturally** present ***gamma*-tocopherol** in the **corn oil,** it **cannot be considered low**, although a certain amount of this compound has been lost during *in vitro* digestion. This bioaccessibility is smaller than that of the toxic oxidation compounds generated.

7) It was also observed that, ***gamma*-tocopherol bioaccessibility** in the samples enriched in this compound is **higher the higher the enrichment level** is.

8) Likewise, the **bioaccessibility** of the **naturally** present ***gamma*-tocopherol** in the oil **samples enriched** in ***alpha*-tocopherol** is nearly **one hundred percent** in the **most enriched samples**, in spite of *alpha*-tocopherol provoking oil component oxidation in line with its higher enrichment degree.

9) **Added *alpha*-tocopherol** exhibits a **low bioaccessibility**, being even null in the less enriched sample, probably due to its behaviour as a prooxidant.

10) From all the above-mentioned results, it can be deduced that in-depth analysis, case by case, of the safety of enriching foods with compounds considered antioxidant should be mandatory, and caution should be taken in the indiscriminate intake of certain supplements. It would also be advisable to **review** the suitability of the **European legislation** that permits enrichment of refined edible oils with *alpha*-tocopherol under the principle of "*quantum satis*".

Aim 2.2. To address by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the antioxidant effectiveness of mono-, di- and tri-phenolic compounds (*gamma*-tocopherol (γ-T), hydroxytyrosol acetate (HTA) and dodecyl gallate (DG)) during the *in vitro* digestion of virgin flaxseed oil. **(Article 7)**

1) **Enrichment** of **virgin flaxseed oil** with different concentrations of **dodecyl gallate, hydroxytyrosol acetate** and ***gamma*-tocopherol**, does **not** appreciably **modify** either the **lipolysis** degree reached during *in vitro* digestion or its **lipolysis pattern** in comparison with the not enriched virgin flaxseed oil. This means that the **phenolic compounds** involved in this study, under the *in vitro* digestion conditions essayed, **do not inhibit** the **activity** of the digestive **enzymes**.

2) The *in vitro* **bioaccessibility** of the **virgin flaxseed oil main components (B_{OMC})** in the unenriched sample and in enriched samples is almost the same, around **fifty percent**.

3) ***In vitro* digestion** of **virgin flaxseed oil**, **provokes** a **small oxidation** degree:
    - **Diminishing** the **concentration** of **linolenic structures** or what is the same, showing a reduction in the molar percentage of these groups in relation to the total moles of all kinds of fatty acids plus acyl groups (AG+FA).
    - **Forming hydroperoxydes** supporting conjugated *Z,E* dienic systems, which are primary oxidation compounds.
    - **Generating** also **volatile secondary oxidation compounds**, such as aldehydes, furan derivatives and ketones, as well as alcohols, all of them well known oxidation markers.

4) It has been observed that the enrichment with the different **phenols reduce** the **oxidation degree** reached during the flaxseed oil digestion and, for this reason, the bioaccessibility of oxidation compounds, although not totally. **Dodecyl gallate shows** the **higher antioxidant efficiency** followed by **hydroxytyrosol acetate** and ***gamma*-tocopherol** successively, in a **dose-dependent** manner.

5) Likewise, the results obtained evidence that **antioxidant efficiency** of added ***gamma*-tocopherols** is related with its concentration through a **logarithmic relation**.

6) It was demonstrated that the **concentrations** of some **minor components** of the virgin flaxseed oil involved in this study, such as cycloartenol, 24-methylenecycloartenol, terpenes and sesquiterpenes, are **not modified** by *in vitro* digestion, showing **high** *in vitro* **bioaccessibility** near the unity.

7) The *in vitro* **bioaccessibility** of the ***gamma*-tocopherol contained** in **flaxseed oil** is very **small** but **increases** in line **with** the enrichment in **phenolic compounds**.

8) Although oxidation is produced during *in vitro* digestion even in the presence of important concentrations of *gamma*-tocopherol, it remains bioaccessible after digestion in the enriched samples in this compound.

9) Taking into account the obtained results, the **importance** of **indicating** the **accurate units** to which the **antioxidant efficiency** is referred should be noted, since the lack of clarity of some works in this respect could be the cause of the divergences between some studies regarding the antioxidant efficiency order of some compounds.

2.3. To study by means of proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry (SPME-GC/MS) the behaviour of olive oil during *in vitro* digestion. Study of the lipolysis extent and pattern and oxidation reactions, as well as of the bioaccessibility of main and minor compounds. Comparison with corn oil and virgin flaxseed oil submitted to the same digestive conditions. **(Manuscript 8)**

1) The **lipolysis extent** produced during the *in vitro* digestion of **olive oil** is **high**, yields an **important release** of **monoglycerides** and **fatty acids** and, consequently, the *in vitro* **bioaccessibility** of the olive **oil main components** is also **high**, and both **greater than** in those edible oils having higher unsaturation degrees, such as **corn** oil and **virgin flaxseed oil**.

2) Likewise, for the first time, very **close quantitative relationships** between **composition** of the **oil**, expressed in molar percentages of the different kinds of acyl groups, and their ***in vitro* bioaccessibility** have been found in which direct relation between saturated and oleic acyl groups and *in vitro* bioaccessibility and inverse relation between that and linoleic and linolenic acyl groups is observed.

3) The results obtained evidenced that some of the obtained **equations** have **predictive ability** and are able to predict with a high level of **approximation** the ***in vitro* bioaccessibility** of the main components of oils not involved in the development of these equations and can help to design lipid diets for different nutritional purposes.

4) It has been demonstrated that, differently to other polyphenolic compounds, ***gamma*-tocopherol**, **hydroxytyrosol acetate** and **dodecyl gallate do not inhibit** the **lipase activity**, which means that they do not modify either olive oil lipolysis

extent reached during *in vitro* digestion or the *in vitro* bioaccessibility of oil main components.

5) The **oxidation extent** reached during *in vitro* digestion of **olive oils** is **much smaller** than that reached during the digestion of other edible oil such as **corn** and **virgin flaxseed oils,** with the consequent repercussions on health due to the toxicity of oxidation compounds. Likewise, the **enrichment** with **phenolic compounds reduces** to minimum values its **oxidation extent**.

6) It has also been shown that the **concentration** of some **minor olive oil components** are **not modified** during the *in vitro* digestion being totally bioaccessible after digestion. This is of great importance due to the different beneficial bioactive capabilities attribute to most of them.

7) Moreover, the results obtained showed that the *in vitro* **bioaccessibility** of the **added phenolic compounds**, which are very interesting bioactive compounds, is **higher** in the digestate of **olive oil than** in those of **corn** and **virgin flaxseed oil**.

8) Finally, due to the very low oxidation level reached during *in vitro* digestion of olive oil, the **bioaccessibility** of the **oxidation compounds** formed, if any, is much **smaller** in the digestate of **olive oil than** in those of **corn** and **virgin flaxseed oil** with the consequent repercussions on health.

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OTHER CONTRIBUTIONS

Some of the results derived from the pre-doctoral research period gave rise to other contributions (poster presentation and / or oral communication) presented in several national and international scientific conferences:

6th International Congress about Own-Checks and Food Safety "Transparency and Communication. Key Factors in Food Safety"

I. AUTHORS: Ibargoitia, M. L., Alberdi-Cedeño, J., & Guillén, M. D.
TITLE: Presencia de compuestos tóxicos en bajas concentraciones en aceites comestibles presentes en el mercado. (*Poster presentation*)
CONFERENCE: 6th International Congress about Own-Checks and Food Safety "Transparency and Communication. Key Factors in Food Safety"
PLACE: Vitoria-Gasteiz, Spain
YEAR: 2016

II. AUTHORS: Ibargoitia, M. L., Alberdi-Cedeño, J., & Guillén, M. D.
TITLE: Novedades relacionadas con el valor nutricional del aceite de maíz y la alimentación saludable. (*Poster presentation*)
CONFERENCE: 6th International Congress about Own-Checks and Food Safety "Transparency and Communication. Key Factors in Food Safety"
PLACE: Vitoria-Gasteiz, Spain
YEAR: 2016

III. AUTHORS: Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D.
TITLE: Formación de compuestos tóxicos en aceites ricos en grupos poliinsaturados bajo condiciones de almacenaje acelerado. (*Poster presentation*)
CONFERENCE: 6th International Congress about Own-Checks and Food Safety "Transparency and Communication. Key Factors in Food Safety"
PLACE: Vitoria-Gasteiz, Spain
YEAR: 2016

IV. AUTHORS: Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D.

TITLE: Efectos perjudiciales del α-Tocoferol adicionado en concentraciones permitidas a aceites vegetales. (*Poster presentation*)
CONFERENCE: 6th International Congress about Own-Checks and Food Safety "Transparency and Communication. Key Factors in Food Safety"
PLACE: Vitoria-Gasteiz, Spain
YEAR: 2016

IX Congreso CyTA-CESIA

V. AUTHORS: Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D.
TITLE: Compuestos bioactivos detectados por primera vez en aceite de maíz. Dipétidos Cíclicos y otros Compuestos Nitrogenados. (*oral comunication*)
CONFERENCE: IX Congreso CyTA-CESIA
PLACE: Madrid, Spain
YEAR: 2017

VI. AUTHORS: Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D.
TITLE: Degradación de compuestos minoritarios de aceite de maíz considerados como antioxidantes bajo condiciones de almacenaje acelerado. (*Poster presentation*)
CONFERENCE: IX Congreso CyTA-CESIA
PLACE: Madrid, Spain
YEAR: 2017

2nd International Symposium on Lipid Oxidation and Antioxidants

VII. AUTHORS: Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D.
TITLE: Evolution of the Oxidation of Corn oil under accelerated Storage Conditions with and without the Addition of Dodecyl Gallate. (*Poster presentation*)
CONFERENCE: 2nd International Symposium on Lipid Oxidation and Antioxidants
PLACE: Karl-Franzens-Universität Graz (Graz, Austria)
YEAR: 2018

VIII. AUTHORS: Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D.
TITLE: α-Tocopherol accelerates the Formation of Primary and of Secondary Oxidation Compounds in Corn oil maintained at Intermediate Temperature. (*Poster presentation*)
CONFERENCE: 2nd International Symposium on Lipid Oxidation and Antioxidants
PLACE: Karl-Franzens-Universität Graz (Graz, Austria)
YEAR: 2018

2nd Food Chemistry Conference

IX. AUTHORS: Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D.
TITLE: Changes provoked on lipids of two corn varieties by nixtamalization and tortilla preparation. A study by ^{1}H NMR. (*Poster presentation*) (3rd prize)
CONFERENCE: 2nd Food Chemistry Conference
PLACE: Sevilla, Spain
YEAR: 2019

XII Iberoamerican Congress of Food, CIBIA 2019

X. AUTHORS: Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D.
TITLE: Improvement of the oxidative stability of olive oil in presence of dodecyl gallate. (*Poster presentation*)
CONFERENCE: XII Iberoamerican Congress of Food, CIBIA 2019
PLACE: University of Algarve (Faro, Portugal)
YEAR: 2019

X Congreso Nacional CyTA-CESIA

XI. AUTHORS: Alberdi-Cedeño, J., Ibargoitia, M. L., & Guillén, M. D.
TITLE: Efecto que el enriquecimiento de aceite de maíz con *alfa* y *gamma* tocoferol provaca en su proceso de digestión bajo condiciones *in vitro*. Un estudio comparativo. (*Poster presentation*)
CONFERENCE: X Congreso Nacional CyTA-CESIA

PLACE: Universidad de León (León, España)

YEAR: 2019

CONTRIBUTION I

PRESENCIA DE COMPUESTOS TÓXICOS EN BAJAS CONCENTRACIONES EN ACEITES COMESTIBLES PRESENTES EN EL MERCADO

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INTRODUCCIÓN

Los aceites comestibles, desempeñan un papel esencial en la industria alimentaria, en la nutrición humana y en la salud de los consumidores. La composición de los aceites depende de muchos factores entre los que se pueden citar el origen geográfico, la especie vegetal y variedad de la que proceden, las condiciones ambientales y tratamientos de los cultivos, así como el procesado empleado para su obtención y las condiciones de almacenaje [1]. Una característica esencial en los aceites, como en cualquier alimento es su seguridad. En este tema se centra esta comunicación referida a aceites comerciales.

OBJETIVOS

Analizar la presencia de compuestos tóxicos procedentes de la oxidación de ácidos grasos y triglicéridos en aceites comerciales presentes en el mercado.

MATERIALES Y METODOS

Los aceites estudiados se adquirieron en supermercados de la zona. El estudio de la presencia de este tipo de compuestos tóxicos se llevo a cabo mediante Microextracción en Fase Solida seguida de Cromatografía de Gases/Espectrometría de Masas (MEFS-CG/EM).

RESULTADOS

Aceites comerciales que se ajustan a la normativa vigente y que según la misma son aptos para consumo humano contienen aldehídos oxigenados *alfa,beta* insaturados, cuya toxicidad es ampliamente conocida. Hay que señalar que no sólo está presente 4-hidroxi-*E*-2-nonenal cuya toxicidad ha sido muy ampliamente estudiada en biomedicina, sino otros como 4-oxo-*E*-2-nonenal con toxicidad igual o superior a la de aquel y que probablemente se genera en el estudio cromatográfico a partir del mucho más reactivo y tóxico 4-hidroperoxi-*E*-2-nonenal. Aparte de estos, algunos aceites comerciales también contienen 4,5-epoxi-2-alquenales compuestos de conocida toxicidad que sin embargo están considerados como GRAS por la administración americana. A estos compuestos se les atribuye responsabilidad en enfermedades como Cáncer, Alzheimar o Parkinson y algunos de ellos se han detectado en tejidos humanos dañados unidos a proteínas. Hay que señalar que en algunos estudios referidos a 4-hidroxi-*E*-2-nonenal se indica que en concentraciones bajas no produce efectos tóxicos [2].

CONCLUSIONES

*La concentración de estos compuestos tóxicos presentes en algunos aceites comerciales es un indicador no solo de la calidad del aceite, sino también de su estado de oxidación y de su seguridad.

*El hecho de que estos compuestos tóxicos estén presentes en algunos aceites comerciales y teniendo en cuenta su toxicidad, se debería analizar la conveniencia de la inclusión de un nuevo parámetro indicador de la calidad y seguridad del aceite, que limite la concentración de estos compuestos.

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PRESENCIA DE COMPUESTOS TÓXICOS EN BAJAS CONCENTRACIONES EN ACEITES COMESTIBLES PRESENTES EN EL MERCADO

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INTRODUCCIÓN

Los aceites comestibles, desempeñan un papel esencial en la industria alimentaria, en la nutrición humana y en la salud de los consumidores. La composición de los aceites depende de muchos factores entre los que se pueden citar el origen geográfico, la especie vegetal y variedad de la que proceden, las condiciones ambientales y tratamientos de los cultivos, así como el procesado empleado para su obtención y las condiciones de almacenaje [1]. Una característica esencial en los aceites, como en cualquier alimento es su seguridad. En este tema se centra esta comunicación.

OBJETIVO

Analizar la presencia de compuestos tóxicos procedentes de la oxidación de ácidos grasos y de triglicéridos en aceites comerciales presentes en el mercado.

MATERIALES Y MÉTODO

MUESTRAS: aceites vegetales adquiridos en supermercados de la zona: oliva (O), oliva virgen extra (OVE), maíz (M), girasol (G), soja refinado (SR), soja virgen (SV) y lino (L)

ESTUDIO DE LAS MUESTRAS: mediante Microextracción en Fase Sólida seguida de Cromatografía de Gases/Espectrometría de Masas (MEFS-CG/EM).

RESULTADOS Y DISCUSIÓN

-Aceites comerciales que se ajustan a la normativa vigente y que según la misma son aptos para consumo humano, contienen aldehídos oxigenados *alfa*,*beta*-insaturados (AOαBIs), cuya toxicidad es ampliamente conocida [2]. La Tabla muestra la abundancia de algunos de ellos en los aceites estudiados.

-No sólo está presente 4-hidroxi-*E*-2-nonenal cuya toxicidad ha sido muy ampliamente estudiada en biomedicina, sino otros como 4-oxo-*E*-2-nonenal con toxicidad igual o superior a la de aquel y que probablemente se genera en el estudio cromatográfico a partir del mucho más reactivo y tóxico 4-hidroperoxi-*E*-2-nonenal.

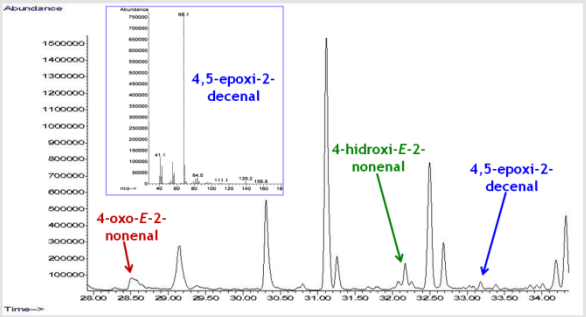
-Además, algunos aceites comerciales también contienen 4,5-epoxi-2-alquenales, compuestos de conocida toxicidad, que sin embargo están considerados como GRAS por la administración americana.

-A todos estos compuestos, se les atribuye responsabilidad en enfermedades como Cáncer, Alzheimer o Parkinson y algunos de ellos se han detectado en tejidos humanos dañados unidos a proteínas. Hay que señalar que en algunos estudios referidos a 4-hidroxi-*E*-2-nonenal se indica que en concentraciones bajas no produce efectos tóxicos [2]. La Figura muestra el cromatograma de uno de los aceites comerciales estudiados, junto con el espectro de masas de alguno de estos compuestos tóxicos.

AOαBIs identificados en los aceites estudiados y su abundancia expresada en cuentas de area del pico base de su espectro de masas por 10^{-4}, junto con su desviación estandar.

	4-Hidroxi-2-hexenal	4-Oxo-*E*-2-nonenal	4-Hidroxi-*E*-2-nonenal	4-Epoxi-*E*-2-decenal	4-Epoxi-2-decenal (iso)
O	--	11.5 ± 2.7	16.5 ± 3.9	4.2 ± 0.2	10.7 ± 0.2
OVE	2.0 ± 0.5	5.1 ± 0.2	9.2 ± 0.2	6.6 ± 0.6	17.0 ± 1.2
M	--	6.4 ± 0.9	14.5 ± 1.2	4.4 ± 1.1	12.2 ± 3.1
G	--	12.6 ± 0.7	20.6 ± 0.4	4.2 ± 0.2	11.7 ± 1.0
SR	--	3.0 ± 1.4	7.4 ± 3.1	0.8 ± 0.4	2.4 ± 0.8
SV	--	8.0 ± 1.0	10.1 ± 0.7	--	--
L	2.3 ± 0.8	7.7 ± 0.7	19.6 ± 5.7	4.5 ± 0.5	11.6 ± 1.0

--: no detectados
Aceites: oliva (O), oliva virgen extra (OVE), maíz (M), girasol (G), soja refinado (SR), soja virgen (SV) y lino (L)



Cromatograma de uno de los aceites comerciales estudiados junto con el espectro de masas de compuestos tóxicos presentes en ella

CONCLUSIONES

* La concentración de estos compuestos tóxicos presentes en algunos aceites comerciales es un indicador no solo de la calidad del aceite, sino también de su estado de oxidación y de su seguridad.

* Debido a que estos compuestos tóxicos están presentes en algunos aceites comerciales, se debería analizar la conveniencia de la inclusión en la legislación de un nuevo parámetro que limite su concentración en estos productos de alto consumo.

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CONTRIBUTION II

NOVEDADES RELACIONADAS CON EL VALOR NUTRICIONAL DEL ACEITE DE MAÍZ Y LA ALIMENTACIÓN SALUDABLE

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INTRODUCCIÓN

Todos los aceites comestibles tienen como componentes mayoritarios triglicéridos. Además de estos componentes principales también contienen una pléyade de componentes minoritarios de naturaleza muy variada entre los que se encuentran esteroles, tocoles, hidrocarburos, ácidos grasos libres y algunos ésteres de estos últimos entre otros. La mayoría de estos componentes son comunes a muchos aceites diferenciándose los aceites, además de por sus composición específica de triglicéridos por las concentraciones de algunos de los constituyentes de esas familias de compuestos. Sin embargo y a pesar del gran esfuerzo investigador dedicado al estudio de la composición de aceites todavía hoy en día quedan componentes de aceites por identificar. En este contexto se enmarca esta comunicación que se refiere a la composición del aceite de maíz

OBJETIVOS

Profundizar sobre la composición del aceite de maíz a través de un estudio no dirigido, empleando una técnica que proporciona información sobre la mayoría de los componentes minoritarios del aceite.

MATERIALES Y METODOS

Las muestras objeto de estudio fueron diferentes muestras de aceites de maíz refinados adquiridos en supermercados locales. El estudio se llevo a cabo mediante Microextracción en Fase Solida seguida de Cromatografía de Gases/Espectrometría de Masas (MEFS-CG/EM).

RESULTADOS

Al no ser un estudio dirigido, se trataron de identificar todos los componentes del aceite de maíz extraíbles mediante la técnica empleada. Los resultados obtenidos fueron tan sorprendentes que con objeto de confirmarlos se amplió el estudio a varios aceites de maíz. Los aceites de maíz estudiados tienen en su composición componentes nitrogenados, no esperables, y no descritos previamente en la literatura científica como componentes de aceite de maíz. Se trata concretamente dipéptidos cíclicos. La identidad de estos componentes es la misma en todos los aceites de maíz estudiados diferenciándose unos aceites de maíz de otros en la concentración. La importancia de estos resultados se debe a que estos compuesto además de tener importancia nutricional por ser dipéptidos, tienen actividad antioxidante probada y han demostrado también actividad antimicrobiana, anticancerígena y neuroprotectora [1-3].

CONCLUSIONES

- Por primera vez se pone de manifiesto que el aceite de maíz tiene entre sus componentes minoritarios un numeroso grupo de dipéptidos cíclicos procedentes de la ciclación de distintos aminoácidos esenciales.
- La presencia de estos compuestos en aceite de maíz aporta a este aceite un valor añadido importante tanto desde el punto de vista nutricional como desde el punto de vista de su capacidad para influir de forma beneficiosa en la salud de los consumidores.

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[1] Brack, C., Mikolasch, A. y S.chauer, F. (2014). Marine Biotech. 16(4), 385-395.
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NOVEDADES RELACIONADAS CON EL VALOR NUTRICIONAL DEL ACEITE DE MAÍZ Y LA ALIMENTACIÓN SALUDABLE

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INTRODUCCIÓN

Todos los aceites comestibles tienen como componentes mayoritarios triglicéridos. Además de estos componentes principales los aceites contienen una pléyade de componentes minoritarios de naturaleza muy variada entre los que se encuentran esteroles, tocoles, polifenoles, hidrocarburos, ácidos grasos libres y algunos ésteres entre otros. Sin embargo y a pesar del gran esfuerzo investigador dedicado al estudio de la composición de aceites todavía, hoy en día, quedan componentes por identificar. En este contexto se enmarca esta comunicación que se refiere a la composición del aceite de maíz.

OBJETIVO

Profundizar sobre la composición del aceite de maíz a través de un estudio no dirigido, empleando una técnica que proporciona información sobre la mayoría de los componentes minoritarios del aceite.

METODOLOGÍA

❖**MUESTRAS:** Diferentes aceites de maíz refinados adquiridos en supermercados de la zona.

❖**ESTUDIO DE LAS MUESTRAS:** El estudio se llevo a cabo mediante Microextracción en Fase Sólida seguida de Cromatografía de Gases/Espectrometría de Masas (MEFS-CG/EM).

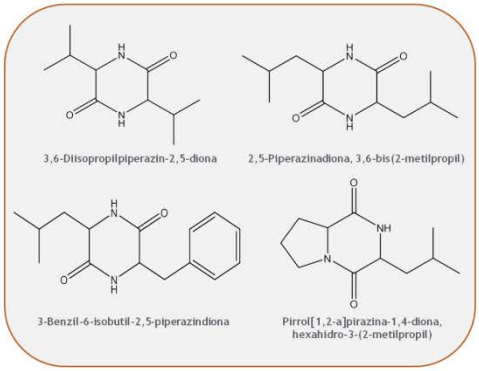
RESULTADOS

✓Al no ser un estudio dirigido, se trataron de identificar todos los componentes del aceite de maíz extraibles mediante la técnica empleada. Los resultados obtenidos fueron tan sorprendentes que con objeto de confirmarlos se amplió el estudio a varios aceites de maíz.

✓Todos los aceites de maíz estudiados contienen **compuestos nitrogenados**, no esperables, y **no descritos** previamente en la literatura científica como componentes del aceite de maíz. Se trata concretamente de **dipéptidos cíclicos.** La **Figura** muestra alguna de las estructuras químicas de los dipeptidos cíclicos encontrados en estos aceites.

✓La identidad de estos componentes es la misma en todos los aceites de maíz estudiados diferenciándose unos aceites de otros en la concentración.

✓El interés de estos resultados se debe a que estos compuestos además de tener importancia nutricional por ser dipéptidos, tienen actividad antioxidante probada y han demostrado también actividad antimicrobiana, anticancerígena y neuroprotectora [1-4].



Dipeptidos cíclicos identificados en las muestras de aceite de maíz refinado.

CONCLUSIONES

➢Por primera vez se pone de manifiesto que el aceite de maíz tiene entre sus componentes minoritarios un numeroso grupo de dipéptidos cíclicos procedentes de la ciclación de distintos aminoácidos esenciales.

➢La presencia de estos compuestos en aceite de maíz aporta a este aceite un valor añadido importante, tanto desde el punto de vista nutricional como desde el punto de vista de su capacidad para influir de forma beneficiosa en la salud de los consumidores.

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CONTRIBUTION III

FORMACIÓN DE COMPUESTOS TÓXICOS EN ACEITES RICOS EN GRUPOS POLIINSATURADOS BAJO CONDICIONES DE ALMACENAJE ACELERADO

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INTRODUCCIÓN

En los últimos años, la presencia de aldehídos oxigenados α,β-insaturados (AOαβIs) en alimentos es causa de preocupación ya que se consideran sustancias genotóxicas y citotóxicas y se les atribuye responsabilidad en enfermedades degenerativas tales como, Alzheimer ó Parkinson entre otras [1]. Estos compuestos se forman en la oxidación de los grupos acilo ω-6 y ω-3, presentes en alimentos en general y en aceites comestibles en particular [2, 3]. Por esto, es de gran interés conocer bajo qué condiciones pueden formarse y que alimentos o aceites los generan con mayor facilidad.

OBJETIVOS

Estudiar la propensión de un aceite vegetal rico en grupos acilo poliinsaturados ω-6 a generar AOαβIs bajo condiciones de almacenaje acelerado.

MATERIALES Y METODOS

La muestra objeto de estudio fue un aceite vegetal rico en grupos acilo poliinsaturados ω-6. El aceite se mantuvo bajo condiciones de almacenaje acelerado (70 ºC) en una estufa con aireación durante un periodo de 12 días. Con el fin de seguir su evolución se tomaron alícuotas cada 24 o 48 horas y se estudiaron mediante Microextracción en Fase Solida seguida de Cromatografía de Gases/Espectrometría de Masas (MEFS-CG/EM).

RESULTADOS

Bajo estas condiciones el aceite se degradó generando alcanales, *E*-2-alquenales, *E,E*- y *E,Z*-2,4-alcadienales; la reactividad y toxicidad de estos compuestos es mayor cuanto mayor es su grado de insaturación. También se formaron AOαβIs cuya toxicidad es superior a la de los anteriores, entre ellos se pueden citar a 4-hidroxi-*E*-2-nonenal, 4-oxo-*E*-2-nonenal y 4,5-epoxi-2-decenal. Tanto unos como otros se generaron a partir de la oxidación de los grupos acilo linoleico.

CONCLUSIONES

El aceite vegetal estudiado, y por extensión los aceites ricos en grupos acilo poliinsaturados ω-6, generan, bajo condiciones de almacenaje acelerado, aldehídos tóxicos entre otros AOαβIs, compuestos a los que se les atribuye responsabilidad en enfermedades degenerativas que afectan al mundo actual.

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FORMACIÓN DE COMPUESTOS TÓXICOS EN ACEITES RICOS EN GRUPOS POLIINSATURADOS BAJO CONDICIONES DE ALMACENAJE ACELERADO

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INTRODUCCIÓN

En los últimos años, la presencia de **aldehídos oxigenados α,β-insaturados** (AOαβIs) en alimentos es causa de preocupación ya que se consideran sustancias **genotóxicas y citotóxicas** y se les atribuye responsabilidad en enfermedades degenerativas tales como, Alzheimer ó Parkinson entre otras [1]. Estos compuestos se forman en la **oxidación de los grupos acilo ω-6 y ω-3**, presentes en alimentos en general y en aceites comestibles en particular [2, 3]. Por esto, es de gran interés conocer bajo qué condiciones pueden formarse y que alimentos o aceites los generan con mayor facilidad.

OBJETIVO

Estudiar la propensión de un aceite vegetal rico en grupos acilo poliinsaturados ω-6 a generar AOαβIs bajo condiciones de almacenaje acelerado.

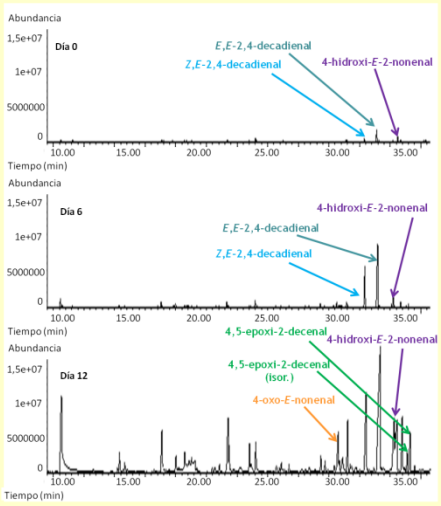
MATERIALES Y MÉTODOS

✓**MUESTRA:** aceite vegetal (M) rico en grupos acilo poliinsaturados ω-6.

✓**CONDICIONES DE ALMACENAMIENTO ACELERADO:** calentamiento a 70 °C en una estufa con aireación durante un periodo de 12 días.

✓**METODO:** toma de alícuotas a lo largo del almacenaje a intervalos regulares de 24-48 horas para su estudio mediante Microextracción en Fase Solida seguida de Cromatografía de Gases/Espectrometría de Masas (MEFS-CG/EM).

RESULTADOS



Cromatogramas de un aceite rico en grupos acilo poliinsaturados ω-6, a lo largo del almacenaje acelerado (días 0, 6 y 12).

- La Figura, muestra los cromatogramas del aceite original y del mismo aceite sometido a condiciones de almacenaje acelerado durante distintos periodos de tiempo (6 y 12 días). En ella se observa que a medida que **aumenta** el **tiempo de calentamiento mayor es la concentración** de **compuestos generados** en la **degradación** del **aceite.**
- Algunos de estos compuestos son, **alcanales**, ***E*-2-alquenales**, ***E,E-*** y ***E,Z*-2,4-alcadienales**. La reactividad y toxicidad de estos compuestos es mayor cuanto mayor es su grado de insaturación.
- Además, también se forman **AOαβIs** cuya toxicidad es superior a la de los anteriores. Entre ellos se pueden citar a, **4-hidroxi-*E*-2-nonenal**, **4-oxo-*E*-2-nonenal** y los **4,5-epoxi-2-decenal**. Tanto unos como otros se generaron a partir de la oxidación de los grupos acilo linoleico.
- La velocidad y mecanismo de formación de cada uno de estos tóxicos condiciona la evolución de su concentración en el aceite.

CONCLUSIONES

El aceite vegetal estudiado, y por extensión los aceites ricos en grupos acilo poliinsaturados ω-6, generan, **bajo condiciones de almacenaje acelerado**, aldehídos tóxicos entre otros, **aldehídos oxigenados α,β-insaturados (AOαβIs)**, compuestos a los que se les atribuye **responsabilidad** en **enfermedades degenerativas** que afectan al mundo actual.

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CONTRIBUTION IV

EFECTOS PERJUDICIALES DEL α-TOCOFEROL ADICIONADO EN CONCENTRACIONES PERMITIDAS A ACEITES VEGETALES

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INTRODUCCIÓN

El α-tocoferol (α-T), es una de las principales formas de la vitamina E, y se considera de gran interés por su conocida capacidad antioxidante. De hecho, su adición a aceites comestibles y otros alimentos está permitida en la Unión Europea sin indicación de límite máximo (principio *quantum satis*) [1]. Sin embargo, recientemente, están apareciendo evidencias de que, dependiendo de la concentración, puede comportarse como pro-oxidante [2,3]. Esta comunicación se enmarca en esta temática, ya que la confirmación de éste hecho tendría gran repercusión en la vida útil de los alimentos enriquecidos en α-T, en su seguridad y en sus efectos en la salud una vez ingeridos.

OBJETIVOS

Analizar el efecto que la adición de diferentes concentraciones de α-T provoca en la estabilidad oxidativa de un aceite vegetal rico grupos acilo poliinsaturados ω-6, sometido a condiciones de almacenamiento acelerado.

MATERIALES Y MÉTODOS

La muestra objeto de estudio fue un aceite vegetal rico en grupos acilo poliinsaturados ω-6 y el mismo aceite enriquecido con α-T en diferentes concentraciones: 0,2, 2 y 5% en peso. Todas las muestras se mantuvieron bajo condiciones de almacenaje acelerado (70 ºC) durante 8 días. Con el fin de seguir su evolución se tomaron alícuotas a lo largo del almacenaje y fueron estudiadas mediante Microextracción en Fase Solida seguida de Cromatografía de Gases/Espectrometría de Masas (MEFS-CG/EM).

RESULTADOS

La adición de α-T en las tres concentraciones estudiadas, disminuye la estabilidad oxidativa del aceite. Se ha observado que se favorece la formación de productos secundarios de la oxidación tóxicos a medida que la concentración de α-T aumenta. Así mismo se ha observado la formación de productos de degradación del α-T, algunos de los cuales se han descrito como citotoxicos [4].

CONCLUSIONES

* El α-T, en las concentraciones y condiciones estudiadas, actúa como pro-oxidante cuando se adiciona a aceite vegetal rico en grupos acilo poliinsaturados ω-6.

* El aceite enriquecido en α-T genera a más velocidad y en mayor concentración sustancias tóxicas que el aceite sin enriquecer, por lo que su seguridad es menor.

* La legislación debería ser modificada a la luz de estos resultados.

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AGRADECIMIENTOS

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EFECTOS PERJUDICIALES DEL α-TOCOFEROL ADICIONADO EN CONCENTRACIONES PERMITIDAS A ACEITES VEGETALES

Universidad del País Vasco · Euskal Herriko Unibertsitatea

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INTRODUCCIÓN

El α-tocoferol (α-T), es una de las principales formas de la vitamina E, y se considera de gran interés por su conocida capacidad antioxidante. De hecho, su adición a algunos aceites comestibles y otros alimentos está permitida en la Unión Europea sin indicación de límite máximo (principio *quantum satis*) [1]. Sin embargo, recientemente, están apareciendo evidencias de que, dependiendo de la concentración, puede comportarse como pro-oxidante [2,3]. Esta comunicación se enmarca en esta temática, ya que la confirmación de éste hecho tendría gran repercusión en la vida útil de los alimentos enriquecidos en α-T, en su seguridad y en sus efectos en la salud una vez ingeridos.

OBJETIVO

Analizar el efecto que la adición de diferentes concentraciones de α-T provoca en la estabilidad oxidativa de un aceite vegetal rico en grupos acilo poliinsaturados ω-6, sometido a condiciones de almacenamiento acelerado.

MATERIALES Y MÉTODOS

- ❑ MUESTRAS: **aceite vegetal rico en grupos acilo poliinsaturados ω-6 (S) y el mismo aceite enriquecido en α-T a diferentes concentraciones, tales como, 0.2% (S+0.2% α-T), 2% (S+2% α-T) y 5% (S+5% α-T) en peso.**
- ❑ CONDICIONES DE ALMACENAMIENTO ACELERADO: **calentamiento a 70 °C en una estufa con aireación durante un periodo de 8 días.**
- ❑ MÉTODO: **toma de alícuotas a lo largo del almacenaje a intervalos regulares de 24-48 horas para su estudio mediante Microextracción en Fase Solida seguida de Cromatografía de Gases/Espectrometría de Masas (MEFS-CG/EM).**

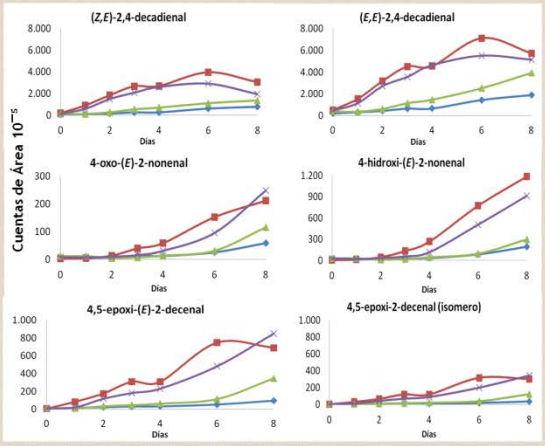
RESULTADOS y DISCUSIÓN

✓En la **Figura** se muestra la evolución de la concentración de alguno de los aldehídos mas reactivos formados como consecuencia de la oxidación de los 4 tipos de aceites estudiados (S; S+0.2% α-T; S+2% α-T; S+5% α-T) ocurrida bajo las mismas condiciones de almacenaje acelerado arriba mencionadas.

✓Puede observarse que la **concentración** de estos **compuestos secundarios** de la oxidación es **mayor** en los **aceites enriquecidos** en **α-T** que en el aceite original.

✓Hay que señalar que **alguno** de los **aldehídos formados**, tales como, los **aldehídos oxigenados α,β-insaturados** (4-oxo-(*E*)-2-nonenal, 4-hidroxi-(*E*)-2-nonenal y los 4,5-epoxi-2-decenal) son **tóxicos** [4].

✓En resumen, el **enriquecimiento** del aceite objeto de estudio con **α-T** en los tres niveles de concentración ensayados provoca en todos los casos una **disminución** de la **estabilidad oxidativa** del **aceite**.



Evolución de la concentración de alguno de los aldehídos mas reactivos formados como consecuencia de la oxidación de los aceites estudiados (♦ S; ▲ S+0.2% α-T; × S+2% α-T; ■ S+5% α-T)

CONCLUSIONES

- **El α-T, en las concentraciones y condiciones estudiadas, actúa como pro-oxidante cuando se adiciona a aceite vegetal rico en grupos acilo poliinsaturados ω-6.**
- **El aceite enriquecido en α-T genera a más velocidad y en mayor concentración sustancias tóxicas que el aceite sin enriquecer, por lo que su seguridad es menor.**
- **La legislación debería ser modificada a la luz de estos resultados.**

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[1] Reglamento (UE) nº 1129/2011 de la Comisión de 11 de noviembre de 2011. (OJ L 295, 12.11.2011, p. 1-177).
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CONTRIBUTION V

COMPUESTOS BIOACTIVOS DETECTADOS POR PRIMERA VEZ EN ACEITE DE MAÍZ. DIPEPTIDOS CÍCLICOS Y OTROS COMPUESTOS NITROGENADOS

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Se informa por primera vez de la presencia de determinados compuestos nitrogenados bioactivos en aceite de maíz. Además de las importantes propiedades que aportan al aceite de maíz, podrían considerarse marcadores de éste al estar ausentes en el resto de aceites estudiados.

Palabras clave – aceites vegetales, aceite de maíz, compuestos nitrogenados bioactivos

INTRODUCCIÓN

A pesar de que los aceites y grasas constituyen un grupo de alimentos que ha sido amplia y profundamente estudiado a lo largo del tiempo por su gran utilización en alimentación y su importante función en la nutrición humana, todavía quedan por conocer muchos de sus componentes. Esto se debe a que son mezclas extraordinariamente complejas cuyo estudio no es sencillo. Un grupo de compuestos minoritarios que hasta ahora ha recibido muy poca atención en aceites han sido los componentes nitrogenados. Esto se debe fundamentalmente a la falta de métodos establecidos para su determinación [1, 2]. Algunos estudios previos han abordado el estudio de proteínas y péptidos en aceites tras su hidrólisis a amino ácidos y posterior cuantificación global mediante HPLC. Sin embargo, en ningún caso se ha realizado la identificación y cuantificación de las moléculas nitrogenadas que realmente están presentes en el aceite.

En este contexto se plateó el estudio de la presencia de compuestos nitrogenados en aceites vegetales mediante el empleo de una metodología nueva [3] que no requiere someter al aceite a proceso previo alguno.

MATERIALES Y MÉTODOS

El estudio se llevó a cabo en once aceites comestibles de origen vegetal variado, mediante Microextracción en Fase Sólida por inmersión directa en la muestra seguida de Cromatografía de Gases/Espectrometría de Masas [3].

RESULTADOS Y DISCUSIÓN

Únicamente en los aceites de maíz estudiados se han encontrado por primera vez dipeptidos cíclicos, oxazolinas y pirrolidinas de ácidos grasos, así como derivados de indol y pirrol, además de otros compuestos nitrogenados. Sin embargo no están presentes en el resto de aceites estudiados. Estos compuestos permiten distinguir los aceites de maíz del resto de aceites y clasificarlos en función de los dipeptidos que contienen. Unos contienen

principalmente dipeptidos derivados de leucina/isoleucina y fenilalanina mientras que otros contienen principalmente dipeptidos derivados de prolina y fenilalanina. Estos compuestos además de contribuir con su capacidad antioxidante al aumento de vida útil de este aceite, le aportan atributos sensoriales interesantes, así como importantes propiedades bioactivas de potencial interés para la salud de los consumidores.

CONCLUSIONES

El estudio ha puesto de manifiesto, por primera vez, la presencia de compuestos nitrogenados bioactivos en aceite de maíz lo que supone un avance importante en el conocimiento de este aceite. Estos componentes proporcionan a este aceite importantes propiedades con repercusión en aspectos tecnológicos, nutricionales y de la salud.

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COMPUESTOS BIOACTIVOS DETECTADOS POR PRIMERA VEZ EN ACEITE DE MAÍZ. DIPÉPTIDOS CÍCLICOS Y OTROS COMPUESTOS NITROGENADOS

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CONTRIBUTION VI

DEGRADACIÓN DE COMPUESTOS MINORITARIOS DE ACEITE DE MAÍZ CONSIDERADOS COMO ANTIOXIDANTES BAJO CONDICIONES DE ALMACENAJE ACELERADO

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Se analiza simultáneamente y con la misma metodología la evolución de la degradación de compuestos minoritarios considerados como antioxidantes, así como la formación de compuestos derivados de ellos y de triglicéridos durante un proceso de termooxidación de aceite de maíz.

Palabras clave – aceite de maíz, compuestos minoritarios, termooxidación

INTRODUCCIÓN

La degradación de aceites bajo condiciones de almacenaje acelerado, se ha venido estudiando mediante métodos clásicos y en los últimos tiempos, los estudios se han centrado en el análisis de la evolución de los compuestos mayoritarios, es decir, de los triglicéridos [1, 2]. En muy pocas ocasiones se ha estudiado la evolución de los componentes minoritarios y hasta donde nosotros sabemos nunca se ha llevado a cabo el estudio de los compuestos en que estos últimos se transforman.

En este contexto, el objetivo de este trabajo es estudiar mediante una nueva metodología [3] la evolución que sufren algunos compuestos minoritarios presentes en el aceite de maíz cuando éste es sometido a condiciones de almacenaje acelerado así como, la formación de compuestos derivados de ellos. Simultáneamente y con la misma técnica se evalúa la generación de compuestos secundarios de la oxidación derivados de los componentes mayoritarios del aceite.

MATERIALES Y MÉTODOS

El estudio se llevo a cabo en aceite refinado de maíz sometido a condiciones de almacenaje acelerado (70 ºC con aireación), mediante Microextracción en Fase Sólida por inmersión directa en la muestra seguida de Cromatografía de Gases/Espectrometría de Masas [3].

RESULTADOS Y DISCUSIÓN

La técnica empleada permitió seguir, a lo largo del proceso y de forma simultánea la cinética de degradación de tocoles (tocoferoles, tocotrienoles y tocomonoenoles), escualeno y distintos esteroles así como, la cinética de formación de algunos compuestos generados en su degradación y de distintos tipos de aldehídos y otros compuestos secundarios de la oxidación derivados de los triglicéridos del aceite.

La simultaneidad de los procesos de degradación y de formación de nuevos compuestos proporciona una visión global del proceso de degradación del aceite y de la resistencia a la oxidación tanto de sus componentes minoritarios como mayoritarios.

CONCLUSIONES

Por primera vez el proceso de oxidación del aceite refinado de maíz ha sido estudiado mediante esta nueva metodología. Esta técnica, que no requiere modificación de la muestra, permite observar simultáneamente tanto la degradación de compuestos minoritarios de interés, como la generación de productos de oxidación derivados de los mismos. Asimismo permite estudiar la cinética de formación de compuestos secundarios de la oxidación derivados de los triglicéridos.

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DEGRADACIÓN DE COMPUESTOS MINORITARIOS DE ACEITE DE MAÍZ CONSIDERADOS COMO ANTIOXIDANTES BAJO CONDICIONES DE ALMACENAJE ACELERADO

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INTRODUCCIÓN

La degradación de aceites bajo condiciones de almacenaje acelerado, se ha venido estudiando mediante métodos clásicos y en los últimos tiempos, los estudios se han centrado en el análisis, mediante RMN de ^1H, de la evolución de los compuestos mayoritarios [1, 2]. Sin embargo, en muy pocas ocasiones se ha estudiado la evolución, en este proceso, de los componentes minoritarios y en muy pocas ocasiones se ha llevado a cabo el estudio de los compuestos en los que estos últimos se transforman.

OBJETIVOS

- Estudiar la evolución que sufren algunos compuestos minoritarios presentes en el aceite de maíz cuando éste es sometido a condiciones de almacenaje acelerado, así como, la formación de sus compuestos derivados, todo ello de forma simultánea.
- Simultáneamente evaluar la generación de compuestos secundarios de la oxidación derivados de los componentes mayoritarios del aceite.

MATERIALES Y MÉTODOS

❖MUESTRAS: aceite de maíz refinado (AMR).

❖CONDICIONES DE ALMACENAJE ACELERADO: calentamiento a 70 ºC en una estufa con aireación.

❖ESTUDIO DE LAS MUESTRAS: Mediante Microextracción en Fase Sólida por Inmersión Directa en la muestra seguida de Cromatografía de Gases/Espectrometría de Masas [3].

RESULTADOS Y DISCUSIÓN

❖Algunos de los componentes minoritarios en los que se ha centrado el interés en este estudio son los TOCOLES y los ESTEROLES.

EVOLUCIÓN DE TOCOLES

✓Bajo estas condiciones oxidativas en una primera etapa la concentración de TOCOLES (γ-, α-, β-, δ-tocoferoles, γ-, α-tocomonoenoles y γ-, α-tocotrienoles) apenas varía o sufre una pequeña disminución, indicando que la velocidad de degradación en esta etapa es pequeña en todos los casos (ver Figura 1; días 0-3).

✓En una segunda etapa la degradación de tocoles transcurre a mayor velocidad, desapareciendo la mayoría de ellos tras 9 días bajo las condiciones indicadas, a excepción de γ-, α- y δ-tocoferol, que permanecen en concentración muy bajas hasta el día 12 (ver Figura 1; días 3-12).

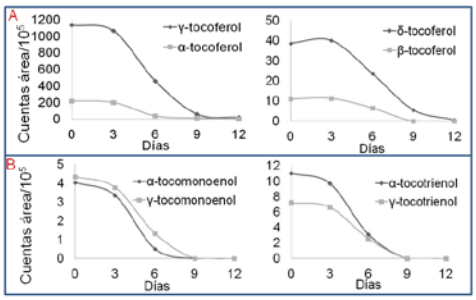

Figura 1. Evolución de la abundancia de los tocoles presentes en AMR, expresada en cuentas de área del pico base/10^5.

ALGUNOS COMPUESTOS FORMADOS EN LA DEGRADACIÓN DE TOCOFEROLES

✓Como compuestos procedentes de tocoferoles se forman entre otros 6,10,14-trimetilpentadecan-2-ona y 4,8,12,16-tetrametilheptadecan-4-olido (ver Figura 2).

✓Como compuestos derivados de α-tocoferol se han detectado los epóxidos en posiciones 5,6- y 2,3- de α-tocoferilquinona (ver Figura 2).

✓El incremento en la concentración de estos compuestos se produce simultáneamente con la disminución de la concentración de los productos de los que derivan.

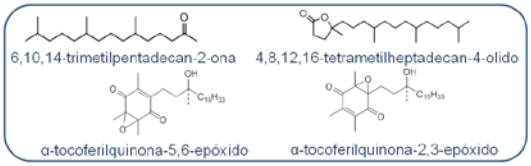

Figura 2. Estructuras químicas de compuestos formados en la degradación de tocoferoles.

EVOLUCIÓN DE ESTEROLES

✓Los ESTEROLES también sufren degradación (ver Tabla 1).

✓Todos ellos evolucionan de manera similar, manteniéndose bastante estables durante los primeros días de tratamiento y degradándose a más velocidad a partir del día 3, sin llegar a desaparecer (ver Tabla 1).

Tabla 1. Evolución de las abundancias de los principales esteroles, expresada en cuentas de área del pico base de su espectro de masas por 10^{-5}, junto a su desviación estándar.

	TIEMPO (días)				
ESTEROLES	0	3	6	9	12
Campesterol	13,6 ± 0,4	13,9 ± 0,6	9,1 ± 0,6	6,2 ± 0,1	1,9 ± 0,2
Estigmasterol	2,8 ± 0,1	2,6 ± 0,3	1,6 ± 0,3	1,2 ± 0,0	0,5 ± 0,4
β-sitosterol	32,7 ± 0,5	31,3 ± 1,9	19,5 ± 1,7	14,2 ± 0,4	5,3 ± 0,0
Δ5-avenasterol	2,5 ± 0,0	2,4 ± 0,2	1,4 ± 0,1	1,3 ± 0,0	0,5 ± 0,3

COMPUESTOS FORMADOS EN LA DEGRADACIÓN DE ESTEROLES

✓Entre otros se ha detectado, el 7-ketositosterol (ver Figura 3), en etapas avanzadas del proceso de oxidación, en concentraciones bajas

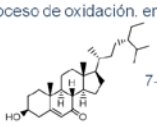

Figura 3. Estructura química de 7-ketositosterol.

COMPUESTOS DERIVADOS DE LOS TRIGLICÉRIDOS

✓Durante el proceso de almacenaje acelerado los componentes mayoritarios del aceite se degradan generando, entre otros, aldehídos tóxicos tales como, 4-hidroxi-(*E*)-2-nonenal, 4-oxo-(*E*)-2-nonenal y 4,5-epoxi-(*E*)-2-decenal.

✓La concentración de aldehídos aumenta lentamente hasta el día 9 a partir del cual, su velocidad de formación se incrementa notablemente. Este aumento coincide con la práctica total desaparición de la mayor parte de los tocoles (ver Figuras 1 y 4).

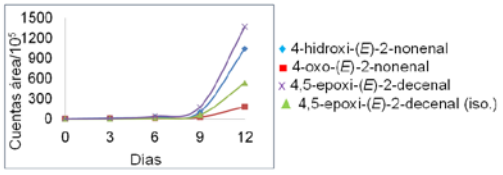

Figura 4. Evolución de la concentración de alguno de los aldehídos más reactivos formados como consecuencia de la oxidación de los triglicéridos.

CONCLUSIONES

➢Con la metodología empleada ha sido posible estudiar de forma simultanea la cinética de degradación de los compuestos minoritarios del aceite de maíz y la de formación de sus compuestos derivados, así como, la cinética de formación de compuestos secundarios procedentes de la degradación de los triglicéridos.

➢Bajo las condiciones ensayadas, los tocoles se degradan prácticamente en su totalidad al final del proceso, permaneciendo sin degradar pequeñas proporciones de esteroles.

➢La mayor velocidad de generación de aldehídos ocurre tras la degradación de la mayoría de los tocoles (día 9).

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CONTRIBUTION VII

EVOLUTION OF THE OXIDATION OF CORN OIL UNDER ACCELERATED STORAGE CONDITIONS WITH AND WITHOUT THE ADDITION OF DODECYL GALLATE

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The antioxidant activity of gallic acid as well as of their alkyl esters is well known, although prooxidant activity has also been reported (1). Gallic acid is a natural component of many foods and its alkyl esters are considered synthetic antioxidants used as additives. Thus, dodecyl gallate, named additive E312, is used at certain concentrations, in the manufacture of several foods, such as fats, oils, sauces and cereals among others, in accordance with Regulation (EC) Nº 1333/2008 (2).

The efficiency of gallic acid, as well as of other alkyl gallates has been studied by using well known assays such as, Total Phenolic Content (TPC), Radical Scavenging (ABTS), Ferric reducing Antioxidant Power (FRAP), Oxygen Radical Absorbance Capacity (ORAC); however, the weakness of these assays to give sound information about the antioxidant ability has been broadly commented. In other studies the parameters used to evaluate the antioxidant ability of the above mentioned compounds are the Peroxide Values (PV), Conjugated Dienes (CD) or Thiobarbituric Acid Reactance (TBAR); however these parameters do not provide information about the oxidation process underwent by the lipid involved.

In this context, this communication informs about the influence of dodecyl gallate on the evolution of the oxidation process of corn oil submitted to accelerated storage conditions. The study is carried out by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) and the degradation rate of the oil main components, as well as, the formation and degradation rates of primary oxidation compounds and the formation rate of secondary oxidation compounds are addressed giving a global picture of the process.

References

(1) Murakam, K., Ito, M., Htay, H. H., Tsubouchi, R., Iwata, S., & Yoshino, M. (2000). *Biomed. Res.*, *21*(5), 291-296.

(2) Regulation (EC) N° 1333/2008 of 16 December 2008 on food additives. *OJ* L 354, 31.12.2008, p. 16–33.

Acknowledgements

This work has been supported by the Spanish Ministry of Economy and Competitiveness (MINECO, AGL2015-65450-R), the Basque Government (EJ-GV. IT916-16), Department of Agriculture, Fisheries and Food of the EJ-GV (Pa 18/04). J. Alberdi-Cedeño thanks the EJ-GV for a predoctoral grant.

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Evolution of the oxidation of corn oil under accelerated storage conditions with and without the addition of dodecyl gallate

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INTRODUCTION

The antioxidant activity of gallic acid as well as of their alkyl esters is well known, although prooxidant activity has also been reported (1). Gallic acid is a natural component of many foods and its alkyl esters are considered synthetic antioxidants used as additives. Thus, dodecyl gallate (GD), (E312), is used as additive at certain concentrations, in the manufacture of several foods, such as fats, oils, sauces and cereals among others, in accordance with Regulation (EC) Nº 1333/2008 (2).
The efficiency of gallic acid, as well as of other alkyl gallates has been studied by using well known assays such as, Total Phenolic Content (TPC), Radical Scavenging (ABTS), Ferric reducing Antioxidant Power (FRAP), Oxygen Radical Absorbance Capacity (ORAC). However, the weakness of these assays to give sound information about the antioxidant ability has been broadly commented. In other studies the parameters used to evaluate the antioxidant ability of the above mentioned compounds are Peroxide Values (PV), Conjugated Dienes (CD) or Thiobarbituric Acid Reactance (TBAR). Nevertheless, these parameters do not provide information about the oxidation process underwent by the lipid involved.

OBJETIVE

Study the influence of dodecyl gallate (GD) on the evolution of the oxidation process of corn oil submitted to accelerated storage conditions.

MATERIALS AND METHODS

❖**SAMPLES:** Corn oil **(CO)** and enriched corn oil with different proportions of dodecyl gallate: **0.02%** **(CO+0.02GD)**; **0.2%** **(CO+0.2GD)**.

❖**ACCELERATED STORAGE EXPERIMENTS:** samples were submitted to accelerated storage conditions (70°C with aeration) until its total polymerization.

❖**STUDY OF THE SAMPLES:** the evolution of the samples was monitored by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) (3).

RESULTS AND DISCUSSION

✓Evolution of linoleic acyl groups

- The degradation of linoleic acyl groups take places earlier and faster in **non-enriched sample (CO)**.
- GD delayes the degradation of linoleic acyl groups. This effect is greater as the concentration of GD increases (**see Figure 1**).

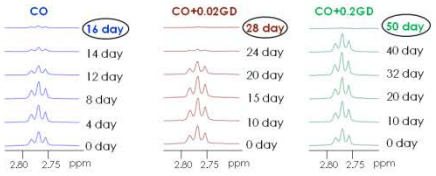

Figure 1. Evolution of the signals of *bis*-allylic protons of linoleic acyl groups at different days throughout the accelerate storage process, in **CO**, **CO+0.02GD** and **CO+0.2GD** samples.

✓Evolution of Z,E- and E,E- conjugated dienic hydroperoxides (primary oxidation compounds)

- GD delayes the formation of **(Z,E)-CD-OOH** and **(E,E)-CD-OOH** (**see Figure 2**).
- In the sample with highest level of GD **(CO+0.2)**, both **(Z,E)-CD-OOH** and **(E,E)-CD-OOH** are generated in less concentration.

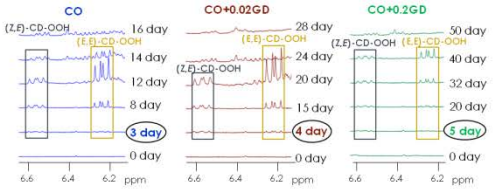

Figure 2. Evolution of the signals of **(Z,E)-CD-OOH** and **(E,E)-CD-OOH** protons at different days throughout the accelerate storage process, in **CO**, **CO+0.02GD** and **CO+0.2GD** samples.

✓Evolution of secondary oxidation compounds

- The formation of **aldehydes** is also delayed in presence of GD (**see Figure 3**).

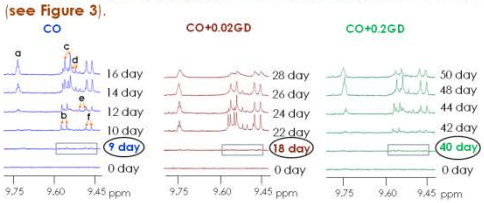

Figure 3. Evolution the signals of aldehydic protons at different days throughout the accelerate storage process, in **CO**, **CO+0.02GD** and **CO+0.2GD** samples.
a: n-alkanals; **b:** 4-hydroperoxy-(*E*)-2-alkenals; **c:** 4-hydroxy-(*E*)-2-alkenals; **d:** 4-epoxy-(*E*)-2-alkenals; **e:** (*E,E*)-2,4-alkadienals; **f:** (*E*)-2-alkenals.

- In the presence of GD, the formation of other secondary oxidation compounds, such as, **ketones**, **epoxides** and **alcohol** is also delayed.

CONCLUSIONS

❑ Dodecyl gallate (**GD**), exhibits potent **antioxidant activity** on corn oil submitted to accelerate storage conditions. This effect is greater as the concentration of GD increases.

❑ **GD delays** the **degradation** of **linoleic acyl groups**.

❑ The formation of both **primary** and **secondary oxidation compounds** is also **delayed**.

❑ The enrichment with **GD does not modify** the **mechanism of the** of the **oxidation process** of corn oil, only provokes its delay.

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(1) Murakam, K., Ito, M., Htay, H. H., Tsubouchi, R., Iwata, S., & Yoshino, M. (2000). *Biomed. Res.*, 21(5), 291-296.
(2) Regulation (EC) Nº 1333/2008 of 16 December 2008 on food additives. *OJ L* 354, 31.12.2008, p. 16-33.
(3) Martínez-Yusta, A., Goicoechea, E., & Guillén, M. D. (2014). *Compr. Rev. Food Sci. Food Saf.*, 13(5), 838-859.

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CONTRIBUTION VIII

α-TOCOPHEROL ACCELERATES THE FORMATION OF PRIMARY AND OF SECONDARY OXIDATION COMPOUNDS IN CORN OIL MAINTAINED AT INTERMEDIATE TEMPERATURE

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The effect of α-tocopherol (α-T) on the oxidation of systems such as vegetable oils, emulsions and purified oil triglycerides has been extensively investigated (1, 2). Nevertheless, the results obtained in these studies are in some cases contradictory. This may be due to different factors such as the lipid composition, the analytical techniques and methods employed, or the conditions to which the systems lipid + α-T were submitted.

Due to the addition of α-T to refined oils is allowed in the EU under the "*Quantum satis*" principle (3), it is important to clarify whether this addition causes beneficial or detrimental effects on the oils from the point of view of their oxidative stability.

In this context corn oil, not enriched and enriched with different concentrations of α-T, was submitted to accelerated storage conditions and the evolution of the different samples was monitored by ^{1}H Nuclear Magnetic Resonance. This technique allows in each run the simultaneous study of the original oil components as well as of the new primary or secondary compounds formed, providing a global view of the status of the sample (4). The obtained results show that the enrichment of this oil with α-T causes the decrease of its oxidative stability and accelerates the formation not only of the primary oxidation compounds but also of the secondary ones, and this effect is greater as greater the concentration of α-T is. These results suggest that it is necessary to go deeper into this matter to clarify if European legislation is adequate in this important issue.

Reference

(1) Huang, S. W., Frankel, E. N., & German, J. B. (1994). *J. Agric. Food Chem.*, *42*(10), 2108-2114. **(2)** Martin-Rubio, A. S., Sopelana, P., Ibargoitia, M. L., & Guillén, M. D. (2018). *Food Chem.*, *245*, 312-323. **(3)** Commission Regulation (EU) No 1129/2011 of 11 November 2011. *OJ* L 295, 12.11.2011, p. 1-177. **(4)** Martínez-Yusta, A., Goicoechea, E., & Guillén, M. D. (2014). *Compr. Rev. Food Sci. Food Saf.*, *13*(5), 838-859.

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α-Tocopherol accelerates the formation of primary and of secondary oxidation compounds in corn oil maintained at intermediate temperature

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INTRODUCTION

The effect of α-tocopherol (α-T) on the oxidation of systems such as vegetable oils, emulsions and purified oil triglycerides has been extensively investigated (1, 2). Nevertheless, the results obtained in these studies are in some cases contradictory. This may be due to different factors such as the lipid composition, the analytical techniques and methods employed, or the conditions to which the systems lipid + α-T were submitted. Due to the addition of α-T to refined oils is allowed in the EU under the "*Quantum satis*" principle (3), it is important to clarify whether this addition causes beneficial or detrimental effects on the oils from the point of view of their oxidative stability.

OBJECTIVE

Study the influence of the addition of α-T on the degradation of original corn oil components, as well as on the new primary or secondary compounds formed at intermediate temperature.

MATERIALS AND METHODS

❑SAMPLES: corn oil (CO) and enriched corn oil with different proportions of α-T: 0.02% (CO+0.02); 0.2% (CO+0.2); 2% (CO+2) and 5% (CO+5).

❑ACCELERATED STORAGE EXPERIMENTS: samples were submitted to accelerated storage conditions (70 ºC with aeration) during sixteen days.

❑STUDY OF THE SAMPLES: the evolution of different samples was monitored by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) (4).

RESULTS AND DISCUSSION

1. EVOLUTION OF LINOLEIC ACYL GROUPS

❑ The rate of degradation of linoleic acyl groups, the main acyl groups in this oil, depends clearly of the α-T concentration.

❑ The higher the α-T concentration, the higher the linoleic degradation rate is, and as a consequence, the greater the decrease of its concentration during a very broad period of time (see Figure 1).

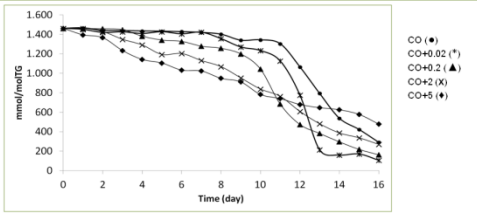

Figure 1. Evolution of the concentration (mmol/molTG) of linoleic acyl group *versus* time (day).

2. FORMATION OF PRIMARY OXIDATION COMPOUNDS

❑ The addition of α-T accelerates the formation of primary oxidation compounds (hydroperoxides), being this greater as higher the concentration of α-T is (Figure 2).

❑ The enrichment of the oil with α-T modified the distribution of **(*Z*,*E*)-CD-** versus **(*E*,*E*)-CD-hydroperoxides**.

❑ The higher the α-T concentration, the greater the formation of **(*Z*,*E*)-CD-OOH** and smaller that of **(*E*,*E*)-CD-OOH** (see Figure 3)

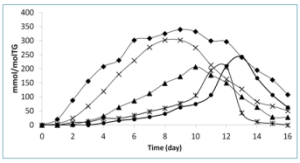

Figure 2. Evolution of the concentration (mmol/molTG) of hydroperoxide group *versus* time (day).
CO (●); CO+0.02 (*);CO+0.2 (▲); CO+2 (x) ;CO+5 (♦).

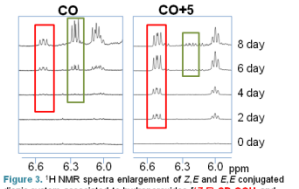

Figure 3. ^{1}H NMR spectra enlargement of *Z*,*E* and *E*,*E* conjugated dienic system associated to hydroperoxides [(*Z*,*E*)-CD-OOH and (*E*,*E*)-CD-OOH)] of **CO** and **CO+5** samples.

3. FORMATION OF SECONDARY OXIDATION COMPOUNDS

❑ The enrichment of corn oil with α-T also accelerates the formation of secondary oxidation compounds, such as, aldehydes, alcohols and epoxides (Figure 4).

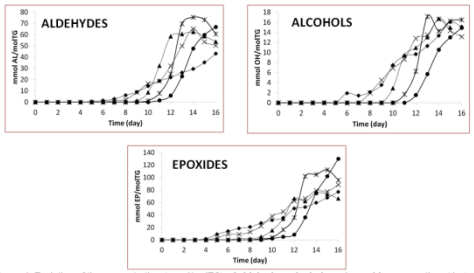

Figure 4. Evolution of the concentration (mmol/molTG) of **aldehydes**, **alcohols** and **epoxides** *versus* time (day).
CO (●); CO+0.02 (*);CO+0.2 (▲); CO+2 (x) ;CO+5 (♦).

❑ Only in the sample with the highest concentration of α-T **(CO+5)**, the presence of **(*Z*,*E*)-2,4-alkadienals** is detected.

CONCLUSIONS

❖Under the conditions studied the enrichment of corn oil with **α-T** provokes decrease of its oxidative stability, due to its behavior as **prooxidant**.
❖**α-T accelerates** the degradation of the linoleic acyl groups as well as the **formation** not only of **primary** but also of **secondary oxidation compounds**, being this effect greater as higher the concentration of α-T is.
❖These results suggest that it is necessary to go deeper into this matter to clarify if European legislation is adequate in this important issue.

REFERENCES
(1) Huang, S. W., Frankel, E. N., & German, J. B. (1994). *J. Agric. Food Chem.*, 42(10), 2108-2114. (2) Martin-Rubio, A. S., Sopelana, P., Ibargoitia, M. L., & Guillén, M. D. (2018). *Food Chem.*, 245, 312-323. (3) Commission Regulation (EU) No 1129/2011 of 11 November 2011. OJ L 295, 12.11.2011, p. 1-177. (4) Martinez-Yusta, A., Goicoechea, E., & Guillén, M. D. (2014). *Compr. Rev. Food Sci. Food Saf.*, 13(5), 838-859.

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CONTRIBUTION IX

CHANGES PROVOKED ON LIPIDS OF TWO CORN VARIETIES BY NIXTAMALIZATION AND TORTILLA PREPARATION. A STUDY BY ^{1}H NMR

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Nixtamalization is a technology developed by the Mesoamerican peoples today commonly used in many American countries. This process provokes chemical changes with important nutritional repercussions on corn nutrients such as niacin bioavailability, enrichment of the corn kernels in calcium ions, among others [1, 2]. Furthermore, while the loss of a certain amount of lipids has been described, this issue requires further study [3]. Likewise, the potential oxidation of corn lipids over the whole process, including tortilla-making, is a subject of great interest [4].

The aim of this work is the study of the effect of nixtamalization and tortilla-making on the changes provoked in corn lipids composition including their potential oxidation.

The study was carried out with corn of two varieties, one native blue, B, (Tziranza variety) and another white, W, (Sonaloa variety) and submitted to nixtamalization process. With the obtained dough, tortillas BT and WT were prepared. The lipids of raw corn kernels (BR and WR) and the lipids contained in tortillas (BT and WT) were extracted using hexane as solvent. They were studied by ^{1}H NMR [5].

Unsaturation degree, molar percentages of the different kinds of fatty acids and acyl groups, concentration of fatty acids, 1,2-diglycerides, methyl esters, phosphatidylcholine, sitosterol+campesterol, 7-avenasterol, sitostanol+campestanol, stigmastanol, ferulates and of oxidation compounds were monitored in the four lipid samples.

Nixtamalization and tortilla-making modify slightly the unsaturation degree of the corn lipids and the molar percentage of the different kinds of acyl groups. However, it reduces the concentration of minor components, especially of fatty acids. Furthermore, a slight oxidation occurs, as the presence of primary oxidation compounds at very low concentration shows.

References

[1] *Food Technol*., 42(10), 198-204 (1988)

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[3] *Cereal. Chem*., 65(3), 262-266 (1988)
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Acknowledgements
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Changes provoked on lipids of two corn varieties by nixtamalization and tortilla preparation. A study by ^{1}H NMR

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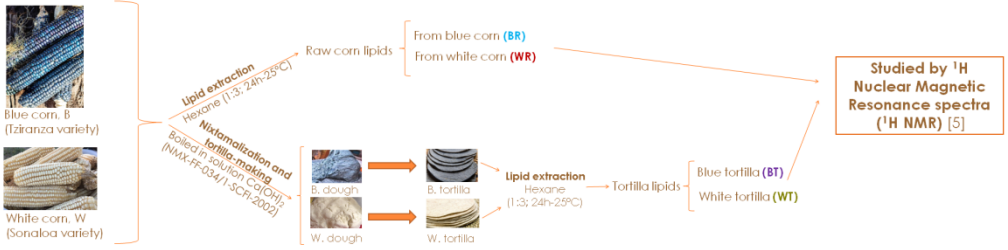
INTRODUCTION

Nixtamalization is a technology developed by the ancient Mesoamerican peoples, which is nowadays of common use in many countries. This process provokes chemical changes in corn components improving its nutritional characteristics, such as niacin bioavailability, the enrichment of the corn kernels in calcium ions, among others [1, 2]. Regarding lipids, the loss of a certain amount of them [3] has been described; however, in spite of their importance, the lipids evolution in this process including tortillas making has received scarce attention [4].

OBJETIVE

The aim of this work is the **study** by ^{1}H NMR spectroscopy of the **effect** of **nixtamalization** and **tortilla-making** on the **changes provoked in corn lipids** composition **including** their **potential oxidation**.

MATERIALS AND METHODS



RESULTS AND DISCUSSION

❖Changes in Main Lipid Components

❖ Corn, BR and WR lipids, as Table 1 shows, are **richer in linoleic than in oleic acyl groups**, being the concentration of the firsts higher in WR than in BR and the opposite occurs for oleic and saturated acyl groups.

Table 1. Molar percentages of the different kinds of acyl groups and fatty acids (AG+FA), in BR, WR, BT and WT samples and their Iodine values. Different letters within each row indicate a significant difference ($p < 0.05$).

	BR	WR	BT	WT
Linolenic (Ln)	1.05 ± 0.05a	1.29 ± 0.06b	0.92 ± 0.00c	1.26 ± 0.00b
Linoleic (L)	41.88 ± 0.39a	49.16 ± 0.47b	41.20 ± 0.13a	49.06 ± 1.99b
Oleic (O)	40.51 ± 0.19a	34.94 ± 0.01b	41.72 ± 0.01a	35.59 ± 2.30b
Saturated (S)	16.56 ± 0.17a	14.61 ± 0.40b	16.16 ± 0.12a	14.09 ± 0.31b
Iodine Values	117.53 ± 2.61a	127.70 ± 1.10b	114.98 ± 0.32a	125.16 ± 0.48b

❖ Nixtamalization and tortilla-making only **reduce slightly the concentration of polyunsaturated acyl groups** which is **evidenced** in the **Iodine Value** as Table 1 shows.

❖Changes in Minor Lipid Components

❖ Corn BR and WR lipids contain as **minor components fatty acids (FA)** and a numerous group of **other compounds in much smaller concentrations. Among these** there are some well known **sterols (sitosterol (S), campesterol (C), Δ5- and Δ7-avenasterol (Δ5A and Δ7A)), stanols (sitostanol (STN) and campestanol (CTN)), 1-2-diglycerides (1,2-DG), methyl esters (ME)** and **phosphatidylcholine (PC); these two latter** were **only detected** in BR and WR **respectively.** Both kinds of **corn lipids also contain ferulates (FE);** to the best of our knowledge, **this is the first time that the presence of this kind of compounds is described in corn lipids** (see Table 2).

Table 2. Concentrations, (mmol/mol TG), of minor components in BR, WR, BT and WT lipids. Different letters within each row indicate a significant difference ($p < 0.05$).

Minor compounds	BR	WR	BT	WT
1,2-DG	14.43 ± 0.81a	17.38 ± 0.44b	8.27 ± 0.01c	3.48 ± 0.48d
ME	0.74 ± 0.02	-	-	-
PC	-	0.45 ± 0.07	-	-
S+C+Δ5A	15.75 ± 1.84ab	19.84 ± 0.34b	13.55 ± 1.08a	14.38 ± 0.44a
Δ7A	1.02 ± 0.07a	1.18 ± 0.01a	1.01 ± 0.15a	0.91 ± 0.06a
STN	1.66 ± 1.46a	2.53 ± 0.07b	1.42 ± 0.17a	1.28 ± 0.06a
CTN	1.46 ± 0.26a	1.72 ± 0.02a	0.54 ± 0.10b	0.52 ± 0.06b
FE	2.55 ± 0.21a	3.27 ± 0.24a	1.21 ± 0.52b	0.98 ± 0.12b
FA	173.44 ± 0.06	190.29 ± 0.01	-	-

❖ Nixtamalization and tortilla-making **eliminate fatty acids** and **reduce** the **concentration of minor components** in such way that those in smaller concentrations such as, ME and PC **can not be detected in tortillas lipids.** Nevertheless, FE are **present in** the lipids of **both tortillas.**

❖Changes in Lipid Oxidation Status. Formation of Oxidation Compounds

❖ In the ^{1}H NMR spectra of BR and WR lipids there are **no** signals of **oxidation compounds** (see **Figure 1**).

❖ However, **during nixtamalization** and **tortilla-making some oxidation** take place. Thus, in the ^{1}H NMR spectrum of **BT** there are signals of **primary oxidation compounds** such as **Z,E-hydroperoxy conjugated dienes (ZE** in **Figure 1)** and of **secondary oxidation compounds** such as **alkanals (AL** in **Figure 1**). Likewise, in the ^{1}H NMR spectrum of **WT** signals of **primary oxidation compounds (ZE)** are observed (see **Figure 1**).

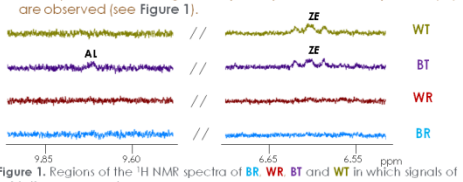


Figure 1. Regions of the ^{1}H NMR spectra of BR, WR, BT and WT in which signals of oxidation compounds appear.

CONCLUSIONS

➢ The **^{1}H NMR** spectroscopy has allowed the **detection and quantification** not only **of main** but also **of minor components of the lipids of raw corn and** of **their derived tortillas.** For the **first time** the **occurrence** of **FERULATES** has been described in these lipids.

➢ **Nixtamalization eliminates** totally the **FATTY ACIDS.** These are **present** in **significant concentrations** in **raw corn,** however **tortillas lipids are free** of **fatty acids.**

➢ Furthermore, during processing from corn to tortilla **the concentration of the other minor components and the unsaturation degree of the lipids are slightly reduced. In addition** a **slight oxidation** degree occurs, shown by the **presence** of **primary** and **secondary oxidation compounds** in tortillas lipids.

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ACKNOWLEDGEMENTS
This work has been supported by MINECO (AGL2015-65450-R) and EJ-GV (IT916-16; PA 19/02). J. Alberdi-Cedeño thanks the EJ-GV for a predoctoral grant and M. Molina thanks the CONACyT for the research fellowship.

CONTRIBUTION X

IMPROVEMENT OF THE OXIDATIVE STABILITY OF OLIVE OIL IN PRESENCE OF DODECYL GALLATE

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I. INTRODUCTION

Vegetable oils are widely used as cooking media and as ingredients and that why their oxidation mechanism, is a complex subject of great interest. Throughout the oxidation process, not only the reduction of nutritional and sensorial quality take place but also new compounds are generated, some of them with well known toxic effect for human health [1]. To delay this process and to extend the shelf life of the products, several natural and synthetic compounds have been widely used due to their potential antioxidant activity [2].

II. OBJETIVE

In this context and with the purpose of extend the shelf life of olive oil, the aim of this work is focused in the study of the influence that different amount of dodecyl gallate (DG) have on the oxidative stability of olive oil submitted to accelerate storage conditions.

III. MATERIALS AND METHODS

The samples were olive oil and the same oil enriched with different amount of DG (0.002, 0.02 and 0.2 % in weight). All the samples were submitted to accelerated storage conditions until their total polymerization. The degradation rate of their main and minor components as well as, the formation rate of different oxidation compounds was monitored by ^{1}H Nuclear Magnetic Resonance [3].

IV. RESULTS

The obtained results show that, the addition of DG in the studied concentrations (0.002%, 0.02% and 0.2%) improve significantly the oxidative stability of olive oil submitted to accelerate storage conditions. It has been observed that the degradation rate of the main acyl groups present is this oil is delayed as the concentration of DG increase. Likewise, the degradation rate of some minor compounds with well known bioactive and antioxidant activity is delayed. It must be taken into account that the generation rate of both primary and secondary oxidation compounds some of them with well known, is also delayed in presence of DG.

V. CONCLUSIONS

The present study demonstrates the potential effectiveness of DG as an antioxidant. The addition of GD in concentrations of 0.002 %, 0.02 % and 0.2 % to olive oil improve its oxidative stability increasing the product shelf life. This effect is greater as the concentration of DG in the medium increases.

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Improvement of the oxidative stability of olive oil in presence of dodecyl gallate

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Abstract

The aim of this work is to study the influence of different amounts of dodecyl gallate (DG) on the oxidative stability and oxidation mechanism of olive oil (OO) submitted to accelerated storage conditions by ^{1}H NMR. DG exhibits potent antioxidant activity. It delays the degradation of both main and minor components present in the oil, as well as, the formation of primary and secondary oxidation compounds. This effect is greater as the concentration of DG increases.

Introduction

Vegetable oils are widely used as cooking media and as ingredients, for this reason their oxidative stability and oxidation mechanism are subjects of great interest. It is well known that their oxidation provokes not only the reduction of their nutritional and sensorial quality but also the formation of new compounds, some of which are well-known for toxic effects on human health [1]. To delay this process and to extend their shelf life, natural and synthetic compounds claimed to have antioxidant ability have been tested [2]. Among these is dodecyl gallate (DG), which has been attributed antioxidant activity [3] but also the promotion of oxidative stress in cells [4].

Material and Methods

❖**SAMPLES:** Olive oil **(OO)** and enriched corn oil with different proportions of dodecyl gallate: **0.002%** **(OO+0.002DG)**; **0.02%** **(OO+0.02DG)**; **0.2%** **(OO+0.2DG)**.

❖**ACCELERATED STORAGE EXPERIMENTS:** samples were submitted to accelerated storage conditions (70ºC with aeration) until its total polymerization.

❖**STUDY OF THE SAMPLES:** the evolution of the samples was monitored by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) [5].

Results

✓*Degradation rate of unsaturated acyl groups*

❖The rate of degradation of oleic and linoleic acyl groups occurs earlier and faster in **OO**, than in the enriched samples (**OO+0.002DG**; **OO+0.02DG** and **OO+0.2DG**).

❖The **higher** the **DG concentration** in the oil, the **higher** the **stability** of its unsaturated acyl groups (Fig. 1).

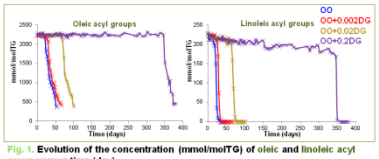

Fig. 1. **Evolution of the concentration (mmol/molTG) of oleic and linoleic acyl group *versus* time (day).**

✓*Degradation of some minor components*

❖The **enrichment** of olive oil with **DG slows down** significantly the rate of the **degradation of sterols** (Fig. 2), to which several healthy and antioxidant properties have been attributed [6]. In all the samples **Δ7-sterols** are **degraded first**, followed by β-sitosterol and 4,4'-dimethyl sterols.

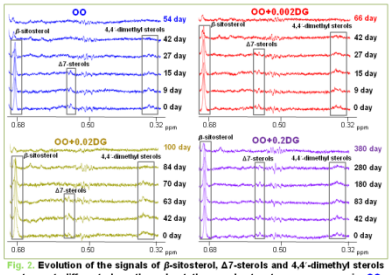

Fig. 2. **Evolution of the signals of β-sitosterol, Δ7-sterols and 4,4'-dimethyl sterols protons at different days throughout the accelerate storage process, in OO; OO+0.002DG; OO+0.02DG and OO+0.2DG samples.**

✓*Formation of primary oxidation compounds*

❖**DG delays** the **generation** of hydroperoxides coming from oleic acyl groups (**OOH-O**) (Fig. 3) and from linoleic acyl groups (**(*Z,E*)-CD-OOH** and **(*E,E*)-CD-OOH**) (Fig. 4).

❖In addition, the **amount** of both types **hydroperoxides** formed **decreases** as **DG concentration increases**.

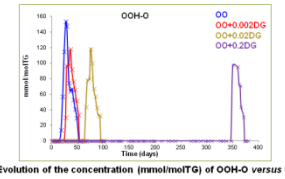

Fig. 3. **Evolution of the concentration (mmol/molTG) of OOH-O *versus* time (day).**

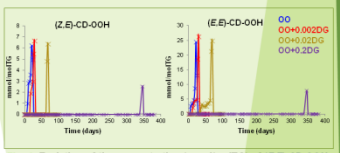

Fig. 4. **Evolution of the concentration (mmol/molTG) of (*Z,E*)-CD-OOH and (*E,E*)-CD-OOH *versus* time (day).**

✓*Formation of secondary oxidation compounds*

❖In the **presence of GD**, the **formation** of **aldehydes** (Fig. 5), and of other secondary oxidation compounds, such as, **ketones, epoxides** and **alcohols** is also **delayed** in line with DG enrichment degree.

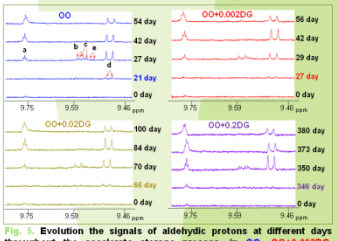

Fig. 5. **Evolution the signals of aldehydic protons at different days throughout the accelerate storage process, in OO, OO+0.002DG, OO+0.02DG and OO+0.2DG samples. a: n-alkanals; b: 4-hydroperoxy-(*E*)-2-alkenals; c: 4-hydroxy-(*E*)-2-alkenals; d: 4-epoxy-(*E*)-2-alkenals; e: (*E*)-2-alkenals.**

Conclusions

❑ The **enrichment** of **olive oil** with the three tested levels of **DG** significantly **improves** its **oxidative stability**. This **effect** is clearly **dose-dependent.**

❑ **DG delays** the **degradation** of **acyl groups** and of **minor components** and also the formation of **primary** and of **secondary oxidation compounds.**

❑ DG can be considered as an effective antioxidant of edible oils, leaving aside other considerations regarding its effects on health.

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CONTRIBUTION XI

EFECTO QUE EL ENRIQUECIMIENTO DE ACEITE DE MAÍZ CON *ALFA* Y *GAMMA* TOCOFEROL PROVOCA EN SU PROCESO DE DIGESTIÓN BAJO CONDICIONES *IN VITRO*. UN ESTUDIO COMPARATIVO

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En este trabajo se estudia el efecto que ejercen diferentes concentraciones de α- y γ-Tocoferol sobre la lipólisis y el grado de oxidación alcanzados por aceite de maíz en digestión in vitro. Se ha observado una clara diferencia en el efecto de ambos compuestos.

Palabras clave – aceite de maíz, digestión *in vitro*, tocoferol

INTRODUCCIÓN

La presencia de antioxidantes naturales en aceites tiene gran importancia, no sólo porque contribuyen a preservar la calidad de los mismos y alargar su vida útil, sino porque su ingesta puede reportar beneficios para la salud. Es por ello que cada vez es más frecuente el uso de compuestos considerados como antioxidantes y saludables en el enriquecimiento de aceites. Algunos de los compuestos naturales ampliamente utilizados con este fin son los tocoferoles, especialmente el alfa- y el gamma- tocoferol (α-T y γ-T).

Durante la digestión *in vitro* de alimentos grasos, además de las reacciones lipolíticas propias de este proceso, pueden tener lugar reacciones de oxidación [1, 2]. Existen pocos estudios que analicen si los compuestos antes citados inhiben, retrasan o favorecen la oxidación de lípidos en el proceso de digestión *in vitro* o al grado de lipólisis que se alcanza.

En este contexto, el objetivo de esta comunicación es estudiar el efecto que diferentes concentraciones de α-T y γ-T provocan en las transformaciones que sufre el aceite de maíz en su digestión *in vitro.*

MATERIALES Y MÉTODOS

El estudio se llevo a cabo con aceite de maíz refinado y con el mismo aceite enriquecido con diferentes concentraciones de α-T y γ-T (0,2%, 2% y 5% en peso). Estas muestras se sometieron a digestión *in vitro* [2, 3]. En las muestras digeridas se estudió el grado de lipólisis alcanzado, así como el estado de oxidación de los lípidos digeridos. Para llevar a cabo el estudio se emplearon Resonancia Magnética Nuclear de Protón (RMN ^{1}H) y Microextracción en Fase Solida seguida de Cromatografía de Gases/Espectrometría de Masas (MEFS-CG/EM).

RESULTADOS Y DISCUSIÓN

Los resultados obtenidos muestran que la adición de α-T y γ-T en las concentraciones estudiadas, no influye en el grado de lipólisis que alcanzan los lípidos del aceite en este proceso digestivo. Sin embargo, se advierte que la oxidación alcanzada por los lípidos digeridos depende del tipo de tocol con el que se enriquece el aceite y también de su concentración. El α-T muestra una clara actividad pro-oxidante, principalmente a altas concentraciones (2% y 5% en peso), favoreciendo, la degradación del ácido linoleico y la formación de compuestos primarios y secundarios derivados de la degradación de este. Sin embargo, el γ-T actúa como antioxidante, disminuyendo la degradación del ácido linoleico, en relación con la que ocurre en la muestra control, y la formación de compuestos de oxidación derivados. Este efecto antioxidante es mayor cuanto mayor es la concentración de γ-T (2% y 5% en peso).

CONCLUSIONES

El enriquecimiento con α-T y γ-T sólo afecta a la oxidación de los componentes del aceite de maíz en el proceso de digestión *in vitro*. El enriquecimiento con α-T aumenta el nivel de oxidación que alcanza el aceite, cuando se somete a condiciones digestión *in vitro*, en relación con el control. Este aumento es mayor cuanto mayor es el grado de enriquecimiento. Sin embargo, lo contrario sucede cuando se enriquece en γ-T.

AGRADECIMIENTOS

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EFECTO QUE EL ENRIQUECIMIENTO DE ACEITE DE MAÍZ CON *ALFA* Y *GAMMA* TOCOFEROL PROVOCA EN SU PROCESO DE DIGESTIÓN BAJO CONDICIONES *IN VITRO*. UN ESTUDIO COMPARATIVO

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INTRODUCCIÓN

La presencia de antioxidantes naturales en aceites tiene gran importancia, no sólo porque contribuyen a preservar la calidad de los mismos y alargar su vida útil, sino porque su ingesta puede reportar beneficios para la salud. Es por ello que cada vez es más frecuente el uso de compuestos considerados como antioxidantes y saludables en el enriquecimiento de aceites.

Algunos de los compuestos naturales ampliamente utilizados con este fin son los tocoferoles, especialmente el alfa- y el gamma-tocoferol (α-T y γ-T).

Durante la digestión *in vitro* de alimentos grasos, además de las reacciones lipolíticas propias de este proceso, pueden tener lugar reacciones de oxidación [1, 2]. Sin embargo, existen pocos estudios que analicen si la presencia de los compuestos antes citados afecta al grado de lipólisis que se alcanza o si inhibe, retrasa o favorece la oxidación de lípidos en el proceso de digestión *in vitro*.

MATERIALES Y MÉTODOS

❖MUESTRAS: aceite de maiz refinado (M) y el mismo aceite enriquecido con diferentes concentraciones de α-T y γ-T (0,2%, 2% y 5% en peso).

❖DIGESTIÓN *IN VITRO*: las muestras de aceite (0,5 gr) fueron digeridas utilizando una versión modificada del modelo de digestión *in vitro* descrita por Versantvoort y col. [3].

❖ESTUDIO DE LAS MUESTRAS: Mediante Resonancia Magnética Nuclear de Protón (RMN ^{1}H) y Microextracción en Fase Solida seguida de Cromatografía de Gases/Espectrometría de Masas (MEFS-CG/EM).

OBJETIVO

Estudiar el efecto que diferentes concentraciones de α-T y γ-T provocan en las transformaciones que sufre el aceite de maiz en su digestión *in vitro*.

RESULTADOS Y DISCUSIÓN

GRADO DE HIDRÓLISIS DURANTE LA DIGESTION IN VITRO

✓La adición de **α-T** y **γ-T** en las concentraciones estudiadas, **influye ligeramente** en el **grado de lipólisis** que alcanzan los lípidos del aceite en este proceso digestivo (ver **Tabla 1**).

Tabla 1. Porcentajes de hidrólisis (%H), de transformación de triglicerido ($\%T_{TG}$) y de bioaccesibilidad de los lípidos ($\%L_{BA}$) determinados mediante RMN de ^{1}H de los extractos lipídicos digeridos de: **MD (aceite de maiz digerido)**; (M+γ-T)D (aceite de maiz enriquecido con γ-T digerido) y (M+α-T)D (aceite de maiz enriquecido con α-T digerido).

Muestras	%H	$\%T_{TG}$	$\%L_{BA}$
MD	56,8 ± 5,2	77,7 ± 5,9	67,1 ± 7,4
(M+0,2%γ-T)D	56,9 ± 3,3	77,8 ± 4,4	66,9 ± 4,0
(M+2%γ-T)D	57,8 ± 2,2	79,3 ± 3,4	69,0 ± 2,4
(M+5%γ-T)D	60,5 ± 5,3	83,0 ± 7,8	72,3 ± 8,1
(M+0,2%α-T)D	58,6 ± 3,7	79,9 ± 4,3	69,6 ± 4,7
(M+2%α-T)D	56,3 ± 1,4	78,2 ± 1,8	67,6 ± 2,4
(M+5%α-T)D	54,6 ± 2,1	75,2 ± 2,0	64,3 ± 3,0

GRADO DE OXIDACIÓN DURANTE LA DIGESTIÓN IN VITRO

✓La **oxidación** alcanzada por los lípidos digeridos **depende** del **tipo** de **tocol** con el que se enriquece el aceite y de **su concentración.**

❑ESTUDIO MEDIANTE RMN ^{1}H

➢*Degradación del ácido linoleico*

➢Durante el proceso de digestión *in vitro* se degrada el ácido linoleico, ácido graso mayoritario del aceite de maiz.

➢El **α-T** muestra una clara **actividad pro-oxidante**, **favoreciendo** la **degradación** del **ácido linoleico,** respecto al control.

➢El **γ-T** actúa como **antioxidante, disminuyendo** la **degradación** del **ácido linoleico,** en relación con la que ocurre en la muestra control.

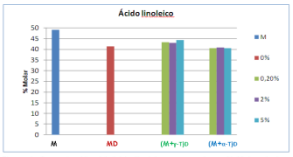

Figura 1. Concentración del ácido linoleico, expresada en % Molar, de las muestras en referencia a GA+AGL (grupos acilo+ácidos grasos libres): **M (aceite de maiz sin digerir)**; **MD (aceite de maiz digerido)**; (M+γ-T)D (aceite de maiz enriquecido con γ-T digerido) y (M+α-T)D (aceite de maiz enriquecido con α-T digerido).

➢*Formación de compuestos primarios de la oxidación*

➢Durante el proceso de digestión *in vitro* del **aceite de maiz sin enriquecer** (MD), se observo una cierta degradación de los lípidos dando lugar a la **formación** de **hidroperóxidos** asociados a **dienos conjugados** (**(*Z*,*E*)-CD-OOH**) (**señal a** en **Fig. 2**)

➢La presencia de **α-T favorece** la **formación** de **hidroperóxidos asociados a dienos conjugados** (**(*Z*,*E*)-CD-OOH** y de **hidróxidos asociados a dienos conjugados** ((*Z*,*E*)-CD-OH y (*E*,*E*)-CD-OH) (**señales a**, b y c en **Fig. 2**), especialmente a altas concentraciones (2 y 5%).

➢El enriquecimiento de aceite de maiz con **γ-T reduce** significativamente la **formación** de **compuestos primarios de la oxidación** durante su digestión *in vitro* (**Fig. 2**). Esta **reducción** es **mayor** cuanto **mayor** es la **concentración** de **γ-T**

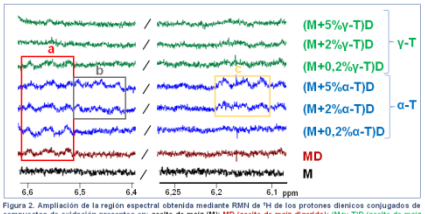

Figura 2. Ampliación de la región espectral obtenida mediante RMN de ^{1}H de los protones dienícos conjugados de compuestos de oxidación presentes en: **aceite de maiz (M)**; **MD (aceite de maiz digerido)**; (M+γ-T)D (aceite de maiz enriquecido con γ-T digerido) y (M+α-T)D (aceite de maiz enriquecido con α-T digerido).

❑ESTUDIO MEFS-CG/EM

➢*Formación de compuestos secundarios de la oxidación*

➢El enriquecimiento con **α-T favorece** la **formación** de compuestos secundarios de la oxidación, tales como **aldehídos**, **furananos** y **derivados de furano**. Este efecto es mayor cuanto mayor es el grado de enriquecimiento (**Tabla 2**).

➢Entre los aldehídos formados hay que **destacar** la **presencia** de **aldehídos oxigenados α,β-insaturados,** compuestos con conocida toxicidad [4].

➢Sin embargo, el **γ-T** actúa como **antioxidante**, **disminuyendo** o incluso **inhibiendo** la **formación** de estos **compuestos de oxidación** derivados de la degradación del ácido linoleico (**Tabla 2**).

➢Los resultados obtenidos por esta técnica están en concordancia con los obtenidos mediante RMN ^{1}H.

Tabla 2. Abundancia expresada en cuentas de área del pico base del espectro de masas por 10^6, junto a su desviación estándar de algunos compuestos secundarios de la oxidación identificados mediante MEFS-CG/EM en el espacio de cabeza de las muestras: **M+JD (aceite de maiz sin digerir y jugos digeridos)**; **MD (aceite de maiz digerido)**; (M+α-T)D (aceite de maiz digerido enriquecido con α-T digerido) y (M+γ-T)D (aceite de maiz enriquecido con γ-T digerido).

Compuestos (peso molecular)	Origen	PB	M+JD	MD	(M+0,2%α-T)D	(M+2%α-T)D	(M+5%α-T)D
Alcanales							
Hexanal (100)	J/M	44	12,9 ± 0,9	76,0 ± 3,1	184,4 ± 12,3	480,6 ± 75,3	604,0 ± 56,0
Heptanal (114)	J/M	70	0,7 ± 0,0	3,0 ± 1,2	6,8 ± 0,1	51,2 ± 11,7	164,0 ± 2,1
Octanal (128)	M	41	---	---	---	34,0 ± 6,4	46,4 ± 5,1
Decanal (156)	M	41	---	---	---	---	4,6 ± 0,5
Alquenales							
(*E*)-2-Heptenal (112)	J/M	41	4,4 ± 0,3	47,4 ± 8,1	64,5 ± 8,7	186,3 ± 28,3	275,0 ± 47,7
(*E*)-2-Octenal (126)	J/M	70	3,1 ± 0,2	---	42,3 ± 2,2	186,3 ± 26,2	346,3 ± 58,9
(*E*)-2-Decenal (154)	M	41	---	---	4,1 ± 0,6	6,7 ± 1,0	10,8 ± 1,8
Alcadienales							
(*Z*,*E*)-2,4-Decadienal (152)	M	81	---	0,6 ± 0,0	2,6 ± 0,8	19,3 ± 3,1	38,4 ± 10,1
(*E*,*E*)-2,4-Decadienal (152)	M	81	---	0,8 ± 0,1	3,3 ± 0,3	15,8 ± 0,5	29,0 ± 13,2
Aldehídos Oxigenados							
4-Hidroxi-(*E*)-2-nonenal (156)	M	57	---	---	---	0,5 ± 0,1	0,7 ± 0,2
4,5-Epoxi-2-decenal (isómero) (168)	M	68	---	---	0,4 ± 0,1	2,7 ± 0,2	4,3 ± 2,2
4,5-Epoxi-(*E*)-2-decenal (168)	M	68	---	---	1,1 ± 0,2	10,9 ± 0,4	21,9 ± 0,5
Furananos y Derivado de Furano							
5-Butil-5H-furanona (140)	M	84	---	---	---	8,7 ± 0,3	16,0 ± 1,4
5-Pentil-2(5H)-furanona (154)	M	84	---	---	---	27,0 ± 3,4	44,3 ± 8,7
2-Pentilfurano (138)	J/M	81	4,7 ± 1,4	218,5 ± 15,4	434,9 ± 21,1	1867,6 ± 427,8	3082,8 ± 214,8
Compuestos (peso molecular)	**Origen**	**PB**	**M+JD**	**MD**	**(M+0,2%γ-T)D**	**(M+2%γ-T)D**	**(M+5%γ-T)D**
Alcanales							
Hexanal (100)	J/M	44	12,9 ± 0,9	76,0 ± 3,1	79,1 ± 11,8	72,4 ± 4,9	45,5 ± 4,9
Heptanal (114)	J/M	70	0,7 ± 0,0	3,0 ± 1,2	4,4 ± 0,7	3,5 ± 0,5	2,4 ± 0,4
Octanal (128)	M	41	---	---	---	---	---
Decanal (156)	M	41	---	---	---	---	---
Alquenales							
(*E*)-2-Heptenal (112)	J/M	41	4,4 ± 0,3	47,4 ± 8,1	40,2 ± 6,4	16,5 ± 8,9	8,8 ± 3,0
(*E*)-2-Octenal (126)	J/M	70	3,1 ± 0,2	---	---	---	---
(*E*)-2-Decenal (154)	M	41	---	---	---	---	---
Alcadienales							
(*Z*,*E*)-2,4-Decadienal (152)	M	81	---	0,6 ± 0,0	0,4 ± 0,0	0,4 ± 0,1	0,3 ± 0,0
(*E*,*E*)-2,4-Decadienal (152)	M	81	---	0,8 ± 0,1	0,5 ± 0,0	0,3 ± 0,1	0,3 ± 0,1
Aldehídos Oxigenados							
4-Hidroxi-(*E*)-2-nonenal (156)	M	57	---	---	---	---	---
4,5-Epoxi-2-decenal (isómero) (168)	M	68	---	---	---	---	---
4,5-Epoxi-(*E*)-2-decenal (168)	M	68	---	---	---	---	---
Furananos y Derivado de Furano							
5-Butil-5H-furanona (140)	M	84	---	---	---	---	---
5-Pentil-2(5H)-furanona (154)	M	84	---	---	---	---	---
2-Pentilfurano (138)	J/M	81	4,7 ± 1,4	218,5 ± 15,4	198 ± 7,9	194,2 ± 5,1	175,9 ± 11,0

CONCLUSIONES

❖El enriquecimiento con **α-T** y **γ-T** provoca una tendencia a disminuir o aumentar respectivamente, de forma leve, el grado de lipolisis alcanzado en la digestión, medido a través del nivel de hidrolisis, %H, del porcentaje de transformación del Triglicérido $\%T_{TG}$ y a través del nivel de bioaccesibilidad de los lipidos $\%L_{BA}$.

❖La presencia de α-T añadido aumenta el nivel de oxidación que ocurre en la digestión de aceite de maiz, siendo este aumento mayor cuanto mayor es el grado de enriquecimiento.

❖La presencia de γ-T añadido inhibe el proceso de oxidación que ocurre en la digestión de aceite de maiz. Esta inhibición es mayor cuanto mayor es el grado de enriquecimiento en γ-T.

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