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Universidad del País Vasco

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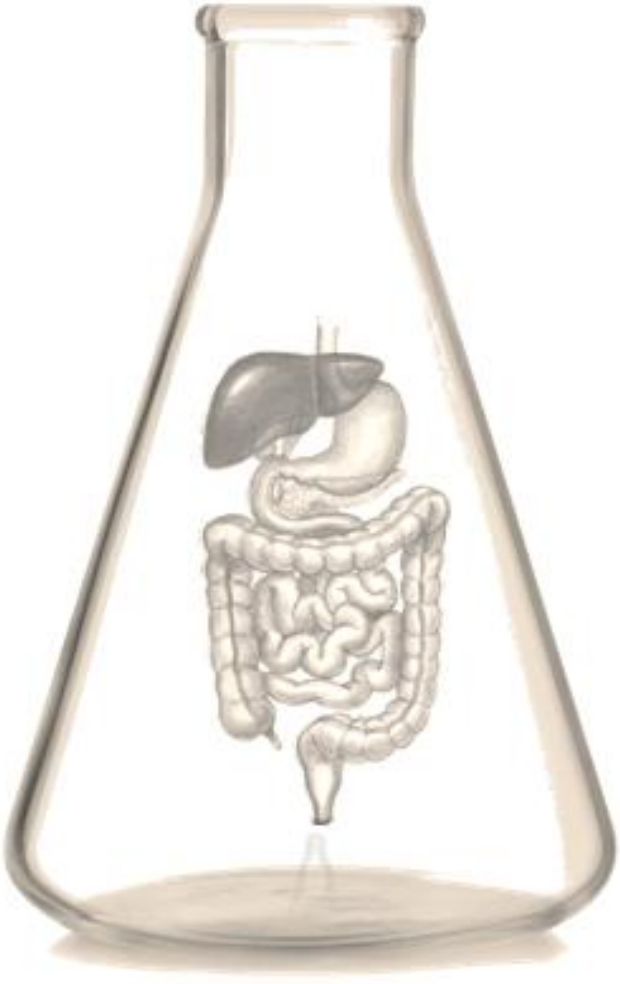
PHILOSOPHIÆ DOCTORAL THESIS

CHANGES IN LIPIDS AND FORMATION OF DERIVED COMPOUNDS DURING FISH COOKING AND *IN VITRO* GASTROINTESTINAL DIGESTION OF EDIBLE OILS, MODEL SYSTEMS AND PROCESSED FISH MEAT



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AIM 3: In vitro gastrointestinal digestion of oils. Simultaneous study of lipid hydrolysis, oxidation and other reactions taking place during the in vitro digestion of oils of vegetable and animal origins, and of other model systems. Influence on their extent of the oil initial oxidation level, of the oil unsaturation degree, of the presence of proteins and of the synthetic antioxidant BHT

ABSTRACT

Cooking process involves the occurrence of several physico-chemical reactions affecting into a lesser or greater extent food quality parameters with consequences from the nutritional, sensory and safety points of view. The main purpose of cooking is the enhancement of digestibility, microbiological safety and sensory attributes, although, under certain conditions, the simultaneous occurrence of non-desirable reactions cannot be ruled out.

Among food components, lipids play important roles not only regarding food texture, flavor and shelf-life, but also nutritional food quality, because they provide certain vitamins, essential fatty acids and cholesterol, among other components. In spite of its relevance, changes undergone by lipids during cooking, especially by the most prone to oxidation, like those polyunsaturated omega-3 contained in fish, require further research bearing in mind the scarce number of studies performed to date and the inconclusive results reported.

Due to this, the **first aim** of the present doctoral thesis was contemplated. This is, to deepen knowledge on the changes, if any, provoked in the lipids and/or volatile profile of fish meat as a result of cooking by means of Proton Nuclear Magnetic Resonance (^{1}H NMR) and Solid Phase Microextraction followed by Gas Chromatography/ Mass Spectrometry (SPME-GC/MS). For this purpose, several cooking methods differing on the temperature and cooking time, the heat transfer medium and on the exposure to oxygen were selected: pan-frying, microwave-frying, boiling, steaming, *sous-vide* cooking, conventional oven baking, salt-crusted oven baking and microwave cooking. The influence of the cooking method applied on the extent of the changes observed was investigated. Moreover, the potential impact of fish species and growing conditions was also tackled. This was approached by using two fish species widely consumed in the Mediterranean area (Gilthead sea bream, *Sparus aurata*; and European sea bass, *Dicentrarchus labrax*), and by using wild and farmed specimens of European sea bass. These latter samples, although belonging to the same fish species, widely differed on their initial lipid content and composition in main and/or minor lipidic components, as well as on their volatile profile.

The information obtained by accomplishing this first aim showed that the cooking process greatly modified the lipids and the volatile profile of fish, although large differences were observed depending on the cooking technique applied, and the fish species and growing conditions. Hence, from a practical point of view, the results obtained can be very valuable; for instance, they could be

helpful for the food industry to produce *ready-to-eat* fish products that would ensure food nutritional and safety quality as much as possible, as well as consumer acceptance.

Due to economical and health implications, many food technologists and scientists have devoted their efforts to study lipid degradation during food processing and storage, paying special attention to the underlying mechanisms and the development of strategies which are capable of delaying it. Nevertheless, the nutritional quality and safety of lipids could also be modified during subsequent digestion. Since this physiological process is an unavoidable step, it seems logical to research on the several chemical reactions that may take place in lipids under gastrointestinal digestive conditions, in order to better understand the effect of lipids on human health. Indeed, a deeper knowledge on this issue is required not only to advance in the study of the fate of the different nutrients, but also for the design of healthier foods and diets.

To date, the study on the changes undergone by lipids along the gastrointestinal tract has been scarcely addressed and hence, they remain unknown. The research performed was mainly focused on lipid hydrolysis reaction and on the factors affecting lipid bioaccessibility and bioavailability, especially in the last years, due to the increasing public concern about obesity. However, after reviewing the available literature in the field of lipid digestion research, it was detected the need for the development of methodologies able to accurately quantify lipolytic products and to assess the extent of lipid digestion in a simple and fast way. The most commonly employed methodologies to study lipolysis reaction are the titration of fatty acids released by means of a pH-stat apparatus and chromatographic techniques, such as High Performance Liquid Chromatography, High Performance Thin Layer Chromatography or Gas Chromatography followed by Mass Spectrometry. These methodologies show some limitations, among which lack of accuracy, unspecificity, time-consuming, and/or the use of large amount of solvents can be cited.

In this context, the **second aim** of this doctoral thesis included the development of a ^{1}H NMR methodology that could provide a global study of the digested lipidic sample in a fast and simple way, and without any chemical modification. Firstly, the identification and assignment of the proton signals corresponding to triglycerides, 1,2- and 1,3-diglycerides, 2- and 1-monoglycerides and fatty acids was performed using standard compounds of different chain length and unsaturation degree. Secondly, several equations based on ^{1}H NMR spectral data were proposed in order to quantify the number of moles of the above-mentioned molecular species when present in complex lipid mixtures. The accuracy of these equations was validated by using mixtures of known composition made up with standard compounds. Afterwards, the usefulness and high versatility of the new methodology proposed to study the extent of lipid digestion extent was proved by using real digested food lipid

samples. They were obtained from the *in vitro* digestion of sunflower oil and fish meat samples using different experimental conditions, in such a way that partially and totally lipolyzed digested samples were collected and studied. The validated new methodology hereby proposed can offer many advantages not only in the field of food technology and nutrition, but also in those of enzymology, pharmacology, medicine and petrochemistry, among others.

In addition, the sound and reliable results obtained by means of this new ^{1}H NMR methodology allowed to further deepen knowledge on the influence of several experimental factors on *in vitro* lipolysis extent reached under gastrointestinal digestive conditions. Thus, in order to lately investigate the potential changes undergone by lipids during digestion, an *in vitro* gastrointestinal digestion protocol widely employed in lipid digestion studies was optimized with regard to *in vivo* lipid digestion performance reported (95% of triglycerides absorbed as fatty acids and monoglycerides). This issue was considered a challenge necessary to overcome because: on the one hand, the lipolysis degree reached and reported in many *in vitro* digestion studies is far below the *in vivo* one, especially regarding fish lipids; and on the other hand, an accurate match of *in vitro* methodologies with *in vivo* naturally occurring events is necessary for consistent statements and predictions. In fact, in digestion research the use of *in vitro* digestion models that simulate the human physiological environment within the digestive tract has been widespread in the last decade due to ethical, economical and practical reasons.

Once optimized the *in vitro* gastrointestinal digestion model to be used, the **third aim** of this doctoral thesis consisted on the study of the lipid hydrolysis, oxidation and other reactions occurring during *in vitro* gastrointestinal digestion of unsaturated edible oils of vegetable and animal origins and the potential influence on the extent of these reactions of the oil initial oxidation level, the oil unsaturation degree, and of the presence of other food components.

Apart from hydrolysis, other chemical reactions affecting lipids might also take place in the gastrointestinal tract because of its high reactive environment. Nonetheless, due to the scarce number of studies and the methodologies usually employed (absorbance in the ultraviolet visible region for determining conjugated dienes, peroxide value, thiobarbituric acid reactive substances test), there is a current lack of knowledge on the extent of ongoing chemical reactions, especially of lipid oxidation, as well as on the specific nature of the oxidation products generated from lipids that could remain bioaccessible for intestinal absorption. Hence, in this doctoral thesis, a systematic and in-depth study of the potential occurrence of lipid oxidation, and other reactions like Maillard-type and esterification ones, in addition to lipolysis, during *in vitro* digestion of edible oils rich in ω-6 and ω-3 lipids was carried out by means of ^{1}H NMR and SPME-GC/MS.

To evaluate how the unsaturation degree of lipids and the initial oxidative status could affect the extent of the above-mentioned reactions, fresh and slightly oxidized oil samples of sunflower oil, as model of lipids rich in ω-6 acyl groups, and flaxseed oil, as model of lipids rich in ω-3 acyl groups, were *in vitro* digested. Likewise, cod liver oil, as model of fish lipids, was also employed in digestion experiments. The influence of the presence in the food bolus of other non-lipidic components that are usually present in food, like proteins or antioxidants, was also tackled by submitting to *in vitro* digestion systems consisting of mixtures made up with slightly oxidized sunflower and flaxseed oils and two proteins (ovalbumin and soy protein isolate) and of mixtures made up with commercial cod liver oil and the antioxidant 2,6-di-*tert*-butyl-hydroxytoluene (BHT, E-321). It must be pointed out that these two proteins were selected because they are widely employed as food additives and are present in several food formulations. Likewise, the synthetic phenolic antioxidant BHT was selected because of its widespread use by the food industry and its ubiquitous presence, including food products of animal origin in whose addition is not allowed by the authorities but results from a carry-over process from commercial feeds. This is the case of the farmed European sea bass samples subjects of study of this thesis.

By accomplishing this third aim, knowledge on the specific oxidation products generated from polyunsaturated lipids under the specific conditions of gastrointestinal digestion was provided. It was observed that not only their amount, but also their nature, widely varied depending on the unsaturation degree and the initial oxidation level of the oil sample. In this sense, lipids rich in ω-3 acyl groups oxidized into a greater extent that those rich in ω-6 ones. The consumption of oxidized oils (even those at the first stages of lipid oxidation) should be avoided as much as possible, because a larger amount of potentially toxic aldehydes was generated during digestion. Among the several kinds of oxidation products generated during *in vitro* digestion of sunflower, flaxseed and cod liver oils, the formation of hydroxy-octadecadienoic acids/acyl groups derived from linoleic chains, of monoepoxy-octadecadienoic acids/acyl groups from linolenic chains, and of 4-hydroperoxy-*(E)*-2-alkenals from long-chain polyunsaturated ω-3 acyl groups was evidenced for the first time.

Furthermore, data obtained clearly showed that the food bolus composition considerably influences the extent and the pathways of oxidation reactions occurring under gastrointestinal conditions. For instance, in the presence of proteins lipid oxidation was greatly limited during *in vitro* digestion and, in addition to oxidation, reduction reactions took place, in such a way that almost all the lipid hydroperoxides were converted to hydroxy-derivatives. Therefore, caution should be taken when selecting markers to evaluate the occurrence and extent of lipid oxidation during digestion. Techniques, such as ^{1}H NMR, that allow the study at once of a broad variety of oxidation products, including not only conjugated dienes, hydroperoxides and carbonyl compounds, but also epoxides and

hydroxides, are required to avoid erroneous conclusions. Likewise, the use of both ^{1}H NMR and SPME-GC/MS techniques provides very useful and complementary information, enabling a global study at a molecular level of the reactions taking place during lipid *in vitro* digestion.

Finally, chemical reactions occurring during *in vitro* gastrointestinal digestion of fish meat, which is a much more complex matrix than bulk oils or protein-oil mixtures, was investigated in the **fourth aim** of this thesis. Attention was also paid to the impact of common technological processing, like salting and smoking, on the extent of the ongoing chemical reactions. For this purpose, European sea bass unprocessed, brine-salted, dry-salted and smoked with two liquid smoke flavourings showing different phenolic content were *in vitro* digested using the optimized digestion protocol. Afterwards, non-digested and digested samples were studied by means of ^{1}H NMR and SPME-GC/MS, as done in the studies performed in the frame of the third aim.

It was observed that, in addition to the expected hydrolysis, lipid oxidation occurred during *in vitro* digestion of fish meat, although to a low extent because only the generation of low amounts of primary oxidation compounds (conjugated dienes supported on chains having also hydroperoxy and hydroxy groups) was evidenced by ^{1}H NMR. The formation of secondary oxidation compounds of low molecular weight coming from fish unsaturated acyl groups (mainly from ω-3 acyl groups or fatty acids) was only proved by means of SPME-GC/MS; this high sensitive technique was able to detect oxidation compounds present in such low concentrations that were not detectable by ^{1}H NMR. Likewise, the increase after *in vitro* digestion of certain volatile compounds highlighted the occurrence of amino acids degradation, Maillard-type reactions between fish nitrogenated components and lipid oxidation products and esterification.

As far as the effect of salting is concerned, data obtained from SPME-GC/MS study showed that this technological processing favours the advance of the above-mentioned chemical reactions during *in vitro* digestion, especially when intense salting processes (dry-salting) are performed. By contrast, no differences were observed between *in vitro* lipolysis of unsalted and salted fish. As far as the effect of liquid smoking is concerned, both ^{1}H NMR and SPME-GC/MS showed that this technological process protects fish lipids from oxidative degradation under gastrointestinal conditions, inhibiting the generation of primary and secondary oxidation compounds arising from polyunsaturated ω-3 and ω-6 lipids that takes place during digestion of unsmoked fish samples.

Moreover, an important result derived from this fourth aim, was the evidence of the bioaccessibility of smoke flavouring components, among which a great variety of phenolic compounds with well-known antioxidant activity can be cited, which could be very relevant from a health point of view since these compounds could limit *in vivo* oxidative damage.

RESUMEN

El proceso de cocinado implica la ocurrencia de diversas reacciones físico-químicas que pueden afectar, en mayor o menor extensión, los parámetros de calidad del alimento, lo cual tiene consecuencias tanto desde el punto de vista nutricional y sensorial, como de la seguridad alimentaria. Aunque el objetivo principal del cocinado es la mejora de la digestibilidad, de la seguridad microbiológica y de las propiedades organolépticas del alimento, bajo ciertas condiciones de procesado culinario, puede tener lugar la ocurrencia simultánea de reacciones no deseables que afecten negativamente a los distintos componentes del alimento.

Entre dichos componentes del alimento, los lípidos juegan un papel esencial en relación no sólo a su textura, flavor y vida útil, sino también a su calidad nutricional, porque proporcionan al organismo vitaminas liposolubles, ácidos grasos esenciales, colesterol, etc. No obstante y a pesar de su relevancia, los cambios provocados por el procesado culinario en los lípidos del alimento, especialmente en aquellos particularmente sensibles a oxidación como pueden ser los grupos acilo ω-3 de cadena larga presentes en el pescado, requieren una mayor atención dado el escaso número de estudios publicados hasta la fecha y los resultados no concluyentes reportados en los mismos.

Es por ello que el **primer objetivo** de la presente tesis doctoral fue profundizar en los cambios provocados en los lípidos y/o en el perfil volátil del pescado como consecuencia del procesado culinario, mediante dos técnicas: la Resonancia Magnética Nuclear de Protón (^{1}H NMR) y la Micro-Extracción en Fase Sólida seguida de la Cromatografía de Gases/Espectrometría de Masas (SPME-GC/MS). Para la consecución de dicho objetivo, diversas técnicas culinarias fueron seleccionadas para el cocinado de pescado: fritura superficial en sartén y en horno microondas, cocción en agua, cocción al vapor, cocción al vacío (también llamado *sous-vide*), asado convencional y asado a la sal en horno, y cocción en horno microondas. Estas técnicas difieren en la temperatura y tiempo de cocinado, en el medio de transferencia del calor, así como en el grado de exposición al oxígeno del alimento. Se estudió la influencia del tipo de cocinado en la extensión de los cambios provocados en el alimento. Igualmente, se prestó especial atención a la potencial influencia de la especie de pescado y del método de producción del mismo (pesca extractiva/acuicultura). Para ello, se emplearon dos especies de pescado ampliamente consumidas en la zona mediterránea (dorada, *Sparus aurata*, y lubina europea, *Dicentrarchus labrax*), así como muestras de origen salvaje y cultivado de lubina europea. Estas últimas muestras, si bien pertenecen a la misma especie, difieren de forma

significativa en cuanto a su contenido lipídico, composición de sus lípidos en componentes mayoritarios y minoritarios, así como en su perfil aromático.

La información obtenida tras el desarrollo de este primer objetivo mostró que el proceso de cocinado puede modificar de forma significativa los lípidos y el perfil volátil del pescado, pero observándose grandes diferencias dependiendo de la técnica culinaria empleada, la especie de pescado y el método de producción del mismo. Por tanto, desde el punto de vista práctico, los resultados obtenidos pueden ser de gran utilidad, por ejemplo, para la Industria Alimentaria interesada en producir productos de pescado listos para consumo que aseguren, lo máximo posible, no sólo su calidad nutricional y de seguridad alimentaria, sino también la aceptación por parte del consumidor.

Hasta el momento, debido a las implicaciones económicas y de salud, una gran parte de los tecnólogos de alimentos y científicos han centrado su interés en el estudio de la degradación lipídica durante el procesado y almacenamiento de los alimentos, prestando especial atención a los mecanismos por los cuales transcurre y el desarrollo de estrategias capaces de limitar dicha degradación. Sin embargo, la calidad nutricional y la seguridad de los lípidos alimentarios podría verse también afectada por el posterior proceso de digestión. Dado que este proceso fisiológico es una etapa ineludible, es de esperar que las posibles reacciones químicas que puedan afectar a los lípidos durante su paso por el tracto gastrointestinal sean también estudiadas con objeto de comprender mejor el efecto de los lípidos en la salud humana. De hecho, un mayor conocimiento sobre este tema permitiría avanzar en el diseño de alimentos y/o dietas más saludables.

Los cambios sufridos por los lípidos durante el proceso de digestión gastrointestinal han sido escasamente estudiados en profundidad, y por consiguiente todavía no se conocen bien. La investigación realizada hasta la actualidad se ha centrado principalmente en la lipólisis y en los factores que influyen en la bioaccesibilidad y biodisponibilidad de los lípidos, especialmente en los últimos años debido a la obesidad, un creciente problema de salud pública. Sin embargo, tras revisar la literatura científica disponible sobre el proceso de digestión de lípidos, se detectó la necesidad de desarrollar nuevas metodologías robustas y fiables para la cuantificación de los distintos productos de la hidrólisis de los triglicéridos y para la evaluación de la extensión de la digestión de lípidos de forma rápida y sencilla. Entre las metodologías actualmente empleadas, cabe mencionar: la valoración de ácidos grasos mediante el equipo pH-stat, la Cromatografía Líquida de Alto Rendimiento (HPLC), la Cromatografía en capa fina (TLC) o la Cromatografía de Gases seguida por Espectrometría de Masas (GC/MS). Estas metodologías presentan ciertas limitaciones, como por

ejemplo: la falta de precisión, de especificidad, el largo tiempo de análisis, y/o el uso de cantidades importantes de solventes.

En este contexto, en el **segundo objetivo** de esta tesis doctoral se planteó el desarrollo de una metodología basada en la ^{1}H NMR que permitiera un estudio global de la muestra de lípidos digeridos de forma rápida, sencilla y sin previa modificación química de la muestra. En primer lugar, se llevó a cabo la identificación y asignación de las señales espectrales correspondientes a triglicéridos, 1,2- y 1,3-diglicéridos, 2- y 1-monoglicéridos, y ácidos grasos, empleando para ello compuestos estándares de distinta longitud de cadena y grado de insaturación. En segundo lugar, se plantearon diversas ecuaciones basadas en datos espectrales obtenidos por ^{1}H NMR con objeto de cuantificar el porcentaje molar de las distintas especies moleculares anteriormente citadas cuando están presentes en mezclas lipídicas complejas. Dichas ecuaciones fueron validadas usando mezclas de composición conocida de los estándares previamente caracterizados. Posteriormente, la utilidad y versatilidad de la nueva metodología propuesta para el estudio de la extensión de la digestión lipídica se probó usando muestras reales de lípidos digeridos (aceite de girasol y lípidos de pescado parcialmente y totalmente digeridos). La nueva metodología propuesta puede ser de gran utilidad no sólo en el campo de la Tecnología de Alimentos y de la Nutrición, sino también en otros como la Enzimología, Farmacología, Medicina, Petro-Química, etc.

Por otra parte, los fiables resultados obtenidos mediante esta nueva metodología basada en la RMN de ^{1}H permitieron avanzar en el conocimiento de la influencia de diversos factores experimentales en la extensión de la hidrólisis de lípidos bajo condiciones *in vitro* de digestión gastrointestinal. Los factores objeto de estudio fueron: ……Se llevó a cabo la optimización de un modelo de digestión *in vitro* ampliamente utilizado en estudios de digestión lipídica en relación a la capacidad de hidrólisis reportada *in vivo* (95% de triglicéridos absorbidos en forma de ácidos grasos y monoglicéridos). Esta cuestión fue considerada un desafío necesario de superar debido por un lado, a que el grado de lipólisis alcanzado *in vitro* y reportado en la mayoría de estudios de digestión está muy por debajo del alcanzado *in vivo*, y por otro lado, a que para obtener resultados consistentes y conclusiones que puedan ser extrapolables al ser humano es necesario simular lo más fiel posible las condiciones gastrointestinales *in vivo*. De hecho, cabe mencionar que en la última década, debido a razones éticas, económicas y prácticas, están siendo extensamente utilizados en investigación modelos de digestión *in vitro* que simulan las condiciones fisiológicas del tracto gastrointestinal humano.

Una vez optimizado el modelo de digestión *in vitro* a emplear, el **tercer objetivo** de esta tesis doctoral consistió en el estudio de las reacciones de hidrólisis, oxidación y otras, que tienen lugar

durante la digestión gastrointestinal *in vitro* de aceites insaturados comestibles de origen vegetal y animal, y la posible influencia del grado de insaturación del aceite, del nivel de oxidación inicial del aceite, así como de la presencia de otros componentes alimentarios, en la extensión de dichas reacciones.

Durante la digestión, además de la hidrólisis de los grupos ester en glicéridos, otras reacciones químicas que afectan a los lípidos pueden ocurrir también debido a la gran reactividad del medio. No obstante, debido al escaso número de estudios publicados y al tipo de metodologías empleadas en los mismos (absorbancia en una región concreta del espectro ultravioleta visible para determinar dienos conjugados, índice de peróxidos, test de substancias reactivas al ácido tiobarbitúrico), actualmente hay un gran desconocimiento en relación a la naturaleza y extensión de la reacciones que tienen lugar, especialmente en relación a la oxidación lipídica. De igual forma, es poco conocida la naturaleza específica de los productos de oxidación lipídica que pudieran ser generados en tales condiciones y por tanto estar bioaccesibles para su absorción intestinal. Es por ello, que en esta tesis doctoral se ha querido llevar a cabo un estudio sistemático y en profundidad sobre la posible ocurrencia de reacciones de oxidación y otras, tales como reacciones de Maillard y de esterificación, además de la lipólisis, durante la digestión *in vitro* de aceites ricos en grupos acilo poliinsaturados mediante ^{1}H NMR de y de SPME-GC/MS.

Para evaluar cómo el grado de insaturación de los lípidos, así como su nivel de oxidación inicial, pudieran influir la extensión de las reacciones anteriormente citadas, muestras comerciales no oxidadas y ligeramente oxidadas de aceite de girasol (como modelo de lípidos ricos en grupos acilo ω-6) y de aceite de lino (como modelo de lípidos ricos en grupos acilo ω-3) fueron sometidas a digestión *in vitro*. Igualmente, aceite de hígado de bacalao (como modelo de lípidos de pescado) también fue sometido a las mismas condiciones. Asimismo, también se estudió el efecto de la presencia en el bolo alimenticio de otros componentes no lipídicos que pueden encontrarse frecuentemente en los alimentos, como proteínas o compuestos con capacidad antioxidante. Para ello, se sometieron a digestión *in vitro* sistemas modelo consistentes en mezclas de aceites de girasol y de lino ligeramente oxidados con dos tipos de proteína (ovoalbúmina y proteína aislada de soja) y en mezclas de aceite de hígado de bacalao con el antioxidante 2,6-di-*tert*-butyl-hydroxytolueno (BHT, E-321) usando dos concentraciones distintas: una muy inferior (20 ppm) al límite legal vigente de 100 ppm y otra muy superior (800 ppm). Ambas proteínas fueron seleccionadas porque son frecuentemente empleadas como aditivos alimentarios y están presentes en muchas formulaciones de alimentos. De igual forma, se empleó el antioxidante fenólico sintético BHT dado su amplio uso por la Industria Alimentaria a nivel mundial y su ubicua presencia, incluyendo productos de origen animal en los cuales su uso no está permitido por las autoridades vigentes, pero resulta de su

bioacumulación a través de los piensos animales dónde sí está permitido (proceso de *carry-over*). Éste es el caso de las muestras cultivadas de lubina europea objeto de estudio en este proyecto de tesis.

A través de la consecución de este tercer objetivo, se pudo avanzar en el conocimiento de los productos de oxidación generados a partir de lípidos poliinsaturados bajo condiciones específicas de digestión gastrointestinal *in vitro*. Se observó que tanto su naturaleza como su concentración, varía notablemente dependiendo del grado de insaturación y del nivel de oxidación inicial de los lípidos. Como cabría esperar, los lípidos ricos en grupos acilo ω-3 se oxidaron en mayor extensión que aquellos ricos en grupos acilo ω-6. El consumo de aceites oxidados, incluso ligeramente, debería de ser evitado en la medida de lo posible, debido a que en condiciones de digestión éstos generan mayores concentraciones de aldehídos potencialmente tóxicos que los aceites sin oxidar. Entre los distintos tipos de productos de oxidación generados durante la digestión *in vitro* de aceite de girasol, de lino y de hígado de bacalao, se ha puesto de manifiesto por primera vez la potencial formación de ácidos grasos/grupos acilos hidroxi-octadecadienoicos derivados de cadenas de linoléico, de ácidos grasos/grupos acilos monoepoxi-octadecadienoicos derivados de cadenas de linolénico, y de 4-hidroperoxi-*(E)*-2-alquenales durante la digestión de aceite de hígado de bacalao.

Los datos obtenidos en el marco de este tercer objetivo también mostraron claramente que la composición del bolo alimenticio ejerce una gran influencia en la extensión y en los mecanismos de las reacciones de oxidación que tienen lugar en condiciones gastrointestinales. Por ejemplo, la presencia de proteína limita notablemente la oxidación lipídica durante la digestión *in vitro*, y además de reacciones de oxidación, provoca la ocurrencia de reacciones de reducción de tal forma que gran parte de los grupos hidroperóxidos presentes en los lípidos se reducen a hidróxidos. Por lo tanto, se debería de tener especial precaución a la hora de seleccionar marcadores de oxidación para evaluar la ocurrencia y la extensión de la oxidación lipídica durante la digestión. En este sentido, son especialmente útiles técnicas como la ^{1}H NMR, que permiten el estudio simultáneo de una gran variedad de productos de oxidación, no sólo dienos conjugados, hidroperóxidos y aldehídos, sino también hidróxidos y epóxidos, lo cual es necesario para evitar conclusiones erróneas. De igual manera, el uso de la SPME-GC/MS ofrece una gran cantidad de información muy útil y complementaria a la obtenida mediante RMN de ^{1}H, permitiendo un estudio global a nivel molecular de las reacciones que tienen lugar durante la digestión *in vitro* de los lípidos.

Finalmente, en el marco del **cuarto objetivo** de la presente tesis doctoral se investigó la digestión gastrointestinal *in vitro* de la carne de pescado, que es una matriz mucho más compleja que los aceites o las mezclas aceites+proteínas estudiadas para el tercer objetivo. El estudio se centró en

el impacto de dos tipos de procesados tecnológicos ampliamente empleados en la Industria Pesquera a nivel mundial, como son el salado y el ahumado, en la naturaleza y extensión de las reacciones químicas que pueden tener lugar en condiciones de digestión. Para ello, se sometieron a digestión gastrointestinal *in vitro* muestras de lubina europea cultivada no procesadas (a modo de control) y muestras previamente sometidas a salado húmedo, a salado seco, y a ahumado usando dos humos líquidos comerciales con distinta concentración en compuestos fenólicos. Posteriormente, las muestras antes y después de la digestión fueron estudiadas mediante ^{1}H NMR de y SPME-GC/MS, al igual que en los estudios realizados durante el desarrollo del tercer objetivo.

En relación a las muestras de pescado no procesado, se observó que en las condiciones estudiadas, además de la esperada lipólisis, la oxidación de lípidos también tuvo lugar, aunque en muy baja extensión porque sólo se pudo evidenciar mediante ^{1}H NMR la generación en muy baja concentración de productos de oxidación primarios (dienos conjugados soportados en cadenas que contienen también grupos hidroperóxido e hidróxido). La formación de compuestos de oxidación secundarios de bajo peso molecular procedentes de la degradación de los grupos acilos insaturados de los lípidos de pescado (principalmente ω-3) fue únicamente puesta de manifiesto a través del estudio mediante SPME-GC/MS del espacio de cabeza de las muestras antes y después de la digestión; la gran sensibilidad de esta técnica permitió detectar compuestos de oxidación secundarios presentes en concentraciones por debajo del límite de detección de la ^{1}H NMR. Además, tras la digestión *in vitro* de pescado, se observó un incremento en la abundancia de ciertos compuestos volátiles, marcadores de la degradación de aminoácidos, de la ocurrencia de reacciones de tipo Maillard entre compuestos nitrogenados del pescado y productos de oxidación lipídica, así como de reacciones de esterificación.

En relación al efecto del salado en la evolución de los lípidos durante la digestión *in vitro* de pescado, los datos obtenidos mediante SPME-GC/MS mostraron que este tipo de procesado favorece el avance de las reacciones de oxidación, Maillard y esterificación anteriormente mencionadas, especialmente cuando el salado es más intenso (salado seco). Sin embargo, mediante ^{1}H NMR no se observaron diferencias en el grado de lipólisis alcanzado en las muestras sin salar y saladas. En cuanto al efecto del ahumado con aromas de humo líquidos, ambas técnicas mostraron un menor avance de la oxidación lipídica durante la digestión de las muestras ahumadas, en comparación con las muestras sin ahumar, lo que puso de manifiesto que la aplicación de este tipo de procesado previo a la digestión de pescado puede evitar la formación de productos de oxidación primarios y secundarios provenientes de la degradación de los grupos acilo poliinsaturados ω-3 y ω-6 de los lípidos de pescado.

Además de esto, un resultado notable derivado de este cuarto objetivo ha sido que se ha demostrado por primera vez en condiciones *in vitro* la potencial bioaccessibilidad de los componentes del humo, entre los cuales se encuentran una gran variedad de compuestos fenólicos con conocida actividad antioxidante. La posible influencia de dichos compuestos en reacciones de estrés oxidativo *in vivo* podría ser de gran relevancia desde el punto de vista de la salud humana.

ABBREVIATIONS

ABTS	2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADI	Acceptable dietary intake
AG	Acyl group
Ald	Aldehyde
ALE	Advanced lipid oxidation endproducts
ANOVA	Analysis of variance
ARA	Arachidonic (C20:4ω6) acyl group/fatty acid
AV	*p*-Anisidine value
BHA	2,6-Di-*tert*-butyl-4-hydroxyanisole
BHQ	2,6-Di-*tert*-butyl-1,4-benzenediol
BHT	2,6-Di-*tert*-butyl-hydroxytoluene
BHT-CHO	3,5-Di-*tert*-butyl-4-hydroxy-benzaldehyde
BHT-CH_2OH	3,5-Di-*tert*-butyl-4-hydroxy-benzyl alcohol
BHT-COOH	3,5-Di-*tert*-butyl-4-hydroxy-benzoic acid
BHT-OH	2,6-Di-*tert*-butyl-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one
BHT-OH(t)	3-*Tert*-butyl-2-hydroxy-β,β,5-trimethyl-benzeneethanol
BHT-OH(t)QM	2-*Tert*-butyl-6-(2-hydroxy-*tert*-butyl)-4-methylene-2,5-cyclohexadien-1-one
BHT-OOH	2,6-Di-*tert*-butyl-4-methyl-4-hydroperoxy-2,5-cyclohexadien-1-one
BHT-Q	2,6-Di-*tert*-butyl-2,5-cyclohexadien-1,4-dione
BHT-QM	2,6-Di-*tert*-butyl-4-methylene-2,5-cyclohexadien-1-one
BJ	Bile juice
CD	Conjugated dienes
CD-OH	Conjugated dienic system supported in a chain having also an hydroxy group
CD-OOH	Conjugated dienic system supported in a chain having also an hydroperoxy group
CMP_L	Complete molecular picture of lipolysis
DBP	2,6-Di-*tert*-butyl-4-phenol
DG	Diglycerides
DHA	Docosahexaenoic (C22:6ω3) acyl group/fatty acid
DJ	Duodenal juice
DNA	Deoxyribonucleic acid
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
DUω-6	Diunsaturated omega-6 acyl groups/fatty acids
Eq	Equation
EPA	Eicosapentaenoic (C20:5ω3) acyl group/fatty acid
FA	Fatty acid
FAME	Fatty acid methyl ester
FA_{PR}	Fatty acids physiologically releasable
FID	Flame ionization detector

FTIR/FT-MIR	Fourier transform mid-infrared spectroscopy
FOX-2	Ferrous ion oxidation-xylenol orange
GC	Gas chromatography
GJ	Gastric juice
Gol	Glycerol
GS	Glyceryl structures
HHE	4-Hydroxy-*(E)*-2-hexenal
H_L	Hydrolysis level
HNE	4-Hydroxy-*(E)*-2-nonenal
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HS-SPME	Headspace-solid phase microextraction
L	Linoleic (C18:2ω-6) acyl group/fatty acid
L_{BA}	Lipid bioaccessibility
LC/APCI-MS	Lliquid chromatography/ atmospheric pressure chemical ionization/ mass spectrometry
Ln	Linolenic (C18:3ω-3) acyl group/fatty acid
MG	Monoglycerides
MPL	Maximum permitted level
MRL	Maximum residue level
MS	Mass spectrometry
MTBSTFA	N-*tert*-butyldimethylsilyl-N-methyl-trifluoroacetamide
N	Number of moles
NOAEL	No observed adverse effect level
nq	Not quantifiable
O	Oleic (C18:1ω9) acyl group/fatty acid
OHE	4-Oxo-(*E*)-2-hexenal
O+OU	Oleic plus other minor unsaturated acyl groups/fatty acids (mainly other monounsaturated ω-7 and ω-9, arachidonic and other minor unsaturated acyl groups/fatty acids)
PAH	Polycyclic aromatic hydrocarbon
Pc	Proportionality constant relating the ^{1}H NMR spectral signal areas and the number of protons that generate them
PCA	Principal Component Analysis
PV	Peroxide Value
S	Stearic (C18:0) acyl group/fatty acid
S+M	Saturated plus modified acyl groups/fatty acids (modified acyl groups/fatty acids are those unsaturated chains that have been modified as a result of oxidation reactions, losing their original typical structure)
SDE	Simultaneous distillation-extraction
Sit+Camp	β-sitosterol plus Δ5-campesterol
SPE	Solid phase extraction
SPME	Solid phase microextraction
St	Sterol
TBARS	Thiobarbituric acid reactive substances

TBHQ	*Tert*-butyl-hydroquinone
TG	Triglyceride
TLC	Thin layer chromatography
TMA	Trimethylamine
TMAO	Trimethylamine oxide
TMDI	Theoretical maximum daily intake
TMS	Tetramethylsylane
T_{TG}	Degree of tranformation of triglycerides
U	Unsaturated acyl groups/fatty acids
UHPLC-ESI-MS	Ultrahigh performance liquid chromatography-electrospray ionization-mass spectrometry
UV	Ultraviolet
Vis	Visible

^{1}H NMR	Proton nuclear magnetic resonance
1-MG	1-Monoglyceride
1,2-DG	1,2-Diglyceride
1,3-DG	1,3-Diglyceride
2-BHT	4,4´-Ethylenebis(2,6-di-*tert*-butyl-phenol)
2-BHT-QM	4,4´-Ethanediylidenebis(2,6-di-*tert*-butyl-2,5-cyclohexadien-1-one)
2-MG	2-Monoglyceride
^{13}C NMR	Carbon nuclear magnetic resonance
^{31}P NMR	Phosphorous nuclear magnetic resonance

·OH	Hydroxyl radical
·OOR	Peroxyl radical

LIST OF MANUSCRIPTS

The present Doctoral Thesis relies on the 13 research papers listed below (**Manuscripts 1-13**):

1. Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2016). The influence of frying technique, cooking oil and fish species on the changes occurring in fish lipids and oil during shallow-frying, studied by ^{1}H NMR. ***Food Research International, 84*, 150-159.**

2. Nieva-Echevarría, B., Manzanos, M. J., Goicoechea, E., & Guillén, M. D. (2016). Effect of boiling, steaming and *sous-vide* cooking on lipids, including cholesterol, phospholipids and vitamin A, and on the volatile components of farmed and wild European sea bass meat. ***Submitted to be published.***

3. Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2016). Changes provoked by salt-crusted oven baking, conventional oven baking and microwave oven cooking on the lipids and volatile components of farmed and wild European sea bass meat and their causes. ***Submitted to be published.***

4. Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2014). A method based on ^{1}H NMR spectral data useful to evaluate the hydrolysis level in complex lipid mixtures**.** ***Food Research International, 66*, 379-387.**

5. Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2015). Usefulness of ^{1}H NMR in assessing the extent of lipid digestion. ***Food Chemistry, 179*, 182-190.**

6. Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2016). A study by ^{1}H NMR on the influence of some factors affecting lipid *in vitro* digestion. ***Food Chemistry, 211*, 17-26.**

7. Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2017). ^{1}H NMR and SPME-GC/MS study of hydrolysis, oxidation and other reactions occurring during *in vitro* digestion of non-oxidized and oxidized sunflower oil. Formation of hydroxy-octadecadienoates. ***Food Research International. Ahead of Print.*** **DOI: 10.1016/j.foodres.2016.11.027**

8. Nieva-Echevarría, B., Goicoechea, E., & Guillén, M. D. (2016). Behaviour of non-oxidized and oxidized flaxseed oils, as models of omega-3 rich lipids, during *in vitro* digestion. Occurrence of epoxidation reactions. ***Submitted to be published.***

9. Nieva-Echevarría, B., Goicoechea, E., & Guillén, M. D. (2016). Effect of the presence of protein on lipolysis and lipid oxidation occurring during *in vitro* digestion of highly unsaturated oils. Selection of lipid oxidation markers, a crucial decision. ***Submitted to be published.***

10. Nieva-Echevarría, B., Manzanos, M. J., Goicoechea, E., & Guillén, M. D. (2015). 2,6-Di-*tert*-butyl-hydroxytoluene and its metabolites in foods. ***Comprehensive Reviews in Food Science and Food Safety, 14*(1), 67-80.**

11. Nieva-Echevarría, B., Goicoechea, E., & Guillén, M. D. (2016). Polyunsaturated lipids and Vitamin A oxidation during cod liver oil *in vitro* gastrointestinal digestion. Antioxidant effect of added BHT. ***Submitted to be published.***

12. Nieva-Echevarría, B., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2017). Fish *in vitro* digestion: Influence of fish salting on the extent of lipolysis, oxidation and other reactions. ***Journal of Agricultural and Food Chemistry. Ahead of Print.*** **DOI: 10.1021/acs.jafc.6b04334**

13. Nieva-Echevarría, B., Goicoechea, E., & Guillén, M. D. (2016). Effect of liquid smoking on lipid hydrolysis and oxidation reactions during fish *in vitro* gastrointestinal digestion. ***Submitted to be published.***

INTRODUCTION

1. PREVIOUS STUDIES ON THE EFFECT OF COOKING ON THE LIPIDS AND VOLATILE PROFILE OF FISH

Fish is the main dietary source of long chain polyunsaturated ω-3 acyl groups, including eicosapentaenoic (EPA, 20:5ω3) and docosahexaenoic (DHA, 22:6ω3) groups. Their intake has been associated with potential health benefits in recent years. For this reason fish consumption is highly recommended in the context of a healthy diet. However, it has to be considered that the fish species and their growing conditions, as well as the post-harvest technological processing, like cooking, can greatly influence not only fish lipids but also its volatile profile (Alasalvar, Taylor, Zubcov, Shahidi, & Alexis, 2002; Orban, Nevigato, Di Lena, Casini, & Marzetti, 2003; Grigorakis, 2007; Fuentes, Fernández-Segovia, Serra, & Barat, 2010; Vidal, Manzanos, Goicoechea, & Guillén, 2012, 2016abc; Vidal, Goicoechea, Manzanos, & Guillén, 2014). In spite of its obvious relevance for food technology, nutritional and sensory properties and thus consumer acceptance, few studies have addressed this topic.

As is well-known, several physico-chemical reactions take place during cooking. Some of the main reactions expected to take place during cooking are: ***lipolysis,*** due to the presence of moisture; ***lipid oxidation,*** because of exposure to heat, to oxygen and to endogenous pro-oxidant species present in fish muscle tissue (Hsieh & Kinsella, 1989); and also the ***degradation of nitrogenated components*** (proteins, amino acids and trimethylamine oxide) through Maillard-type or other reactions. Moreover, in the case of frying, ***lipid migration*** phenomenon in the food/culinary oil system may occur via absorption of culinary oil or fat into the food and leaching of liposoluble molecules out of the food to the frying oil.

The occurrence of these reactions can affect the lipids and volatile profile of fish to a greater or lesser degree depending on their extent, which in turn depends on several factors, such as the availability of the corresponding substrates of each reaction and the cooking method conditions, like temperature, time, medium employed to transfer heat to food (air, oil and/or water, which in turn can be liquid or steam), and food exposure to oxygen. In the case of fried fish, the frying conditions, the nature of the culinary oil or fat and the food lipid content have proved to be decisive parameters for lipid changes in the fried food and in the frying oil (Echarte, Zulet & Astiasaran, 2001; Bakar, Rahimabadi, & Che Man, 2008; Moradi et al., 2011; Martínez-Yusta & Guillén, 2014abc, 2016).

1.1. Changes on fish lipids provoked by cooking

As far as the changes provoked by cooking on fish lipids are concerned, contradictory effects on the composition of acyl groups were observed for the same cooking method. For instance, Nurhan

(2007), Weber, Bochi, Ribeiro, Victorio, & Emanuelli (2008) and Larsen, Quek, & Eyres (2010) indicated that oven baking and microwave cooking did not affect the proportions of polyunsaturated acyl groups in fish lipids, whereas the opposite was reported by Chung, Choi, Cho, & Kim (2011) and Zhang et al. (2013). Likewise, a lack of consensus can be found among the data reported on cooked fish lipid oxidation status. For example, Tokur (2007) and Chung et al. (2011) evidenced a significant increase of conjugated dienes (CD) and of peroxide value (PV) in fish lipids after oven baking, whereas Weber et al. (2008) did not. Bakar et al. (2008) found significantly increased peroxide value in king mackerel lipids after steaming, whereas no increase was observed by Al-Saghir et al. (2004) in salmon lipids after steaming.

The disagreements between the results reported by the above-mentioned authors might be due to: i) the different cooking experimental conditions (*i.e.* time and temperature) applied to the same culinary treatment; ii) the different fish species studied; or even to iii) the lack of precision in the multistep time consuming methodologies (analysis of fatty acid methyl esters (FAMEs) by gas chromatography) used to analyze lipid composition (Eder, 1995) and to iv) the well-known drawbacks of the classical methodologies employed to assess lipid oxidation level, like measurement of conjugated dienes absorbance at 234 nm, determination of anisidine (AV) and peroxide values, and/or thiobarbituric acid reactive substances (TBARS) test (Connell, 1975; Addis, 1986; Saito & Udagawa, 1992; Haywood et al., 1995; Frankel, 2005). Thus, further research on this topic using more specific techniques would be required in order to clarify if polyunsaturated fish lipids undergo oxidation during cooking and if so to what extent it takes place, by identifying the nature and abundance of the oxidation products generated from their degradation.

1.2. Changes on fish volatile profile provoked by cooking

Previous studies have shown that a great variety of volatile compounds are formed during fish cooking, and their nature and abundance depend on the kind of culinary technique performed, the fish species studied and the fish growing conditions (farmed *versus* wild) (Milo & Grosch, 1996; Prost, Serot, & Demaimay, 1998; Hallier, Prost, & Serot, 2005; Methven, Tsoukka, Oruna-Concha, Parker, & Mottram, 2007; Frank, Poole, Kirchhoff, & Forde, 2009; Liu, Zhao, Xiong, & Zhang, 2009; Chung et al., 2011; Moreira, Valente, Castro-Cunha, Cuhna, & Guedes de Pinho, 2013). Nevertheless, no comparative studies on the changes provoked by different cooking methods on fish volatile profile have been performed to date. Thus, little is known about the potential differences among cooking methods regarding the formation of odour and flavour-contributing volatile compounds in fish, among which there are those arising from the occurrence of Maillard-type reactions (Whitfield, 1992).

In this context, the first three objectives of the present doctoral thesis were contemplated. On the one hand, the **Objective 1.1.** addressed, by means of ^{1}H NMR, the changes occurring in fish lipids and in the frying oil during shallow-frying, analyzing the potential influence of the frying technique, the cooking oil and the fish species on their extent. Although some exceptions can be found (Sanchez-Muniz, Viejo, & Medina, 1992; Sioen et al., 2006; Amira et al., 2010; Martínez-Yusta & Guillén, 2014abc, 2016), it must be noted that most previous studies of fish frying were focused only on the changes occurring in fish lipids, disregarding those taking place at the same time in the frying oil. In addition, most of them reported only the changes occurring in major but not minor lipid components. Therefore, as many aspects still remain unknown, it is of paramount importance to study the frying process from a global point of view in order to understand all the changes that it provokes not only in food, but also in the frying oil or fat.

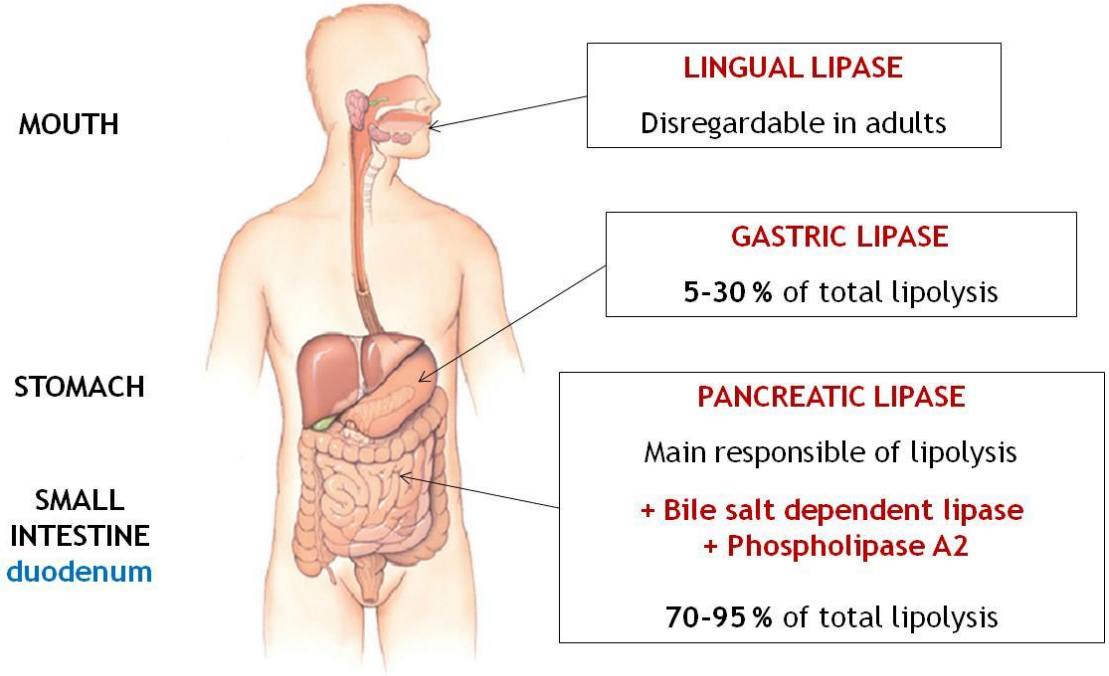
On the other hand, the **Objectives 1.2.** and **1.3.** tackled the changes in the lipids of farmed and wild European sea bass (including some minor components like cholesterol, phospholipids and vitamin A) and also in their volatile profile, as a result of several common cooking methods: boiling, steaming, *sous-vide* cooking, salt-crusted oven baking, conventional oven baking and microwave oven cooking. For this purpose, two innovative techniques will be employed, Proton Nuclear Magnetic Resonance spectroscopy (^{1}H NMR) and Solid Phase Microextraction followed by Gas Chromatography/ Mass Spectrometry (SPME-GC/MS). These widely used cooking methods were selected because they greatly differ on the mechanism of heating food. In this sense, it would be of great interest to go into the effect of the several culinary techniques in depth, from a food technological, nutritional and sensory point of view.

2. LIPID HYDROLYSIS REACTION UNDER DIGESTIVE CONDITIONS

Food lipids play important roles not only from a technological point of view, because they are related to food texture, flavor and shelf-life, but also from a nutritional one, because they include not only triglycerides, but also certain vitamins, essential fatty acids, and cholesterol, among other compounds. Nowadays, a deeper knowledge of the digestion process is required, not only for the design of healthier foods, but also to advance in the study of the fate of the different nutrients. Indeed, the management of lipid release and absorption has become a challenge in the last years (McClements, Decker, & Park, 2009).

2.1. Lipid digestion process

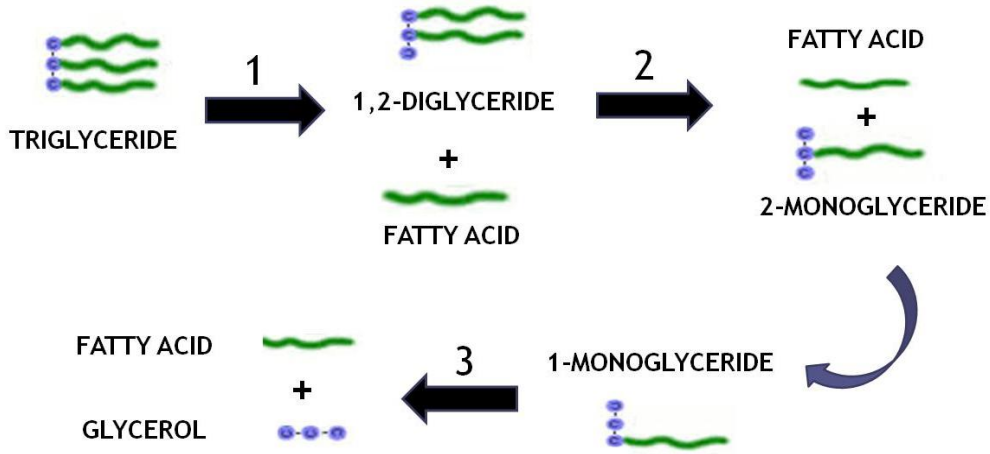
Triglycerides (TG) are the major components of fats and oils, and once ingested, they are submitted to a process of hydrolysis which is mainly catalyzed by lipases present in gastric, and especially in duodenal digestive juices (see Introduction-**Figure 1**). This latter juice is a complex mixture in which at least three different types of lipases are secreted by the pancreas, colipase-dependent lipase being mainly responsible for the digestion of dietary lipids. Carboxyl ester hydrolase (also named bile salt dependent lipase), which hydrolyzes water-soluble esters, cholesterol esters and lipovitamins, and phospholipase A2, which hydrolyzes phospholipids, are considered minor components of pancreatic juice (Reis, Holmberg, Watzke, Leser & Miller, 2009). In spite of wide compositional variations (Kalantzi, Goumas, Kalioras, Abrahamsson, Dressman, & Reppas, 2006; Clarysse, Tack, Lammert, Duchateau, Reppas, & Augustijns, 2009), the concentration of pancreatic lipases *in vivo* is reported to always be in large excess over substrate, which ensures a complete lipid digestion (Reis et al., 2009). Indeed, human body shows a high efficiency for lipid digestion, being more than 95% of ingested TG absorbed as monoglycerides (MG) or fatty acids (FA) (Golding & Wooster, 2010).



Introduction-**Figure 1**. Schematic representation of lipid digestion process *in vivo.*

Lipolysis reaction of a TG consists in a two-step reaction ruled by enzyme regiospecificty, and yields one molecule of 2-monoglyceride (2-MG) and two FA. Complete hydrolysis of TG into three FA

and glycerol (Gol) can also be achieved (see Introduction-**Figure 2**), after isomerization of 2-MG into 1-monoglyceride (1-MG) (Desnuelle & Savary, 1963; Mattson & Volpenhein, 1964). In turn, MG and FA are solubilized in bile-salt aggregates and then absorbed across the intestinal epithelium after lipolysis (Mu & Høy, 2004).



Introduction-**Figure 2**. Schematic representation of *in vivo* hydrolysis reaction of triglycerides.

2.2. Assessment of lipid digestion extent

The extent of lipid digestion process reached can be characterized by the quantification of each one of the different molecular species above mentioned (TG, DG, MG, FA, Gol) and/or by determining different parameters defined below and named: hydrolysis level, degree of TG transformation, lipid bioaccessibility level and percentage of FA physiologically releasable. These various approaches have been proposed by different authors due to several different interpretations of the concept of lipid digestion extent for its determination. In some cases, lipid digestion is considered from the chemical point of view, whereas in others the bioaccesibility of the products generated is taken into account, in an attempt to see matters from a physiological point of view.

- ***Hydrolysis in the chemical sense.*** Several authors evaluate lipid hydrolysis level (**H_L%**) by the percentage of FA released in relation to the total number of moles of acyl groups (AG) plus FA present in the sample (Rodriguez et al., 2008; Capolino et al., 2011; Helbig, Silleti, Timmerman, Hamer, & Gruppen, 2012; Zhu, Ye, Verrier, & Singh, 2013). This approach considers that total lipolysis (100%) is achieved when all the glycerides initially present (TG, DG, MG) are converted into FA and Gol.

- ***Lipid digestion as the relative disappearance of the substrate*.** In this approach, the degree of lipid digestion is defined as the relative disappearance of the substrate, which is to say the degree of TG transformation (**T_{TG}%**) considering the substrate for digestion as made up of TG exclusively (Armand et al., 1999; Vinarov et al., 2012ab). In this case, complete lipolysis (100%) involves the hydrolysis of at least one ester bond in each TG molecule initially present.

- ***Lipid digestion and bioaccessiblity*** (**L_{BA}%**)***.*** Another more physiological approach is also used to evaluate the extent of lipid digestion. Some authors, focusing on the notion of bioaccessibility, have determined the level of lipolysis as the number of moles of acyl groups bound to MG and of FA in relation to the total number of moles of acyl groups plus fatty acids present in the sample (Capolino et al., 2011; Kenmogne-Domguia, Meynier, Viau, Llamas, & Genot, 2012). In fact, although further hydrolysis is possible, the complete absorption of a TG only requires its conversion into MG and two FA. In this case, a value of 100% involves the transformation of each TG into absorbable molecules, which may be either MG or FA.

- ***Fatty acids in relation to those which may be released in the conversion of TG into MG.*** The percentage of FA physiologically releasable (**FA_{PR}%**) assumes that each TG molecule can generate two FA and one MG and that no further hydrolysis of MG occurs (Pafumi et al., 2002; Li & McClements, 2010; Li, Hu, & McClements, 2011; Lamothe, Corbeil, Turgeon, & Britten, 2012; Marze, Meynier, & Anton, 2013). In this case, a value of 100% involves the transformation of each TG into MG and FA. However, this assumption simplifies the real lipolysis reaction, since it does not take into account that a TG molecule can also suffer complete hydrolysis and may give rise to one molecule of GoI and three molecules of FA.

2.3. Current methodologies employed to study lipolysis reaction

Monitoring hydrolysis advance is an important task in lipid digestion research, and in consequence, the development of methodologies which are able to accurately assess the extent of lipolysis reaction is needed.

The technique most commonly employed to estimate the extent of lipid digestion during *in vitro* digestion is the ***titration of fatty acids*** released by means of a pH-stat apparatus, in which titration with NaOH is carried out (Fatouros, Bergenstahl, & Mullertz, 2007; Brogård, Troedsson, Thuresson, & Ljusberg-Wahren, 2007; Li & McClements, 2010; Thomas, Holm, Rades, & Müllertz, 2012; Helbig, et al., 2012; Marze, et al., 2013; Zhu et al., 2013). However, it has been pointed out that the accuracy of the pH-stat titration technique in quantifying the FA released during lipid digestion is highly dependent on the ionization of each FA and its availability to be titrated, which is in turn

dependent on several factors, including chain length, the pH of the medium and the bile salt and electrolyte concentrations (Sek, Porter, Kaukonen, & Charman, 2002; Thomas et al., 2012; Zhu et al., 2013). In fact, this methodology is usually performed to monitor lipolysis only during the intestinal step and by using simple solutions (buffers) that do not mimic the composition of *in vivo* digestion juices, in order to avoid any interference from the complex media (Di Maio & Carrier, 2011). Other authors have already highlighted the inability of the pH-stat method to give reliable results when complex matrices are studied or when simulated digestive juices reproducing physiological composition are used (Hur, Decker, & McClements, 2009). Moreover, limited information on the lipolysis reaction can be obtained since quantification of partial glycerides (DG and MG) is not possible.

Chromatographic techniques, such as High Performance Liquid Chromatography (HPLC), High Performance Thin Layer Chromatography (HPTLC) or Gas Chromatography (GC) followed by Mass Spectrometry (MS), have also been applied to quantify the different lipolytic products generated (Armand et al., 1999; Sek, Porter, & Charman, 2001; Capolino et al., 2011; Hur, Joo, Lim, Decker, & McClements, 2011; Kenmogne-Domguia et al., 2012b, Helbig et al., 2012; Zhu et al., 2013). Nevertheless, these methodologies are time-consuming, usually imply many preparation steps, including calibration with standard compounds, and also involve large amounts of polluting organic solvents. Moreover, some authors have reported unspecificity or discrepancies among data obtained when some of the above-mentioned techniques are compared (Sek et al., 2002; Helbig et al., 2012; Thomas et al., 2012).

Regarding ***Nuclear Magnetic Resonance***, few studies have demonstrated the usefulness of ^{13}C and ^{31}P ***NMR*** in determining the content of DG, MG and FA (Gunstone, 1991; Vlahov, 1996, 2006; Ng, 2000; Spyros & Dais, 2000; Spyros, Philippidis, & Dais, 2004). However, these spectroscopic techniques may require long relaxation delays and lenghty accumulations to achieve a satisfactory signal to noise ratio necessary for accuracy of quantification or a previous derivatization of the labile hydrogens of partial glycerides in the sample with 2-chloro-4,4,5,5-tetramethyldioxaphospholane, as well as the use of internal standards for calibration. Previous studies have employed ^{1}H ***NMR*** to quantify DG, MG and FA in relation to TG when they are minor components in vegetable fats and oils (Sacchi, Paolillo, Giudicianni, & Addeo, 1991; Compton, Vermillion, & Laszlo, 2007; Jin, Kawasaki, Kishida, Tohji, Moriya, & Enomoto, 2007; Satyarthi, Srinivas, & Ratnasamy, 2009; Kumar et al., 2011; Skiera, Steliopoulos, Kuballa, Holzgrabe, & Diehl, 2012; Sopelana, Arizabaleta, Ibargoitia, & Guillén, 2013). Nevertheless, its usefulness to quantify all the lipolytic products arising from hydrolysis reaction in complex mixtures and to assess the extent of lipid digestion remained to be proven.

Therefore, in this context, the **Objectives 2.1.** and **2.2.** of the present PhD thesis were formulated in order to develop and validate a new methodology based on ^{1}H NMR spectral data allowing the qualitative and quantitative study of lipid hydrolysis reaction.

2.4. Use of *in vitro* digestion models for the study lipid digestion

Research into the influence of the digestion process on lipids, in order to better understand its effect on human health, is a current trend in food technology and nutrition. For ethical, practical and economic reasons, *in vitro* approaches have emerged as powerful tools when studying the physico-chemical events that take place within the gastrointestinal tract, at least as an initial screening step (Hur, Lim, Decker, & McClements, 2011). In recent years, several authors have estimated *in vitro* the bioaccessibility and bioavailability of certain compounds which are either toxic or beneficial for human health (Garrett, Failla, & Sarama, 1999; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005; Goicoechea et al., 2008; Roman, Burri, & Singh, 2012; Colle, Van Buggenhout, Lemmens, Van Loey, & Hendrickx, 2012). However, gastrointestinal digestion is a very complex and dynamic process where ingested food components are submitted to mechanical forces and to digestive juices until transformation into small bioavailable molecules, some of which can also be metabolized by the gut microbiota. Thus, an accurate reflection of the human physiological environment within the digestive tract in order to mimic naturally occurring events is very difficult and the performance of *in vitro* digestion can be influenced by several experimental factors.

The *in vitro* digestion models proposed in the literature greatly differ in their complexity level, varying from static to dynamic, and from one step procedures to models that simulate sequentially all the digestive process, that is those taking place in the mouth, stomach and gut, including colonic fermentation (Molly, Woestyne, & Verstraete, 1993; Minekus, Marteau, Havenaar, & Huis in 't Veld, 1995; Versantvoort, Van de Kamp, & Rompelberg, 2004; Kong & Singh, 2010; Li et al., 2011). Depending on the research topic and objectives of the study, a wide variety of conditions has been assayed. Therefore, differences can be observed between the proportions of samples/digestive fluids, the composition of digestive juices, the transit times performed in each step, or the intensity of the mechanical forces applied (Hur et al., 2011b). Recently, an international attempt to homogenize experimental conditions for *in vitro* digestion was made in the frame of COST Action FA1005 INFOGEST (Minekus et al., 2014; Egger et al., 2016).

The first requirement for all the *in vitro* methodologies should be to mimic *in vivo* macronutrient digestion extent (Hur et al., 2009; Golding & Wooster, 2010). As far as *in vitro* lipid digestion studies are concerned, lipolysis levels reported in the literature are usually far lower than

those occurring *in vivo,* especially with regard to fish lipids (Martin, Nieto-Fuentes, Señoráns, Reglero, & Soler-Rivas, 2010; Larsson, Cavonius, Alminger, & Undeland, 2012; Marze et al., 2013; Zhu et al., 2013; Tullberg et al., 2016). The high resistance of long-chain polyunsaturated acyl groups to *in vitro* hydrolysis by pancreatic lipase could explain the low rates of lipolysis reported for fish oils (Bläckberg, Hernell, Bengtsson, & Olivecrona, 1979). Thus, the improvement of lipolysis under *in vitro* conditions is a challenge that deserves a deeper knowledge of the factors affecting lipases activity.

In this context, the effect of different experimental factors on lipid *in vitro* digestion extent was outlined in the **Objective 2.3.** of the present PhD thesis, using, as a starting point, the method described by Versantvoort et al. (2004, 2005). This *in vitro* gastrointestinal digestion model was initially designed for assessing bioavailability of food mycotoxins. Nonetheless, since then, it has been employed for several purposes, mainly related to lipid research, like the study of microstructural changes in emulsified lipids (Hur et al., 2009), the fate of toxic compounds coming from lipid oxidation (Goicoechea et al., 2008, 2011), the influence of cheese matrix on lipid digestion (Lamothe et al., 2012), the effects of antioxidants on lipid oxidation during digestion (Tarvainen, Phuphusit, Suomela, Kuksis, & Kallio, 2012), the digestion of fish oil emulsions (Marze et al., 2013), and milk macronutrient decomposition (Kopf-Bolanz, Schwander, Gijs, Vergères, Portmann, & Egger, 2012).

3. CHANGES UNDERGONE BY LIPIDS UNDER GASTROINTESTINAL DIGESTIVE CONDITIONS AND FACTORS AFFECTING IT

Due to its economic and health-related implications, many food scientists have devoted their efforts to studying lipid degradation during food processing and storage, paying special attention to the underlying mechanisms and the development of strategies which are capable of delaying it (Guillén, Cabo, Ibargoitia, & Ruiz, 2005; Decker, Elias, & McClements, 2010; Guillén & Uriarte, 2012abcd; Mártinez-Yusta, Goicoechea, & Guillén, 2014). However, the digestion process is also an important and determinant step, in which the nutritional quality and safety of lipids could be modified, and to date this topic has been scarcely addressed, in spite of its great relevance.

3.1. Hydrolysis reaction

Among the several chemical reactions undergone by lipids during their transit through the gastrointestinal tract, hydrolysis reaction has been the most widely studied to date. Nonetheless, few studies have focused on the factors affecting hydrolysis level reached during digestion (Márquez-Ruiz, Garcia-Martinez, & Holgado, 2008). Among these latter, the oxidative status of the ingested TG

can be cited (Márquez-Ruiz, Guevel, & Dobarganes, 1998; Sánchez-Muñiz, Arroyo, Sánchez-Montero, & Cuesta, 2000). It was observed that the higher the molecular weight of the substrate (TG monomers, dimers or polymers), the lower pnacreatic lipase activity. In addition, it was evidenced that the hydrolysis of the non-oxidized TG (monomers) present in the frying oils was impaired by the concomitant presence of dimers and polymers. Nevertheless, these studies provided a "partial view" of the digestion process, because they focused on just *in vitro* lipolysis under simple incubation conditions with lipases, leaving aside the possible influence of all the other components of digestive fluids, or because they studied just the effect of the presence of pure oxidation compounds, disregarding the crucial influence of other dietary components simultaneously present in food.

3.2. Lipid oxidation

The potential occurrence of lipid oxidation during digestion cannot be discarded. Unsaturated lipids could undergo oxidative degradation during the digestion process, especially in the gastric step where lipids can be exposed to pro-oxidant conditions, like the acid pH of gastric fluid, the presence of oxygen incorporated to food during mastication, as well as of heme groups in certain proteins and of food-released transition metals, among others (Halliwell, Zhao, & Whiteman, 2000; Kanner & Lapidot, 2001).

However, this issue has been studied very little. A few *in vivo* and *in vitro* digestion studies on lipid emulsions and meat products can be found in literature (Gorelik et al., 2005; Gorelik, Ligumsky, Kohen, & Kanner, 2008; Kuffa, Priesbe, Krueger, Reed, & Richards, 2009; Larsson et al., 2012; Lorrain, Dangles, Loonis, Armand, & Dufour, 2012; Kenmogne-Domguia, Meynier, Boulanger, & Genot, 2012; Kristinova, Storrø, & Rustad, 2013; Gobert, Rémond, Loonis, Buffière, Santé-Lhoutellier, & Dufour, 2014; Kenmogne-Domguia, Moisan, Viau, Genot, & Meynier, 2014; Steppeler, Haugen, Rødbotten, & Kirkhus, 2016).

It has been shown that the advance of lipid oxidation can be greatly influenced by several factors. Among these may be cited: the presence of heme proteins, free iron or minor dietary compounds showing antioxidant properties (Kanner & Lapidot, 2001; Gorelik et al., 2008; Kenmogne-Domguia et al., 2012a; Larsson et al., 2012), as well as lipid composition, initial oxidative status and food fat content (Larsson et al., 2012; Kristinova et al., 2013; Kenmogne-Domguia et al., 2014; Steppeler et al., 2016). It must be noted that in most of these studies complex food matrices such as emulsions or cooked meat products already containing oxidation inititators or showing a certain degree of oxidation were digested. This may complicate the interpretation and comparison of the results obtained due to the interactions, often unknown, of other sample components with the lipid oxidation process. In this sense, additional systematic studies addressing the *in vitro* digestion of

simpler lipid matrices, such as bulk oils, could better clarify the susceptibility of lipids to oxidation under gastrointestinal conditions and the extent to which this reaction can be influenced by several factors.

Moreover, the lipid oxidation level of the samples in the above-mentioned studies was mainly assessed by indirect measurements (oxygen uptake and loss of "antioxidant" compounds) and by classical techniques, which might offer limited accuracy and specificity (Frankel, 2005; Schaich, 2016), such as: determination of lipid hydroperoxides by iodometric titration or by ferrous ion oxidation in the presence of xylenol-orange (FOX2) or thiocyanate, and measurement of conjugated dienes absorbance or of Thiobarbituric Acid Reactive Substances (TBARS test). In addition, these measurements and techniques are unable to provide information either about the specific nature of the lipid oxidation products generated.

It must be noted that in three recent studies the determination of three specific oxidation markers (malondialdehyde, 4-hydroxy-*(E)*-2-nonenal and 4-hydroxy-*(E)*-2-hexenal) by chromatographic techniques, after their extraction and derivatization, was also carried out (Kenmogne-Domguia et al., 2014; Steppeler et al., 2016; Tullberg et al., 2016). Nonetheless, these techniques are very laborious, require chemical transformation of the sample, involve the use of large amounts of solvents, and provide a very partial view of the lipid oxidation process, during which a high number of compounds of very different molecular weight and nature can be generated. Moreover, it must be noted that in order to get a "real picture" of the complex lipid oxidation reactions taking place, as many oxidation markers as possible should be considered because lipid oxidation can take place without the occurrence of these three specific compounds. So the use of other innovative techniques allowing a more complete study of lipid oxidation products in a simple and fast way would be required. Furthermore, in the above-mentioned studies the degree of lipolysis reached in the digestates was not investigated, in spite of its paramount importance in obtaining sound statements. In fact, as is well known, FA are more prone to oxidation than acyl groups supported on TG (Holman & Elmer, 1947).

In this context, the **Objectives 3.1.** and **3.2.** were addressed, this is, a study in depth of the *in vitro* digestion of fresh and slightly oxidized sunflower and linseed oils, as a models of ω-6 and ω-3 rich lipids respectively, by means of ^{1}H NMR and SPME-GC to get a more global view of the changes occurring during this complex process. As the intake of polyunsaturated ω-3 acyl groups has been encouraged in recent years, because of the health benefits which derive from this, a deeper knowledge of their potential chemical transformation during digestion can be considered of great relevance from a nutritional and food safety point of view. Indeed, the specific nature of the

compounds that may arise from the oxidation under digestive conditions of oils rich in polyunsaturated ω-3 acyl groups still remains unknown.

3.3. Potential influence of the presence of proteins on lipid hydrolysis and oxidation reactions under gastrointestinal digestive conditions

The extent of lipid hydrolysis and oxidation could be greatly influenced by the presence of proteins in the food bolus. During digestion, much in the same way as lipids, dietary proteins are hydrolyzed into smaller absorbable molecules (peptides and amino acids) by gastric pepsine and intestinal proteases. As a result, amino acids and peptides with different functional and bioactive properties (Elias, Kellerby, & Decker, 2008) could be present in the lumen.

Some recent studies reported the potential antioxidant activity of peptides released during simulated digestion of several protein containing-foods, such as cooked eggs (Remanan & Wu, 2014) and carp muscle (Borawska, Darewicz, Vegarud, Iwaniak, & Minkiewicz, 2015), but using only chemical assays, like oxygen radical absorbance capacity (ORAC), 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or 2,2´-diphenyl-1-picrylhydrazyl (DPPH) free radical assays, whose drawbacks and limitations make them unable to reflect what is occurring *in vivo* or to provide further knowledge on the impact of peptides and amino acids on lipid oxidation pathways (Prior, Wu, & Schaich, 2015). Moreover, it is still unclear if amino acids/peptides/proteins can protect lipids from oxidation, or if the former react with oxidized lipids, causing a less presence of lipid oxidation products, and thus simulating more slowly advancing oxidation, which masks the real progress of this reaction.

In order to further deepen knowledge about the potential effect of protein on chemical reactions undergone by lipids during digestion, the **Objective 3.3.** was outlined. In fact, most foods are composed not only of lipids, but also of other components, like proteins. Increasing knowledge on the potential inhibition of lipid oxidation by digestion-released amino acids/peptides would be of a great interest, not only from a nutritional point of view, but also from a food safety one, because this information would be very useful for the design of healthier and safer foodstuffs, or even diets.

3.4. Potential influence of the presence of the synthetic antioxidant BHT on lipid hydrolysis and oxidation reactions under gastrointestinal digestive conditions.

Data reported to date suggest that unsaturated lipids undergo oxidation under gastrointestinal conditions. However, this reaction could be limited by the presence of minor food components exhibiting antioxidant ability. Pioneer studies on the *in vitro* or *in vivo* gastric or duodenal digestion of oxidized turkey meat, myoglobin-added linoleic acid, and beef-sunflower oil mixture reported a

lower advance of lipid oxidation in the samples containing polyphenols than in the control ones; these polyphenols were either naturally present in red wine, apple, plum and artichoke, or were added pure standard compounds like resveratrol, epicatechin and caffeic acid (Kanner & Lapidot, 2001; Gorelik et al., 2005, 2008; Kerem, Chetrit, Shoseyov, & Regev-Shoshani, 2006; Lorrain et al., 2012; Gobert et al., 2014).

Among the antioxidants of synthetic origin commonly used as additives in the food industry for delaying lipid oxidation in bulk oils, 2,6-di-*tert*-butylhydroxytoluene (BHT, E-321) can be cited. In Europe the single use of BHT in fish oils is currently restricted up to 100 ppm (Commission Regulation (EU) No 1129/2011 of 11 November 2011 to establish a Union list of food additives). In a recent study, the ability of this food additive to inhibit lipid oxidation during *in vitro* and *in vivo* digestion of cooked turkey meat has been assayed (Kuffa et al., 2009). The authors reported a significant decrease of PV and TBARS values in the presence of BHT at 200, 2000 and 10000 ppm under *in vitro* gastric conditions. In addition, in *in vivo* trials, a significantly lower concentration of conjugated dienes (measured by absorbance value at 234 nm) was reported in the blood of pigs 3-4 hours after their being fed with cooked turkey meat containing 2000 ppm of BHT than in that of the controls. By contrast, Tarvainen et al. (2012) did not observe differences in the amount of unspecified lipid oxidation products generated during *in vitro* gastrointestinal digestion of rapeseed oil containing or not BHT at 100 and 1000 ppm, determined by ultrahigh performance liquid chromatography-electrospray ionization-mass spectrometry (UHPLC-ESI-MS). These contradictory results concerning the antioxidant effect of BHT may be due to: i) the different experimental conditions performed (gastric *vs* gastrointestinal digestion); ii) the different methodologies employed to assess lipid oxidation extent; or iii) to the different concentrations of BHT assayed, among other reasons. Moreover, in none of these two studies was attention paid to the occurrence of BHT-derived metabolites, which might be relevant due to the potential toxicity attributed to some of them, especially to quinone methide derivatives (Nagai, Ushiyama, & Kano, 1993; Thompson, Carlson, Sun, Dwyer-Nield, & Malkinson, 2001; Meier, Gomez, Kirichenko, & Thompson, 2007).

In this context, on the one hand, the **Objective 3.4.** of the present PhD thesis was contemplated in order to review the literature data available on BHT and its metabolites (occurrence, origin, possible dual role as antioxidant/pro-oxidant, fate in foodstuffs, transformation into metabolites, toxicological implications, dietary BHT exposure studies and established limits, additional sources of exposure, and analytical determination in foods). And on the other hand, in order to shed light on the potential effect of BHT on *in vitro* gastrointestinal digestion of cod liver oil the **Objective 3.5.** was addressed. Since from a chemical point of view, any antioxidant could also be able to exert a pro-oxidant activity, concentrations of BHT either lower or far beyond that permitted

by European authorities will be employed, in order to check BHT behaviour under digestion conditions.

4. *IN VITRO* GASTROINTESTINAL DIGESTION OF FISH AND THE POTENTIAL INFLUENCE OF SOME TECHNOLOGICAL PROCESSES LIKE SALTING AND SMOKING ON THE LIPIDS PERFORMANCE UNDER DIGESTIVE CONDITIONS

Due to their high content in polyunsaturated acyl group, fish lipids may be especially prone to oxidation under digestive pro-oxidant conditions, which might impair their nutritional quality and safety. Thus, considering the results obtained in previous *in vitro* digestion studies on marine oils (Larsson et al., 2012; Kristinova et al., 2013; Kenmogne-Domguia et al., 2014; Tullberg et al., 2016), some oxidation could be expected to occur during *in vitro* digestion of fish meat. Nevertheless, the latter is a much more complex matrix than fish oil: the presence of other components together with fish lipids in the food bolus, such as proteins or endogenous fish antioxidants, among others, might greatly influence the advance of oxidation reactions occurring under digestive conditions.

In a recent study, the formation of malondialdehyde (measured as TBARS value) and 4-hydroxy-*(E)*-2-hexenal was reported during *in vitro* digestion of salmon (Steppeler et al., 2016). However, further knowledge concerning the nature of lipid oxidation products that may be generated during fish digestion would be useful. Moreover, other chemical reactions in addition to oxidation might take place and deserve further attention (Goicoechea et al., 2008, 2011). Thus, a detailed study of the occurrence of lipid oxidation and other reactions during fish meat digestion has not been undertaken to date; nor has the potential effect, if any, on the extent of these reactions of common technological processes applied to fish, such as salting and smoking, been studied.

Salting is a technological process that has been traditionally carried out on fish in order to extend their shelf-life. Its preservative effect relies on the decreased water activity that this process provokes, which prevents microbiological growth. However, certain studies reported that salting can reduce the oxidative stability of fish lipids under frozen, refrigerated or thermo-oxidative conditions (Aubourg, & Ugliano, 2002; Guillén & Ruiz, 2004; Guillén, Ruiz, & Cabo, 2004; Vidal, Goicoechea, Manzanos, & Guillén, 2015). Using spectroscopic techniques, like Fourier Transform Mid-Infrared (FT-MIR) and ^{1}H NMR, it was observed that salting did not provoke any immediate oxidation, but when submitted to pro-oxidative conditions, fish lipid oxidation evolved at a greater rate in salted than in unsalted fish fillets. Nevertheless, when farmed and wild specimens of European sea bass were salted and studied by means of SPME-GC/MS, very slight lipid oxidation could be observed in wild

samples immediately after the most intense salting process (Vidal, Manzanos, Goicoechea, & Guillén, 2016b). This pro-oxidant effect could be attributed to the loss of water-soluble antioxidants and to the increase of pro-oxidant agent concentration in contact with the lipid phase (Kanner, Harel, & Jaffe, 1991). Nevertheless, the degree of salting might also be determinant (Aubourg & Ugliano, 2002).

In this context, the **Objective 4.1.** of this doctoral thesis was addressed, to investigate fish meat *in vitro* digestion, paying special attention to the hydrolysis reaction and to the occurrence of lipid oxidation, without forgetting that other chemical reactions are also possible. Furthermore, the evaluation of the potential differences occurring during digestion of unsalted and salted fillets, as well as the influence of the degree of salting need to be addressed.

Smoking is another preservation technique applied to fish from ancient times up to the present day. In recent decades, the use of commercial smoke flavourings, complex mixtures of smoke components retained in a liquid or solid carrier, instead of traditional smoking techniques using burning or smoldering materials has increased because of their many advantages. Among these can be cited: ease of application and a better control not only of sensory characteristics of smoked products, but also of the presence of smoke-derived toxic compounds, such as polycyclic aromatic hydrocarbons (Guillén & Ibargoitia, 1998).

The extension of fish shelf-life and oxidative stability due to the smoking process has been addressed in previous studies, in which special attention was paid to the effect of different smoke flavouring compositions and application methods (Alcicek, 2011; Vidal, Goicoechea, Manzanos, & Guillén, 2016; Vidal, Manzanos, Goicoechea, & Guillén, 2016c). Nevertheless, the performance of smoked fish during human digestion still remains unknown. It could be expected that the well-known beneficial effects of the smoke components during fish meat storage, would also be evidenced *in vivo* during fish meat gastrointestinal digestion, because smoke phenolic components such as dihydroxybenzenes, 2,6-dimethoxy- and 2-methoxy-phenols have proved antioxidant activity (Bortolomeazzi, Sebastianutto, Toniolo, & Pizzariello, 2007; Soldera, Sebastianutto, & Bortolomeazzi, 2008; Huang, Chang, Sung, Vong, & Wang, 2011). However, it must be noted that most of the studies on the antioxidant activity of smoke phenolic compounds have been carried out using chemical assays, like radical scavenging assays using ABTS and DPPH, whose drawbacks limit their usefulness in reflecting what takes place in *in vivo* or in real food systems (Prior et al., 2005).

Therefore, the **Objective 4.2.** was considered to evaluate the effect of the smoking process with two commercial liquid smoke flavourings on lipid hydrolysis and oxidation occurring during *in vitro* gastrointestinal digestion of European sea bass, and to determine to what extent the flavouring

composition may influence these reactions. This study will provide a global view of how the mechanisms and extent of fish lipid hydrolysis and oxidation processes could be affected, if any, by the presence of smoke-derived compounds showing potential antioxidant activity.

AIMS AND OBJECTIVES

The present PhD dissertation relies on four main aims, which will be achieved throughout the development of several specific objectives that are listed below.

AIM 1. To study the effect of several cooking methods on fish lipids and on the formation of volatile compounds, as well as the potential influence of fish species and growing conditions

For this purpose, the following specific objectives were formulated:

1.1. To investigate by means of ^{1}H NMR the potential influence of frying technique (pan-frying/microwave-frying), cooking oil (extra-virgin olive oil/sunflower oil) and fish species (farmed European sea bass/farmed Gilthead sea bream) on the changes occurring in fish lipids and in cooking oil during fish shallow-frying (**Manuscript 1**).

1.2. To study by means of ^{1}H NMR and SPME-GC/MS the potential effect of boiling, steaming and *sous-vide* cooking on the lipids and volatile profile of farmed and wild European sea bass (**Manuscript 2**).

1.3. To address by means of ^{1}H NMR and SPME-GC/MS the potential effect of salt-crusted oven baking, conventional oven baking and microwave cooking on the lipids and volatile profile of farmed and wild European sea bass (**Manuscript 3**).

Increasing knowledge on the effect of cooking on food quality parameters is of paramount importance from a nutritional, food safety, technological and sensory point of view. Accomplishing this **first aim** will shed light on the extent of the changes, if any, produced in the lipids and volatile profile of fish meat as a result of the different cooking methods. The information obtained could be very valuable, among others, for the food industry to produce *ready-to-eat* fish products that will ensure food quality and safety as much as possible, as well as consumer acceptance.

AIM 2. To study the *in vitro* gastrointestinal digestion of lipids by means of ^{1}H NMR, and the influence of several factors affecting the extent of *in vitro* lipolysis

For this purpose, the following specific objectives were tackled:

2.1. To develop and validate a new methodology based on ^{1}H NMR spectral data to evaluate the hydrolysis level in complex lipid mixtures (quantification of triglycerides, 1,3- and 1,2-diglycerides, 2- and 1-monoglycerides and fatty acids) (**Manuscript 4**).

2.2. To demonstrate the usefulness of ^{1}H NMR when studying the extent of lipid hydrolysis reached during digestion of food samples (sunflower oil/minced fish meat), determining the hydrolysis level, the degree of triglyceride transformation, the lipid bioaccessibility level and the percentage of fatty acids physiologically releasable (**Manuscript 5**).

2.3. To investigate the effect of different experimental factors (gastric acidification, intestinal transit time, presence of gastric lipase, sample/digestive fluids ratio, intestinal enzymes concentration and bile concentration) on lipid *in vitro* digestion extent (**Manuscript 6**).

Reaching this **second aim** would be very relevant for the field of lipid digestion research, because there is an urgent need for the development of methodologies able to overcome the limitations of the currently employed techniques regarding the accurate quantification of lipolytic products and the versatility to assess lipolysis reaction extent in any of its definitions. ^{1}H NMR approach will allow a global study of the digested lipidic sample in a fast and simple way, and without any chemical modification of the sample. This will be very useful to study in depth the impact of experimental factors on lipid *in vitro* digestion in order to optimize *in vitro* digestion protocols with regard to *in vivo* lipolysis level, which is required to obtain sound and reliable results.

AIM 3. To study simultaneously lipid hydrolysis, oxidation and other reactions occurring during the *in vitro* gastrointestinal digestion of oils of vegetable and animal origins, and of other model systems, as well as the potential influence on their extent of the oil initial oxidation level, the oil unsaturation degree, of the presence of proteins and of the synthetic antioxidant BHT

For this purpose, the following specific objectives were addressed:

3.1. To tackle by means of ^{1}H NMR and SPME-GC/MS the chemical reactions taking place during *in vitro* gastrointestinal digestion of non-oxidized and slightly oxidized sunflower oils, as models of ω-6 rich lipids (**Manuscript 7**).

3.2. To investigate by means of ^{1}H NMR and SPME-GC/MS the chemical reactions taking place during *in vitro* gastrointestinal digestion of non-oxidized and slightly oxidized flaxseed oils, as models of ω-3 rich lipids (**Manuscript 8**).

3.3. To study by means of ^{1}H NMR and SPME-GC/MS the potential effect of protein (ovalbumin/soy protein isolate) on the chemical reactions taking place during *in vitro* gastrointestinal digestion of slightly oxidized sunflower and flaxseed oils (**Manuscript 9**).

3.4. To review the available literature on the synthetic antioxidant 2,6-di-*tert*-butylhydroxytoluene (BHT, E-321) and its metabolites (occurrence, origin, possible dual role as antioxidant/pro-oxidant, fate in foodstuffs, transformation into metabolites, toxicological implications, dietary BHT exposure studies and established limits, additional sources of exposure, and analytical determination in foods) (**Manuscript 10**).

3.5. To investigate by means of ^{1}H NMR and SPME-GC/MS the hydrolysis and oxidation reactions affecting polyunsaturated acyl groups and naturally present vitamin A during *in vitro* gastrointestinal digestion of cod liver oil, and the potential effect of low and high concentrations of added BHT (20/800 ppm) on the above-mentioned reactions (**Manuscript 11**).

This **third aim** will allow an in-depth and systematic study of the chemical reactions affecting lipids during gastrointestinal digestion and of the several factors affecting the extent of these reactions. The use of ^{1}H NMR and SPME-GC/MS will provide a holistic view of the evolution of polyunsaturated lipids during this complex process, including potential oxidative degradation. This will provide, for the first time, knowledge on the specific nature of the oxidation compounds that can be generated under digestive conditions, which is needed to select properly the oxidation markers for these specific conditions.

AIM 4. To study the effect of common technological processes, like salting and smoking, on lipid hydrolysis, oxidation and other reactions occurring during fish *in vitro* gastrointestinal digestion

For this purpose, the following specific objectives were considered:

4.1. To investigate by means of ^{1}H NMR and SPME-GC/MS the chemical reactions taking place during *in vitro* gastrointestinal digestion of European sea bass, and the effect of fish salting (brine-salting/dry-salting) on their extent (**Manuscript 12**).

4.2. To study by means of ^{1}H NMR and SPME-GC/MS the effect of smoking with two liquid smoke flavourings on lipid hydrolysis and oxidation occurring during *in vitro* gastrointestinal digestion of European sea bass, and to evaluate to what extent the flavouring composition may influence these reactions (**Manuscript 13**).

The achievement of this **fourth aim** will be of paramount importance to gain further knowledge concerning the relationships existing between food composition, technological processing and human health. For the first time, an evaluation of how common technological processes could affect the chemical reactions ongoing during fish *in vitro* gastrointestinal digestion of will be investigated.

EXPERIMENTAL DESIGN

AIM 1

Fish cooking methods

Effect on lipids and formation of volatile compounds

Influence of fish species and growing conditions

(MANUSCRIPTS 1-3)

OBJECTIVE 1.1.

To investigate by means of ^{1}H NMR the potential influence of frying technique (pan-frying/microwave-frying), cooking oil (extra-virgin olive oil/sunflower oil) and fish species (farmed European sea bass/farmed Gilthead sea bream) on the changes occurring in fish lipids and in cooking oil during fish shallow-frying

(**Manuscript 1**)

1.1.1. Oil sample subjects of study

Two cooking oils, extra-virgin olive oil (named **evo**) and sunflower oil (named **s**), were acquired in a local supermarket.

In order to evaluate the *effect of heating* on the oils, extra-virgin olive and sunflower oils were submitted to the same frying conditions in the microwave-oven and in the pan in the *absence of food*. These heated samples were named: **evoP**, extra-virgin olive oil submitted to pan-frying conditions without food; **evoM**, extra-virgin olive oil submitted to microwave-frying conditions without food; **sP**, sunflower oil submitted to pan-frying conditions without food; and **sM**, sunflower oil submitted to microwave-frying conditions without food.

After being used to *fry fish fillets*, oil samples were also collected and named: **evoPA**, extra-virgin olive oil used to pan-fry sea bream (*Sparus aurata*); **evoPL**, extra-virgin olive oil used to pan-fry sea bass (*Dicentrarchus labrax*); **sPA**, sunflower oil used to pan-fry *S. aurata*; **sPL**, sunflower oil used to pan-fry *D. labrax*; **evoMA**, extra-virgin olive oil used to microwave-fry *S. aurata*; **evoML**, extra-virgin olive oil used to microwave-fry *D. labrax*; **sMA**, sunflower oil used to microwave-fry *S. aurata*; and **sML**, sunflower oil used to microwave-fry *D. labrax*. It must be noted that oils were used just once for frying and never reused.

1.1.2. Fish lipid sample subjects of study

Fresh specimens of farmed gilthead sea bream (**A**, *n*=8) and of farmed European sea bass (**L**, *n*=8) were acquired in a local supermarket on the day of the experiment. Just before frying, fishes were gutted, cleaned and filleted. The average weight of sea bream fillets (*n*=16) was 332.7±21.7 g and that of sea bass fillets (*n*=16) 295.4±29.6 g; all fillets presented very similar dimensions (width and length). From each specimen, one fillet was submitted to cooking and the other one was kept raw (**R**) as a control.

The lipid extracts obtained from the control fillets were named: **AR**, lipids of raw sea bream; and **LR**, lipids of raw sea bass. The lipid extracts obtained from shallow-fried fish fillets were named: **APevo**, lipids of *S. aurata* pan-fried in extra-virgin olive oil; **LPevo**, lipids of *D. labrax* pan-fried in extra-virgin olive oil; **APs**, lipids of *S. aurata* pan-fried in sunflower oil; **LPs**, lipids of *D. labrax* pan-fried in sunflower oil; **AMevo**, lipids of *S. aurata* microwave-fried in extra-virgin olive oil; **LMevo**, lipids of *D. labrax* microwave-fried in extra-virgin olive oil; **AMs**, lipids of *S. aurata* microwave-fried in sunflower oil; and **LMs**, lipids of *D. labrax* microwave-fried in sunflower oil.

1.1.3. Shallow-frying techniques

Two different shallow-frying techniques were employed, conventional pan-frying (**P**) using a domestic pan (28 cm internal diameter) over an electric heating unit, and microwave-frying (**M**) using a domestic ceramic baking dish (28 cm internal diameter) in a household microwave oven (Samsung Combi CE 117KB) operating at 900 W. In order to obtain comparable results and to mimic domestic conditions, some experimental conditions were the same in both techniques: oil temperature (170ºC), cooking time (2.5 min each fillet side) and oil surface/oil volume ratio (28 cm diameter/100 mL). These conditions were maintained for all frying experiments. Before fish frying, oil temperature was checked with a dual purpose infrared and penetration thermomether (104-IR, Testo instruments, Lenzkirch, Germany), that can measure both oil/food surface and core temperatures. One fish fillet was fried each time and two independent experiments were carried out for consistency of results. The mean core temperature reached in pan-fried fillets was 60±5ºC and in microwave-fried ones 95±3ºC; crust formation was observed on the surface of the former but not on the latter. After cooking, all fried fillets were drained for 15 s to remove excess oil and then minced in a grinder, vacuum-packed and stored at -80ºC for up to 24 h for subsequent study.

1.1.4. Fish lipid extraction method

Lipids of fish fillets before and after frying were extracted using carbon disulphide as solvent (CS_2, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA) in a proportion of 1:2 (w/v) in an ultrasonic bath for 1 h, as in a previous study (Guillén & Ruiz, 2004). This solvent was selected because of its ability to extract lipids and its high volatility. Afterwards, solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature in order to avoid lipid oxidation.

1.1.5. Proton Nuclear Magnetic Resonance spectra acquisition

The ^{1}H NMR spectra of the oils unheated, heated and after their use in fish shallow-frying, as well as of the fish lipids extracted from raw and fried fillets, were recorded on a Bruker Avance 400

spectrometer operating at 400 MHz. As in previous edible oil studies carried out in our laboratory (Guillén & Ruiz, 2004), 200 µl of lipid samples were mixed in a 5 mm diameter tube with 400 µl deuterated chloroform ($CDCl_3$), which contains 0.2% of non-deuterated chloroform, and a small proportion of tetramethylsilane (TMS) used as reference compound for calibrating chemical shift at 0.0 ppm (Euroisotop, Paris, France). In order to select the most appropriate values to obtain accurate quantitative results in the shortest possible period of time, a very broad range of recycling times and relaxation delays were tested in the acquisition of the ^{1}H NMR spectra. Thus, the acquisition parameters selected as being the most appropriate were the following: spectral width 6410 Hz, relaxation delay 3 s, number of scans 64, acquisition time 4.819 s and pulse width 90º. Each lipid sample was analyzed in duplicate. 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 their use for quantitative purposes possible. ^{1}H NMR spectra were plotted at a fixed value of absolute intensity to be valid for comparative purposes. Spectra were processed using MNova program (Mestrelab Research, Santiago de Compostela, Spain).

1.1.6. Determination from ^{1}H NMR data of the molar percentage of main acyl groups and of the concentration of some minor components, hydrolytic and thermo-oxidation compounds in the oils and in fish lipids

As the area of the ^{1}H NMR signal is proportional to the number of protons that generates it, and because the proportionality constant is the same for all types of hydrogen atoms, it is possible to determine in an accurate way the absolute concentration and also the molar percentages of the different kinds of acyl group chains present in the oils and in fish lipids. These determinations were carried out in agreement with previous studies (Guillén, Carton, Goicoechea, & Uriarte, 2008; Martínez-Yusta & Guillén, 2014abc, 2016). It is worth considering that the contribution of diglycerides present in the oils and of phosphatidylcholine in fish lipids is very small (due to the fact that their molar abundance is a hundred times lower than that of triglycerides). Thus, the molar percentage of total omega-3 (ω-3); of docosahexaenoic (DHA, C22:6ω3); of eicosapentaenoic (EPA, C20:5ω3) plus arachidonic (ARA, C20:4ω6) (EPA+ARA); of diunsaturated omega-6 acyl groups (DUω-6), mainly linoleic (C18:2ω6); of total unsaturated (U); of oleic (C18:1ω9) plus other unsaturated (O+OU) acyl groups, the latter being mainly other monounsaturated ω-7 and ω-9, ARA and other minor unsaturated acyl groups; and of omega-1 (ω-1) acyl groups were estimated as follows:

$$\omega\text{-}3\% = 100*(4*A_B)/(9*A_I) \quad \text{[Objective 1.1.-eq.1]}$$

$$DHA\% = 100*A_{F2}/(3*A_I) \quad \text{[Objective 1.1.-eq.2]}$$

$$EPA+ARA\% = 100*(2*A_{D2})/(3*A_I) \quad \text{[Objective 1.1.-eq.3]}$$

$DU\omega\text{-}6\% = 100*(2*A_G)/(3*A_l)$ [Objective 1.1.-eq.4]

$U\% = 100*(2*A_E+A_{F2})/(6*A_l)$ [Objective 1.1.-eq.5]

$O+OU\% = (U\%)-(\omega\text{-}3\%)-(\omega\text{-}1\%)-(DU\omega\text{-}6\%)$ [Objective 1.1.-eq.6]

$\omega\text{-}1\% = 100*(2*A_P)/(3*A_l)$ [Objective 1.1.-eq.7]

where $\mathbf{A_X}$ is the area of signal X (see the signal assignment in **Table 1** of **Manuscript 1**). Then, saturated plus modified (**S+M**) acyl groups were calculated from U%, by difference to 100%. It must be pointed out that modified acyl groups are those unsaturated chains that have been modified as a result of oxidation reactions, losing their original typical structure. It also must be noted that as the ARA content in these farmed fishes is usually very low (Orban et al., 2003), the whole area of signal D2 was considered due to EPA. Due to the overlapping of signals D1-D2 and G-H, the spectra of pure standard trieicosapentaenoin, triarachidonin, trilinolein and trilinolenin, acquired from Larodan AB (Malmö, Sweden), were recorded and taken into account for a correct determination of A_{D2} and A_G.

Moreover, quantification of some minor components present in the oils (β-sitosterol plus Δ5-campesterol (**Sit+Camp**) and Δ7-avenasterol) and in fish lipids (cholesterol and phosphatidylcholine), expressed as mmol/mol of triglyceride (mmol/molTG), was also carried out using the following equations:

$St\ (mmol/molTG) = 1000*(4*A_{St})/(3*A_l)$ [Objective 1.1.-eq.8]

$Phosphatidylcholine\ (mmol/molTG) = 1000*(4*A_O)/(9*A_l)$ [Objective 1.1.-eq.9]

where $\mathbf{A_{St}}$ is the area of the signal of the methylic proton at the carbon atom C-18 of each sterol (Sit+Camp, Δ7-avenasterol, cholesterol) and $\mathbf{A_X}$ the area of signal X (see the signal assignment in **Table 1** of **Manuscript 1**).

Regarding hydrolytic and thermo-oxidation compounds, the concentrations of 1,2-diglycerides, aldehydes (**Ald**) and *(E)*-9,10-epoxystearate, expressed also as mmol/mol of TG, were also determined as follows:

$1,2\text{-}diglycerides\ (mmol/molTG)=1000*(2*A_{3.72})/A_l$ [Objective 1.1.-eq.10]

$Ald\ (mmol/molTG)=1000*(4*A_{Ald})/A_l$ [Objective 1.1.-eq.11]

$(E)\text{-}9,10\text{-}epoxystearate\ (mmol/molTG)=1000*(2*A_{2.63})/A_l$ [Objective 1.1.-eq.12]

where $\mathbf{A_{3.72}}$ is the area of the signal at 3.72 ppm due to -C$\underline{H_2}$OH protons of 1,2-diglycerides, $\mathbf{A_{Ald}}$ is the area of aldehydic proton signals Q, R, S or T (see the signal assignment in **Table 1** of **Manuscript 1**), and $\mathbf{A_{2.63}}$ is the area of the signal at 2.63 ppm due to -C$\underline{H}$O$\underline{H}$C- protons of *(E)*-9,10-epoxystearate.

1.1.7. Statistical Analysis

Statistical analysis was performed using the Statistical package SPSS v.19 (IBM, NY, USA). The significance of the differences on the several determinations among groups were determined by one-way variance analysis (ANOVA) followed by post hoc Tukey b test at 0.05 threshold.

OBJECTIVE 1.2.

To study by means of ^{1}H NMR and SPME-GC/MS the potential effect of boiling, steaming and *sous-vide* cooking on the lipids and volatile profile of farmed and wild European sea bass

(**Manuscript 2**)

1.2.1. Fish samples

Fresh specimens of farmed (**F**, *n*=6) and wild (**W**, *n*=6) European sea bass were acquired in a local supplier within 48 h of their catch. On the day of purchase and just before cooking, fish specimens were gutted, cleaned, filleted and skinned. Average weight of farmed and wild sea bass fillets was 251.5±21.0 g (*n*=12) and 281.5±40.6 g (*n*=12), respectively. From each specimen, one fillet was kept raw (R) as a control, and the other one was submitted to cooking. Raw fillets of farmed sea bass were named $\mathbf{F_R}$ (*n*=6) and those of wild sea bass $\mathbf{W_R}$ (*n*=6).

1.2.2. Cooking methods

Also on the day of purchase, cooking processes were carried out. Three cooking methods were employed, fixing cooking times and temperatures according to real household conditions. Each fish fillet was cooked independently and cooking experiments were carried out in duplicate for consistency of results. After cooking, the core temperatures of the fillets were checked with a thermometer (104-IR, Testo instruments, Lenzkirch, Germany).

Boiling (BO). This was performed using a domestic stainless steel casserole (24 cm internal diameter) over an electric heating unit; each fillet was immersed in 2 L of boiling water (100ºC) for 10 min. After boiling, farmed ($\mathbf{F_{BO}}$, *n*=2) and wild ($\mathbf{W_{BO}}$, *n*=2) fillets core temperature was 88±2ºC.

Steaming (ST). This was carried out using a steaming casserole set (24 cm internal diameter); 2 L of boiling water (100ºC) were placed in the bottom of the casserole and the fillet was placed on the perforated middle layer (covered with the lid) and submitted to steaming for 10 min. After steaming, farmed ($\mathbf{F_{ST}}$, *n*=2) and wild ($\mathbf{W_{ST}}$, *n*=2) fillets core temperature was 91±0ºC.

Sous-vide cooking (SV). Each fillet was vacuum-packed in a polypropylene (PP) heat-resistant (up to 120ºC) bag designed for this culinary technique, using a vacuum sealer (VAC-20S model, Edesa, Mondragon, Spain). Then, plastic bags were submerged for 20 min in 20 L of water pre-heated at 85ºC, using a thermostatic water bath (Precisdig model, Selecta, Barcelona, Spain). Afterwards, the

plastic bag was opened and the core temperature of farmed (**F_{SV}**, *n*=2) and wild (**W_{SV}**, *n*=2) fillets measured (83±2ºC).

Immediately after cooking, each fish fillet was minced in a grinder and the volatile profile of minced sea bass meat was studied by SPME-GC/MS. The remaining ground meat of each fillet was vacuum-packed immediately and stored at -80ºC for up to 24 h for the subsequent extraction of their lipids and study by ^{1}H NMR.

1.2.3. Lipid extraction

Lipids of sea bass fillets before and after cooking were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA) in a proportion of 1:2 (w/v) in an ultrasonic bath for 1 h. This solvent was selected because of its ability to extract lipids, its suitable polarity for an exhaustive extraction and its high volatility. Afterwards, solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature in order to avoid lipid oxidation.

1.2.4. ^{1}H NMR spectra acquisition, standards and derived data

The ^{1}H NMR spectra of the lipid extracts of raw (**F_R**, **W_R**), cooked farmed (**F_{BO}**, **F_{ST}**, **F_{SV}**) and wild (**W_{BO}**, **W_{ST}**, **W_{SV}**) sea bass meat were recorded in duplicate on a Bruker Avance 400 spectrometer operating at 400 MHz. To do this, the sample preparation to acquire the corresponding spectra, the acquisition conditions and the study of the spectral data were the same as those described before in subsection 1.1.5. of the experimental design of Objective 1.1.

Compounds, such as cholesterol, cholesterol-5β,6β-epoxide, 5α-cholestan-3β-ol, phosphatidylcholine, retinyl palmitate, retinyl acetate and retinol acquired from Sigma Aldrich, cholestadien-5β,6β-epoxy-3β-ol and 5-cholesten-3β,7β-diol acquired from Steraloids (Newport, RI, USA), and cholesterol-5α,6α-epoxide acquired from Cymit Química S.L. (Barcelona, Spain), were used as standard compounds for identification purposes in ^{1}H NMR spectra.

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, the area of some spectral signals make it possible to estimate the molar proportion of the several kinds of acyl groups and the concentration of some minor components present in the lipid extracts of raw and cooked sea bass fillets.

***Estimation of the molar percentages of acyl groups*.** The molar percentage of saturated and of total unsaturated acyl groups, the latter including total ω-3, diunsaturated ω-6 (DUω-6, mainly linoleic C18:2ω6), ω-1, and oleic plus other minor unsaturated acyl groups (mainly other monounsaturated ω-7 and ω-9, arachidonic and other minor unsaturated acyl groups) (O+OU), were calculated using equations developed in previous studies (Guillén & Ruiz, 2004; Vidal et al., 2012). Likewise, the molar percentages of long chain polyunsaturated acyl groups, such as docosahexaenoic (DHA, C22:6ω3) and eicosapentaenoic (EPA, C20:5ω3) plus arachidonic (ARA, C20:4ω6) acyl groups were estimated.

Estimation of the concentration of minor lipidic components. The concentration, expressed as mmol/mol of triglyceride (mmol/molTG), of certain minor lipidic components of interest was also estimated using the following equations (see subsection 1.1.6. of the experimental design of Objective 1.1.):

Cholesterol (mmol/molTG) = $4000*A_a/(3*A_l)$ [Objective 1.2.-eq.1]

Phosphatidylcholine (mmol/molTG) = $4000*A_d/(9*A_l)$ [Objective 1.2.-eq.2]

Phosphatidylethanolamine (mmol/molTG) = $2000*A_c/A_l$ [Objective 1.2.-eq.3]

Vitamin A (retinyl ester) (mmol/molTG) = $4000*A_b/(3*A_l)$ [Objective 1.2.-eq.4]

where $\mathbf{A_a}$, $\mathbf{A_d}$, $\mathbf{A_c}$, $\mathbf{A_b}$ and $\mathbf{A_l}$ are the areas of the ^{1}H NMR spectral signal a (singlet at 0.68 ppm), signal d (singlet at 3.35 ppm), signal c (singlet at 3.15 ppm), signal b (singlet at 1.89 ppm) and signal I (doublet of double doublets at 4.22 ppm) respectively; the signal assignment is given in **Table 1** of **Manuscript 2** (Choi, Kim, Wilson, Erkelens, Trijzelaar, & Verpoorte, 2004; Guillén & Ruiz, 2004; Mannina et al., 2008; Vidal et al., 2012).

1.2.5. Study by SPME-GC/MS of the volatile profile of raw and cooked sea bass meat

Solid Phase Microextraction (SPME). The extraction of the volatile components of the headspace of raw and cooked minced sea bass meat (0.5 g in 10 mL screw-cap vial) was accomplished automatically in duplicate using a CombiPAL autosampler (Agilent Technologies, Santa Clara, CA, USA). The fibre used was coated with DVB/CAR/PDMS (Divinylbenzene/Carboxen/ Polydimethylsiloxane, 50/30 µm film thickness, 1 cm long), acquired from Supelco (Sigma-Aldrich), which was inserted into the headspace of the sample and was maintained for 60 min (50ºC). Variables such as the type of fibre (polarity and thickness of the coating) and the extraction conditions (sample and headspace volumes, extraction time and temperature) had been previously tested in our laboratory to select the best extraction conditions.

Gas chromatography/mass spectrometry (GC/MS) study. The fibre containing the extracted components was desorbed for 10 min in the injection port (splitless mode with 5-min purge time) of a gas chromatograph model GC 7890A equipped with a mass selective detector 5975C inert MSD with Triple Axis Detector (Agilent Technologies) and a computer operating with the ChemStation program. The column used was a fused-silica capillary column (60 m long x 0.25 mm inner diameter x 0.25 µm film thickness, from Agilent J & W Advanced Capillary GC Columns), coated with a non-polar stationary phase (HP-5MS, 5% phenyl methyl siloxane). The operating conditions were as follows: the oven temperature was set initially at 50ºC (5 min hold) and increased to 290ºC at 4ºC/min (2 min hold); the temperatures of the ion source and the quadrupole mass analyser were kept at 230ºC and 150ºC respectively; helium was used as carrier gas at a pressure of 18.611 psi; injector temperature was held at 250ºC; mass spectra were recorded at an ionization energy of 70 eV and the data acquisition mode employed was Scan (mass spectra range from 40 to 550 m/z). After the first desorption, the fibre was routinely submitted to desorption conditions for a second time to clean up and to determine if the first process was complete. In order to avoid carry-over problems, after each run the fibre was submitted to 20-min bake-out at 250ºC in the Fibre Cleaning and Conditioning Station of the CombiPAL autosampler. A reference sample of known composition was periodically analyzed in order to verify the extraction efficiency and repeatability of the SPME fibre and the performance of the chromatographic run.

Most of the compounds were identified by using commercial standards (asterisked compounds in **Tables 3**, **4** and **S1** of **Manuscript 2**) acquired from Sigma-Aldrich. When standards were not available, matching of the mass spectra with those obtained from a commercial library higher than 85% (Wiley W9N08, Mass Spectral Database of the National Institute of Standards and Technology (NIST)), was taken as identification criteria. The semiquantification 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 the same ion peak of the mass spectrum of another compound, an alternative ion peak was selected for the quantification of the former. 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. Compounds having lower abundance values than 50000 area counts were considered as traces.

1.2.6. Statistical analysis

The significance of the differences in the molar percentages of the several kinds of acyl groups and in the concentration of minor lipidic components estimated from ^{1}H NMR data among the lipid extracts of raw and cooked sea bass fillets were determined by one-way variance analysis (ANOVA)

followed by Tukey b test at $p<0.05$, using SPSS v.22 software (IBM, NY, USA). Principal component analysis (PCA) performed on the abundances of certain volatile markers of lipid oxidation identified by SPME-GC/MS was carried out by using SIMCA v.13.0.3 software (Umetrics/MKS, Umeå, Sweden).

OBJECTIVE 1.3.

To address by means of ^{1}H NMR and SPME-GC/MS the potential effect of salt-crusted oven baking, conventional oven baking and microwave cooking on the lipids and volatile profile of farmed and wild European sea bass

(**Manuscript 3**)

1.3.1. Fish samples

Fresh specimens of farmed (**F**, *n*=6) and wild (**W**, *n*=6) European sea bass were acquired from a local supplier within 48 h of their being caught. On the day of purchase and just before cooking, specimens were gutted, cleaned, filleted and skinned. The average weight of farmed sea bass fillet (*n*=12) was 256.5±25.0 g and that of wild one (*n*=12) was 253.4±18.9 g. From each specimen, one fillet was kept raw (R) as a control and the other one was submitted to cooking. Raw fillets of farmed and wild sea bass were named $\mathbf{F_R}$ (*n*=6) and $\mathbf{W_R}$ (*n*=6), respectively.

1.3.2. Cooking methods

Three cooking methods were carried out in this study: salt-crusted oven baking, conventional oven baking and microwave oven cooking. These widely used cooking methods were selected because they differ greatly in the food-heating mechanism. Thus, in the case of microwave cooking, the alternating electric field of microwaves provokes the rotation of dipole molecules contained in food (mainly water), whose friction heats the food from the inside to the outside; it is a very fast thermal treatment in which the maximum heating temperature is limited by the boiling point of water molecules. In the case of oven baking, infrared radiation heats the air molecules present inside the oven, which in turn heat the food surface by convection and then the inside is heated by conduction; therefore, longer heating times are needed and higher cooking temperatures can be reached.

Fish cooking time and temperature (or microwave oven potency) were set according to real household conditions. Each fish fillet was cooked independently and cooking experiments were carried out in duplicate for consistency of results. After cooking, the core temperature of the fillets was checked with a thermometer (104-IR, Testo instruments, Lenzkirch, Germany).

Salt-crusted oven baking (SC). Each fish fillet was laid on a coarse sea salt bed placed on a pyrex baking dish and entirely covered with a thick salty paste made of coarse sea salt and water. Salt was acquired in a local supermarket. The baking dish was placed in a pre-heated household oven

(model HT-610 ME, Teka, Santander, Spain) and cooked at 185ºC for 25 min. Then, the salt-crust was broken and the fish fillet scooped out. After salt-crusted oven baking, farmed ($\mathbf{F_{SC}}$, *n*=2) and wild ($\mathbf{W_{SC}}$, *n*=2) fillets core temperature was 91±1ºC.

Conventional oven baking (CB). A fish fillet was placed on a pyrex baking dish and baked in a pre-heated household oven (model HT-610 ME, Teka) at 185ºC for 25 min. After conventional oven baking, farmed ($\mathbf{F_{CB}}$, *n*=2) and wild ($\mathbf{W_{CB}}$, *n*=2) fillet core temperature was 93±1ºC.

Microwave oven cooking (MW). Each fish fillet was placed on a porcelain dish, covered with a plastic lid, and cooked at 900 W for 5 min in a household microwave oven (Samsung Combi CE 117 KB). After microwave cooking, farmed ($\mathbf{F_{MW}}$, *n*=2) and wild ($\mathbf{W_{MW}}$, *n*=2) fillet core temperature was 98±0ºC.

Immediately after cooking, each fish fillet was minced in a grinder and the volatile profile of minced sea bass meat was studied by SPME-GC/MS. The remaining ground meat of each fillet was vacuum-packed immediately and stored at -80ºC for up to 24 h for the subsequent extraction of their lipids and study by ^{1}H NMR.

1.3.3. Fish lipid extraction

Lipids of raw and cooked sea bass samples were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA), following the same methodology described in subsection 1.2.3. of the experimental design of Objective 1.2. The proportion of lipids in raw and cooked sea bass was calculated and expressed as g per 100 g of wet weight.

1.3.4. ^{1}H NMR spectra acquisition, standards and derived data

The ^{1}H NMR spectra of the lipid extracts of raw ($\mathbf{F_R}$, $\mathbf{W_R}$) and cooked farmed ($\mathbf{F_{SC}}$, $\mathbf{F_{CB}}$, $\mathbf{F_{MW}}$) and wild ($\mathbf{W_{SC}}$, $\mathbf{W_{CB}}$, $\mathbf{W_{MW}}$) sea bass samples were recorded in duplicate on a Bruker Avance 400 spectrometer operating at 400 MHz. The sample preparation methodology and the acquisition parameters were the same as those described in subsection 1.1.5. of the experimental design of Objective 1.1.

For identification purposes, the ^{1}H NMR spectrum of the following standard compounds: cholesterol, cholesterol-5β,6β-epoxide, 5α-cholestan-3β-ol, phosphatidylcholine, retinyl palmitate, retinyl acetate, retinol, α-tocopherol, and squalene acquired from Sigma Aldrich; cholestadien-5β,6β-epoxy-3β-ol and 5-cholesten-3β,7β-diol acquired from Steraloids (Newport, RI, USA); and cholesterol-5α,6α-epoxide acquired from Cymit Química S.L. (Barcelona, Spain) were recorded.

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, the area of some spectral signals were used to estimate the molar proportion of the several kinds of acyl groups and the concentration of some minor components in relation to triglycerides present in the lipid extracts of raw and cooked samples. The equations employed were the same as those detailed in subsection 1.2.4. of the experimental design of Objective 1.2.

1.3.5. Study by SPME-GC/MS of the headspace composition of raw and cooked sea bass meat

The methodology followed for the study by SPME-GC/MS of the headspace composition of raw and cooked sea bass meat is the same as that described in subsection 1.2.5. of the experimental design of Objective 1.2.

1.3.6. Statistical analysis

The significance of the differences on the molar percentages of the several kinds of acyl groups and on the concentration of the several minor lipidic components estimated from ^{1}H NMR data among the lipid extracts of raw and cooked sea bass fillets were determined by one-way variance analysis (ANOVA) followed by Tukey b test at 0.05 threshold, using the Statistical package SPSS (v.22, IBM, NY, USA). Principal component analysis (PCA) performed on the average abundances of volatiles identified in duplicate by SPME-GC/MS and obtained from two cooking experiments was carried out by using SIMCA software (v.13.0.3 for Windows, Umetrics/MKS, Umeå, Sweden).

AIM 2

In vitro **gastrointestinal digestion of lipids**

Development and validation of a new method based on ^{1}H NMR for the study of lipid hydrolysis during digestion

Study in depth of the influence of several factors affecting the extent of *in vitro* lipolysis

(MANUSCRIPTS 4-6)

OBJECTIVE 2.1.

To develop and validate a new methodology based on ^{1}H NMR spectral data to evaluate the hydrolysis level in complex lipid mixtures (quantification of triglycerides, 1,3- and 1,2-diglycerides, 2- and 1-monoglycerides and fatty acids)

(**Manuscript 4**)

2.1.1. Standards and mixtures

For the development of the approach, standard compounds, such as tridocosahexaenoin, trieicosapentaenoin, trilinolenin, trilinolein and triolein (Larodan AB, Malmö, Sweden), as well as triestearin, 1,2-diolein, 1,3-dilinolein, 1-monolinolein, 2-monoolein, docosahexaenoic acid, eicosapentaenoic acid, linoleic acid and oleic acid (Sigma Aldrich, St. Louis, MO, USA) were used.

For the validation of the approach, different mixtures (**Mx**) of the above-mentioned standard compounds were prepared. The composition of these mixtures, named **Mx1** to **Mx10**, is given in Objective 2.1.-**Table 1**. They differ widely in the molar percentage of glycerides and fatty acids, in order to cover a broad range of potential hydrolysis levels. It has to be noted that mixtures **Mx1** and **Mx2** only contain triglycerides and that mixtures **Mx3** to **Mx10** are much more complex, also containing di-, mono-glycerides and fatty acids. Mixtures from **Mx3** to **Mx5** contain typical glycerides and fatty acids present in oils and fats of vegetable and terrestrial animal origin, whereas mixtures **Mx6** to **Mx10** contain glycerides and fatty acids present in marine lipids, which include typical polyunsaturated ω-3 acyl groups of fish lipids like docosahexaenoate and eicosapentaenoate.

Objective 2.1.-**Table 1**. Molar percentages (%) of the different standard compounds in the several mixtures (**Mx1-10**) prepared by weight.

Standard compounds	**Mixtures**									
(%)	**Mx1**	**Mx2**	**Mx3**	**Mx4**	**Mx5**	**Mx6**	**Mx7**	**Mx8**	**Mx9**	**Mx10**
TG (DHA)	-	6.0	-	-	-	7.2	5.4	-	-	-
TG (EPA)	-	7.9	-	-	-	-	-	5.2	-	-
TG (Ln)	34.4	18.8	-	-	-	21.9	-	-	5.1	-
TG (L)	33.6	50.7	24.6	-	-	21.5	16.6	-	-	-
TG (O)	12.8	16.6	21.6	6.1	-	8.2	14.6	3.9	-	-
TG (S)	19.2	-	-	-	-	12.2	-	-	-	-
Total TG	**100.0**	**100.0**	**46.2**	**6.1**	-	**71.0**	**36.6**	**9.1**	**5.1**	-
1,2-DG (O)	-	-	6.5	19.1	7.1	-	4.4	12.3	5.9	6.2
1,3-DG (L)	-	-	-	-	13.0	-	-	-	10.9	11.5
Total DG	-	-	**6.5**	**19.1**	**20.1**	-	**4.4**	**12.3**	**16.8**	**17.7**
1-MG (L)	-	-	-	30.2	21.6	-	-	19.4	18.0	19.0
2-MG (O)	-	-	4.0	-	-	-	2.7	-	-	-
Total MG	-	-	**4.0**	**30.2**	**21.6**	-	**2.7**	**19.4**	**18.0**	**19.0**
FA (DHA)	-	-	-	-	-	-	27.1	-	-	-
FA (EPA)	-	-	-	-	-	29.0	-	30.6	11.4	12.0
FA (L)	-	-	43.3	-	30.8	-	29.2	-	25.7	27.1
FA (O)	-	-	-	44.6	27.5	-	-	28.6	23.0	24.2
Total FA	-	-	**43.3**	**44.6**	**58.3**	**29.0**	**56.3**	**59.2**	**60.1**	**63.3**

Abbreviations: 1-MG: 1-monoglyceride; 2-MG: 2-monoglyceride; 1,2-DG: 1,2-diglyceride; 1,3-DG: 1,3-diglyceride; DHA: docosahexaenoate (C22:6ω-3); EPA: eicosapentaenoate (C20:5ω-3); FA: fatty acid; L: linoleate (C18:2ω-6); Ln: linolenate (C18:3ω-3); O: oleate (C18:1ω-9); S: stearate (C18:0); TG: triglyceride.

2.1.2. ^{1}H NMR spectra acquisition

Pure standard compounds and all the above-mentioned mixtures (200 mg) were dissolved in 400 µl of deuterated chloroform, which contains tetramethylsilane (TMS) as internal reference (Cortec, Paris, France). The ^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz. The acquisition conditions were the same as those described in subsection 1.1.5. of the experimental design of Objective 1.1.

^{1}H NMR spectral signal areas of the different kinds of protons are proportional to the number of protons that generate them, and the proportionality constant is the same in all cases. This allows one to carry out quantitative determinations.

OBJECTIVE 2.2.

To demonstrate the usefulness of ^{1}H NMR when studying the extent of lipid hydrolysis reached during digestion of food samples (sunflower oil/minced fish meat), determining the hydrolysis level, the degree of triglyceride transformation, the lipid bioaccessibility level and the percentage of fatty acids physiologically releasable

(**Manuscript 5**)

2.2.1. Samples

The usefulness of ^{1}H NMR to assess the extent of lipid digestion was tested in the *in vitro* digestion of two foods, considered as model foods, namely sunflower oil (**S**) and European sea bass (**F**). Sunflower oil was selected as representative of fats and oils coming from vegetables and terrestrial animals, and sea bass lipids of those coming from marine origins. It has to be noted that their lipid composition comprise different level of complexity. Sunflower oil is mainly made up of triglycerides supporting linoleic, saturated and oleic acyl groups. However, sea bass lipids are much more complex, including highly-polyunsaturated ω-3 acyl groups in addition to the above-mentioned acyl groups. Moreover, sunflower oil and fish are two very different matrices; the former consists exclusively of lipids whereas the latter also contains water, proteins and other minor components.

These foods were purchased from a local supermarket. Before *in vitro* digestion experiments, fish was gutted, cleaned, filleted, skinned and grinded. Sunflower oil and minced fish flesh were submitted to *in vitro* digestion under different experimental conditions in order to obtain samples having different lipid hydrolysis levels. In this way, three different samples were studied from each food: the unlipolyzed samples (**SUL**, **FUL**), that is samples before being submitted to digestion; partially lipolyzed samples (**SPL**, **FPL**); and totally lipolyzed samples (**STL**, **FTL**).

2.2.2. *In vitro* gastrointestinal digestion

Digestion experiments were carried out following the *in vitro* digestion model described by Versantvoort et al. (2004, 2005), already employed in some previous studies (Goicoechea et al., 2008, 2011). This model implies a three-step procedure which simulates digestive processes in the mouth, stomach and small intestine by adding sequentially the corresponding digestive juices. The transit times employed for oral, gastric and duodenal *in vitro* digestion were 5 min, 2 h and 4 h, respectively.

Digestive juices (saliva, gastric juice, duodenal and bile juice) were prepared artificially in accordance with Versantvoort et al. (2005), but some slight modifications were carried out in order to obtain samples digested to different degrees of lipolysis. In the sunflower oil *in vitro* digestion, 100 U/mL of lipase from *Aspergillus niger* in the gastric juice, 9.6 g/L of lipase (lipase from porcine pancreas) in the duodenal juice and 60 g/L of bile (bovine bile) in the bile juice were used. The partial lipolyzed **SPL** sample was obtained using 0.5 g of sunflower oil, whereas the totally lipolyzed **STL** sample was obtained using 0.25 g.

As far as fish lipid samples were concerned, the partially lipolyzed **FPL** sample was obtained using the above-mentioned concentrations of enzymes and of bile and 4.5 g of minced fish. The totally lipolyzed **FTL** sample was obtained using the same amount of fish flesh, but on this occasion with 100 U/mL of lipase from *Aspergillus niger* in the gastric juice, a lipase (lipase from porcine pancreas) concentration in the duodenal juice of 1.5 g/L and a bile (bovine bile) concentration in the bile juice of 15 g/L.

All the reagents for the preparation of the digestive juices were acquired from Sigma-Aldrich (St. Louis, MO, USA). Each digestion experiment was carried out in quadruplicate.

2.2.3. Lipid extraction

Lipid extraction was carried out on fish flesh before subjecting it to *in vitro* digestion and also in the digested samples of sunflower oil and minced fish. The lipids from minced fish muscle were extracted using dichloromethane (CH_2Cl_2, HPLC grade, Sigma-Aldrich), following the same methodology described in subsection 1.2.3. of the experimental design of Objective 1.2. The extraction was performed in duplicate and lipid extracts from minced sea bass were named **FUL**. Digested samples were submitted to a liquid-liquid extraction, using the solvent above-mentioned in a proportion of 2:3 (v/v). Afterwards, solvent was eliminated by means of a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. Lipid extracts obtained from *in vitro* digested samples were named **SPL**, **STL**, **FPL** and **FTL**, like the original digested samples.

In all cases, dichloromethane was selected as solvent because of its ability to extract lipids, its high volatility and its suitable polarity. Different extraction conditions and solvents had been previously tested in our laboratory to ensure the exhaustive extraction of all the lipolytic products arising from triglyceride hydrolysis.

2.2.4. ^{1}H NMR spectra acquisition

The ^{1}H NMR spectra of sunflower oil and fish lipids before digestion (**SUL**, *n*=2; **FUL**, *n*=2) and of the corresponding digested lipid extracts (**SPL**, *n*=4; **STL**, *n*=4; **FPL**, *n*=4; **FTL**, *n*=4) were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz. To do this, the sample preparation to acquire the corresponding spectra, the acquisition conditions and the study of the spectral data were the same as those described in subsection 1.1.5. of the experimental design of Objective 1.1.

2.2.5. Equations derived from ^{1}H NMR spectral data used to quantify lipolytic products and lipid digestion extent

As mentioned before, the signal areas in the spectra are proportional to the number of protons that generate them. Given this, the number of moles of the different kinds of molecules present (triglycerides (TG), diglycerides (DG), monoglycerides (MG), fatty acids (FA)) in the lipid samples can be calculated by the following equations, developed and validated in a previous study (see **Manuscript 4**):

$$N_{2\text{-}MG} = Pc*A_K/4 \quad \text{[Objective 2.2.-eq.1]}$$

$$N_{1\text{-}MG} = Pc*A_L \quad \text{[Objective 2.2.-eq.2]}$$

$$N_{1,2\text{-}DG} = Pc*(A_{I+J}-A_L)/2 \quad \text{[Objective 2.2.-eq.3]}$$

$$N_{1,3\text{-}DG} = Pc*(A_{4.04\text{-}4.38}-2*A_{4.26\text{-}4.38}-2*A_L)/5 \quad \text{[Objective 2.2.-eq.4]}$$

$$N_{TG} = Pc*(2*A_{4.26\text{-}4.38}-A_{I+J}+2*A_L)/4 \quad \text{[Objective 2.2.-eq.5]}$$

$$N_{FA} = (Pc*A_{2.26\text{-}2.40}-6*N_{TG}-4*N_{1,2\text{-}DG}-4*N_{1,3\text{-}DG}-2*N_{1\text{-}MG}-2*N_{2\text{-}MG})/2 \quad \text{[Objective 2.2.-eq.6a]}$$

$$N_{FA} = (Pc*10*A_{2.26\text{-}2.37}+Pc*5*A_{2.37\text{-}2.44}-60*N_{TG}-40*N_{1,2\text{-}DG}-40*N_{1,3\text{-}DG}-18*N_{1\text{-}MG}-13*N_{2\text{-}MG})/20 \quad \text{[Objective 2.2.-eq.6b]}$$

where **N** is the number of moles of the corresponding compound, **Pc** is the proportionality constant relating the ^{1}H NMR spectral signal areas and the number of protons that generate them, **A** is the area of the ^{1}H NMR spectral signal involved (see signal assignment in **Table 1** of **Manuscript 5**), and $\mathbf{A_{4.04\text{-}4.38}}$, $\mathbf{A_{4.26\text{-}4.38}}$, $\mathbf{A_{2.26\text{-}2.40}}$, $\mathbf{A_{2.26\text{-}2.37}}$ and $\mathbf{A_{2.37\text{-}2.44}}$ are the areas of the signals ranging from 4.04 to 4.38, 4.26 to 4.38, 2.26 to 2.40, 2.26 to 2.37, and 2.37 to 2.44 ppm respectively. It has to be noted that equation 6a is intended for lipids from vegetable or terrestrial animal origins, and equation 6b for marine lipids.

These equations were applied to the two different methods employed in order to express the quantification of the products generated during lipolysis, as well as to assess the extent of lipid digestion.

OBJECTIVE 2.3.

To investigate the effect of different experimental factors (gastric acidification, intestinal transit time, presence of gastric lipase, sample/digestive fluids ratio, intestinal enzymes concentration and bile concentration) on lipid *in vitro* digestion extent

(**Manuscript 6**)

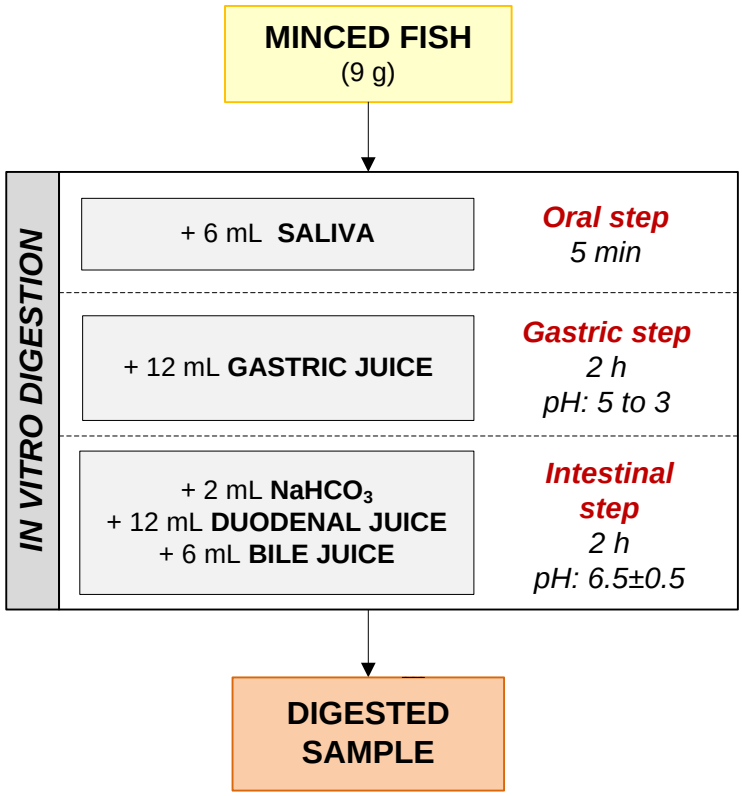
2.3.1. Samples, reagents and enzymes

Farmed European sea bass specimens were purchased from a local supermarket. After cleaning, gutting, filleting and skinning, they were submitted to *in vitro* digestion. The fillets average weight was 252.9±22.0 g and their average lipid content of 8.2±1.0% (ww).

Reagents and enzymes for the preparation of digestive juices were acquired from Sigma-Aldrich (St. Louis, MO, USA): *Aspergillus oryzae* α-amylase (10065); pepsin from porcine gastric mucosa (P7125); lipases from *Aspergillus niger* (534781) and *Candida rugosa* (62316); pancreatin from porcine pancreas (P1750); lipase type II crude from porcine pancreas (L3126) and bovine bile extract (B3883).

2.3.2. *In vitro* gastrointestinal digestion experiments

The starting point for this study was the *in vitro* digestion model developed by Versantvoort et al. (2004, 2005) for fed state (see Objective 2.3.-**Figure 1**). The composition of digestive juices (saliva, gastric, duodenal and bile juice) is given in Objective 2.3.-**Table 1**.



Objective 2.3.-**Figure 1**. Schematic representation of the *in vitro* gastrointestinal digestion model developed by Versantvoort et al. (2004, 2005) used as a starting point in this study.

Just before the *in vitro* digestion experiments, the juices were heated to 37±2ºC. The fish sample was prepared by mincing in a grinder, to simulate mechanical disintegration that occurs in the mouth. The digestion experiment started with the addition of 6 mL of saliva to 9 g of minced sea bass sample. After 5 min of incubation, 12 mL of simulated gastric juice (GJ) were added and the mixture was rotated head-over-heels at 40 rpm for 2 h at 37±2ºC. Thirty minutes after starting the gastric digestion, pH was set between 2 and 3 with HCl (37%), simulating the gradual acidification of the chyme that occurs *in vivo*. After 2 h of gastric digestion, 2 mL of sodium bicarbonate solution (1 M), 12 mL of duodenal juice (DJ) and 6 mL of bile juice (BJ) were added. Subsequently, pH was set between 6 and 7, and the mixture was rotated again at 40 rpm and incubated at 37±2ºC for 2 h.

Objective 2.3.-**Table 1**. Composition of the juices employed in the model described by Versantvoort et al. (2004, 2005) used as a starting point in this study.

Components	**Saliva**	**Gastric Juice (GJ)**	**Duodenal Juice (DJ)**	**Bile Juice (BJ)**
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	-	-
α-amylase (g/L)	0.29	-	-	-
Pepsin (g/L)	-	2.50	-	-
Pancreatin (g/L)	-	-	9.00	-
Pancreatic lipase (g/L)	-	-	1.50	-
Bile (g/L)	-	-	-	30.00
pH	6.8±0.2	1.3±0.2	8.1±0.2	8.2±0.2

The influence of some experimental factors on the fish lipolysis was evaluated. These were: gastric pH, intestinal residence time, presence of lipase in the GJ, sample/digestive fluids ratio, enzymatic composition of the DJ and bile concentration in the BJ. Although each variable can be affected by the others, the influence of each experimental factor on the lipolysis extent was studied sequentially, keeping the rest of the experimental conditions constant but including the selected conditions for the factor previously tested. This selection was made considering the improvement of lipolysis, the reflection of physiological conditions, as well as practical and economical reasons. Each digestion experiment was carried out in triplicate, except for that using a lower amount of sample/digestive fluids ratio that was performed in duplicate (4.5 g of fish meat: 6 mL of saliva: 12 mL of GJ with lipase of *A. niger* added: 12 mL of DJ proposed by Versantvoort: 6 mL of BJ with bile at 30 g/L).

2.3.3. Lipid extraction and ^{1}H NMR spectra acquisition

Lipids contained in minced fish and in digested samples were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA), following the same methodology described in subsection 2.2.3. of the experimental design of Objective 2.2.

2.3.4. Equations derived from ^{1}H NMR spectral data employed for the quantification of the several lipolytic products in the digestates and the extent of lipid digestion

Bearing in mind that the signal areas in the spectra are proportional to the number of protons that generate them and the proportionality constant is the same for all kinds of protons, the number of moles (**N**) of 2-monoglycerides (2-MG), 1-monoglycerides (1-MG), 1,2-diglycerides (1,2-DG), triglycerides (TG), fatty acids (FA) and glycerol (Gol) in each sample can be expressed as follows (see **Manuscript 4**):

$$N_{2\text{-}MG} = Pc*A_K/4 \quad \text{[Objective 2.3.-eq.1]}$$

$$N_{1\text{-}MG} = Pc*A_L \quad \text{[Objective 2.3.-eq.2]}$$

$$N_{1,2\text{-}DG} = (Pc*A_{I+J}-2*N_{1\text{-}MG})/2 \quad \text{[Objective 2.3.-eq.3]}$$

$$N_{TG} = (Pc*A_{N+O+P}-2*N_{1,2\text{-}DG}-2*N_{1\text{-}MG})/4 \quad \text{[Objective 2.3.-eq.4]}$$

$$N_{FA} = (Pc*10*A_{2.26\text{-}2.37}+Pc*5*A_{2.37\text{-}2.44}-60*N_{TG}-40*N_{1,2\text{-}DG}-18*N_{1\text{-}MG}-13*N_{2\text{-}MG})/20 \quad \text{[Objective 2.3.-eq.5]}$$

$$N_{Gol} = (N_{FA}-N_{1,2\text{-}DG}-2*N_{2\text{-}MG}-2*N_{1\text{-}MG})/3 \quad \text{[Objective 2.3.-eq.6]}$$

where **Pc** is the proportionality constant relating the number of protons that generate a signal, **A** is the area of the signal involved (see signal assignment in **Table 2** of **Manuscript 6**) and $\mathbf{A_{2.26–2.37}}$ and $\mathbf{A_{2.37–2.44}}$ are the areas of signals at 2.26–2.37 and 2.37–2.44 ppm respectively.

2.3.5. Statistical Analysis

The significance of the differences on the molar percentages of the different kinds of lipolytic products present in the digestates, and on the hydrolysis level, triglyceride transformation and lipid bioaccessibility were determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p<0.05$, using SPSS v.19 (IBM, NY, USA).

AIM 3

***In vitro* gastrointestinal digestion of oils**

Simultaneous study of lipid hydrolysis, oxidation and other reactions taking place during the *in vitro* digestion of oils of vegetable and animal origins, and of other model systems

Influence on their extent of the oil initial oxidation level, of the oil unsaturation degree, of the presence of proteins and of the synthetic antioxidant BHT

(MANUSCRIPTS 7-11)

OBJECTIVE 3.1.

To tackle by means of ^{1}H NMR and SPME-GC/MS the chemical reactions taking place during *in vitro* gastrointestinal digestion of non-oxidized and slightly oxidized sunflower oils, as models of omega-6 rich lipids

(**Manuscript 7**)

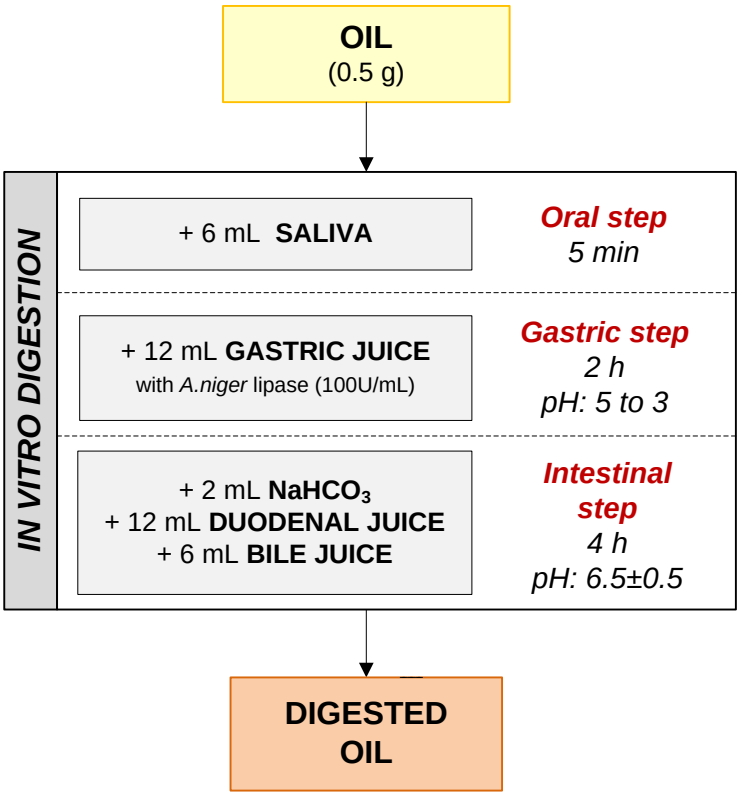
3.1.1. Sunflower oil samples: non-oxidized and slightly oxidized

Sunflower oil (**S**) was acquired in a local supermarket. To obtain the slightly oxidized sunflower oil (**Sx**), 10 g of oil were weighed in crystal Petri dishes and placed in a convection oven (Memmert GmbH+Co, Schwabach, Germany) at 70ºC with circulating air for 4 days; this heating time was selected because at that moment only signals related to primary oxidation compounds (hydroperoxides supported on chains having also conjugated dienes) were observed in their ^{1}H NMR spectra, in agreement with previous studies in which sunflower oil was submitted to similar accelerated storage conditions (Guillén & Ruiz, 2005a).

3.1.2. *In vitro* gastrointestinal digestion

Sunflower oil samples (0.5 g), either slightly oxidized (**Sx**) or not (**S**), were digested following the *in vitro* gastrointestinal model developed by Versantvoort et al. (2004, 2005), which was slightly modified as described in detail in **Manuscript 6**. This model implies a three-step procedure which simulates digestive processes in the mouth, stomach, and small intestine by adding sequentially the corresponding digestive juices (see Objective 3.1.-**Figure 1**). The transit times employed for oral, gastric and duodenal *in vitro* digestion were 5 min, 2 h and 4 h, respectively. Digestive juices (saliva, gastric juice, duodenal and bile juice) were prepared in accordance with the original model, although some modifications were performed in order to reach a higher level of lipolysis: addition of *Aspergillus niger* lipase to the gastric juice at 100 U/mL and use of bovine bile extract at 18.75 g/L instead of 30.00 g/L in the bile juice. All the reagents for the preparation of the digestive juices were acquired from Sigma-Aldrich (St. Louis, MO, USA).

Blank samples, corresponding to the same mixture of digestive juices in the absence of oils, were also subject of study in each experiment. For consistency of results the experiment was carried out in quadruplicate. The digested samples obtained from non-oxidized sunflower oils were named **DS** (n=4), and from the digestion of slightly oxidized samples **DSx** (n=4).



Objective 3.1.-**Figure 1**. Schematic representation of the *in vitro* digestion methodology followed in this study.

3.1.3. Lipid extraction and ^{1}H NMR spectra acquisition

Lipids of the digestates (**DS**, **DSx**) were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich) and following the same methodology as described in subsection 2.2.3. of the experimental design of Objective 2.2. Afterwards, to ensure a complete protonation of fatty acids and/or the dissociation of the potential salts formed between fatty acids and cations, the remaining water phase was acidified to pH≈2 with HCl (37%) and a second extraction was carried out. Both 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. With the aim of evaluating if the performed extraction was complete, the water phase was freeze-dried and later extracted with CH_2Cl_2 for subsequent analysis by ^{1}H NMR spectroscopy.

The ^{1}H NMR spectra of 4 starting oil samples of each kind (**S**, **Sx**) and of the lipid extracts obtained from the 4 digestion experiments (**DS**, **DSx**) were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz; thus, 8 spectra of each kind of sample were obtained. To do this, the sample preparation to acquire the corresponding spectra, the acquisition

conditions and the study of the spectral data were the same as those described in subsection 1.1.5. of the experimental design of Objective 1.1.

3.1.4. Quantification of the lipolytic products generated during *in vitro* digestion from ^{1}H NMR spectral data

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, the area of some spectral signals make it possible to determine the molar percentage of fatty acids (FA) and all glycerides present in the sample, which is to say 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). For this purpose, the number of moles (**N**) of each kind of glyceride structures present in the sample was expressed as follows:

$$N_{2\text{-}MG} = Pc*A_K/4 \quad \text{[Objective 3.1.-eq.1]}$$

$$N_{1\text{-}MG} = Pc*A_L \quad \text{[Objective 3.1.-eq.2]}$$

$$N_{1,2\text{-}DG} = Pc*(A_{I+J}-2A_L)/2 \quad \text{[Objective 3.1.-eq.3]}$$

$$N_{TG} = Pc*(2A_{4.26\text{-}4.38}-A_{I+J}+2A_L)/4 \quad \text{[Objective 3.1.-eq.4]}$$

$$N_{1,3\text{-}DG} = Pc*(A_{4.04\text{-}4.38}-2A_{4.26\text{-}4.38}-2A_L)/5 \quad \text{[Objective 3.1.-eq.5]}$$

where **Pc** is the proportionality constant existing between the area of the ^{1}H NMR signals and the number of protons that generate the signal; $\mathbf{A_K}$, $\mathbf{A_L}$ and $\mathbf{A_{I+J}}$ are the areas of the corresponding signals indicated in **Table 1** and **Figure 1** of **Manuscript 7**; and $\mathbf{A_{4.04\text{-}4.38}}$ and $\mathbf{A_{4.26\text{-}4.38}}$ represent the area of the spectrum signals at 4.04-4.38 ppm and 4.26-4.38 ppm, respectively.

In this study three different quantitative approaches based on ^{1}H NMR data are proposed, which differ in the way of determining number of moles of fatty acids (N_{FA}). In the first approach, named *approach I*, $\mathbf{N_{FA}}$ will be determined using the area (A) of ^{1}H NMR signal F (A_F) corresponding to methylenic protons located in the α-position in relation to the carbonyl group of acyl groups (AG) and to the carboxylic group of FA, as in previous studies (see **Manuscripts 4** and **5**). In *approach II*, the area of signal D (A_D) will be employed to determine $\mathbf{N_{FA}}$ (it is also due to methylenic protons, but in the β-position in relation to the carbonyl group of AG and to the carboxylic group of FA). Finally, in *approach III*, the area of signal A (A_A) due to all methylic protons of FA and AG will be used to determine N_{FA}. Thus, the number of moles of fatty acids and glycerol ($\mathbf{N_{Gol}}$) in the sample can be expressed as follows:

$$N_{FA} = (Pc*A_F-6N_{TG}-4N_{1,2\text{-}DG}-4N_{1,3\text{-}DG}-2N_{1\text{-}MG}-2N_{2\text{-}MG})/2 \quad \text{[Objective 3.1.-eq.6-I]}$$

$N_{FA} = (Pc*A_D-6N_{TG}-4N_{1,2\text{-}DG}-4N_{1,3\text{-}DG}-2N_{1\text{-}MG}-2N_{2\text{-}MG})/2$ [Objective 3.1.-eq.6-II]

$N_{FA} = (Pc*A_A-9N_{TG}-6N_{1,2\text{-}DG}-6N_{1,3\text{-}DG}-3N_{1\text{-}MG}-3N_{2\text{-}MG})/3$ [Objective 3.1.-eq.6-III]

$N_{Gol} = (N_{FA}-N_{1,2\text{-}DG}-N_{1,3\text{-}DG}-2N_{2\text{-}MG}-2N_{1\text{-}MG})/3$ [Objective 3.1.-eq.7]

In this way, the total number of moles of glyceride structures (**NT_{GS}**) and the total number of moles of acyl groups plus fatty acids (**NT_{AG+FA}**) present in the samples can be determined as follows, depending on the approach used (I, II or III):

$NT_{GS} = Pc*A_F/6$ [Objective 3.1.-eq.8-I]

$NT_{GS} = Pc*A_D/6$ [Objective 3.1.-eq.8-II]

$NT_{GS} = Pc*A_A/9$ [Objective 3.1.-eq.8-III]

$NT_{AG+FA} = Pc*A_F/2$ [Objective 3.1.-eq.9-I]

$NT_{AG+FA} = Pc*A_D/2$ [Objective 3.1.-eq.9-II]

$NT_{AG+FA} = Pc*A_A/3$ [Objective 3.1.-eq.9-III]

Using these equations the molar percentages of the different kinds of glyceryl structures in relation to NT_{GS} (**TG%**, **1,2-DG%**, **1,3-DG%**, **2-MG%**, **1-MG%** and **Gol%**) and the molar percentages of acyl groups present in the different glycerides (AG) and of fatty acids (FA) in relation to NT_{AG+FA} present (**FA%**, **AG_{TG}%**, **$AG_{1,2\text{-}DG}$%**, **$AG_{1,3\text{-}DG}$%**, **$AG_{2\text{-}MG}$%** and **$AG_{1\text{-}MG}$%**), can be determined, as in previous studies (see **Manuscripts 5** and **6**).

3.1.5. Quantification of the molar percentages of the several kinds of acyl groups and fatty acids present in the lipid samples from ^{1}H NMR spectral data

In order to investigate if changes measurable by ^{1}H NMR in the lipid composition of the oil samples occur due to oxidation or other reactions during *in vitro* digestion, the molar percentage of the several kinds of AG or FA in relation to NT_{AG+FA} was also determined using certain spectral signals. As far as we know this is the first time that equations based on ^{1}H NMR spectra are proposed to do these determinations in hydrolyzed lipidic samples. Thus, the molar percentage of total unsaturated AG or FA (**U%**), linoleic AG or FA (**L%**), oleic AG or FA (**O%**), and of saturated plus modified AG or FA (**S+M%**) were calculated as follows:

$U\% = 100*(Pc*A_E/4)/NT_{AG+FA}$ [Objective 3.1.-eq.10]

$L\% = 100*(Pc*A_G/2)/NT_{AG+FA}$ [Objective 3.1.-eq.11]

$O\% = U\%-L\%$ [Objective 3.1.-eq.12]

$(S+M)\% = 100-U\%$ [Objective 3.1.-eq.13]

It must be pointed out that modified AG or FA are those unsaturated chains (linoleic, oleic) that have been modified as a result of oxidation reactions, losing their original typical structure. The molar percentage of these modified chains together that of saturated ones are grouped as (S+M)%.

3.1.6. Quantification of the oxidation compounds present in the lipid samples from ^{1}H NMR spectral data

The concentration of *(Z,E)* and *(E,E)* isomers of conjugated dienes (**CD**) supported in chains having also a hydroperoxy (**OOH**) or a hydroxy (**OH**) group, present either in the oil samples (**S**, **Sx**) or in their corresponding digested lipid extracts (**DS**, **DSx**), can be expressed as mmol/mol of AG plus FA present (mmol/molAG+FA) by these equations:

$$\textit{(E,E)}\text{-CD-OH (mmol/molAG+FA)} = 1000*(Pc*A_{6.18})/NT_{AG+FA} \quad \text{[Objective 3.1.-eq.14]}$$

$$\textit{(E,E)}\text{-CD-OOH (mmol/molAG+FA)} = 1000*(Pc*A_{6.27})/NT_{AG+FA} \quad \text{[Objective 3.1.-eq.15]}$$

$$\textit{(Z,E)}\text{-CD-OH (mmol/molAG+FA)} = 1000*(Pc*A_{6.45})/NT_{AG+FA} \quad \text{[Objective 3.1.-eq.16]}$$

$$\textit{(Z,E)}\text{-CD-OOH (mmol/molAG+FA)} = 1000*(Pc*A_{6.58})/NT_{AG+FA} \quad \text{[Objective 3.1.-eq.17]}$$

where $\mathbf{A_{6.18}}$ is the area of signal d at 6.18 ppm corresponding to one proton of the *(E,E)*-conjugated double bond supported on chains having also a hydroxy group, $\mathbf{A_{6.27}}$ is the area of signal b at 6.27 ppm due to one proton of the *(E,E)*-conjugated double bond supported in chains having also a hydroperoxy group, $\mathbf{A_{6.45}}$ is the area of signal a at 6.45 ppm corresponding to one proton of the *(Z,E)*-conjugated double bond supported on chains having also a hydroxy group, $\mathbf{A_{6.58}}$ is the area of signal c at 6.58 ppm due to one proton of the *(Z,E)*-conjugated double bond supported in chains having also a hydroperoxy group, and $\mathbf{NT_{AG+FA}}$ can be obtained using the equations 9-I, 9-II or 9-III.

3.1.7. Study by SPME-GC/MS of the headspace composition

The methodology employed for the study by SPME-GC/MS of the volatile components of the headspace of the samples subject of study (0.5 g in 10 mL screw-cap vial) is the same as that described in subsection 1.2.5. of the experimental design of Objective 1.2.

The samples, all of them studied in duplicate, were the following: the starting oils (**S**, **Sx**); the corresponding digestates (**DS**, **DSx**); the juices submitted to digestion conditions in the absence of oil (**J**); and the mixtures **S+J** and **Sx+J** made of the starting oils and juices submitted to digestion conditions in absence of oils, having the same oil:juices proportions as in the digestates (DS, DSx). The S+J and Sx+J mixtures were studied for comparative purposes, in order to simulate, to a certain extent, a similar a matrix to that of digestates. As 4 digestion experiments were carried out, altogether the headspaces of 8 samples of each kind were studied.

3.1.8. Statistical analysis

The significance of the differences on the molar percentages of several kinds of AG/FA, on the molar percentages of the different kinds of lipolytic products, and on the concentration of the several oxidation compounds, were determined either among the three different approaches for quantification by one-way variance analysis (ANOVA) followed by Tukey b test, or between two specific samples by t-student test at $p<0.05$, using SPSS v.22 software (IBM, NY, USA).

OBJECTIVE 3.2.

To investigate by means of ^{1}H NMR and SPME-GC/MS the chemical reactions taking place during *in vitro* gastrointestinal digestion of non-oxidized and slightly oxidized flaxseed oils, as models of omega-3 rich lipids

(**Manuscript 8**)

3.2.1. Fresh and oxidized flaxseed oil samples

This study was carried out using fresh virgin flaxseed oil (**F**), acquired in a local supermarket, and slightly oxidized flaxseed oil (**Fx**). In order to obtain the oxidized samples (**Fx**), some aliquots of flaxseed oil were submitted to accelerated storage conditions: 10 g of oil were weighed in crystal Petri dishes and placed in a convection oven (Memmert GmbH+Co, Schwabach, Germany) at 70ºC with circulating air for 36 h. This heating time was selected in order for the flaxseed oil samples to reach the first stages of oxidation, in other words when their ^{1}H NMR spectra show signals related to mainly primary oxidation compounds, in accordance with previous studies in which flaxseed oil was submitted to the same accelerated storage conditions (Guillén & Ruiz, 2005b).

3.2.2. *In vitro* gastrointestinal digestion

Flaxseed oil samples (0.5 g), either non-oxidized or slightly oxidized, were digested as previously described in subsection 3.1.2. of the experimental design of Objective 3.1.

Blank samples, corresponding to the mixture of juices submitted to digestive conditions in the absence of oil sample (**J**), were also undertaken in each experiment for further analysis. For consistency of results the experiment was carried out in quadruplicate. The digested samples obtained from non-oxidized (fresh) flaxseed oils were named **DF** (*n*=4), and from the digestion of slightly oxidized samples **DFx** (*n*=4).

3.2.3. Lipid extraction and ^{1}H NMR spectra acquisition

Lipids of the digestates (**DF**, **DFx**) were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA) and following the same methodology as described in subsection 3.1.3. of the experimental design of Objective 3.1.

The ^{1}H NMR spectra of the starting oils and of the corresponding digested lipid extracts were acquired using a Bruker Avance 400 spectrometer operating at 400 MHz. To do this, the sample preparation to acquire the corresponding spectra, the acquisition conditions and the study of the

spectral data were the same as those described in subsection 1.1.5. of the experimental design of Objective 1.1.

3.2.4. Quantification from ^{1}H NMR spectral data of lipolytic products, lipid composition, and some oxidation compounds present in the starting oil samples and 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, it is possible from the area of some spectral signals to determine the molar percentage or the concentration of several components present not only in the starting oil samples, but also in the lipid extracts of the digestates.

Lipolytic compounds. The molar percentages of the different kinds of glycerides, this is triglycerides (**TG%**), diglycerides (**1,2-DG%**, **1,3-DG%**), monoglycerides (**2-MG%**, **1-MG%**) and glycerol (**Gol%**), in relation to the total number of glyceryl structures present in the samples were determined using the equations developed, validated and explained in detail in previous studies (see **Manuscripts 4** and **5**).

Lipid composition. The various kinds of acyl groups (AG) and fatty acids (FA) of flaxseed oil samples and those of the corresponding digested lipid extracts were also studied quantitatively by ^{1}H NMR. So the molar percentage of total unsaturated AG/FA (**U%**), linolenic AG/FA (**Ln%**), linoleic AG/FA (**L%**), oleic AG/FA (**O%**), and of saturated plus modified AG/FA (**S+M%**) in relation to the total number of moles of AG plus FA present ($\mathbf{NT_{AG+FA}}$) were calculated as follows:

$$NT_{AG+FA} = Pc*A_F/2 \quad \text{[Objective 3.2.-eq.1]}$$

$$U\% = 100*(Pc*A_E/4)/NT_{AG+FA} \quad \text{[Objective 3.2.-eq.2]}$$

$$Ln\% = 100*(Pc*A_H/4)/NT_{AG+FA} \quad \text{[Objective 3.2.-eq.3]}$$

$$L\% = 100*(Pc*A_G/2)/NT_{AG+FA} \quad \text{[Objective 3.2.-eq.4]}$$

$$O\% = U\%-L\%-Ln\% \quad \text{[Objective 3.2.-eq.5]}$$

$$(S+M)\% = 100-U\% \quad \text{[Objective 3.2.-eq.6]}$$

where $\mathbf{A_F}$, $\mathbf{A_E}$, $\mathbf{A_H}$ and $\mathbf{A_G}$ are the areas of signals F, E, H and G indicated in **Table 1** and **Figure 1** of **Manuscript 8**. It must be noted that due to partial overlapping of signals H and G, a previous correction of both areas must be undertaken to properly assess the area corresponding to each of them. This correction was performed by using trilinolein as standard compound (Sigma-Aldrich).

Oxidation compounds. The concentration of *(Z,E)*-conjugated dienic systems associated with hydroperoxy group in octadeca-di/tri-enoates (***(Z,E)*-CD-OOH**) and that of monoepoxides present in the starting oil samples (**F**, **Fx**) and in the digested lipid extracts (**DF**, **DFx**), if any, were estimated as mmol/mol of AG plus FA present in the samples (mmol/molAG+FA), using the following equation:

(Z,E)-CD-OOH (mmol/molAG+FA) = $1000*(Pc*A_c)/NT_{AG+FA}$ [Objective 3.2.-eq.7]

Monoepoxides (mmol/molAG+FA) = $1000*[Pc*(A_e)/2]/NT_{AG+FA}$ [Objective 3.2.-eq.8]

where $\mathbf{A_c}$ is the area of signal c at 6.58 ppm, and $\mathbf{A_e}$ is the area of signal e at 2.94 ppm (see **Table 1** and **Figure 2** of **Manuscript 8**), after subtracting the overlapped area corresponding to the side band of bis-allylic protons signals G and H (see intact side band at 2.65 ppm in **Figure 2** of **Manuscript 8**).

3.2.5. Study by SPME-GC/MS of the headspace composition

The methodology employed for the study by SPME-GC/MS of the volatile components of the headspace of the samples subject of study (0.5 g in 10 mL screw-cap vial) is the same as that described in subsection 1.2.5. of the experimental design of Objective 1.2. These samples were the following: non-oxidized and slightly oxidized flaxseed oil samples (**F**, **Fx**), their corresponding digestates (**DF**, **DFx**), the juices submitted to digestion conditions in the absence of food (**J**), and mixtures made up of starting oil samples and juices submitted to digestion conditions in the same proportions as in the digestates (**F+J**, **Fx+J**). These latter mixtures were studied to simulate a matrix in the non-digested samples that was similar to that obtained after digestion, and thus comparable.

3.2.6. Statistical analysis

The significance of the differences on the molar percentages of the different kinds of lipolytic products and on the several kinds of AG/FA present in non-digested and digested samples were determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p<0.05$, using SPSS v.22 software (IBM, NY, USA).

OBJECTIVE 3.3.

To study by means of ^{1}H NMR and SPME-GC/MS the potential effect of protein (ovalbumin/soy protein isolate) on the chemical reactions taking place during *in vitro* gastrointestinal digestion of slightly oxidized sunflower and flaxseed oils

(**Manuscript 9**)

3.3.1. Samples involved in the study

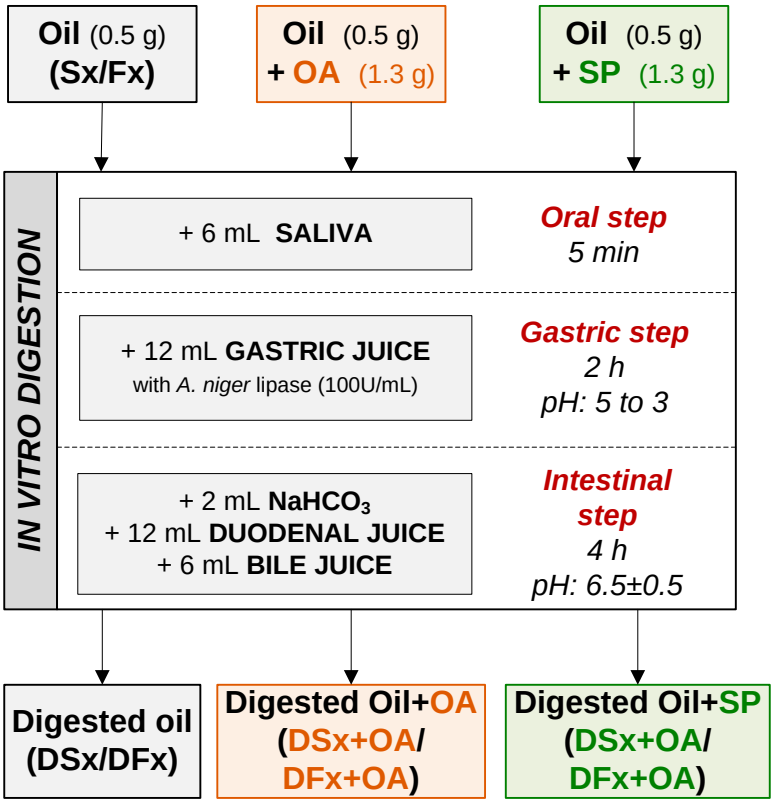
Sunflower oil and flaxseed oil were acquired in a local supermarket. Their acyl groups composition, determined by ^{1}H NMR as indicated in subsection 3.3.5. of the experimental design of this Objective 3.3., was the following: sunflower oil contained 50.5±0.5% linoleic, 38.7±0.3% oleic and 10.8±0.1% saturated acyl groups; and flaxseed oil was composed of 49.4±0.2% linolenic, 22.0±0.1% oleic, 20.9±0.3% linoleic and 7.8±0.3% saturated acyl groups. In order to obtain the slightly oxidized sunflower (**Sx**) and flaxseed oil (**Fx**) samples, 10 g of each oil were weighed in crystal Petri dishes and placed in a convection oven (Memmert GmbH+Co, Schwabach, Germany) at 70ºC with circulating air for 4 (**Sx**) and 1.5 days (**Fx**), in accordance with previous studies in which both oils were submitted to the same oxidative conditions (Guillén & Ruiz, 2005ab).

The proteins subject of study were ovalbumin (**OA**) and soy protein isolate (**SP**) acquired from a protein manufacturer (Apasa SA, Astigarraga, Spain). Both proteins were selected because they are widely employed as ingredients in many food formulations.

3.3.2. *In vitro* gastrointestinal digestion

Oil samples (0.5 g) were *in vitro* digested in the absence (**DSx**, **DFx**) or in the presence of protein (1.3 g), either ovalbumin (**DSx+OA**, **DFx+OA**) or soy protein isolate (**DSx+SP**, **DFx+SP**), following the same *in vitro* gastrointestinal model described in subsection 3.1.2. of the experimental design of Objective 3.1. (see Objective 3.3.-**Figure 1**).

The amounts of oil and protein in the sample for digestion (0.5 and 1.3 g respectively) were selected because they correspond approximately to those present in 4.5 g of fish containing around 11% lipids and 29% proteins (w/w); these latter are the typical proportions present in European sea bass, a fish species widely studied before by our research group (see **Manuscripts 5** and **6**).



Objective 3.3.-**Figure 1**. Schematic representation of the *in vitro* digestion methodology followed in this study.

For consistency of results, the digestion experiments of the oils and of the oil+protein systems were carried out in triplicate (*n*=3). In each digestion experiment, blank samples corresponding to the mixture of juices submitted to digestive conditions in the absence of food (**J**, *n*=3) were also taken for further analysis.

3.3.3. Lipid extraction of the digestates

Lipids of the digestates were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich, St. Louis, MO, USA) and following the same methodology as described in subsection 3.1.3. of the experimental design of Objective 3.1.

3.3.4. ^{1}H NMR spectra acquisition

The ^{1}H NMR spectra of the starting oil samples (**Sx**, **Fx**) and of the lipid extracts of digested oils (**DSx**, **DFx**) and digested oil+protein systems (**DSx+OA**, **DFx+OA**, **DSx+SP**, **DFx+SP**) were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz; thus, from each kind of sample 6 spectra were obtained. To do this, the sample preparation to acquire the corresponding

spectra, the acquisition conditions and the study of the spectral data were the same as those described in subsection 1.1.5. of the experimental design of Objective 1.1.

3.3.5. Quantification from ^{1}H NMR spectral data of lipolytic products, of the several kinds of acyl groups and fatty acids, and of the oxidation compounds present in the starting samples and 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 i) the molar proportions of the different glycerides; ii) three typical parameters used to describe the extent of lipolysis; iii) the molar proportions of the various kinds of acyl groups/fatty acids; and iv) the concentration of several oxidation compounds present in the starting oils and in the lipid extracts of the digestates.

***Concerning the various types of glycerides present*.** The molar percentages of triglycerides (**TG%**), diglycerides (**1,2-DG%**, **1,3-DG%**), monoglycerides (**2-MG%**, **1-MG%**) and glycerol (**Gol%**) in relation to the total number of glyceryl structures present in the lipid samples were determined using the equations developed, validated and explained in detail in **Manuscripts 4** and **5**.

Concerning certain parameters used to describe the extent of lipolysis. To date, three parameters have been widely used in digestion studies to describe the extent of lipid hydrolysis. The first one, named Hydrolysis level ($\mathbf{H_L}$), is defined as the percentage of fatty acids (FA) released in relation to the total number of moles of acyl groups (AG) plus FA present; the second one, called degree of Transformation of TG ($\mathbf{T_{TG}}$), only considers the proportion of TG that have undergone hydrolysis of the ester bond of at least one AG in relation to the intact TG initially present; and the third one, named Lipid bioaccessiblity ($\mathbf{L_{BA}}$), is a more physiological approach that takes into account the proportion of MG and FA (which are the absorbable molecules) in relation to the total number of AG+FA present in the sample. These three parameters were calculated by using equations developed before in **Manuscript 5**.

***Concerning lipid composition*.** The molar percentages of total unsaturated AG or FA (**U%**), linolenic AG or FA (**Ln%**), linoleic AG or FA (**L%**), oleic AG or FA (**O%**), and saturated plus modified AG or FA (**S+M%**), in relation to the total number of moles of AG+FA present in the starting oils and digested lipid extracts, were estimated as previously described in subsection 3.1.5. of the experimental design of Objective 3.1. and in subsection 3.2.4. of the experimental design of Objective 3.2.

Concerning oxidation compounds. The concentration of *(Z,E)*- and *(E,E)*-conjugated dienic systems supported in chains having also hydroperoxy (**CD-OOH**) or hydroxy groups (**CD-OH**), of monoepoxides and of alkanals, expressed as mmol/mol of AG plus FA present, was also estimated as previously described in subsection 3.1.6. of the experimental design of Objective 3.1. and in subsection 3.2.4. of the experimental design of Objective 3.2.

3.3.6. Study by SPME-GC/MS of the headspace composition of the digestates

The methodology employed for the study by SPME-GC/MS of the volatile components of the headspace of the samples subject of study (0.5 g in 10 mL screw-cap vial) is the same as that described in subsection 1.2.5. of the experimental design of Objective 1.2. The samples, all of them analyzed in duplicate, were the following: slightly oxidized oil samples (**Sx**, **Fx**), their corresponding digestates obtained in the 3 digestion experiments carried out either without (**DSx**, **DFx**) or with ovalbumin (**DSx+OA**, **DFx+OA**) or soy protein isolate (**DSx+SP**, **DFx+SP**), and the juices submitted to digestion conditions in the absence of food (**J**). In addition, to gain knowledge about the possible origin of volatile compounds, both proteins were also submitted separately to the same digestion conditions in the absence of oils (**DOA**, **DSP**).

3.3.7. Statistical Analysis

The significance of the differences on the several determinations made among the samples were determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p<0.05$, using SPSS software v.22 (IBM, NY, USA).

OBJECTIVE 3.4.

To review the available literature on the synthetic antioxidant 2,6-di-*tert*-butylhydroxytoluene (BHT) and its metabolites

(**Manuscript 10**)

This Manuscript 10 is a review of the current state-of-the-art of the occurrence and origin of BHT, of its possible dual role as antioxidant/pro-oxidant, of its fate in foodstuffs, of its transformation into metabolites, of its toxicological implications, of the dietary BHT exposure studies and established limits, of the additional sources of exposure, and of the analytical determination of BHT and its derived metabolites in foods. For this purpose, the experimental design involved:

- Bibliographic research
- Analysis and critic study of related works
- Description of the current knowledge on BHT and its derived metabolites in foods

OBJECTIVE 3.5.

To investigate by means of ^{1}H NMR and SPME-GC/MS the hydrolysis and oxidation reactions affecting polyunsaturated acyl groups and naturally present vitamin A during *in vitro* gastrointestinal digestion of cod liver oil, and the potential effect of low and high concentrations of added BHT (20/800 ppm) on the above-mentioned reactions

(**Manuscript 11**)

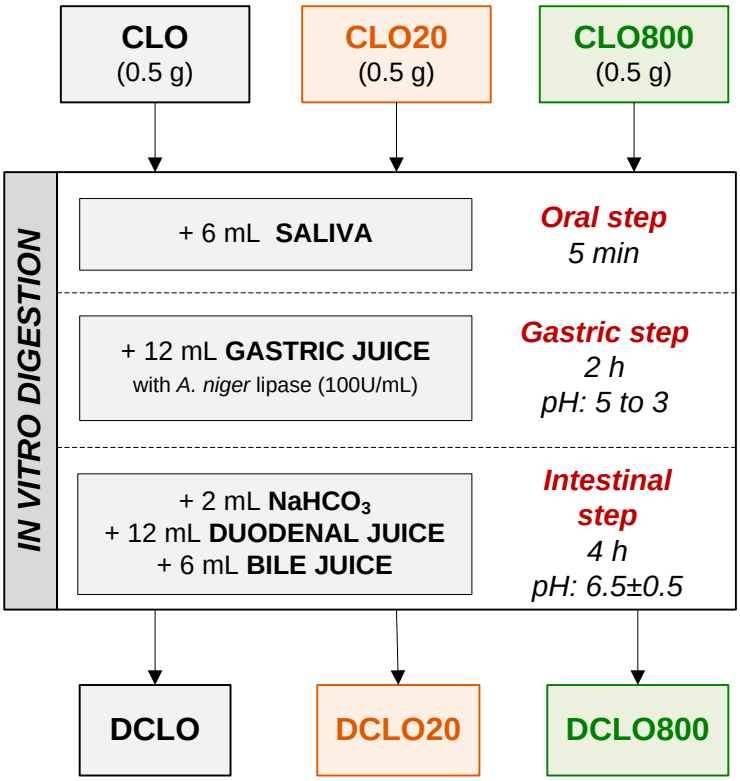
3.5.1. Cod liver oil samples

Samples subject of study were commercial cod (*Gadus morhua*) liver oil, intended for human consumption and acquired in a pharmacist's, either non-enriched (**CLO**) or enriched with the synthetic antioxidant 2,6-di-*tert*-butyl-hydroxytoluene (BHT) (Sigma-Aldrich, St. Louis, MO, USA) at 20 ppm (**CLO20**) and at 800 ppm (**CLO800**).

3.5.2. *In vitro* gastrointestinal digestion experiments

Oil samples (0.5 g), enriched (**CLO20**, **CLO800**) or not (**CLO**) with BHT, were digested were digested as previously described in subsection 3.1.2. of the experimental design of Objective 3.1. (see Objective 3.5.-**Figure 1**).

For consistency of results, the *in vitro* digestion was carried out in quadruplicate. The digested samples obtained from BHT-free cod liver oil were named **DCLO** (n=4), and those obtained from cod liver oil enriched with BHT at 20 ppm were named **DCLO20** (n=4) and those at 800 ppm, **DCLO800** (n=4). Blank samples, corresponding to the mixture of juices submitted to *in vitro* digestion conditions in the absence of oil (**J**) were also undertaken in each experiment for further analysis (n=4).



Objective 3.5.-**Figure 1**. Schematic representation of the *in vitro* digestion methodology followed in this study.

3.5.3. Lipid extraction, ^{1}H NMR spectra acquisition and standards

Lipids of the digestates (**DCLO**, **DCLO20**, **DCLO800**) were extracted using dichloromethane as solvent (CH_2Cl_2, HPLC grade, Sigma-Aldrich) and following the same methodology as described in subsection 3.1.3. of the experimental design of Objective 3.1.

The ^{1}H NMR spectra of the starting oils (**CLO**, **CLO20**, **CLO800**) and of the lipid extracts of the digestates (**DCLO**, **DCLO20**, **DCLO800**) were acquired in duplicate using a Bruker Avance 400 spectrometer operating at 400 MHz; thus, 8 spectra of each kind of sample were obtained. To do this, the sample preparation to acquire the corresponding spectra, the acquisition conditions and the study of the spectral data were the same as those described in subsection 1.1.5. of the experimental design of Objective 1.1.

Compounds, such as retinyl palmitate, retinyl acetate, retinol and 2,6-di-*tert*-butylhydroxytoluene (BHT), acquired from Sigma Aldrich, together with 4-hydroxy-*(E)*-2-nonenal, 4-hydroxy-*(E)*-2-hexenal and 4-hydroperoxy-*(E)*-2-nonenal, acquired from Cayman Chemical (Ann Arbor, MI, USA), were used as standard compounds for identification purposes in ^{1}H NMR spectra.

3.5.4. Quantification from ^{1}H NMR spectral data of lipolytic products, of the several kinds of acyl groups and fatty acids, of vitamin A, of some of their oxidation compounds and of BHT, present in the starting oils and in the corresponding 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 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 lipolytic products and of the several kinds of acyl groups and fatty acids, as well as the concentration of retinyl esters (vitamin A), of some oxidation compounds and of BHT, present in the starting oils and in the lipid extracts of the digestates.

***Concerning the various types of glycerides present*.** The molar percentages of triglycerides (**TG%**), 1,2- and 1,3-diglycerides (**1,2-DG%**, **1,3-DG%**), 2- and 1-monoglycerides (**2-MG%**, **1-MG%**) and glycerol (**Gol%**) in relation to the total number of glyceryl structures present in the samples were determined based on the equations developed, validated and explained in detail in **Manuscripts 4** and **5**. All these equations are detailed in the Supplementary Material of **Manuscript 11**.

Concerning the several kinds of acyl groups (AG) and fatty acids (FA). The molar percentage of the several kinds of acyl groups present in the starting oils was determined by ^{1}H NMR spectral data as in previous studies (Guillén & Ruiz, 2004; Guillén et al., 2008). Regarding digested lipid extracts, the molar percentage of ω-3 AG/FA was estimated in relation to the total number of moles of AG plus FA present (NT_{AG+FA}) in either the starting oils or the lipid extracts of digestates, in agreement with **Manuscript 4** and previous studies (Siddiqui, Sim, Silwood, Toms, Iles, & Grootveld, 2003; Guillén & Ruiz, 2004). All these equations are detailed in the Supplementary Material of **Manuscript 11**.

Concerning vitamin A. The concentration of retinyl esters, expressed as mmol/mol of AG plus FA present (mmol/molAG+FA), was estimated by means of the following equation:

$$\text{Retinyl esters (mmol/molAG+FA)} = 1000*(Pc*A_m/2)/NT_{AG+FA} \quad \text{[Objective 3.5.-eq.1]}$$

where **Pc** is the proportionality existing between the area of the ^{1}H NMR signals and the number of protons that generate them, $\mathbf{A_m}$ is the area of signal m at 4.72 ppm due to the two methylenic protons in α-position in relation to the carbonyl group of retinyl esters (see signal assignment in **Table 1**, **Figure 2** and **Figure S1** in Supplementary Material of **Manuscript 11**) and $\mathbf{NT_{AG+FA}}$ is determined using [eq.S15] detailed in Supplementary Material of **Manuscript 11**.

Concerning oxidation compounds. The concentrations of *(Z,E)*-conjugated dienic systems supported in chains having also an hydroperoxy group (***(Z,E)*-CD-OOH**) and of certain aldehydes, expressed as mmol/mol of AG plus FA present, were estimated as follows:

$$\textit{(Z,E)}\text{-CD-OOH (mmol/molAG+FA)} = 1000*(Pc*A_{6.58})/NT_{AG+FA} \quad \text{[Objective 3.5.-eq.2]}$$

$$\text{Aldehyde (mmol/molAG+FA)} = 1000*(Pc*A_{Ald})/NT_{AG+FA} \quad \text{[Objective 3.5.-eq.3]}$$

where $\mathbf{A_{6.58}}$ is the area of signal c centered at 6.58 ppm as one proton of the conjugated dienic system of *(Z,E)*-CD-OOH, $\mathbf{NT_{AG+FA}}$ is determined using [eq.S15] detailed in Supplementary Material of **Manuscript 11**, and $\mathbf{A_{Ald}}$ is the area of the signals j, k or l due to the aldehydic proton of 4-hydroxy-, 4-hydroperoxy-*(E)*-2-alkenals or *(Z,E)*-2,4-alkadienals, respectively (see **Table 1** of **Manuscript 11**).

Concerning BHT concentration. The concentration of BHT in CLO800 samples and in their corresponding digested lipid extracts was also estimated, as mmol/mol of AG plus FA present in the samples, by using the following equation:

$$\text{BHT (mmol/molAG+FA)} = 1000*(Pc*A_n/2)/NT_{AG+FA} \quad \text{[Objective 3.5.-eq.4]}$$

where $\mathbf{A_n}$ is the area of signal n due to the two aromatic protons of BHT (see **Table 1** of **Manuscript 11**) and $\mathbf{NT_{AG+FA}}$ is determined using [eq.S15] detailed in Supplementary Material of **Manuscript 11**.

3.5.5. Study of the headspace composition of the digestates

The methodology employed for the study by SPME-GC/MS of the volatile components of the headspace of the samples subject of study (0.5 g in 10 mL screw-cap vial) is the same as that described in subsection 1.2.5. of the experimental design of Objective 1.2.

The samples, all of them studied in duplicate, were the following: non-enriched cod liver oil (**CLO**) and BHT-enriched samples at 20 and 800 ppm (**CLO20**, **CLO800**); their corresponding digestates (**DCLO**, **DCLO20**, **DCLO800**); the juices obtained after being submitted to digestion conditions in the absence of oil (**J**); and mixtures made up of starting oils and juices submitted to digestion conditions in the same proportions as in the digestates (**CLO+J**, **CLO20+J**, **CLO800+J**). These latter mixtures were studied with the aim of simulating a matrix in the sample before digestion similar to that obtained after digestion, and thus to a certain extent comparable. As 4 digestion experiments were carried out, altogether the headspaces of 8 samples of each kind were studied.

3.5.6. Statistical analysis

The significance of the differences among the starting oils and the corresponding digestates on the molar percentage of the different kinds of lipolytic products, on the molar percentage of ω-3 AG or FA, and on the concentration of vitamin A and of the several oxidation compounds present, were determined by one-way variance analysis (ANOVA) followed by Tukey b test at $p<0.05$, using SPSS v.22 software (IBM, NY, USA).

AIM 4

***In vitro* gastrointestinal digestion of processed fish**

Influence of common technological processes like salting and smoking on the lipids performance under digestive conditions

(MANUSCRIPTS 12-13)

OBJECTIVE 4.1.

To investigate by means of ^{1}H NMR and SPME-GC/MS the chemical reactions taking place during *in vitro* gastrointestinal digestion of European sea bass, and the effect of fish salting (brine-salting/dry-salting) on their extent

(**Manuscript 12**)

4.1.1. Fish specimens

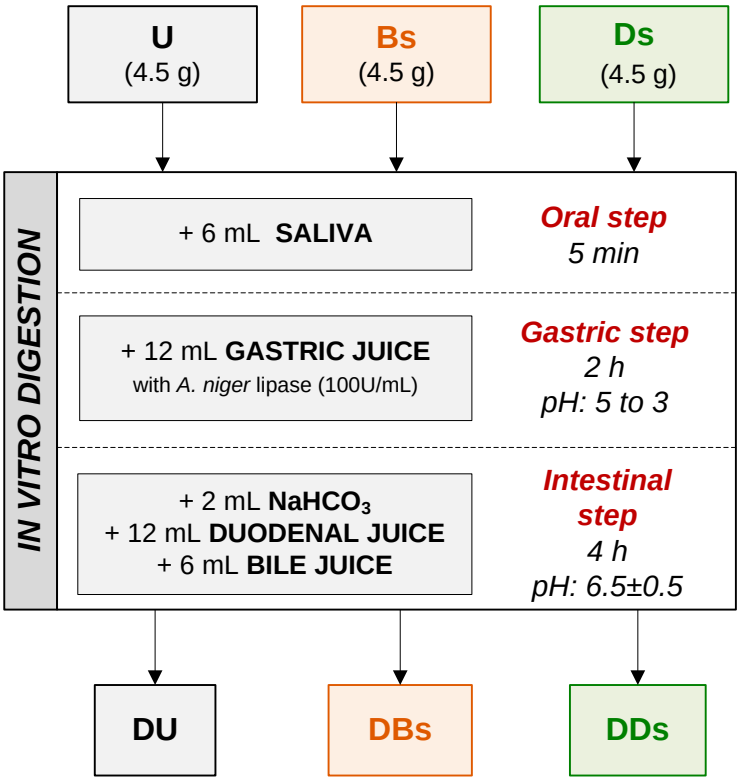
Four specimens of farmed European sea bass were acquired from a local supplier within 48 h of harvest. The average body weight of the specimens was 1337.3±51.1 g. On the same day of purchase specimens were gutted, cleaned, filleted and skinned. From each specimen, one fillet was maintained unsalted (**U**, *n*=4) and the other fillet was submitted to salting. The average weight of sea bass fillet (*n*=8) was 261.2±12.1 g.

4.1.2. Fish salting

Two salting methods were carried out in this study: dry-salting and brine-salting. The dry-salted fillet (**D_S**, *n*=2) was obtained by covering it completely with coarse sea salt for 8 h at 4ºC, whereas the brine-salted one (**B_S**, *n*=2) was immersed for 15 min in a 15% brine-solution of salt at room temperature with a brine-to-fish proportion of 1:6 (w/v). Afterwards, both fillets were rinsed with water to remove the remaining surface salt or brine. Salted fish fillets were vacuum-packed, frozen and stored at -80ºC for up to 24 h until *in vitro* digestion experiments.

4.1.3. *In vitro* gastrointestinal digestion

Minced fish samples (4.5 g), either previously salted or not, were digested in duplicate, as previously described in subsection 3.1.2. of the experimental design of Objective 3.1. (see Objective 4.1.-**Figure 1**). Thus, the digestates obtained from the *in vitro* digestion of unprocessed sea bass were named **DU** (*n*=8); those obtained from brine-salted sea bass samples were named **DB_S** (*n*=4) and from dry-salted ones **DD_S** (*n*=4). In addition, blank samples, corresponding to digestive juices submitted to *in vitro* digestion conditions in the absence of fish (**J**), were also undertaken (*n*=4) for further analysis.



Objective 4.1.-**Figure 1**. Schematic representation of the *in vitro* digestion methodology employed in this study.

4.1.4. Fish lipid extraction and study by ^{1}H NMR

Lipid extraction. Lipids from sea bass fillets before digestion (**U**, **B_S**, **D_S**), from the digested fish samples (**DU**, **DB_S**, **DD_S**) and from the juices submitted to digestion conditions in the absence of food (**J**) were extracted with dichloromethane (CH_2Cl_2, Sigma-Aldrich, St. Louis, MO, USA), following the same methodology described in subsection 2.2.3. of the experimental design of Objective 2.2. The average lipid content per fillet was 7.5±1.2% (ww).

^{1}H NMR spectra acquisition and derived data. ^{1}H NMR spectra of lipid extracts were recorded in duplicate on a Bruker Avance 400 spectrometer operating at 400 MHz. To do this, the sample preparation to acquire the corresponding spectra, the acquisition conditions and the study of the spectral data were the same as those described in subsection 1.1.5. of the experimental design of Objective 1.1.

The lipid composition of unsalted and salted fish fillets before digestion, expressed as molar percentage of the different kind of acyl groups, and the content of the several kinds of lipolytic

products present in digested lipid extracts, were determined from ^{1}H NMR spectral data, as in **Manuscript 5** and in a previous study (Vidal et al., 2012).

4.1.5. Study by SPME-GC/MS of the headspace composition

The methodology employed for the study by SPME-GC/MS of the volatile components of the headspace of the samples subject of study (0.5 g in 10 mL screw-cap vial) is the same as that described in subsection 1.2.5. of the experimental design of Objective 1.2. The samples were the following: sea bass samples before digestion (**U**, **B_S**, **D_S**); digestive juices employed and submitted to digestion conditions in the absence of fish (**J**); mixtures made up of juices submitted to digestive conditions and of unsalted, brine-salted or dry-salted sea bass before digestion mixed in the same proportions as in the digestates (**U+J**, **B_S+J**, and **D_S+J**); and fish digestates (**DU, DB_S**, **DD_S**).

4.1.6. Statistical Analysis

Statistical analysis was performed using the Statistical package SPSS v.22 (IBM, NY, USA). The significance of the differences on the molar percentages of lipolytic products among samples was determined by one-way variance analysis followed by post hoc Tukey b test at 0.05 threshold.

OBJECTIVE 4.2.

To study by means of ^{1}H NMR and SPME-GC/MS the effect of smoking with two liquid smoke flavourings on lipid hydrolysis and oxidation occurring during *in vitro* gastrointestinal digestion of European sea bass, and to evaluate to what extent the flavouring composition may influence these reactions

(**Manuscript 13**)

4.2.1. Fish specimens

Four specimens of farmed European sea bass were acquired from a local supplier within 48 h of harvest. The average body weight of the specimens was 1352.1±48.7 g. On the same day of purchase fishes were gutted, cleaned, filleted and skinned just before smoking. From each specimen, one fillet was maintained unprocessed as control (**U**, *n*=4) and the other fillet was submitted to smoking. The average weight of the fillet (*n*=8) was 240.3±13.1 g. The average molar percentages of the several kinds of acyl groups in fish fillets, determined by ^{1}H NMR (Vidal et al., 2012), were the followings: 21.4±0.7% of saturated acyl groups and 78.6±0.7% of total unsaturated acyl groups; the latter consisting of 41.8±0.7% of oleic (plus other minor unsaturated acyl groups), 22.5±0.7% of diunsaturated ω-6 (mainly linoleic), 14.2±0.6% of polyunsaturated ω-3 acyl groups, which include 5.8±0.3% of DHA and 5.1±0.4% of EPA, and 0.1±0.0% of ω-1 acyl groups.

4.2.2. Liquid smoking procedure

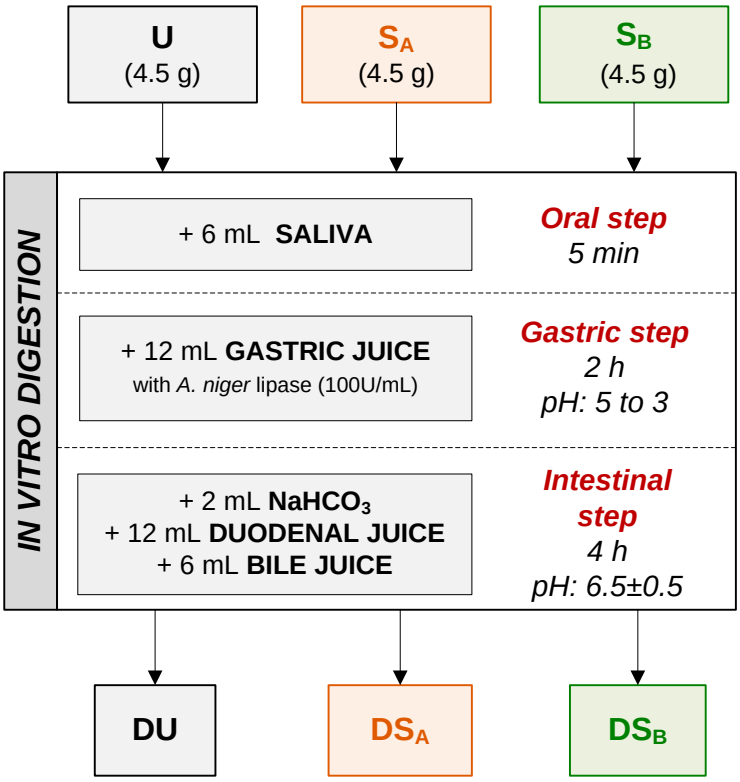
The smoking procedure consisted in the immersion of the fish fillet for 1 min in a solution of smoke flavouring in water (1:6, v/v) with a fish-to-flavouring solution proportion of 1:16 (w/v). Afterwards, smoked fillets were kept at room temperature for 2 h to facilitate the interaction between smoke components and fish flesh. In this study two commercial aqueous smoke flavourings (A and B) were used. Fish fillets smoked with flavouring A were named $\mathbf{S_A}$ (*n*=2) and those smoked with flavouring B were named $\mathbf{S_B}$ (*n*=2).

These smoke flavourings A and B were selected because: i) they differ in their phenolic concentration (19 g/L and 24 g/L respectively, according to the manufacturer); ii) they have been employed in previous works carried out in our laboratory on oxidative stability and evolution during refrigerated storage of smoked European sea bass and thus, their performance under these conditions is known (Vidal et al., 2016ce); and iii) the composition of the dichloromethane extracts of

flavourings A and B is also known, having been studied in detail by GC/MS in the above-mentioned works (Vidal et al., 2016ce).

4.2.3. *In vitro* gastrointestinal digestion

Unsmoked and smoked fish fillets were minced in a grinder and then, 4.5 g of each sample were *in vitro* digested following the same methodology described in subsection 3.1.2. of the experimental design of Objective 3.1. (see Objective 4.2.-**Figure 1**).



Objective 4.2.-**Figure 1**. Schematic representation of the *in vitro* digestion methodology employed in this study.

For consistency of results, digestion experiments were performed in duplicate. Thus, the digestates obtained from the *in vitro* digestion of unsmoked sea bass were named **DU** (*n*=8) and those obtained from smoked sea bass samples using flavouring A were named **DS_A** (*n*=4) and from flavouring B, **DS_B** (*n*=4). In addition, blank samples, corresponding to juices submitted to *in vitro* digestion conditions in the absence of fish meat (**J**, *n*=4) were also analyzed.

4.2.4. Fish lipid extraction and study by ^{1}H NMR

Lipids from fish samples before (**U**, **S_A**, **S_B**) and after digestion (**DU**, **DS_A**, **DS_B**) were extracted with dichloromethane (CH_2Cl_2, Sigma-Aldrich, St. Louis, MO, USA), following the same methodology described in subsection 2.2.3. of the experimental design of Objective 2.2.

The ^{1}H NMR spectra of the lipid extracts were recorded in duplicate on a Bruker Avance 400 spectrometer operating at 400 MHz. To do this, the sample preparation to acquire the corresponding spectra, the acquisition conditions and the study of the spectral data were the same as those described in subsection 1.1.5. of the experimental design of Objective 1.1.

Bearing in mind that, as mentioned before, 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, it is possible from the area of some spectral signals to estimate the molar percentage or the concentration of several components present in fish lipid extracts before and after digestion. Hence, the hydrolysis degree reached in the digestates was assessed using the equations developed, validated and explained in detail in **Manuscripts 4** and **5**.

Furthermore, the concentration of *(Z,E)*-conjugated dienic systems associated with hydroperoxy groups and with hydroxy groups (***(Z,E)*-CD**), if any, was estimated as mmol/mol of acyl group (AG) plus fatty acid (FA) present in the samples, in relation to total number of moles of AG plus FA present (**NT_{AG+FA}**), as follows:

$$NT_{AG+FA} = Pc*A_{0.84\text{-}1.00}/3 \quad \text{[Objective 4.2.-eq.1]}$$

$$\textit{(Z,E)}\text{-CD (mmol/molAG+FA)} = 1000*[Pc*(A_{6.58+}A_{6.48})]/NT_{AG+FA} \quad \text{[Objective 4.2.-eq.2]}$$

where **Pc** is the proportionality constant existing between the area of the ^{1}H NMR signals and the number of protons that generate the signal, **$A_{0.84\text{-}1.00}$** is the area of the signals at 0.84-1.00 ppm due to the methylic protons (-CH_3) of all AG and FA present, and (**$A_{6.58+}A_{6.48}$**) is the sum of the areas of signals a at 6.58 ppm and b at 6.48 ppm, the former signal being generated by one proton of *(Z,E)*-conjugated dienes supported in chains having also hydroperoxy groups, and the latter signal by one proton of *(Z,E)*-conjugated dienes supported in chains also having hydroxy groups (Gardner & Weisleder, 1972; Murakami, Shirahashi, Nagatsu, & Sakakibara, 1992).

4.2.5. Study by SPME-GC/MS of the headspace composition of the digestates

The methodology employed for the study by SPME-GC/MS of the volatile components of the headspace of the samples subject of study (0.5 g in 10 mL screw-cap vial) is the same as that described in subsection 1.2.5. of the experimental design of Objective 1.2. Samples were the

following: digestates obtained in the two digestion experiments from unprocessed sea bass (**DU**) and from smoked samples using flavourings A and B (**DS_A**, **DS_B**), together with the juices submitted to digestion conditions in the absence of fish (**J**); in addition, the headspace composition of fish fillets before digestion (**U**, **S_A**, **S_B**) was also analyzed.

4.2.6. Statistical Analysis

The significance of the differences on the molar percentages of lipolytic products among samples and on the abundance of volatiles compounds detected in the digestates was determined by one-way variance analysis (ANOVA) followed by post hoc Tukey b test or by t-Student test at 0.05 threshold, using the Statistical package SPSS v.22 (IBM, NY, USA).

RESULTS AND DISCUSSION

SUMMARY

AIM 1: Fish cooking methods. Effect on lipids and formation of volatile compounds. Influence of fish species and growing conditions

OBJECTIVE 1.1.

To investigate by means of ^{1}H NMR the potential influence of frying technique (pan-frying/microwave-frying), cooking oil (extra-virgin olive oil/sunflower oil) and fish species (farmed European sea bass/farmed Gilthead sea bream) on the changes occurring in fish lipids and in cooking oil during fish shallow-frying (**Manuscript 1**)

In this work, a detailed study of food shallow-frying was undertaken by means of ^{1}H NMR for the first time. Fillets of farmed Gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) were shallow-fried under domestic conditions using two frying methods (microwave- and pan-frying) and two cooking oils (sunflower and extra-virgin olive). The lipids of raw and fried fish fillets were extracted and their ^{1}H NMR spectra recorded, together with that of the cooking oils before and after fish frying. Moreover, both oils were heated under the same conditions in the absence of food, and their ^{1}H NMR spectra studied in order to elucidate potential changes which occurred in the main and minor components of fish lipids and of the oils used for frying, either in the absence or in the presence of fish fillets.

Occurrence of lipid migration during fish shallow-frying

The results obtained confirmed that there is a migration of main and some minor lipidic components between the two systems involved (oil/fish fillet), this exchange being linked to the proportion of each component in the original lipidic medium. Therefore, after fish frying, extra-virgin olive oil is richer in ω-3, ω-1, linoleic and saturated acyl groups and poorer in oleic acyl groups than the original oil. Likewise, after fish frying, sunflower oil is richer than the original in all kinds of acyl groups except for linoleic groups. In addition, after frying fish, both oils are enriched in the fish minor lipid component cholesterol. Concerning fish lipids, their composition also changes during frying, becoming richer in those acyl groups and minor components that are in higher concentration in the frying oil than in fish lipids, while poorer in those acyl groups and minor components that are in higher concentration in the raw fish lipids than in the original oils. Migration of healthy vegetable sterols Δ7-avenasterol, β-sitosterol, Δ5-campesterol from sunflower oil to fish lipids was highlighted, as well as migration of β-sitosterol, Δ5-campesterol from extra-virgin olive oil to fish lipids.

Considering all the frying techniques, oils and fish species studied, a contribution ranging from 19.4 to 28.1% of the **migrated fish lipids** to the final molar percentage of the different kinds of acyl groups in the fried oil was estimated. The highest contribution of fish lipids to the changes observed in the fried oil was observed in those fish fillets which contained the highest initial fat content and which underwent the highest fat loss as a result of the frying process, evidencing a remarkable leaching of their lipids. By contrast, the smallest contribution of fish lipids to the changes observed in the fried oil was observed in those fish fillets showing the lowest initial fat content.

The contribution of **migrated oil** to the molar percentage of the different acyl groups in the fried fish lipids was also important, although somewhat lower than that of fish lipids to the fried oil, ranging in most of the cases from 15 to 25%. The exception is the case of sea bass fillets microwave-fried in sunflower oil, in which the contribution of the oil is very high (≈43%). These results are in agreement with the fact that the latter fish fillets showed the lowest initial fat content and that they were the only ones in which the total fat content increased after frying, highlighting that when microwave-frying these fillets the oil uptake took place to a greater extent than the leaching out of the fish lipids.

Likewise, it could be observed that the **total fat content** of sea bream fillets significantly decreased after shallow-frying, whereas in the case of sea bass ones, it remained almost unchanged or increased. This seemed to be related to the initial fat content of the fish fillet.

Occurrence of lipid thermo-oxidation during fish shallow-frying

The occurrence of thermo-oxidation reactions under shallow-frying conditions was also evidenced by the degradation of the main unsaturated acyl group of the cooking oil and the occurrence of secondary oxidation products, being much more pronounced during **pan-frying** than during microwave-frying and in **sunflower oil** than in extra-virgin olive oil. As expected, no signals related to primary oxidation compounds (hydroperoxides containing a conjugated dienic system) were detected in any of the lipidic samples studied. However, secondary oxidation compounds were detected in the cooking oils, but not in the fried fish lipids. Regarding **heated oils** in the absence of food, alkanals, *(E)*-2-alkenals, *(Z,E)*- and *(E,E)*-2,4-alkadienals were detected in pan-heated sunflower oil, whereas alkanals, *(E)*-2-alkenals and *(E)*-9,10-epoxystearate in pan-heated extra-virgin olive oil. By contrast, none of these oxidation products were detected in microwave-heated extra-virgin olive oil and in microwave-heated sunflower oil significantly lower (p<0.05) amount of *(Z,E)*-2,4-alkadienals than in pan-heated sunflower oil were found. It must be noted that no changes in the concentration of sterols was noticed with the heating under shallow-frying conditions in the absence of food.

Concerning the occurrence of lipid oxidation products in the **oils used for shallow-frying fish**, these aldehydes were in much lower concentrations than those detected in the oils heated in the absence of food. This fact could be explained by several reasons: i) when the fish fillet is introduced into the system, the oil temperature goes down for a while and as a consequence the thermo-oxidation process can be reduced, whereas in the absence of food it maintains constant (≈170ºC) for the 5 min heating experiment; ii) the presence of the fish fillet provokes a greater movement of the surrounding oil, which facilitates the escape of volatile aldehydes to the atmosphere; iii) the occurrence of Maillard type reactions between aldehydes and fish proteins takes place; and iv) there is a potential dilution effect due to the leaching of fish lipids into the frying media.

Moreover, certain **influence of the fish species** on the abundance of **aldehydes** detected in the fried oils was evidenced. Indeed, *(E,E)*-2,4-alkadienals and *(E)*-2-alkenals were found in the sunflower oil employed to pan-fry sea bass, whereas only *(E,E)*-2,4-alkadienals were found in the sunflower oil employed to pan-fry sea bream, which was attributed to the lower molar percentage of DUω-6 (mainly linoleic) and a higher one of ω-3 acyl groups in this latter. It is well-known that the degradation of linoleic acyl groups generates *(E)*-2-alkenals of higher molecular weight than those arising from polyunsaturated ω-3 acyl groups, which are very volatile and escape more easily towards the atmosphere due to their lower boiling points. This was confirmed by the study of the headspace composition of the fried oils by SPME-GC/MS. No aldehydes nor epoxides were detected in the pan-fried extra-virgin olive oil. In this sense, extra-virgin olive oil is safer and more suitable than sunflower oil for fish shallow-frying.

Occurrence of lipid hydrolysis during fish shallow-frying

The heating provoked in general a decrease of 1,2-diglycerides concentration in the cooking oils, which was more accentuated with the fish frying process. As the evolution of 1,2-diglycerides in fish lipids during frying did not follow a clear trend, the changes observed in their concentration in oils during fish frying are probably associated with degradation processes, in addition to lipid exchange between fish fillet and oil.

In summary, in this study the frying technique, the nature of the cooking oil and the fish species have been evidenced to have a great influence on the changes occurring during food shallow-frying.

OBJECTIVE 1.2.

To study by means of ^{1}H NMR and SPME-GC/MS the potential effect of boiling, steaming and *sous-vide* cooking on the lipids and volatile profile of farmed and wild European sea bass (**Manuscript 2**)

This study shed light on the changes provoked by boiling, steaming and *sous-vide* cooking on the lipids (including cholesterol, phospholipids and vitamin A) and on the volatile profile of farmed and wild European sea bass meat. Firstly, the lipid and volatile components of raw farmed and wild sea bass meat were investigated by means of ^{1}H NMR and SPME-GC/MS and discussed with the aim of properly characterizing the starting fish samples. Secondly, the same study on cooked farmed and wild sea bass was undertaken, to evaluate the changes provoked by the three cooking methods.

Raw European sea bass. Farmed *versus* wild specimens

Raw farmed and wild sea bass had not only very different ***total lipid content*** but also a very different ***lipid composition***. This is mainly attributed to the different diet of farmed and wild sea bass. Thus, the total *lipid content* of farmed specimens was significantly higher (≈5-fold) than that of the wild ones. Moreover, regarding the *molar proportions of the several kinds of acyl groups*, the lipid extracts of farmed sea bass contained a higher molar percentage of total unsaturated acyl groups than those of the wild samples, the proportion of DUω-6 acyl groups (mainly linoleic) being much higher (p<0.05) in the former, whereas that of ω-3 acyl groups being much lower (p<0.05). Furthermore, differences, regarding the *molar concentration of certain minor lipidic components* in relation to the moles of triglycerides, were noticed between both kinds of sea bass; the lipid extracts of farmed sea bass showed much lower (p<0.05) concentration of cholesterol, phosphatidylcholine, phosphatidilethanolamine and vitamin A (retinyl ester) than the lipid extracts of wild samples. Nevertheless, due to the much lower lipid proportion of wild than of farmed samples, it must be pointed out that the total content of ω-3 acyl groups, as well as of cholesterol, phospholipids and vitamin A ingested per 100 g of farmed or wild sea bass meat would be quite similar. Hence, from a nutritional point of view, both kinds of sea bass meat might not differ very much.

It was also worth noting the low **variability** of farmed sea bass **lipid composition**, mainly due to the controlled growing conditions and commercial feeds. However, a relatively high degree of variability is found in wild sea bass lipids, which might be explained by the greater heterogeneity in the diet composition of wild specimens and in the environmental conditions of the geographical fishing ground, as well as by other factors, such as sex and age.

In relation to the ***volatile profile***, a higher number of volatile components were detected in farmed than in wild sea bass samples, as could be expected from the markedly higher lipid content in the former than in the latter. One of the main differences is related to the great abundance of *hydrocarbons* in the headspace of farmed sea bass meat. These include aliphatic hydrocarbons, alkylbenzenes, some naphthalene derivatives and terpenic hydrocarbons. Another important difference lies in the abundance of the *synthetic antioxidant 2,6-di-tert-butyl-4-hydroxytoluene* (BHT, E-321), present in very high abundance in farmed samples but absent in wild ones. The near or total absence of these compounds in the headspace of wild sea bass is supposed to be due to the higher exposure to these compounds via commercial feeds and living environment, as well as to a higher *in vivo* retention of these lipophilic metabolites in farmed than in wild specimens due to the ≈5-fold higher lipid content. However, the headspace of farmed and wild sea bass meat hardly differed in volatile compounds coming from *lipid oxidation processes* (like alcohols, aldehydes, ketones, furans and acids) and in those *related to fish nitrogenated components degradation* (trimethylamine and ethanethiol). Abundance data of these compounds are very low in both kinds of sea bass, indicating a very low level of lipid and protein degradation via either autooxidation, enzymatic or microbial activity in the starting samples.

Taking into account the above-mentioned differences, although both kinds of specimens belong to the same fish species, it seemed as if they belonged to different ones. Due to this, after cooking further differences were found between farmed and wild sea bass samples.

Cooked sea bass. Analysis of the effect of some chemical reactions expected to occur during cooking on the lipids and volatile profile of farmed and wild sea bass meat

Influence of cooking on sea bass lipid content. As a consequence of the loss of fish meat water during heat treatment, the cooked sea bass meat has a higher proportion of lipids than the corresponding raw meat. Regardless of the kind of sea bass, the highest weight loss occurred in boiled fillets and the lowest in steamed ones, *sous-vide* cooked samples showing intermediate values.

Influence of cooking on sea bass lipid hydrolysis. None of the cooking techniques provoked the hydrolysis of triglycerides, phospholipids and retinyl esters (vitamin A) present in farmed and wild sea bass lipids to an extent detectable by ^{1}H NMR.

***Influence of cooking on sea bass lipid oxidation*.** None of the cooking techniques provoked the oxidation of unsaturated acyl groups supported in triglycerides and phospholipids, of cholesterol and of vitamin A present in farmed or wild sea bass lipids to an extent detectable by ^{1}H NMR.

By contrast, the study of the headspace composition of cooked fish meat by means of SPME-GC/MS indicated that during cooking a very slight oxidation of unsaturated acyl groups took place, yielding several volatile secondary oxidation compounds of low molecular weight that enriched the aromatic profile of sea bass. Nevertheless, differences on the nature and abundance of lipid oxidation-derived volatiles generated were observed depending on the cooking method applied, on the fish growing conditions and on the content of BHT in farmed sea bass.

- Cooking methods: *boiling* did not provoke lipid oxidation or, if it occurred it was not observable, presumably because the potential new compounds generated were lost by leaching out into the aqueous cooking media; as boiled sea bass meat is so poor in odour-active volatile compounds, it can be said that it could be very suitable for consumers who do not like fishy aroma. Considering *steaming* and *sous-vide cooking*, both methods provoked a slight oxidation of unsaturated acyl groups, leading to the formation of alcohols, aldehydes, ketones, alkylfurans and acids that modified the headspace of cooked sea bass. Based on the abundances found, lipid oxidation reactions occurred to a similar extent during these two culinary techniques.

- Fish growing conditions: a higher generation of volatile compounds was evidenced during cooking of farmed than of wild sea bass, which was expected due to the higher lipid content of the former. Moreover, due to the higher proportion of DUω-6 in relation to ω-3 acyl groups in farmed than in wild samples, in addition to volatile compounds derived from ω-3 acyl groups (1-penten-3-ol, *5(Z)*-octa-1,5-dien-3-ol, propanal, *(E)*-2-pentenal, *(Z)*-4-heptenal, 2,4-heptadienals, 1-penten-3-one, 3,5-octadien-2-one and 2-ethylfuran), high abundances of those derived from ω-6 ones (1-octen-3-ol, 1-pentanol, hexanal, *(E)*-2-hexenal and 2-pentylfuran) were also found in the headspace of cooked farmed sea bass meat.

- BHT content in farmed sea bass: it was also evidenced that the initial BHT content of each farmed sea bass specimen had a great impact on the advance of oxidation reactions taking place during the culinary treatment, and thus on the generation of odour active compounds. The higher the abundance of BHT, the lower the advance of lipid oxidation reactions during cooking was.

***Influence of cooking on sea bass nitrogenated components degradation*.** As a result of the high temperatures applied during cooking, the degradation of fish nitrogenated components, such as proteins, amino acids or the nitrogenated base trimethylamine oxide (TMAO) was proved by SPME-GC/MS. Among the volatile compounds generated from these reactions, there were: Strecker aldehydes and some of its derivatives, including sulphur-containing compounds, and trimethylamine

(TMA). Differences on the abundance of these volatile compounds were also noticed depending on the cooking method applied and the fish growing conditions.

- Cooking methods: it was observed that *boiling* provoked fish nitrogenated components degradation to a lower extent than steaming or *sous-vide* cooking. Although the leaching out into the aqueous media of the potentially generated compounds cannot be ruled out. Again, reactions involved in the formation of protein-related volatile compounds seem to occur to a similar extent during steaming or *sous-vide* cooking.

- Fish growing conditions: cooked farmed samples showed slightly higher number and abundances of all these volatile compounds than cooked wild ones, but these differences were not as remarkable as in the case of compounds coming from lipid oxidation.

Occurrence of off-reactions during *sous-vide* cooking

In addition to the chemical reactions occurring in fish components during cooking, the **degradation of polypropylene vacuum-seal bag** during *sous-vide* cooking was evidenced. Four specific branched hydrocarbons were only detected in *sous-vide* cooked sea bass meat, being 2,4-dimethyl-1-heptene the main one. The occurrence of this latter, which is a well-known by-product of polypropylene degradation, highlighted some degree of migration of plastic-derived compounds into sea bass during *sous-vide* cooking. It also should be noted that the bags employed in this study were supposed to be heat resistant, manufactured for vacuum-cooking at temperatures up to 120ºC, and that sea bass subject of study was *sous-vide* cooked at only 85ºC.

From these results, it seems evident that steaming and *sous-vide* cooking provide similar organoleptic profiles, although steaming is a cleaner, faster, cheaper and more environmentally friendly than *sous-vide* cooking.

OBJECTIVE 1.3.

To address by means of ^{1}H NMR and SPME-GC/MS the potential effect of salt-crusted oven baking, conventional oven baking and microwave cooking on the lipids and volatile profile of farmed and wild European sea bass (**Manuscript 3**)

This Objective 1.3. tackled the possible effect of microwave cooking, salt-crusted and conventional oven baking on certain aspects of farmed and wild European sea bass quality, focused on their lipids and their volatile profiles. For this purpose, ^{1}H NMR and SPME-GC/MS were the techniques employed. Special attention was paid to the different chemical reactions ongoing during cooking that may affect lipid and volatile profile of sea bass, analyzing potential differences on their extent related either to the culinary treatment applied or to fish growing conditions, namely farmed or wild.

As in the previous Objective 1.2., as a starting point, raw farmed and wild sea bass samples were characterized to know the potential initial differences existing on their lipids and volatile profile. Similar results concerning the lipid content, the composition in main and minor lipidic components, and the volatile profile of farmed and wild samples were obtained. Therefore, below are only summarized the changes occurring on the lipids and volatile profile of farmed and wild sea bass during the three cooking techniques studied, which were provoked by lipid hydrolysis and oxidation, and by degradation of fish nitrogenated compounds (including Maillard-type reactions).

Changes in the lipid content after cooking. The proportion of lipids in farmed and wild sea bass meat after conventional oven baking and microwave oven cooking significantly increased, mainly due to the loss of water from fish meat via dripping or evaporation under heating conditions. However, salt-crusted oven baking did not provoke any change in the lipid content, indicating that the presence of the salty crust prevents the fish fillet surface from dehydrating.

Occurrence of lipolysis reactions during cooking. As in the case of boiling, steaming and *sous-vide* cooking studied in the Objective 1.2., ^{1}H NMR did not detect that hydrolysis of either triglycerides or minor lipidic components (phospholipids and retinyl esters) had taken place in sea bass lipids after microwave cooking, salt-crusted and conventional oven baking.

Occurrence of lipid oxidation during cooking. ^{1}H NMR results showed that none of the culinary techniques performed led to a detectable decrease in the *molar proportion of unsaturated acyl groups*, not even in the most polyunsaturated ones. The formation of oxidation products derived from ω-3 and ω-6 acyl groups of sea bass lipids was only evidenced by the SPME-GC/MS study of the

headspace composition of cooked sea bass meat. Although the extent of lipid oxidation reactions occurring during cooking was very limited, its major relevance might be in relation with fish sensory attributes. Among the volatile compounds with well-know aromatic properties, there were acids, alcohols, aldehydes, ketones and alkylfurans, whose generation depended on the fish growing conditions of sea bass and on the cooking method applied.

- Fish growing conditions: in agreement with the previous study of the Objective 1.2. on the effect of boiling, steaming and *sous-vide* cooking, after microwave cooking, salt-crusted and conventional oven baking the headspaces of farmed sea bass was enriched to a greater extent in lipid-oxidation derived metabolites than that of the wild ones during cooking. This is due to the higher lipid content of farmed sea bass meat than of wild one. Moreover, due to the lower ω-3/ω-6 lipids ratio in farmed than in wild samples, the relative abundance of volatile compounds coming from ω-3 lipids in relation to those coming from DUω-6 ones is much higher in cooked wild samples than in cooked farmed ones. Hence, the abundance ratios of propanal:hexanal and of 2-ethylfuran:2-pentylfuran are higher in wild cooked sea bass headspaces than they are in farmed cooked. Likewise, unsaturated aldehydes coming from ω-6 acyl group oxidation, like *(E)*-2-hexenal and *(E)*-2-heptenal, were only detected in cooked farmed samples.

- Cooking methods: salt-crusted baking provoked unsaturated acyl groups oxidation to a slightly lesser extent than did conventional baking and microwave cooking, especially in wild samples. At any rate, it must be pointed out that the influence of the cooking method on the extent of lipid oxidation reaction was very slight in relation to that of sea bass growing conditions.

Occurrence and effect of sea bass nitrogenated component degradation. During sea bass cooking, degradation of fish nitrogenated components (like proteins/peptides/amino acids, TMAO) also occurred generated, yielding TMA, as well as volatile markers of initial (Strecker aldehydes and some derivatives) and more advanced stages (heterocyclic aromatic compounds) of Maillard reaction. Again, sea bass *growing conditions* and the *cooking method* applied markedly influenced the formation of this kind of compounds during cooking.

- Fish growing conditions: the headspace of cooked farmed sea bass was richer in number and abundance of these volatiles than was the wild cooked samples headspace, due to the lower lipid content of these latter, which leads to less generation of potential precursors of Maillard-type reactions (carbonyl compounds).

- Cooking methods: *conventional oven baking* was the cooking method that provoked the most noticeable changes in the headspace of both farmed and wild sea bass meat, enhancing the formation of volatile compounds related to Maillard-type reactions, including alkylthiophenes, pyrroles, pyrazines and 2-ethylpyridine. Indeed, these potent odourants compounds were not detected after microwave cooking or salt-crusted oven baking. Bearing in mind that heterocyclic aromatic compounds provide desirable roasted, nutty, popcorn, and toasted-like notes in cooked fish meat, conventional oven baking might be a very suitable culinary technique for consumers looking for foods with marked odour notes. In the case of *salt-crusted oven baking*, the extent of Maillard-type reactions seemed to be limited by the presence of the salt-crust covering fish fillet during baking, which provokes a wet environment surrounding fish, and in the case of *microwave cooking,* it is limited by the cooking temperature reached, which is not enough to cause browning reactions in such a short cooking time. Anyway, microwave cooking provoked a slightly higher degradation of nitrogenated components than salt-crusted oven baking, in spite of the much shorter cooking time (5 *vs* 25 min).

AIM 2: ***In vitro*** **gastrointestinal digestion of lipids. Development and validation of a new method based on ^{1}H NMR for the study of lipid hydrolysis during digestion. Study in depth of the influence of several factors affecting the extent of** ***in vitro*** **lipolysis**

OBJECTIVE 2.1.

To develop and validate a new methodology based on ^{1}H NMR spectral data to evaluate the hydrolysis level in complex lipid mixtures (quantification of triglycerides, 1,3- and 1,2-diglycerides, 2- and 1-monoglycerides and fatty acids) (**Manuscript 4**)

In this Objective 2.1. a detailed study of the ^{1}H NMR spectra of pure standard compounds, as well as of 10 mixtures containing different known proportions of triglycerides (TG), diglycerides (1,2-DG, 1,3-DG), monoglycerides (1-MG, 2-MG) and fatty acids (FA), was carried out. The mixtures prepared covered a very broad range of concentrations and simulated edible oils and fats of vegetable and animal origins, including fish, with different levels of hydrolysis. 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, being possible their use for quantitative purposes.

Assignment of proton signals of glycerides and fatty acids. The assignment of the spectral signals to the several protons of glycerides and FA was performed using different standard compounds corresponding to TG, 1,2-DG, 1,3-DG, 1-MG, 2-MG and FA of different chain length and unsaturation degree. Results showed that glycerides have specific signals due to the protons present in their glyceryl backbone. In addition, small differences in the multiplicity and chemical shifts of signals due to protons in α- and β-position in relation to the carbonyl group of acyl groups, and to the carboxyl group in the case of FA, were noticed. Thus, the simple observation of the ^{1}H NMR spectrum of lipid samples provides important information about the nature and proportion of lipolytic products present.

Quantification of glycerides and fatty acids using ^{1}H NMR spectral data. Based on the proportionality existing between the area of the ^{1}H NMR signals and the number of protons that generate them, the molar percentages of the different kinds of compounds constituting a complex lipid mixture were estimated using different equations involving the areas of different spectral signals. Two quantitative approaches were carried out, depending on the signals selected for

integration, either using signals generated by the lowest (approach "a") or by the highest (approach "b") number of protons.

Thus, the number of moles (N) in the sample of those components having specific non-overlapped signals in the spectrum, such as 2-MG, 1-MG and 1,2-DG were determined as follows:

$N_{2\text{-}MG} = Pc*A_Q$ [Summary-eq.1a]

$N_{2\text{-}MG} = Pc*A_K/4$ [Summary-eq.1b]

$N_{1\text{-}MG} = Pc*A_L$ [Summary-eq.2]

$N_{1,2\text{-}DG} = Pc*A_R$ [Summary-eq.3a]

$N_{1,2\text{-}DG} = Pc*A_J/2$ [Summary-eq.3b]

where **Pc** is the proportionality constant relating the number of protons that generate a proton signal and its area, and **Ax** is the area of the corresponding signal X, whose assignment is shown in Table 2 of **Manuscript 4**.

However, if 1-MG are also present in the sample, the specific signal **J** of 1,2-DG and the specific signal **I** of 1-MG overlap. Therefore, the following alternative equation was proposed:

$N_{1,2\text{-}DG} = (Pc*A_{I+J}-2*N_{1\text{-}MG})/2$ [Summary-eq.3b']

Quantification of TG requires one to consider that half of its specific signal **O** overlaps with half of the specific signal **P** of 1,2-DG:

$N_{TG} = (Pc*2*A_{4.26\text{-}4.38}-2*N_{1,2\text{-}DG})/4$ [Summary-eq.4]

where $\mathbf{A_{4.26\text{-}4.38}}$ represents the area of the spectrum signals comprised between 4.26 and 4.38 ppm.

In the absence of 1-MG and 1,3-DG, the following equation 5 can be used:

$N_{TG} = (Pc*A_{O+P}-2*N_{1,2\text{-}DG})/4$ [Summary-eq.5]

In the absence of only 1,3-DG, the number of moles of TG can be estimated as follows:

$N_{TG} = (Pc*A_{N+O+P}-2*N_{1,2\text{-}DG}-2*N_{1\text{-}MG})/4$ [Summary-eq.6]

The determination of the number of moles of 1,3-DG also requires bearing in mind that there is a high overlapping of specific signal **M** of 1,3-DG with the specific signal **N** of 1-MG, the specific signal **O** of TG and the specific signal **P** of 1,2-DG. In spite of this, the following equation can be applied:

$$N_{1,3\text{-}DG} = (Pc*A_{M+N+O+P}-4*N_{TG}-2*N_{1\text{-}MG}-2*N_{1,2\text{-}DG})/5 \quad \text{[Summary-eq.7]}$$

The determination of the number of moles of FA can be carried out from the area of the signals of the protons supported on carbon atoms in α-position in relation to the carbonyl and carboxyl groups of acyl chains and FA respectively. In the case of lipids coming exclusively from edible oils of vegetable and terrestrial animal origin, this determination can be made by using this equation:

$$N_{FA} = (Pc*A_{2.26\text{-}2.40}-6*N_{TG}-4*N_{1,2\text{-}DG}-4*N_{1,3\text{-}DG}-2*N_{1\text{-}MG}-2*N_{2\text{-}MG})/2 \quad \text{[Summary-eq.8]}$$

where $\mathbf{A_{2.26\text{-}2.40}}$ is the area of the spectrum signals at 2.26-2.40 ppm.

When fish lipids are involved, some corrections are required to properly quantify FA, due to the slight overlapping between the signals of the protons supported on both carbon atoms in α- and β-position in relation to the carboxyl/carbonyl group of DHA acid and acyl group and that of the protons supported on carbon atoms in α-position in 1-MG, 2-MG and EPA fatty acid. In this case the equation proposed was the following:

$$N_{FA} = (Pc*10*A_{2.26\text{-}2.37}+Pc*5*A_{2.37\text{-}2.44}-60*N_{TG}-40*N_{1,2\text{-}DG}-40*N_{1,3\text{-}DG}-18*N_{1\text{-}MG}-13*N_{2\text{-}MG})/20 \quad \text{[Summary-eq.9]}$$

where $\mathbf{A_{2.26\text{-}2.37}}$ and $\mathbf{A_{2.37\text{-}2.44}}$ are the areas of the signals appearing at 2.26-2.37 and at 2.37-2.44 ppm respectively.

Therefore, the total number of moles of different molecules (N_T) in the sample can be determined as the sum of the moles of all the different compounds present, in which all terms enclose the same proportionality constant:

$$N_T = N_{TG}+N_{1,2\text{-}DG}+N_{1,3\text{-}DG}+N_{2\text{-}MG}+N_{1\text{-}MG}+N_{FA} \quad \text{[Summary-eq.10]}$$

Finally, the molar percentage of any of the different compounds present (X) can be estimated using the following general equation:

$$X\% = 100*N_X/N_T \quad \text{[Summary-eq.11]}$$

Validation of the approach. The accuracy of the new ^{1}H NMR methodology proposed was tested using the several mixtures of known composition prepared. The molar proportions of the different compounds present determined using the above-mentioned equations were compared to those obtained by weight. The level of agreement between both series of data was very high, confirming the validity of ^{1}H NMR to quantify lipolytic products. The error in the determination of the molar percentages of the compounds present in the 10 mixtures ranged from 0 to 9%, and only in one case did it reach 17%. Moreover, very similar results were obtained with the two approaches

carried out, either using the signals generated by the lowest (approach “a”) or by the highest (approach “b”) number of protons. It should be noted that the accuracy of the determinations can be influenced by the quality of the ^{1}H NMR spectrum, the base line correction and the spectral signal integration.

OBJECTIVE 2.2.

To demonstrate the usefulness of ^{1}H NMR when studying the extent of lipid hydrolysis reached during digestion of food samples (sunflower oil/minced fish meat), determining the hydrolysis level, the degree of triglyceride transformation, the lipid bioaccessibility level and the percentage of fatty acids physiologically releasable (**Manuscript 5**)

In this Objective 2.2. ^{1}H NMR was proved to be, for the first time, a very useful technique in monitoring the extent of lipid hydrolysis in digestion processes. Sunflower oil and minced fish meat, as model foods, were subjected to different *in vitro* digestion experiments and the lipolysis levels reached were evaluated using ^{1}H NMR spectral data.

Information extracted from the simple observation of the ^{1}H NMR spectrum. Very valuable information about the extent of the lipolysis could be extracted from the simple observation of ^{1}H NMR spectrum, enabling a rapid discrimination among samples having different hydrolysis degree. When comparing the ^{1}H NMR spectra of the unlipolyzed sample with those of partially and totally lipolyzed ones, noticeable differences were observed. Certain signals remained almost unchanged, whereas specific signals of TG (signals **O**, **S**) disappeared gradually. These almost disappeared in the spectrum of totally lipolyzed sample, indicating that nearly all the TG were hydrolyzed. At the same time and as the hydrolysis advances, specific signals due to partial glycerides appeared in the spectral region ranging from 3.60 to 5.30 ppm. The newly formed glycerides during *in vitro* digestion were predominantly 1,2-DG (signals **J**, **P**, **R**) and 2-MG (signals **K**, **Q**), in agreement with the regiospecificity of digestive lipases used. The presence, though in low intensity, of the spectral signals corresponding to 1-MG (signals **I**, **L**, **N**), especially in totally lipolyzed sample, was explained by the isomerization of 2-MG molecules which have been reported to be very unstable and to isomerize easily into 1-MG in an aqueous medium at neutral or alkaline pH and at moderate temperatures (26-40ºC). It is also noteworthy the presence of the multiplet corresponding to 1,3-DG at 4.05-4.21 ppm (signal **M**) in the spectrum of partially lipolyzed sunflower oil. Due to the positional specificity of the lipases used in the *in vitro* digestion protocol, this could be mainly explained by the occurrence of 1,2-DG isomerization, as also reported *in vivo*.

Likewise, differences in the chemical shift and multiplicity of proton signals due to the methylenic protons in α- and β-position in relation to the carbonyl group of acyl groups and to the carboxyl group of FA (signals **D** and **F**) can also be appreciated. These signals showed higher chemical shifts, as higher the level of lipolysis was. However, due to the great degree of overlapping of these

signals, the observation of these proton signals can also give information about the extent of lipolysis, but not about the nature of the lipolytic products generated.

Determination of lipolytic products generated during in vitro digestion. ^{1}H NMR allowed estimating quantitatively the products generated during TG lipolysis by two different ways.

- Quantitative description in function of acyl groups plus fatty acids: the digestion products were quantified as the molar percentages of acyl groups (AG) supported on the different glyceride structures (TG, DG, MG) and also the molar percentage of FA, using the following equations:

$AG_{TG}\% = 100*3*N_{TG})/NT_{AG+FA}$ [Summary-eq.12]

$AG_{1,2\text{-}DG}\% = 100*2*N_{1,2\text{-}DG})/NT_{AG+FA}$ [Summary-eq.13]

$AG_{1,3\text{-}DG}\% = 100*2*N_{1,3\text{-}DG})/NT_{AG+FA}$ [Summary-eq.14]

$AG_{2\text{-}MG}\% = 100*N_{2\text{-}MG}/NT_{AG+FA}$ [Summary-eq.15]

$AG_{1\text{-}MG}\% = 100*N_{1\text{-}MG}/NT_{AG+FA}$ [Summary-eq.16]

$FA\% = 100*N_{FA}/NT_{AG+FA}$ [Summary-eq.17]

$NT_{AG+FA} = 3*N_{TG}+2*N_{1,2\text{-}DG}+2*N_{1,3\text{-}DG}+N_{2\text{-}MG}+N_{1\text{-}MG}+N_{FA}$ [Summary-eq.18]

where **N** is the number of moles of each kind of digestion product and $\mathbf{NT_{AG+FA}}$ is the total number of moles of acyl groups plus FA present in the sample.

- Quantitative description in function of glyceryl structures: this way determines the proportion of TG molecules that remained intact and that were hydrolyzed partially into DG, MG, and totally into glycerol (Gol). The equations to apply are the following:

$TG\% = 100*N_{TG}/NT_{GS}$ [Summary-eq.19]

$1,2\text{-}DG\% = 100*N_{1,2\text{-}DG}/NT_{GS}$ [Summary-eq.20]

$1,3\text{-}DG\% = 100*N_{1,3\text{-}DG}/NT_{GS}$ [Summary-eq.21]

$2\text{-}MG\% = 100*N_{2\text{-}MG}/NT_{GS}$ [Summary-eq.22]

$1\text{-}MG\% = 100*N_{1\text{-}MG}/NT_{GS}$ [Summary-eq.23]

$N_{Gol} = (N_{FA}-N_{1,2\text{-}DG}-N_{1,3\text{-}DG}-2*N_{2\text{-}MG}-2*N_{1\text{-}MG})/3$ [Summary-eq.24]

$Gol\% = 100*N_{Gol}/NT_{GS}$ [Summary-eq.25]

$NT_{GS} = N_{TG}+N_{1,2\text{-}DG}+N_{1,3\text{-}DG}+N_{2\text{-}MG}+N_{1\text{-}MG}+N_{Gol}$ [Summary-eq.26]

where $\mathbf{NT_{GS}}$ is the total number of moles of glyceryl structures present in the sample.

It must be noted that, except for TG, very different values are obtained due to the different meaning of each way of quantifying. The second way of expressing lipid digestion products is very useful to monitor the progression of the lipolysis reaction during digestion because the proportion of

the different lipolytic products reflects the stoichiometry of reaction, ensuring thus a successful application of mass balances.

Determination from ^{1}H NMR spectral data of the extent of lipid digestion. Due to several different interpretations of the concept of lipid digestion extent, various approaches have been proposed in literature for its determination: hydrolysis level (H_L), degree of TG transformation (T_{TG}), lipid bioaccessibility level (L_{BA}) and percentage of fatty acids physiologically releasable (FA_{PR}). The high versatility of the new ^{1}H NMR methodology proposed allowed the estimation of the extent of lipid digestion in all the different approaches proposed in a fast and simple way by using the following equations:

$$H_L\% = 100*N_{FA}/NT_{AG+FA} = 100*N_{FA}/(3*N_{TGi}) \quad \text{[Summary-eq.27]}$$

$$T_{TG}\% = 100*(N_{TGi}-N_{TG})/N_{TGi} \quad \text{[Summary-eq.28]}$$

$$L_{BA}\% = 100*(N_{1\text{-}MG}+N_{2\text{-}MG}+N_{FA})/NT_{AG+FA} \quad \text{[Summary-eq.29]}$$

$$FA_{PR}\% = 100*N_{FA}/(2*N_{TGi}) \quad \text{[Summary-eq.30]}$$

where $\mathbf{N_{TGi}}$ is the number of moles of TG initially present in the sample, which can be estimated as $\mathbf{NT_{GS}}$ when lipids before digestion consist almost exclusively of TG.

The estimation of all these parameters in the unlipolyzed, partially and totally lipolyzed sunflower and fish lipid samples showed that very different values can be obtained, being the extent of lipolysis remarkably overestimated when using the parameter FA_{PR}%. It must be noted that this latter is usually employed when using pH-stat apparatus to monitor *in vitro* lipolysis reaction. In this context, the wide variation in the definition of lipid digestion extent makes it difficult to compare the results of different studies and thus to advance in the knowledge about lipid digestion. Therefore, a consensus definition on lipolysis during digestion would be of great interest, as well as the use of sound techniques to determine lipolysis extent.

OBJECTIVE 2.3.

To investigate the effect of different experimental factors (gastric acidification, intestinal transit time, presence of gastric lipase, sample/digestive fluids ratio, intestinal enzymes concentration and bile concentration) on lipid *in vitro* digestion extent (**Manuscript 6**)

This work focused on the impact of several experimental factors, like gastric acidification, intestinal transit time, presence of gastric lipase, sample/digestive fluids ratio, concentration and nature of the enzymes in the intestinal juice, and bile concentration, on the extent of *in vitro* lipolysis when using the gastrointestinal model developed by Versantvoort et al. (2004, 2005). To evaluate the changes in the *in vitro* digestion lipolysis as consequence of the variations in several experimental factors, the determination of the lipolysis extent firstly in the starting method and also in all the *in vitro* digestion experiments essayed were carried out.

Determination of lipolysis extent in sea bass digestates using Versantvoort conditions. From the simple observation of digested lipid spectra it was deduced that the lipolysis extent reached was rather limited and that an important amount of TG remained unhydrolyzed (69.2±3.3%), far below the hydrolysis performance reported *in* vivo (95% of TG absorbed as MG and FA). 1,2-DG was the most abundant partial glyceride generated and complete hydrolysis of TG into FA and Gol occurred to a slight extent. Thus, greater hydrolysis of the ester bonds occurred in TG than it did in DG and in MG, which was explained by the positional specificity of pancreatic lipase. The H_L% reached with the starting method is very low, being released less than 20% of acyl groups; T_{TG}% accounted for approximately to 30% and L_{BA}% reached was close to 20%. The small difference between H_L% and L_{BA}% indicated that the molar percentage of AG supported on MG was much smaller than that of FA.

Effect of gastric pH acidification. The acidification of the chyme to pH 2.5±0.5 was performed after 60 min of the addition of gastric juice, instead of 30 min (Versantvoort conditions), with the aim of mimicking human conditions. This change did not cause any significant difference to the lipolysis extent but, as it is closer to physiological conditions, this modification was maintained in the subsequent digestion experiments.

Effect of intestinal transit time. An increase of intestinal residence time from 2 to 4 h was tested in order to ensure a greater lipolysis extent because higher residence time occurs *in vivo.* Results showed that a slight increase (p>0.05) in the extent of lipid *in vitro* digestion took place: approximately 6% more of TG molecules were hydrolyzed mainly yielding MG and Gol, so releasing potentially absorbable lipid structures (MG, FA). It was also observed that the isomerization of 2-MG

to 1-MG was favoured. Thus, a 4 h-intestinal transit time was performed in the subsequent digestions experiments.

Effect of the addition of lipase to the gastric juice. Although pancreatic lipase is mainly responsible for fat digestion, lipolysis can start in the stomach catalyzed by an acid-stable gastric lipase, which hydrolyzes 5-35% of TG. This partial hydrolysis is believed to be of paramount importance for triggering the subsequent digestion in the small intestine, because gastric lipase promotes the pre-emulsification of lipids by altering the interfacial composition of lipid droplets through newly formed products, mainly DG and FA. Taking into account that human gastric lipase remains active in the duodenum, the performance of the addition of *Aspergillus niger* lipase at 100 U/mL in the gastric juice on fish lipolysis was evaluated at the end of the whole digestion. This fungal lipase was selected because it shows a similar regiospecificity, a wide optimum pH range (2.5-5.5) and resistance against proteases. Data showed that *in vitro* lipolysis extent reached increased noticeably: TG% remaining unhydrolyzed decreased significantly (p<0.05) from 63.0 to 48.9%, in agreement with *in vivo* hydrolytic efficiency attributed to human gastric lipase. Consequently, 1,2-DG%, 2-MG% and Gol% increased in a similar proportion. A significant increase was also observed in the three parameters used to describe lipolysis, being higher in T_{TG}% than in H_L% and L_{BA}%. This indicated that the hydrolysis in the ester bond occurred to a greater extent in TG than in partial glycerides. Thus, like pancreatic lipase, this fungal lipase preferentially attacks the ester bonds of TG than those of partial glycerides.

In accordance to previous studies, higher concentration of *A. niger* lipase (200 U/mL) was also assayed. However, no significant differences were observed. This limited increase in the lipolysis extent regardless of the amount of gastric lipase used could be explained by the hindrance of protonated long chain FA accumulated at the surface of lipid droplets, which could inhibit further lipolysis. Thus, *A. niger* lipase was added at 100 U/mL in the gastric juice for the subsequent experiments.

Effect of food/digestive fluids ratio. A lower food/digestive fluids ratio than that initially proposed was tested, in line with previous *in vitro* digestion studies. The reduction of the sample amount from 9 to 4.5 g provoked a significant (p<0.05) decrease of TG% (≈10%). Likewise, 1,2-DG%, 2-MG% and 1-MG% were reduced due to the improvement of complete hydrolysis of glycerides into Gol and FA.

Effect of the enzymatic composition of the duodenal juice. Different types of duodenal juices, widely varying in ionic composition and type and amount of enzymes added, have been proposed in

literature for *in vitro* digestion models. To investigate the effect of the nature and concentration of the enzymes of duodenal juice on lipid digestion, *in vitro* digestion experiments of 4.5 g of minced fish were carried out in parallel, employing the juice proposed in the original model and 3 different approaches. These differed only in the content of pancreatin and porcine pancreatic lipase. Quantitative data showed that the increase in the concentration of duodenal enzymes did not lead to any significant increase of TG hydrolysis during *in vitro* digestion. This limited impact on lipolysis extent suggested that the amount and type of enzymes present in the originally proposed duodenal juice was already in excess over substrate and that did not require any modification.

Effect of the bile concentration in the bile juice. The amount of bile present in the small intestine fluctuates over digestion time, and this value is also influenced by food composition and by individual characteristics; values ranging from 5 to 15 mM of bile have been found *in vivo*. Thus, the impact of different bile concentrations (0, 7.5, 15.0, 18.75, 30.0 and 60.0 g/L) in the bile juice on the extent of *in vitro* lipolysis was investigated. The use of low bile concentrations (0-18.75 g/L) led to significantly lower (p<0.05) TG% than the use of high ones (30, 60 g/L). This decrease of TG yielded mainly 2-MG and Gol, indicating that lower bile concentration greatly favoured the hydrolysis not only in TG, but also in partial glycerides, especially in DG. These results confirmed the key role of bile salts in controlling lipase activity in *in vitro* static models where digestion products generated are not removed from the media, in contrast with what occurs *in vivo.* Among the bile concentration tested, 18.75 g/L (equivalent to 5 mM in the chyme according to the original model) was selected. Even if this concentration still remains lower than that used in *in vitro* protocols simulating fed state, it can be considered within the physiological range.

Proposed conditions for a higher in vitro lipolysis extent. Considering all the above discussed, an optimized *in vitro* digestion method that allows one to obtain a TG hydrolysis level close to that occurring *in vivo,* was proposed for fish lipid digestion. The modifications to carry out regarding the starting model included: delaying gastric pH acidification (from 30 to 60 min), increasing intestinal transit time (from 2 to 4 h), adding *A. niger* lipase to the gastric juice (100 U/mL), decreasing food/digestive fluids ratio (using 4.5 instead of 9 g of food), and decreasing bile concentration (from 30 to 18.75 g/L). Using these conditions, approximately 95% of TG initially present were hydrolyzed, which is very close to *in vivo* lipid digestion performance. No significant modification of 1,2-DG% was noticed in relation to the starting method, whereas the occurrence of potentially absorbable glycerides (2-MG, 1-MG) increased significantly. Moreover, approximately 44% of the initial TG were completely hydrolyzed, in agreement with that observed *in vivo.*

The parameters H_L%, T_{TG}% and L_{BA}% were 3- or 4-fold higher than that reached with the starting model, pointing out the significant improvement of fish lipid digestion by varying, within a physiological range, the above-mentioned experimental factors.

The holistic view of this ^{1}H NMR study provides information of paramount importance to design sound *in vitro* digestion models to determine the bioaccessibility and bioavailability of lipophilic compounds.

AIM 3: ***In vitro*** **gastrointestinal digestion of oils. Simultaneous study of lipid hydrolysis, oxidation and other reactions taking place during the *in vitro* digestion of oils of vegetable and animal origins, and of other model systems. Influence on their extent of the oil initial oxidation level, of the oil unsaturation degree, of the presence of proteins and of the synthetic antioxidant BHT**

OBJECTIVE 3.1.

To tackle by means of ^{1}H NMR and SPME-GC/MS the chemical reactions taking place during *in vitro* gastrointestinal digestion of non-oxidized and slightly oxidized sunflower oils, as models of omega-6 rich lipids (**Manuscript 7**)

In this Objective 3.1. a deep and complete study about the evolution of fresh and slightly oxidized sunflower oils during *in vitro* digestion was addressed. Changes in the lipolysis degree, lipid composition and oxidation level as a result of digestion process were studied by ^{1}H NMR. For this purpose, 3 quantitative approaches which differed in the way of determining the number of moles of fatty acids (N_{FA}) were used.

- **Approach I**, in which N_{FA} was determined using the area of ^{1}H NMR signal corresponding to methylenic protons located in the α-position in relation to the carbonyl group of acyl groups (AG) and to the carboxylic group of FA (approach proposed in previous Objectives 2.1. and 2.2.).
- **Approach II**, in which N_{FA} was determined using the area of the ^{1}H NMR signal due to methylenic protons in the β-position in relation to the carbonyl group of AG and to the carboxylic group of FA.
- **Approach III**, in which N_{FA} was determined using the area of the ^{1}H NMR signal due to methylic protons of FA and AG.

Thus, several equations were newly developed for the quantification of lipolytic products, of the molar percentages of the several kinds of acyl groups and fatty acids, and of the oxidation compounds present in the starting oil samples and the corresponding digested lipid extracts.

Moreover, the headspace of the oil samples before and after digestion was studied by SPME-GC/MS. For this purpose, mixtures made of the starting oils and juices submitted to digestion conditions in absence of oils, having the same oil:juices proportions as in the digestates, were prepared in order to simulate, to a certain extent, a similar a matrix to that of digestates. This

technique provided information about the volatile secondary oxidation products generated by digestion process and also about other volatile compounds, markers of Maillard-type and esterification reactions.

Information obtained from ^{1}H NMR study

Changes in fresh sunflower oil as a result of in vitro digestion. The ^{1}H NMR study of fresh sunflower oil and the corresponding digested lipid extracts evidenced great changes in relation to the hydrolytic and oxidative status:

- Hydrolytic status: after *in vitro* digestion of non-oxidized sunflower oil, approximately 17% of TG initially present remained intact and ≈30% were completely hydrolyzed to glycerol and fatty acids. Among the partial glycerides generated, 2-MG (≈27%) showed the highest values, followed by 1,2-DG (≈15%), 1-MG (≈10%) and finally 1,3-DG (≈1%). Thus, the lipid digestion performance obtained under *in vitro* conditions was smaller but relatively close to that reported *in vivo.*

- Oxidative status: on the one hand, a decrease in the molar percentage of total unsaturated AG/FA (U%) of 1.5-2.8% after digestion occurred, suggesting that a loss of nutritive value may also occur. However, this decrease was statistically significant (p<0.05) only when using approach I. On the other hand, newly proton signals due to protons located in *(Z,E)*-conjugated dienes supported on chains having also hydroperoxy groups (*(Z,E)*-CD-OOH) appeared, evidencing the generation of this kind of primary oxidation compounds in low amounts (2.2±0.5 mmol/molAG+FA) during *in vitro* digestion of fresh sunflower oil.

Changes in slightly oxidized sunflower oil as a result of in vitro digestion. The ^{1}H NMR study of slightly oxidized sunflower oil and the corresponding digested lipid extracts evidenced great changes in relation to the hydrolytic and oxidative status:

- Hydrolytic status: after *in vitro* digestion of slightly oxidized sunflower oil samples, more TG remained intact than in those of non-oxidized ones (23 *vs* 17%), and lower 2-MG% and Gol% were also found. These results indicated that the higher the oxidation level of the oil sample is, the lower its digestibility. Likewise, lower values for hydrolysis level (H_L%, 54 *vs* 60%), lipid bioaccessibility level (L_{BA}%, 66 *vs* 72%) and degree of triglycerides transformation (T_{TG}%, 77 *vs* 85%) were obtained. Therefore, the presence of oxidation compounds in slightly oxidized sunflower oil seemed to partially inhibit the activity of the lipases used in the *in vitro* digestion model used (which was that previously optimized in Objective 2.3.).

- Oxidative status: regarding the molar percentage of unsaturated AG/FA, it was observed a more noteworthy decrease (2.1-4.6%) in comparison to that found after the digestion of fresh oil. This decrease was statistically significant (p<0.05) using approaches I and II, but not if using approach III for quantification. At any rate, the higher the initial oxidation level of the sample, the higher extent of lipid oxidation during its *in vitro* digestion, which implies the higher degradation of unsaturated acyl groups. Regarding the formation of oxidation products, it must be pointed out that slightly oxidized sunflower oil samples already contained *(Z,E)*- and *(E,E)*-CD-OOH. However, after *in vitro* digestion, the occurrence of *(Z,E)*- and *(E,E)*-conjugated dienic systems supported on chains having also hydroxy groups (CD-OH) was evidenced. Taking into account the concentrations obtained and assuming that CD-OH come from the reduction of CD-OOH, the results suggested that during digestion some of the CD-OOH molecules originally present were reduced to CD-OH, that others remained intact, and also that new ones were generated, although at a lower rate than that of CD-OH formation. As far as aldehydes are concerned, no aldehydic proton signals were detected in the spectral region at 9.2-10.2 ppm of digested lipid extracts spectra, suggesting that if primary oxidation compounds broke down into secondary ones, this took place to such a small extent that it was not detectable by ^{1}H NMR.

Information obtained from SPME-GC/MS study

Volatile markers of lipid oxidation. SPME-GC/MS study of non-digested (mixtures) and digested samples headspaces confirmed that the formation of volatile secondary oxidation compounds coming from DUω-6 AG/FA (linoleic) took place during *in vitro* gastrointestinal digestion. In line with the information obtained from ^{1}H NMR study, a higher generation of these volatile compounds was noticed after digestion of slightly oxidized sunflower oil than of fresh one. Among the volatile aldehydes generated, there were reactive α,β-unsaturated aldehydes, like *(E)*-2-alkenals and 2,4-alkadienals. It is especially worth noting that the abundance of *(E,E)*-2,4-nonadienal was much greater than that of *(Z,E)*- and *(E,E)*-2,4-decadienals, in contrast to what was previously reported during sunflower oil storage or heating at 70ºC and at frying temperatures, where 2,4-alkadienals of 10 carbon atoms were the most abundant ones. However, these reactive aldehydes were present in very low abundances in the headspace of the digestates, thus they might not represent a health risk. It must also be considered that human body contains diverse detoxifying mechanisms in the gut that probably would counteract the adverse effects of these lipid oxidation compounds.

Volatile markers of Maillard-type and esterification reactions. Volatile compounds coming from different chemical reactions, such as Maillard-type and esterification reactions, were also found in the headspace of the digested sunflower oil samples:

- Nitrogenated compounds: most of them were related to the components of digestive juices, among which Maillard reaction precursors, like glucose and proteins (enzymes and others), can be found. Nevertheless, the generation of 2-pentylpyridine during digestion, which is known to come from the reaction of 2,4-decadienals and amino acids, evidenced the occurrence of Maillard-type reactions involving sunflower oil oxidation products.

- Esters: the increase of octanoic acid ethyl ester abundance after *in vitro* digestion proved that esterification reactions also took place, in addition to hydrolysis, oxidation and Maillard-type reactions.

OBJECTIVE 3.2.

To investigate by means of ^{1}H NMR and SPME-GC/MS the chemical reactions taking place during *in vitro* gastrointestinal digestion of non-oxidized and slightly oxidized flaxseed oils, as models of omega-3 rich lipids (**Manuscript 8**)

The aim of this work was to investigate the chemical reactions taking place during *in vitro* digestion of flaxseed oil, as a model of ω-3 rich lipids, paying special attention to lipid oxidation. For this purpose, fresh and slightly oxidized flaxseed oils were submitted to *in vitro* gastrointestinal digestion. The hydrolytic and oxidative status of the starting oil samples and of the digested lipid extracts were studied by ^{1}H NMR. Then, for a more global study of the processes taking place during *in vitro* digestion, the headspace composition of digested flaxseed oil samples and mixtures made of the starting oils and juices submitted to digestion conditions in absence of oils, having the same oil:juices proportions as in the digestates, were studied.

Information obtained from ^{1}H NMR study

Changes undergone by non-oxidized flaxseed oil during in vitro digestion

- Hydrolytic status: quantitative data derived from ^{1}H NMR spectra showed that, during *in vitro* digestion of fresh flaxseed oil, approximately 80% of TG underwent a hydrolysis reaction, 37% of them being partially hydrolyzed to monoglycerides (mainly 2-MG) and 27% completely hydrolyzed to Gol and three FA.

- Oxidative status: lower molar percentages of linolenic and linoleic AG/FA were found in the lipid extracts of digested flaxseed oil than in the starting oil, whereas that of oleic ones remained unchanged. This indicated that not only a potential loss of essential AG/FA might occur under gastrointestinal conditions, but also that those AG/FA showing higher degree of unsaturation would be more affected. The decrease in the molar percentage of total unsaturated AG/FA during *in vitro* digestion of fresh flaxseed oil was statistically significant (p<0.05) and estimated to be ≈4.4%. This degradation of unsaturated chains led to the generation of to *(Z,E)*-CD-OOH, which can be supported either in octadecatrienoates (derived from linolenic AG/FA) or in octadecadienoates (derived from linoleic AG/FA). Their concentration was estimated to be 3.2±0.8 mmol/molAG+FA, which is a rather low value.

Changes undergone by slightly oxidized flaxseed oil during in vitro digestion

- Hydrolytic status: as expected, *in vitro* digestion provoked the hydrolysis of oxidized flaxseed oil, although the extent was somewhat lower than that obtained in fresh flaxseed oil digestates: approximately 6% less TG were hydrolyzed and lower 2-MG% and Gol% were found. As a consequence, lower lipid bioaccessibility was reached.

- Oxidative status: after digestion, the molar percentage of total unsaturated AG/FA markedly decreased from to 88 to 81% approximately, affecting mainly the most unsaturated AG/FA, namely the linolenic group. The degradation undergone by these latter was much higher than in the case of fresh flaxseed oil, indicating that oxidation reactions under digestive conditions took place to a greater extent in oxidized than in non-oxidized samples. With regard to the formation of oxidation products, the starting oxidized oil samples contained *(Z,E)*-CD-OOH at 8.2±0.2 mmol/molAG+FA and also a small amount of monoepoxy-octadecadienoates (5.2±1.8 mmol/molAG+FA) but after *in vitro* digestion, the estimated concentration of *(Z,E)*-CD-OOH was ≈2-fold higher. In addition to them, *(Z,E)*-CD-OH were also formed though in lower amounts. The corresponding *(E,E)* isomers may also be present, although the high overlapping in this spectral region did not allow the proper identification of their characteristic doublet of doublets at 6.18 ppm. It is also especially worth noting the great increase of monoepoxy-octadecadienoates, whose concentration was ≈3-fold higher than before digestion. To the best of our knowledge, this is the first time that the generation of monoepoxides supported on AG/FA during *in vitro* gastrointestinal digestion has been evidenced. Finally the formation of alkanals during *in vitro* digestion of slightly oxidized flaxseed oil was also evidenced by ^{1}H NMR.

Information obtained from SPME-GC/MS study

By means of SPME-GC/MS, volatile secondary oxidation compounds that were in such low concentrations as not to be detectable by ^{1}H NMR were identified in digested samples.

Volatile markers of the occurrence of lipid oxidation and its extent. The number and abundance of aldehydes (typical volatile markers of lipid oxidation) increased after *in vitro* digestion of fresh flaxseed oil. As expected, the highest increases were noticed for compounds arising from linolenic chains: pentanal, *(E)*-2-butenal, *(E)*-2-pentenal, 2,4-hexadienal, and 2,4-heptadienals. The same trend, although more pronounced, was observed during *in vitro* digestion of slightly oxidized samples, especially regarding alkadienals and alkatrienals, like 2,4-heptadienals, 2,4-hexadienal, nonatrienals and decatrienals. It was also particularly worth noting the increase of 4,5-epoxy-2-heptenals, a well-known oxygenated α,β-unsaturated aldehyde derived from 2,4-heptadienals, and

hence, from ω-3 lipids. In addition to the linolenic-derived aldehydes, others derived mainly from linoleic chains (hexanal, *(E)*-2-hexenal, *(E)*-2-heptenal, 2,4-nonadienals, 2,4-decadienals) and oleic ones (octanal, nonanal, *(E)*-2-nonenal) also increased notably their abundances during the digestion of oxidized flaxseed oil samples.

Volatile markers of Maillard-type reactions. Compounds, such as 2-ethylpyridine and 2-pentylpyridine, increased their abundance after digestion. These two nitrogenated compounds have been described as deriving from the reaction of amino acids with 2,4-heptadienals and 2,4-decadienals, respectively. The higher abundances found for 2-ethylpyridine than for 2-pentylpyridine are in agreement with the content of their corresponding precursors in flaxseed oil (linolenic and linoleic chains, respectively).

Several considerations about the obtained results

Differences between the transformations undergone by flaxseed oil and sunflower oil during in vitro digestion

- In relation to the lipolysis degree reached: the results obtained on *in vitro* digestion of flaxseed oil are very similar to those obtained when the *in vitro* digestion of sunflower oil was addressed under the same experimental conditions (see previous Objective 3.1.). However, slightly lower (p>0.05) *in vitro* lipolysis was found in non-oxidized flaxseed than in non-oxidized sunflower oil digestates. This might be explained by several factors, such as oil density or viscosity, that may influence the emulsification of lipids during *in vitro* digestion.

- In relation to the changes observed on the proportions of the several kinds of AG and FA: the *in vitro* digestion of sunflower oil also provoked a decrease of the molar percentage of total unsaturated AG/FA, being more affected those AG/FA showing the highest unsaturation degree (linoleic). However, in flaxseed oil samples this change took place to a higher extent than in sunflower oil, in agreement with the higher tendency to oxidation of the former due to its high content of polyunsaturated ω-3 acyl groups.

- In relation to the oxidation products generated: the generation of *(Z,E)*-CD-OOH was also evidenced by ^{1}H NMR in fresh sunflower oil digestates, although in a lower concentration than that found in the digested fresh flaxseed oil, in line with the previously-commented lower decrease of the molar percentage of total unsaturated AG/FA in the former. Regarding, slightly oxidized sunflower oil digestates, in addition to the above-mentioned compounds, the formation of *(Z,E)*- and *(E,E)*-CD-OH was evidenced by ^{1}H NMR, but not that of alkanals or of

epoxides as took place during *in vitro* digestion of slightly oxidized flaxseed oil. Likewise, no oxygenated α,β-unsaturated aldehydes were detected by SPME-GC/MS in the headspace of digested sunflower oil samples, whereas in the headspace of slightly oxidized flaxseed oil digestates, 4,5-epoxy-2-heptenals were found. Therefore, under digestive conditions lipids rich in polyunsaturated ω-3 acyl groups underwent a greater oxidation than those rich in ω-6 ones, generating oxidation products not only more potentially reactive, but also of different natures.

***Some remarks on the selection of markers to study the occurrence and extension of lipid oxidation*.** This work evidenced that not only hydroperoxides or conjugated dienic systems could be useful as lipid oxidation markers under gastrointestinal conditions, but so could epoxides, especially in the case of oils rich in polyunsaturated ω-3 acyl groups. Quantitative data obtained suggested that epoxides were generated at a higher rate than CD-OOH, which could suggest a possible simultaneous generation. Moreover, it was proved that hydroperoxides and epoxides might be more suitable markers than aldehydes to assess the occurrence and extent of lipid oxidation under gastrointestinal conditions. In this sense, the analysis of only one kind of oxidation product might poorly reflect and could even underestimate the extent of oxidation reactions during digestion. Thus, caution should be taken when selecting oxidation compounds target of analysis in digestion studies, because other lipid oxidation markers not considered to date could be more appropriate than those currently employed.

Potential toxicological and nutritional consequences derived from lipid oxidation during digestion

- Toxicological implications: regarding the intake of non-oxidized lipids is concerned, these can be expected to be rather limited for several reasons: i) the low amount of potentially toxic oxidation products that would be generated during digestion; ii) the potential occurrence of "detoxification reactions" through Maillard-type reactions with other food components, thus limiting the bioaccessibility of oxidation products; and iii) the presence of several detoxifying enzymes in the gut (gastrointestinal tract immune system) that limit the increase of *in vivo* oxidative stress. By contrast, regarding the intake of partially oxidized lipids, this should be avoided because the additional oxidative degradation taking place during digestion can yield to higher amounts of potentially toxic oxidation products of different nature.

- From a nutritional point of view: the greatly decreased polyunsaturated ω-3 AG/FA, as well as the reduced lipid digestibility during digestion of oxidized samples in relation to the non-oxidized ones, should be taken into consideration. Reactions between lipases and reactive oxidation products might impair the biological functionality of the former. Therefore, reusing

frying fats and oils, which is a common practice not only at household level but also at industrial one, should be avoided as much as possible due to the resulting increase of the oxidative status of the cooking oil and of the cooked food lipid, which take part in migration processes during food frying.

OBJECTIVE 3.3.

To study by means of ^{1}H NMR and SPME-GC/MS the potential effect of protein (ovalbumin/soy protein isolate) on the chemical reactions taking place during *in vitro* gastrointestinal digestion of slightly oxidized sunflower and flaxseed oils (**Manuscript 9**)

To further deepen knowledge about the potential influence of other food bolus components on the transformations undergone by lipids during digestion, this work addressed the effect of two different kinds of proteins widely employed as ingredients in many food formulations during *in vitro* gastrointestinal digestion of sunflower and flaxseed oils. Instead of fresh oils, slightly oxidized samples were selected because lipid oxidation advances to a greater extent during their *in vitro* digestion, which would contribute to clarifying the possible effect of protein on the chemical reactions taking place. As in Objectives 3.1. and 3.2., ^{1}H NMR and SPME-GC/MS were the techniques employed to obtain a global view of the changes in the extent of lipid hydrolysis and oxidation occurring during the digestion process.

Differences in the extent of lipolysis reached during *in vitro* digestion of slightly oxidized oils due to the presence of protein

The presence of ovalbumin or soy protein isolate proteins during *in vitro* digestion of slightly oxidized oils provoked a significant increase in the hydrolysis of TG into 1,2-DG; in addition, a higher proportion of 2-MG was observed. The decrease of TG% was ≈11-13% for sunflower oil samples, and a bit higher for flaxseed oil samples ≈15-18%. Nevertheless, oil+protein digestates showed lower 1-MG% and Gol% in comparison with oil digestates, indicating that isomerization reactions of 2-MG during digestion are slightly limited, perhaps due to their buffer capacity of these two proteins or their hydrolysates; and that a preferential hydrolysis of AG supported in TG over those supported in DG and MG took place.

Thus, lipolysis in the presence of proteins would be favoured at the beginning of digestion process, when the less polar glycerides (TG and DG) are the most abundant, over that of subsequent stages, when more polar structures with a greater tendency towards emulsification (MG and FA) are present in the media. Indeed, no significant differences were observed regarding lipid bioaccessibility (L_{BA} parameter) between oil digestates and oil+protein digestates.

Differences in the extent of lipid oxidation during *in vitro* digestion of slightly oxidized oils due to the presence of protein as seen by ^{1}H NMR

The occurrence of lipid oxidation during digestion was studied by monitoring the changes in the proportions of the several kinds of AG/FA and the generation of oxidation products of very different natures.

Changes in the proportions of the several kinds of AG/FA. In the presence of protein, the degradation of highly unsaturated AG/FA was reduced, although not avoided. This indicated that lipid oxidation also took place during digestion but to a lesser extent than in the absence of proteins. The molar percentage of linoleic AG/FA in the lipid extracts of sunflower oil+protein digestates was slightly higher (≈0.9%) than in those of sunflower oil digestates. In the case of flaxseed oil samples, the lower degradation of unsaturated AG/AF provoked by the presence of proteins is much more remarkable (p<0.05). The lipid extracts of flaxseed oil+protein digestates contained ≈3.7% more linolenic chains than those of flaxseed oil digestates, and ≈3.6% less of saturated plus modificated AG/FA. Therefore, the intake of unsaturated lipids together with proteins would be more suitable from a nutritional point of view, since it would minimize the loss of the nutritive value of ω-6 and ω-3 lipids.

Generation of oxidation products during in vitro digestion. The starting oil samples were already slightly oxidized: both slightly oxidized sunflower and flaxseed oil samples contained a certain amount of *(Z,E)*- and *(E,E)*-CD-OOH. In addition to these compounds, flaxseed oil samples also contained a small amount of monoepoxy-octadecadienoates. In line with that observed in previous Objectives 3.1. and 3.2., *in vitro* digestion of these starting oils in the absence of proteins provoked an increase in their lipid oxidative status.

- Regarding sunflower oil samples: the estimated concentration of total conjugated dienic systems significantly (p<0.05) increased from 14.0 to 17.3 mmol/molAG+FA, due not only to the generation of *(Z,E)*- and *(E,E)*-CD-OH but also to that of CD-OOH.

- Regarding flaxseed oil samples: the occurrence of new oxidation compounds under digestion conditions was even more evident than in sunflower oil samples. The estimated total amount of conjugated dienic structures supported in octadeca-tri/di-enoates associated with either hydroperoxy or hydroxy groups increased from 13.9 to 39.6 mmol/molAG+FA after digestion. Moreover, the estimated concentration of monoepoxides was 3-fold higher than before digestion and a very small amount of alkanals (0.7 mmol/molAG+FA) was generated.

Furthermore, the occurrence of other oxidations products cannot be discarded. Proton signals newly detected in the spectra of the lipid extracts of digested flaxseed oil samples could be related to diepoxides or triepoxides derived from linolenic or linoleic chains, according to previous studies.

During *in vitro* digestion of oil+protein systems, clear differences were detected in the nature and amount of the oxidation compounds generated not only in relation to the starting oil, but also in relation to the oil digestates in the absence of protein. One of the main differences observed was that reduction of hydroperoxides to hydroxides took place.

- Regarding sunflower oil+protein samples: after digestion, the total amount of conjugated dienic structures remained almost unchanged or slightly increased in comparison to the starting oil sample. But comparing to the digestates of sunflower oil in the absence of protein, this concentration is significantly (p<0.05) lower. It was observed that most CD-OOH originally present in the starting oil were reduced to less reactive and more stable CD-OH during digestion of oil+protein systems. Moreover, no *(E,E)*-CD-OOH were detected in the lipid extracts of sunflower oil+protein digestates, highlighting their total reduction to hydroxides during digestion.

- Regarding flaxseed oil+protein samples: in comparison with the starting oil, an increase of the total amount of conjugated dienic structures, monoepoxides and alkanals was observed, indicating a higher lipid degradation level after than before digestion (much more evident than in the case of sunflower oil+protein samples). Nonetheless, as in the case of sunflower oil+protein digestates, the advance of lipid oxidation occurred to a much lesser extent than during *in vitro* digestion of the oil alone: significantly *(p*<0.05) lower concentrations were found of Total CD, monoepoxides and alkanals than in the lipid extracts of flaxseed oil digested alone were found. These results totally agree with those previously commented on the lower degradation of linolenic and linoleic groups during digestion of flaxseed oil+protein systems than during that of flaxseed oil. Furthermore, as in the case of sunflower oil+protein digetates, all the CD-OOH originally present in the starting flaxseed oils were reduced to *(Z,E)*- and *(E,E)*-CD-OH and only a small amount of *(Z,E)*-CD-OOH was newly formed. Likewise, no *(E,E)*-CD-OOH were detected, confirming their total reduction to hydroxides.

In summary, these results suggested the **potential antioxidant activity** of amino acids/peptides released from ovalbumin and soy protein isolate under gastrointestinal digestive conditions, being the reduction of hydroperoxides to more stable hydroxides one of the possible mechanisms for delaying lipid oxidation. Although in general no significant differences were

observed between the effect of both kinds of proteins, quantitative data obtained indicated that during digestion ovalbumin provoked a slightly higher delay of lipid oxidation than soy protein isolate; the lowest decrease of the unsaturation degree and the lowest generation of total conjugated dienic systems occurred in the oil+ovalbumin digestates.

Differences in the extent of lipid oxidation during *in vitro* digestion of slightly oxidized oils due to the presence of protein as seen by SPME-GC/MS

The headspace composition of oil and oil+protein digestates were analyzed by SPME-GC/MS and compared in order to study in a more global way lipid oxidation process during digestion in the absence and presence of protein. The information obtained confirmed that amino acids/peptides released during digestion showed antioxidant properties, affecting not only the extent of lipid oxidation, but also reactions pathways.

Differences on the occurrence of aldehydes. Alkanals, alkenals, alkadienals, alkatrienals and oxygenated α,β-unsaturated aldehydes presented higher abundances in the headspace of oil digestates than in that of oil+protein digestates. This lower bioaccessibility of potentially toxic aldehydes after digestion of oils in the presence of proteins could be explained not only by a lower advance of oxidation extent, but also by the potential reaction of aldehydes with amine groups or amino acid side chains of hydrolyzed ovalbumin and soy protein isolate.

Differences on the occurrence of ketones and alcohols. Unsaturated ketones and alcohols also showed higher abundances in the headspace of oil digestates than in that of oil+protein digestates. Since alcohols might be less reactive towards amino acids and peptides than aldehydes, their lower abundance might confirm in a more sound way that a lower advance of lipid oxidation occurred in the presence of protein. Nonetheless, it could be observed that saturated ketones were found in higher abundance in the headspace of oil+protein digestates than in those of oil digestates. This suggested that, in the presence of protein, different oxidation pathways took place.

Differences on the occurrence of furan and its derivatives. Higher abundances of 2-ethylfuran and 2-pentylfuran were found in the headspace of oil+protein digestates than in that of oil digestates. Although it is well-known that these two alkylfurans can be formed directly from linolenic and linoleic AG/FA oxidation respectively, their formation was enhanced in the presence of protein hydrolysates.

Selection of markers of lipid oxidation during digestion, a critical issue

This study clearly evidenced that, depending on the oxidative conditions, the nature of the lipids involved and the presence of other food components, lipid oxidation evolves in a different way, giving rise to very different kinds of oxidation products. Hence, the selection of markers to determine the extent of this process under digestive conditions is a crucial issue to avoid erroneous conclusions. It was shown that the lipid oxidation level of slightly oxidized flaxseed oil+protein digestates was higher than that of the starting flaxseed oil because higher concentrations of hydroxides, epoxides and alkanals were found in the former. If oxidative status was assessed by means of peroxide value, which measures only lipid hydroperoxides, it would have been concluded that flaxseed oil+protein digestates had a smaller lipid oxidation level than the starting oil. Thus, in the presence of proteins, hydroxides are more suitable oxidation markers than hydroperoxides in assessing lipid oxidation during digestion. Thus, the best option is to use innovative techniques like ^{1}HNMR that, without sample modification, allows the simultaneous determination of a wide range of lipid oxidation products, providing as global as possible a view of the real oxidative status of the sample.

OBJECTIVE 3.4.

To review the available literature on the synthetic antioxidant 2,6-di-*tert*-butylhydroxytoluene (BHT) and its metabolites (**Manuscript 10**)

BHT is a synthetic phenolic antioxidant which has been widely used as an additive in the food, cosmetic, and plastic industries for the last 70 years. Although it is considered safe for human health at authorized levels, its ubiquitous presence and the controversial toxicological data reported are of great concern for consumers. In recent years, special attention has been paid to these 14 metabolites or degradation products: BHT-CH_2OH, BHT-CHO, BHT-COOH, BHT-Q, BHT-QM, DBP, BHT-OH, BHT-OOH, TBP, BHQ, BHT-OH(t), BHT-OH(t)QM, 2-BHT, and 2-BHT-QM. These derived compounds could pose a human health risk from a food safety point of view, but they have been little studied. In this context, this review dealt with the occurrence, origin, and fate of BHT in foodstuffs, its biotransformation into metabolites, their toxicological implications, their antioxidant and pro-oxidant properties, the analytical determination of metabolites in foods, and human dietary exposure. Moreover, non-controlled additional sources of exposure to BHT and its metabolites were highlighted. These include their carry-over from feed to fish, poultry and eggs, their presence in smoke flavourings, their migration from plastic pipelines and packaging to water and food, and their presence in natural environments, from which they can reach the food chain.

Occurrence of BHT and its origin

BHT is one of the most commonly employed food antioxidants and its use in Europe is restricted to different dosages depending on the food involved. Regarding fats and oils for the professional manufacture of heat-treated foods, in frying oil and frying fat (excluding olive pomace oil), in lard, in fish oil, and in beef, poultry, and sheep fats, it can be added singly or in combination with gallates, BHA, or TBHQ in amounts of up to 100 mg/kg. In addition, BHT may also be added to animal feeds, food packaging materials, pharmaceuticals, pesticides, rubbers, plastic pipelines, biodiesel fuel, lubricants, paints and inks, personal care products, and cosmetics as a stabilizer or anti-skinning agent.

BHT and its metabolites as antioxidants or pro-oxidants

Antioxidant activity. BHT possesses a labile hydrogen atom in the hydroxy group that can be donated and reduce the free radicals. Thus, BHT itself is oxidized and the subsequent derived radical is stabilized by electronic delocalization in the benzene ring. This way, BHT can stop radical oxidation

propagation, retarding lipid oxidation. Concerning BHT-metabolites, BHT-OOH, BHT-CHO, and BHT-Q have shown lower antioxidant activity at 70ºC than BHT.

Pro-oxidant activity. There is very limited information available concerning the conditions and the mechanisms under which BHT exerts a pro-oxidant behaviour. At high aeration rate, BHT could react with molecular oxygen rather than with the reactive oxygen species present, yielding BHT-phenoxyl radical and superoxide. In addition, the phenolic radical itself may undergo redox recycling, which can be a critical factor depending on the reductant involved. However, BHT-phenoxyl radical has been reported to be relatively stable. Furthermore, BHT-metabolites, such as BHT-Q and BHT-QM, could also act as pro-oxidants

Fate of BHT in foods

Only a few studies have addressed the fate of BHT in foods during processing at high temperatures. Under frying conditions, the loss of BHT might be very fast and their activity very low, which has been attributed not only to volatilization and steam distillation caused by the water boiled out of the cooked food, but also to rapid degradation. Concerning *volatilization*, BHT has shown higher volatilization at 110 and 185ºC than other phenolic antioxidants like BHA, TBHQ and propyl-gallate. Regarding *transformation*, BHT may react directly with oxygen giving rise to BHT-OOH as the main oxidation product, which is unstable and, in turn, could generate other metabolites, among which BHT-CHO, BHT-OH, and BHT-Q. Another study reported the degradation at 185ºC of pure BHT into 5-methyl-7-*tert*-butyl-2,2-dimethyl-2,3-dihydrobenzo(b)furan, 2,6-bis(1,1-dimethylethyl)-4-methyl-1-methoxybenzene, TBP, and 2 dimeric derivatives (one of them, 2-BHT). In turn, 2-BHT oxidation can give rise to 2-BHT-QM, a stilbenequinone derivative.

It also has been proved that the compounds generated from BHT during food processing can vary on the food nature in which it is contained and on the heating conditions. Likewise, the concomitant administration of BHA has shown to vary BHT-oxidation pathways and also *in vivo*, leading to different kinds of metabolites (BHT-QM and 2-BHT-QM).

Biotransformation of BHT

The metabolism of BHT is very complex and it has been investigated in different animal species and also in humans. The nature and concentration of intermediate metabolites identified depend on the animal species.

Digestion. Changes undergone during *in vivo* digestion have not been studied, but under *in vitro* gastrointestinal conditions, BHT and its toxic metabolite BHT-QM remained bioaccessible.

Absorption and distribution. A rapid absorption from the gastrointestinal tract and subsequent distribution to the liver and body fat has been observed. Its distribution to other organs, such as stomach, intestines, gall bladder, urinary bladder, kidney, spleen and salivary gland has also been proved in mice.

Metabolism. The major route of BHT degradation is oxidation catalyzed by cytochrome P450 and two major metabolic routes occur:

- Oxidation of alkyl substituents: BHT can show oxidation in the *p*-methyl group and/or in one or both of the *tert*-butyl groups; in humans, this latter predominates. When *oxidation of the p-methyl group* takes place, BHT-COOH (generated from BHT-CHO and BHT-CH_2OH) may be the main metabolite. Further metabolism of BHT-COOH in rat liver can lead to DBP, BHQ and BHT-Q. In the case of *tert-butyl groups oxidation*, BHT-OH(t) and its derivative BHT-OH(t)QM are formed.

- Oxidation of the aromatic ring: among others, BHT-Q and BHT-OOH are formed, which in turn can generate BHT-QM. Generation of BHT-QM has not been proved in humans.

Excretion. BHT and its metabolites are excreted in man mainly in the urine whereas in rodents 50-80% is eliminated in the feces. The major metabolites indentified in human, rat and mice have been BHT-COOH and its ester glucuronide.

***Accumulation*.** Presence of BHT in fat, skin, liver, viscera and edible portions including eggs have been found in several animals fed with BHT. With regard to humans, it has been estimated that the bioconcentration factor of BHT in human adipose tissue is around 45 times higher than that calculated for rats.

Toxicological implications of BHT and its metabolites

Due to the widespread use of BHT in foods and in many other products in the last 70 years, and to the consequent human long-term exposure, a great number of toxicity studies have been carried out in several animal species in order to assess its safety. However, extrapolation to humans is difficult because the metabolites generated could be different and the conditions of exposure may not mimic those occurring in humans. Furthermore, controversial results regarding the toxicity of BHT and its metabolites can be found: beneficial, deleterious and even no effects on laboratory

animals have been attributed to it. With regard to humans, there is an evident lack of studies which relate BHT intake to disease. A prospective cohort study carried out in The Netherlands found no association between the consumption of mayonnaise and creamy salad dressings with BHT and stomach cancer risk. Even a statistically non-significant decrease in stomach cancer risk was observed with increasing BHT intake, although the intake of other food products containing BHT was not considered.

Acute oral toxicity of BHT has been considered low in animals. Regarding *short-term subchronic toxicity* studies, inconsistencies in the findings have been obtained. Likewise, BHT does not represent a *genotoxic risk*, because most of the studies carried out to that date had shown BHT was not able to induce mutations or to damage deoxyribonucleic acid (DNA). Nevertheless, ability to cause DNA cleavage has been attributed to BHT-Q, BHT-CHO and BHT-OOH. However, carcinogenesis risk and cell apoptosis would be dependent on the intensity of the damage and the ability of the cell to repair it. The Panel on Food Additives and Nutrient Sources Added to Food of the European Food Safety Authority (EFSA) recognized in 2012 that these positive genotoxicity results may be due to the pro-oxidative chemistry of BHT. As far as *carcinogenicity* and *chronic toxicity* of BHT and its metabolites in rodents, contradictory results have also been reported. Several studies have demonstrated the potential of BHT to act either as a tumor promotor or as a tumor suppressor, modulating the carcinogenicity of some well-known carcinogens. It must be pointed out that to date BHT is classified in group 3 of carcinogens (*not classifiable as to its carcinogenicity to human*s) by the International Agency for Research on Cancer (IARC).

Regarding toxic effects of BHT metabolites, very few studies have been carried out. Quinone methide derivatives may form adducts with several proteins, including enzymes that protect cells from oxidative stress. Among these, BHT-QM is considered to play a significant role in hepatoxicity, pneumotoxity and skin tumor promotion in mice, and BHT-OH(t)QM, which is chemically more reactive than BHT-QM, has been recognized as the principal metabolite responsible for lung tumor promotion activity of BHT in mice.

Dietary exposure studies and established limits

The Panel on Food Additives and Nutrient Sources Added to Food of EFSA established an acceptable dietary intake (ADI) of 0-0.25 mg/kg body weight/day in 2012. Dietary intake studies carried out to date have shown that BHT exposure is unlikely to exceed the current ADI of 0-0.25 mg/kg body weight/day. However, some exceptions can be found in children. In addition, it must be noted that the selection of food categories containing BHT and the methodological approaches were

very different. In most studies, the estimates were based on the distribution of food intakes observed in dietary surveys, assuming BHT to be present at the maximum permitted levels (MPLs) in all foods in which it is authorized. Other less common options were the use of concentration data of BHT in foods provided by the food industry or analytically determined. An over-estimation could be derived from the consideration of MPLs of BHT in authorized food products, as well as under-estimation due to the fact that the foodstuffs in which BHT is not allowed but might be present are not considered.

Additional sources of exposure and regulations

Due to the variety of uses of BHT and its ubiquitous presence, there are other additional sources of exposure that should also be taken into account, such as the carry-over from animal feed to food, its presence in smoke flavourings, its migration from plastic pipelines and packaging to drinking water and other foodstuffs, or its presence in the natural environments from which it can reach the food chain.

Analytical determination of BHT metabolites in foods

Not only are the techniques and the ranges of abundance of the metabolites very varied, but so are the matrixes under study, with water being the most frequently examined and with GC/MS being the most widely used analytical technique.

OBJECTIVE 3.5.

To investigate by means of ^{1}H NMR and SPME-GC/MS the hydrolysis and oxidation reactions affecting polyunsaturated acyl groups and naturally present vitamin A during *in vitro* gastrointestinal digestion of cod liver oil, and the potential effect of low and high concentrations of added BHT (20/ 800 ppm) on the above-mentioned reactions (**Manuscript 11**)

The present work studied in depth and for the first time the *in vitro* gastrointestinal digestion of cod liver oil by means of ^{1}H NMR and SPME-GC/MS. The occurrence of hydrolysis and oxidation reactions, affecting polyunsaturated acyl groups and naturally present vitamin A, were subject of study, as well as the specific nature of the oxidation products generated. In addition, the effect of the addition of the synthetic antioxidant BHT at 20 and 800 ppm on the above-mentioned reactions was tackled. For this purpose, cod liver oil samples, either enriched or not with BHT at 20 and 800 ppm, were submitted to *in vitro* digestion. Since from a chemical point of view, any antioxidant could also be able to exert a pro-oxidant activity, concentrations of BHT either lower or far beyond that permitted by European authorities (100 ppm) were employed. Likewise, special attention was paid to the occurrence of compounds arising from the oxidation of BHT itself.

Characteristics of the starting oil samples

Hydrolysis level. The commercial cod liver oil subject of study was mainly made up of TG (97%), 1,2-DG and Gol. As expected, the BHT-enriched samples showed the same molar percentages of the different kinds of glycerides present than the non-enriched one.

Composition in main and minor lipidic components. The molar percentage of the several kinds of acyl groups of non-enriched and BHT-enriched cod liver oil samples was: 74.8±0.4% of total unsaturated acyl groups and 25.2±0.4% of saturated acyl groups; the former consisting of 40.1±0.8% of monounsaturated (mainly oleic), 25.8±0.0% of polyunsaturated ω-3 (which included 9.1±0.2% of docosahexaenoic and 9.8±0.3% of eicosapentaenoic acyl groups), 8.5±0.7% of diunsaturated ω-6 (mainly linoleic), and 0.4±0.0% of ω-1 acyl groups.

Moreover, according to the label provided by the manufacturer, cod liver oil also contained as minor components 1078 UI/g of vitamin A and 146 UI/g of vitamin D_3. As far as vitamin A was concerned, proton signals of retinyl esters were detected in the ^{1}H NMR spectra of the starting oils, being their concentration estimated to be 0.4±0.0 mmol/molAG+FA.

Oxidation level. ^{1}H NMR study evidenced that commercial starting cod liver oil used contained a small amount of *(Z,E)*-CD-OOH (1.8±0.1 mmol/molAG+FA). Although this concentration is quite low,

bearing in mind that the cod liver oil acquired was intended for human consumption, special attention should be taken during the processing and storage of fish oils in order to avoid as much as possible the potential intake of oxidized compounds. As expected, no differences in the oxidation level of cod liver oil was noticed after the addition of BHT.

Study of hydrolysis and oxidation of main lipidic components of cod liver oil during *in vitro* digestion and effect of the presence of BHT

Extent of triglycerides hydrolysis. A high lipolysis degree was reached under the *in vitro* gastrointestinal digestion conditions of this study; approximately 75% of TG of non-enriched cod liver underwent an hydrolysis reaction, with Gol, 1,2-DG and 2-MG the main resulting glycerides, generated in similar proportion (≈20-24%). 1-MG and 1,3-DG were also generated, but in much lower proportions. The addition of BHT in the range of concentrations tested did not modify the hydrolysis level reached during *in vitro* digestion.

Occurrence of polyunsaturated acyl groups oxidation. This was proved by means of both ^{1}H NMR and SPME-GC/MS.

- Changes in the unsaturation degree of cod liver oil: a small but significantly (p<0.05) decreased molar percentage of ω-3 AG/FA after *in vitro* digestion of cod liver oil was observed (from 25.8 to 24.7%). However, when BHT was added at 20 and 800 ppm to CLO, no decrease was noticed, indicating that, at the concentrations assayed, BHT slowed down the advance of oxidation reactions under gastrointestinal conditions, avoiding the degradation of polyunsaturated AG/FA into a degree detectable by ^{1}H NMR.

- Occurrence of primary and secondary oxidation products: ^{1}H NMR study showed that during *in vitro* digestion of non-enriched cod liver oil primary (*(Z,E)*-CD-OOH) and several secondary oxidation products (monoepoxides derived from polyunsaturated ω-3 AG/FA, 4-hydroxy-*(E)*-2-alkenals, 4-hydroperoxy-*(E)*-2-alkenals and *(Z,E)*-2,4-alkadienals) were generated. This was the first time that the generation of oxygenated α,β-unsaturated aldehydes containing an hydroperoxy group during fish oil digestion was evidenced. The addition of BHT at 20 and 800 ppm clearly limited the generation of the above-mentioned compounds. In the digestates of samples enriched with BHT at 20 ppm, only a non-significant increase of *(Z,E)*-CD-OOH and a small generation of epoxides was observed. In samples enriched with BHT at 800 ppm, the formation of primary and secondary oxidation products was totally inhibited.

  SPME-GC/MS study showed that *in vitro* digestion of non-enriched cod liver oil provoked a marked increase in the number and abundance of acids, esters, alcohols, aldehydes ketones

and furan derivatives. These included volatile markers of *diunsaturated ω-6 AG/FA* (mainly linoleic) and of *polyunsaturated ω-3 AG/FA*, being the formation of these latter much more pronounced than that of the former. It was very worth noting the generation of ω-3 lipids-derived reactive aldehydes, such as 2,4,6-nonatrienals, 2,4,7-decatrienals and oxygenated α,β-unsaturated aldehydes (4-hydroxy-*(E)*-2-hexenal, 4-oxo-*(E)*-2-hexenal and 4,5-epoxy-2-heptenals), although the abundances detected in the headspace were very low in comparison to that of alkanals and *(E)*-2-alkenals. Thus, it could be thought that oxygenated α,β-unsaturated aldehydes present were mainly supported in truncated FA or AG, and thus their molecular weight is too high to be volatile. With regard to the effect of BHT, the abundances of esters, alcohols, aldehydes, ketones, and furans found confirmed that: i) the addition of BHT greatly limited cod liver oil oxidation during digestion; ii) at low concentration of BHT (20 ppm) oxidation reactions still occurred during digestion; and iii) at high concentration of BHT (800 ppm) fish lipid oxidation was almost, but not totally, inhibited, as shown by the slight increase of the abundances of certain volatile markers derived from fish ω-3 and ω-6 lipids.

Study of hydrolysis and oxidation of vitamin A during *in vitro* digestion of cod liver oil and effect of the presence of BHT

Extent of retinyl esters hydrolysis. The results obtained did not allow a proper estimation of the extent of retinyl esters hydrolysis in cod liver oil digestates. The potential occurrence of retinyl esters hydrolysis into retinol could only be deduced by ^{1}H NMR by the decrease of proton signals due to retinyl esters and the appearance of those of retinol. However, the proton signals of these molecules totally overlap, except for one signal that, if present in the ^{1}H NMR spectra of digested lipid extracts, it would overlap with those of TG and 1,2-DG, and as a result it would not be distinguishable. Taking into account that the addition of BHT at the concentrations tested did not affect triglycerides hydrolysis, the same could be expected to occur in the case of vitamin A esters. Thus, if hydrolysis took place, this was only partial and at most affected half of the retinyl esters initially present because they still remained after *in vitro* digestion of samples enriched with BHT at 800 ppm.

Occurrence of vitamin A oxidation and nature of derived oxidation products. The oxidation of this minor lipidic component of cod liver oil under gastrointestinal digestion conditions was evidenced by the two techniques used.

- <u>Under the light of ^{1}H NMR:</u> during *in vitro* digestion of non-enriched cod liver oil, the degradation of vitamin A was evidenced, reaching a concentration below the limit of detection

by ^{1}H NMR. Therefore, the bioaccessibility of vitamin A naturally present in cod liver oil was clearly reduced under gastrointestinal conditions, and the amount present in cod liver oil was not enough to limit oxidation of polyunsaturated ω-3 and ω-6 lipids. Added BHT was not able to withstand a significant loss of vitamin A during digestion; at 20 ppm of BHT, no retinyl ester remained available after *in vitro* digestion and, at 800 ppm of BHT, the concentration of retinyl esters remaining after *in vitro* digestion was estimated to be half of that initially present.

- Under the light of SPME-GC/MS: seven vitamin A oxidation-derived metabolites were detected in the headspaces of digested samples whereas absent before digestion. These were: β-ionone, 2,2,6-trimethylcyclohexanone, β-cyclocitral, *(E)*-5,6-epoxy-β-ionone, ionene, β-homocyclocytral and dihydroactinidiolide. The highest abundances were found in the digestates of non-enriched cod liver oil, followed by those of samples enriched with BHT at 20 ppm, and by those of samples enriched with BHT at 800 ppm. To our knowledge, this was the first evidence of the occurrence of the above-mentioned vitamin A metabolites not only during fish oil oxidation, but also of their generation under mild oxidative conditions such as digestion.

Transformation of BHT during *in vitro* digestion and occurrence of derived-metabolites

The antioxidant activity of BHT involved the oxidation of the compound itself, so a decrease in its abundance after *in vitro* digestion was evidenced by ^{1}H NMR and by SPME-GC/MS. It must be noted that, in the case of samples enriched with BHT at 800 ppm, this decrease was not observed by SPME-GC/MS, which might be due to the saturation of the SPME fibre. Nonetheless, as ^{1}H NMR spectroscopy does not present this limitation, the decrease in BHT abundance after *in vitro* digestion could be quantified by this latter technique and was estimated to be almost 2-fold.

As a result of the degradation of BHT during digestion, the main volatile oxidation products generated were BHT-OH and BHT-Q; the formation of BHT-QM only took place during digestion at high concentrations like 800 ppm. Further research on the other non-volatile metabolites of BHT would be required, considering the abundances of these four metabolites found, which are quite low in comparison with the order of decrease of BHT abundances. These non-volatile oxidation products of BHT, which might be the major ones, cannot be studied by SPME-GC/MS.

AIM 4: ***In vitro*** **gastrointestinal digestion of processed fish. Influence of common technological processess like salting and smoking on the lipids performance under digestive conditions**

OBJECTIVE 4.1.

To investigate by means of ^{1}H NMR and SPME-GC/MS the chemical reactions taking place during *in vitro* gastrointestinal digestion of European sea bass, and the effect of fish salting (brine-salting/dry-salting) on their extent (**Manuscript 12**)

In this work, a study of the various chemical reactions which take place during fish *in vitro* digestion and the potential effect of fish salting on their extent was addressed for the first time. For this purpose, unsalted, brine-salted or dry-salted farmed European sea bass samples were *in vitro* gastrointestinal digested. Fish samples before and after digestion were studied by means of ^{1}H NMR, which is able to provide information about the hydrolysis degree and the nature of primary and secondary lipid oxidation products, and by means of SPME-GC/MS, which is able to detect volatile secondary oxidation products and other markers coming from different processes, like esterifications, Maillard reactions and others.

Information obtained from ^{1}H NMR study

Lipid composition and oxidation status of fish samples before digestion. None of the salting processes performed provoked a lipid oxidation detectable by ^{1}H NMR technique. This was evidenced on the one hand by the lack of changes on the molar percentages of the several kinds of acyl groups in the lipid extracts of sea bass, and on the other hand, by the lack of proton signals related to primary or secondary oxidation compounds in the ^{1}H NMR spectral regions in which could be visible, if present.

Extent of lipolysis reaction during fish in vitro digestion. The molar percentages of fatty acids and acyl groups bounded to the different kinds of glycerides in relation of the total number of moles of fatty acids plus acyl groups present in the lipid extracts of non-digested and digested fish samples were determined by ^{1}H NMR to evaluate the extent of lipid hydrolysis after *in vitro* digestion of unsalted, brine- and dry-salted sea bass. Similar values (p>0.05) were obtained for the three kinds of fish digestates; approximately 95% of triglycerides underwent a hydrolysis reaction and the average

value of fatty acids released was near 62%. These results indicated that neither the salting process nor the intensity of this latter affected the advance of fish lipid hydrolysis.

Generation of lipid oxidation products during fish in vitro digestion. The potential occurrence of lipid oxidation during digestion was evaluated by comparing the spectra of fish lipids extracted before and after digestion. It was evidenced that new signals due to primary oxidation compounds appeared in the spectra of lipid extracts after *in vitro* digestion of unsalted sea bass. These were: signals due to *(Z,E)*-CD-OOH (hydroperoxy-dienes) and, also in very low intensity, signals due to *(Z,E)*-CD-OH (hydroxy-dienes). It must be noted that none of these signals were detected in the spectra of the lipids extracted from the juices submitted to the same digestion process but in the absence of fish, proving that oxidation process took place to a very low extent during *in vitro* digestion of fish. Indeed, these oxidation products are considered to be formed at initial stages of the process. The same kind of lipid oxidation products, and at similar (p>0.05) concentrations, were generated during *in vitro* digestion of unsalted and salted sea bass. Therefore, information provided by ^{1}H NMR suggests that fish salting does not favour oxidation reactions occurring under *in vitro* gastrointestinal conditions.

In this context, it must me noted that the formation of lipid-derived hydroxy-dienes has been previously observed during *in vivo* digestion in rats. The fact that lipid-derived hydroxy-dienes are also formed during *in vitro* digestion would indicate that similar oxidation pathways and derived oxidation compounds are generated in both *in vivo* and *in vitro* systems, corroborating the usefulness of the results obtained using *in vitro* methodologies.

Information obtained from SPME-GC/MS study

Headspace composition of fish samples before digestion. SPME-GC/MS study of unsalted, brine- and dry-salted sea bass headspaces showed that the starting sea bass samples had similarly low oxidation levels, in line with the information extracted from ^{1}H NMR study.

In order to obtain non-digested and digested fish samples showing a similar matrix and with the same lipid content, whose headspace composition could thus be compared, mixtures made of fish samples before digestion and juices submitted to digestive conditions in the absence of food, in the same proportions as in the digestates, were prepared and their headspace studied.

Occurrence of lipid oxidation during in vitro digestion of unsalted sea bass. When comparing the headspace composition of unsalted sea bass digestates and that of mixtures made of unsalted sea bass and juices, a significant increase of typical volatile markers arising from polyunsaturated ω-3

and ω-6 acyl groups oxidation (including alcohols, acids, aldehydes, ketones and furan derivatives) was noted. The main origin of these compounds generated was fish lipids, and not those lipids present in negligible amounts in juices. These results confirm those above-mentioned in the ^{1}H NMR study, this is, lipid oxidation takes place during *in vitro* digestion of sea bass.

Although a great number of α,β-unsaturated aldehydes were detected in the headspace of fish digestates, it must be pointed out that no toxic oxygenated α,β-unsaturated aldehydes, like 4-hydroxy-*(E)*-2-hexenal or 4-hydroxy-*(E)*-2-nonenal, were found. If generated, they would probably have reacted through the oxygenated groups or the double bond with phospholipids, fish proteins or digestive enzymes, yielding Schiff bases, Michael adducts or other derived compounds.

Differences in the oxidation extent reached during in vitro digestion of unsalted and salted fish. A greater degree of oxidation took place under gastrointestinal conditions in the case of salted fish than in that of unsalted ones, leading inevitably to a higher loss of nutritive value of fish lipids and to increased generation of potentially reactive aldehydes. In fact, most of the volatile compounds related to lipid oxidation process presented significantly higher (p<0.05) abundances in the headspace of salted fish than in the unsalted ones, such as 1-hexanol, 1-octen-3-ol, and 5*(Z)*-octa-1,5-dien-3-ol, which could be derived from ω-3 acyl groups oxidation; octanoic acid; almost all the alkanals, *(E)*-2-alkenals and 2,4-alkadienals, being worth noting those above-mentioned coming from ω-3 acyl groups and *(Z)*-4-heptenal, which has been reported to come from 2,6-nonadienal; ketones like 3-octen-2-one, which was only detected in digested salted samples, and 3,5-octadien-2-one, a well-known fish lipid oxidation product; and 2-(2-pentenyl)furan, that can be generated from ω-3 acyl groups.

Influence of fish salting degree on the advance of oxidation reactions during digestion. Samples submitted to the most intense salting process (dry-salting) underwent oxidation to a highest extent during *in vitro* digestion. Although very similar, almost all the alkanals, *(E)*-2-alkenals and ketones identified, showed higher values in the headspace of digested dry-salted and than in those of brine-salted digestates. Moreover, oxidation pathways occurring during *in vitro* digestion of dry-salted samples significantly (p<0.05) favoured the generation of the fish lipid oxidation markers 1-octen-3-ol and hexanal, and also of 2,5-octanedione.

Evolution during in vitro digestion of the abundance of antioxidants present in sea bass. *In vitro* digestion process provoked a very noteworthy decrease in the abundance of the synthetic antioxidant BHT initially present in the farmed sea bass samples acquired. This was explained by its performance as antioxidant during *in vitro* digestion process, corroborating that oxidation reactions

took place during *in vitro* digestion of sea bass and that the amount of this phenolic compound was not able to completely avoid them.

Moreover, four BHT-derived metabolites (also initially present in farmed sea bass meat) were detected in the headspace of sea bass digestates: BHT-Q, BHT-OH, BHT-QM, and BHT-CHO. However, by comparing the abundances of these compounds in the digestates and in the mixtures, it could be evidenced that, during *in vitro* digestion, the abundance of BHT-QM decreased whereas that of BHT-OH, BHT-Q and BHT-CHO increased. These later 3 metabolites have been reported to arise from the hydroperoxy-derivative of BHT, named BHT-OOH. Nonetheless, the formation of other non-volatile BHT metabolites cannot be discarded because the increase in the abundance of these BHT-derived volatile metabolites was very low in relation to the decrease observed in BHT initial abundance.

Occurrence of amino acid degradation. Evidence of Maillard type reactions markers. The occurrence and/or the higher abundance of branched and aromatic aldehydes, sulphur-derivatives, and nitrogenated compounds in digested samples suggested that the *in vitro* digestion process promoted their formation by means of various reactions involving the loss of essential amino acids. *Strecker aldehydes* of methionine, valine, isoleucine, leucine, and phenylalanine were detected, together with some of their *derivatives*, like benzaldehyde and the sulphur compounds methanethiol and dimethylsulfide. It must be noted that sulphur containing compounds were found in very low abundances, primarily because of the low content of methionine and cysteine in sea bass protein (up to 2.7 g/100 g of protein). By contrast, significantly higher (p<0.05) abundances of aromatic aldehydes were detected after *in vitro* digestion, which is in agreement with a previous study carried out by our research group evidencing that during the intestinal step a selective release of aromatic amino acids (or residues) by the proteolytic enzymes takes place. Furthermore, *nitrogen derivatives* typically associated with Maillard reactions were detected in the headspace of sea bass digestates, proving that these reactions take place under gastrointestinal digestive conditions. However, only 2-ethylpyridine could be exclusively attributed to fish origin and not to digestive juice components.

Some of these amino acid-derived compounds were found significantly higher (p<0.05) abundances in the headspaces of digested salted samples than in those of unsalted ones. Results suggested thus that degradation reactions of amino acids occurring during *in vitro* digestion of sea bass may be enhanced by the salting process, although no clear effect of the degree of salting was observed.

Evidence of esterification reactions during in vitro digestion. Esterification reactions between ethanol and octanoic acid were evidenced during *in vitro* digestion of sea bass, being also more pronounced in the case of salted samples.

About the consequences of fish salting from a food safety point of view

Taking into account the abundance of potentially reactive α,β-unsaturated aldehydes, the digestates obtained from unsalted sea bass can be considered the healthiest ones, followed by those obtained from the fish submitted to the lowest salting degree (brine-salting), and finally by those obtained from the fish submitted to the highest salting degree (dry-salting). Nonetheless, as these compounds are present in very low abundances in the headspace of the fish digestates, they might not represent a health risk. It must be considered that human body contains diverse detoxifying mechanisms in the gut that probably would counteract the adverse effects of these lipid oxidation compounds. Special attention must be paid to the effect of salting on fish species with a higher fat content than European sea bass, like salmon, sardine, tuna or herring, because a higher level of oxidation during digestion can be expected.

OBJECTIVE 4.2.

To study by means of ^{1}H NMR and SPME-GC/MS the effect of smoking with two liquid smoke flavourings on lipid hydrolysis and oxidation occurring during *in vitro* gastrointestinal digestion of European sea bass, and to evaluate to what extent the flavouring composition may influence these reactions (**Manuscript 13**)

This Objective 4.2. aimed to provide a global view of how the mechanisms and extents of fish lipid hydrolysis and oxidation processes could be affected, if any, by the presence of smoke-derived compounds showing potential antioxidant activity. In addition, the potential influence of the composition of the liquid smoke flavouring on the delay of lipid oxidation during digestion, if any, was also addressed. For this purpose, farmed sea bass fillets were smoked using two commercial liquid smoke flavourings of known composition, which have been previously used by our research group and differed in their phenolic content (19 g/L and 24 g/L). Afterwards, unsmoked and smoked samples were *in vitro* digested and non-digested and digested samples were studied by ^{1}H NMR and SPME-GC/MS.

In vitro digestion of unsmoked and smoked sea bass studied by ^{1}H NMR

Differences on the lipolysis extent. The extent of lipolysis reaction reached in the three kinds of fish digestates was assessed by estimating the molar percentages molar percentages of fatty acids and acyl groups bounded to the different kinds of glycerides in relation of the total number of moles of fatty acids plus acyl groups present in digested lipid extracts. The results clearly indicated that the smoking process did not provoke any change in the lipid hydrolysis during fish meat *in vitro* digestion. Approximately 94% of TG were transformed into 1,2-DG, MG and glycerol, yielding ≈64% of FA. The estimated average molar percentage of the total chains that would be bioaccessible was near 77%.

Differences on the lipid oxidation extent. The ^{1}H NMR spectra of non-digested and digested fish lipids were compared in order to verify the occurrence of non-overlapped signals in certain spectral regions: 2.6-3.2 ppm, where proton signals due to epoxides appear; 5.9-6.7 ppm, where signals corresponding to protons of conjugated dienes are observable; and 9.2-10.0 ppm, where aldehydic proton signals are visible.

- Before digestion: no signals related to lipid oxidation products were observed in any of the spectra of unsmoked and smoked fish lipid extracts. Only signals near 6.40, 6.57 and at 6.75-6.85 ppm related to aromatic protons of phenolic compounds coming from the smoke

flavourings were visible in the spectra of smoked fish lipid extracts. It must be noted that the intensities of these signals were higher in the spectra of lipids extracted from the sea bass fillets smoked using the smoke flavouring B with the highest phenolic content.

- After digestion of unsmoked sea bass: new signals appeared in the spectrum of the lipid extracts of unsmoked sea bass digestates, evidencing the occurrence of *(Z,E)*-CD-OOH and that of *(Z,E)*-CD-OH. The low amounts of primary oxidation compounds formed (3.3±0.9 mmol/molAG+FA) and the absence of other kinds of oxidation products in concentration beyond the limit of detection of ^{1}H NMR indicated that the extent of fish lipid oxidation was rather low.

- After digestion of smoked sea bass: under the same simulated gastrointestinal conditions, smoked sea bass lipids did not generate *(Z,E)*-CD-OOH or *(Z,E)*-CD-OH. Therefore, smoking process provoked a clearly lower oxidation advance in the fish fillets during digestion, exerting some antioxidant activity. Moreover, aromatic protons signals of smoke-derived phenolic components were still detected, even showing slightly higher intensities than before digestion. This could be explained by the bounding of some smoked-derived compounds within sea bass muscle tissue before digestion and their release during the digestion process, resulting in their higher extraction.

In summary, it is evidenced that the smoking process has as its consequence not only the increase of fish meat shelf-life, but also protection against oxidation occurring during digestion, with the subsequent health and nutritional benefits.

In vitro digestion of unsmoked and smoked sea bass studied by SPME-GC/MS

In order to confirm the results obtained from ^{1}H NMR study regarding the oxidation level of unsmoked and smoked sea bass digestates, their headspace was also studied by SPME-GC/MS. This technique, much more sensitive than ^{1}H NMR, can provide an additional approach to the study of lipid oxidation extent during digestion of unsmoked and smoked samples, by comparing the nature and abundance of volatile compounds present in the headspace of the three kinds of digestates.

Differences regarding volatile markers of lipid oxidation. Volatile compounds typically arising from lipid oxidation process (alcohols, aldehydes, ketones and furans) were detected in much higher abundances in the headspace of unsmoked fish digestates than in smoked ones. For instance, significantly higher (p<0.05) abundances were observed for compounds typically derived from the degradation of ω-3 (1-penten-3-ol, 5*(Z)*-octa-1,5-dien-3-ol, pentanal, *(E)*-2-butenal, *(E)*-2-pentenal,

(E,E)-2,4-hexadienal, 2,4-heptadienals, *(E,E)*-2,6-nonadienal, 1-penten-3-one, 3,5-octadien-2-one, 2-ethylfuran and 2-(2-pentenyl)furan), and ω-6 lipids (1-octen-3-ol, *(E)*-2-heptenal, 2,4-decadienals and 2-pentylfuran). Furthermore, no 2,4-alkadienals were found in the headspace of smoked fish digestates. Thus, it is evidenced that polyunsaturated AG/FA of unsmoked sea bass lipids underwent a greater oxidation during *in vitro* digestion than those of smoked sea bass lipids.

Regarding the presence of BHT and derived metabolites. The quantification of BHT abundance in farmed sea bass samples before digestion showed that all the samples contained BHT in a similar order (202.5±24.5*10^6 area counts). However, after digestion, BHT was barely detected in unsmoked sea bass digestates, whereas it occurred in very high abundances in the smoked ones. Taking into account the antioxidant activity of BHT and its absence in both liquid smoke flavourings employed, the results obtained suggested that: i) during *in vitro* digestion unsmoked sea bass lipids underwent oxidation to a higher extent than those from smoked samples, and as consequence, BHT was oxidized and its abundance greatly lowered in unsmoked sea bass digestate; ii) the amount of BHT initially present in raw farmed sea bass was not high enough to avoid fish lipid oxidation during digestion; iii) in smoked samples the oxidation of BHT was hindered by the smoke-derived compounds present in fish fillet and/or by those newly produced in the fish fillet as consequence of the smoking process.

As for BHT metabolites derived from the oxidation of BHT, unsmoked sea bass digestates presented significanty higher (p<0.05) abundances of BHT-OH and BHT-Q in comparison with smoked digestates.

Influence of the liquid smoke flavouring composition on delay of lipid oxidation under gastrointestinal digestive conditions. Abundance data of lipid oxidation-derived volatiles detected in the two kinds of smoked digestates evidenced that the higher the concentration of phenolic compounds in liquid smoke flavouring used, the higher delay of lipid oxidation during *in vitro* digestion is. This fact was not highlighted by ^{1}H NMR study, evidencing thus the limitation of this technique when studying samples showing very low levels of oxidation. In this case, the use of SPME-GC/MS in combination with ^{1}H NMR would be suitable.

Bioaccessibility of smoke-derived compounds with potential antioxidant activity

For the first time the potential bioaccessibility of smoked-derived compounds was highlighted in this study. This might be a subject of great interest from either the nutritional, food safety or human health point of view, bearing in mind the antioxidant activity reported for many of them. Among those detected after *in vitro* gastrointestinal digestion, there were mainly phenol,

methoxyphenol and dimethoxyphenol derivatives, followed by ketones, furan and pyran derivatives, acids and esters, pyridine derivatives and alky-aryl ethers. Most of them came from the liquid smoke flavouring used, although pyridine and furfural derivatives could also be generated through Maillard-type reactions between fish fillet and smoke flavouring components. As expected, the highest abundances of phenols (guaiacol, methylguaiacol, ethylguaiacol, propylguaiacol, syringol, methylsyringol, ethylsyringol, cresol, eugenol, isoeugenol, thymol, etc.) were found in the headspace of digested sea bass smoked using the smoke flavouring showing the higest phenolic content. In addition to them, 5-(hydroxymethyl)-2-furfural (HMF), a typical smoke component with well-known antioxidant ability, was also detected.

In summary, it is evidenced that the smoking process had as its consequence not only the increase of fish meat shelf-life, but also protection against oxidation occurring during digestion, with the subsequent health and nutritional benefits. Among the wide variety of commercial liquid smoke flavourings, the use of those showing higher phenolic content would be more suitable to limit the advance of the potential oxidation reactions taking place in the gastrointestinal tract to a greater extent.

CONCLUSIONS

AIM 1: Fish cooking methods. Effect on lipids and formation of volatile compounds. Influence of fish species and growing conditions

In the **OBJECTIVE 1.1.** (**Manuscript 1**):

1. During fish shallow-frying under domestic conditions, **migration** of fish lipids to culinary oil occurs, as does migration of the oil components to the fish fillet. As consequence, the composition of the **oils used for frying** becomes richer in those acyl groups and minor components that are in higher concentration in fish lipids than in the original oil, while poorer in those acyl groups and minor components that are in smaller concentration in fish lipids than in the original oil. Concerning **fish lipids**, their composition also changes during frying, becoming richer in those acyl groups and minor components that are in higher concentration in the frying oil than in fish lipids, while poorer in those acyl groups and minor components that are in higher concentration in the raw fish lipids than in the original oils.

2. The submission of the oils to the heating conditions of shallow-frying provokes **thermo-oxidation** to a very low extent. However, the number and concentration of secondary oxidation products found in oils after fish frying are lower than those found after heating under the same conditions with the absence of food.

3. The **frying technique**, the nature of the **cooking oil** and the **fish species** have a **great influence** on the **changes** occurring during during fish shallow-frying.

   - **Influence of the frying technique**: The oil uptake usually takes place to a greater extent during microwave-frying than during pan-frying. Heating by microwave provokes a lower thermodegradation than heating in a pan.
   - **Influence of the cooking oil:** The selection of the cooking oil is of paramount importance due to its impact not only on the fish lipid profile of main and minor components, but also on the possible generation of potentially toxic compounds in the oil during frying. As expected, sunflower oil shows a smaller resistance to degradation than extra-virgin olive oil not only during heating but also during fish shallow-frying. No thermo-oxidation was observed in extra-virgin oil used for fish frying, which makes it safer and more suitable than sunflower oil for fish shallow-frying.
   - **Influence of the fish species**: After frying, the lipid content of gilthead sea bream fillets greatly decreased, whereas that of sea bass ones increased or remained almost unchanged, which can be attributed to the higher initial fat content in the former. Moreover, different

oxidation products are generated and thus detected in the fried oils depending on the lipid composition of the fish species.

In the **OBJECTIVES 1.2.** (**Manuscript 2**) and **1.3.** (**Manuscript 3**):

4. ^{1}H NMR study evidenced that boiling, steaming, *sous-vide* cooking, salt-crusted oven baking, conventional oven baking and microwave cooking **do not affect significantly** sea bass lipid composition, including lipidic components especially prone to oxidation like long-chain polyunsaturated ω-3 acyl groups, cholesterol and vitamin A.

5. SPME-GC/MS study evidenced that during the above-mentioned cooking methods sea bass volatile profile is notably enriched in volatile compounds coming from **lipid oxidation** and from **fish nitrogenated components degradation**, including Maillard-type reactions. However, **fish growing conditions** and the **cooking method influenced** qualitatively and quantitatively their generation.

   - A remarkable higher number and abundance of compounds were generated during the cooking of farmed sea bass than of wild samples, due to the significantly higher lipid content of the former. Therefore, the discrimination of sea bass samples considering their volatile profile can be made not only before, but also after cooking.
   - Boiling is the culinary technique that modified to the least extent the initial characteristics of the volatile profile of raw sea bass, which could make it suitable for consumers who do not like fishy aromas.
   - Conventional oven baking enhanced the generation of volatile compounds arising from initial (Strecker aldehydes and derivatives) and advanced (heterocyclic aromatic compounds) stages of Maillard-type reactions to a greater extent than the rest of culinary techniques studied. Pyrroles, alkylpyrazines, alkylthiophenes and 2-ethylpyridine were only detected in conventional oven baked samples.
   - In the case of salt-crusted oven baking, it has been evidenced a degree of protection offered by salt-crust against dehydration, lipid oxidation and degradation of nitrogenated components.

6. ^{1}H NMR and SPME-GC/MS provided **very valuable information** not only from the nutritional point of view, but also from the technological one, helping the food industry to produce *ready-to-eat* fish products that will ensure food quality and safety as much as possible, as well as consumer acceptance.

AIM 2: ***In vitro*** **gastrointestinal digestion of lipids. Development and validation of a new method based on ^{1}H NMR for the study of lipid hydrolysis during digestion. Study in depth of the influence of several factors affecting the extent of *in vitro* lipolysis**

In the **OBJECTIVE 2.1.** (**Manuscript 4**):

1. ^{1}H NMR allows the qualitative and quantitative study of the molar proportions of triglycerides, 1,2- and 1,3-diglycerides, 1- and 2-monoglycerides and fatty acids in complex lipid mixtures. For this purpose different equations based on ^{1}H NMR spectral data have been proposed for the first time.

2. In contrast to other methodologies employed to study lipolysis, this approach allows a global study of the lipid sample, providing simultaneous detailed information on all kinds of compounds present (including positional isomers), in a simple and fast way and without any previous chemical modification of the sample.

In the **OBJECTIVE 2.2.** (**Manuscript 5**):

3. The usefulness of ^{1}H NMR to study the advance of lipid digestion process in real *in vitro* digested samples has been evidenced. Moreover, this new methodology shows a high versatility in assessing the extent of lipolysis reaction in any of its current definitions: hydrolysis level, degree of triglyceride transformation, lipid bioaccessibility and proportion of fatty acids physiologically releasable.

4. During *in vitro* digestion of sunflower oil and fish meat samples, 1,2-diglycerides, 2-monoglycerides, fatty acids and glycerol are the main hydrolysis products arising from triglycerides. However, 1,3-diglycerides and 1-monoglycerides are detected in very low proportions, suggesting the occurrence of isomerization reactions under these conditions. Furthermore, the complete hydrolysis of triglycerides into fatty acids and glycerol under *in vitro* digestion conditions can take place into a remarkable extent, in such a way the occurrence of this phenomenon should not be ruled out in *in vitro* digestion studies.

In the **OBJECTIVE 2.3.** (**Manuscript 6**):

5. Further knowledge on the impact of experimental factors on lipid *in vitro* digestion is of paramount importance in gaining a better insight into the limitations of the protocols currently employed in *in vitro* studies, and thus understanding the results obtained.

6. The addition of gastric lipase, the decrease of food/digestive fluids ratio and the decrease of bile concentration significantly improved the lipolysis level reached with the static digestion model proposed by Versantvoort et al. (2005). Bile concentration was found to be a key factor for controlling *in vitro* lipolysis.

7. With the modifications proposed, a lipolysis degree similar to that reported *in vivo* is reached; approximately 95% of triglycerides undergo hydrolysis reaction and 80% of fish lipids acyl groups are bioaccessible.

8. An accurate match of naturally occurring events is necessary for consistent statements and predictions. Therefore, the optimization of *in vitro* digestion experimental conditions should be carried out for each kind of sample when required, especially in bioaccessibility and bioavailability studies.

AIM 3: ***In vitro*** **gastrointestinal digestion of oils. Simultaneous study of lipid hydrolysis, oxidation and other reactions taking place during the *in vitro* digestion of oils of vegetable and animal origins, and of other model systems. Influence on their extent of the oil initial oxidation level, of the oil unsaturation degree, of the presence of proteins and of the synthetic antioxidant BHT**

In the **OBJECTIVE 3.1.** (**Manuscript 7**):

1. The high reactivity of the gastrointestinal tract chemical environment and the usefulness of innovative techniques, like ^{1}H NMR and SPME-GC/MS, in providing a global view of the phenomena taking place, have been evidenced.

2. During *in vitro* gastrointestinal digestion of sunflower oil not only hydrolysis takes place but also lipid oxidation, Maillard-type and esterification reactions. Nevertheless, their advance is greatly influenced by the initial oxidation level of the oil sample:

   - Lipolysis occurs to a smaller extent during *in vitro* digestion of slightly oxidized sunflower oil samples than of fresh ones.
   - Oxidation reactions take place to a greater extent during *in vitro* digestion slightly oxidized sunflower oil samples than of fresh ones. This is evidenced by a higher decrease of unsaturated acyl groups/fatty acids and by the higher generation of oxidation products, among which *(Z,E)*-hydroperoxy-, and *(Z,E)*- and *(E,E)*-hydroxy-octadecadienoic acids/acyl groups, as well as volatile secondary oxidation compounds typically arising from ω-6 acyl groups degradation.

3. For first time, the formation under gastrointestinal digestive conditions of hydroxy-octadecadienoic acids/acyl groups derived from linoleic chains is evidenced by ^{1}H NMR.

4. The consumption of oxidized oils (even those at the first stages of lipid oxidation) should be avoided as much as possible, because a larger amount of potentially toxic aldehydes is expected to be generated during digestion, remaining thus bioaccessible for absorption or even exerting negative effects in the gastrointestinal tract itself.

In the **OBJECTIVE 3.2.** (**Manuscript 8**):

5. ^{1}H NMR study shows a slightly lower digestibility of oxidized flaxseed oil samples in comparison to non-oxidized ones, although in both cases a high *in vitro* lipolysis degree is reached.

6. Under gastrointestinal digestive conditions, oxidation of flaxseed oil occurs yielding the decrease of unsaturated acyl groups (especially linolenic and linoleic) and the formation of octadecatri/dienoic acids or acyl groups with *(Z,E)*-conjugated dienic systems having also hydroperoxy groups. In the case of slightly oxidized flaxseed oil samples digestion, the decrease of polyunsaturated acyl groups or fatty acids was much more pronounced, yielding in addition to the above-mentioned compounds: epoxides, octadeca-di/tri-enoic acids or acyl groups with *(E,E)*-conjugated dienic systems having also hydroperoxy groups and with *(Z,E)*- and *(E,E)*-conjugated dienic systems having also hydroxyl group, and alkanals in concentrations detectable by ^{1}H NMR.

7. For the first time, the formation of monoepoxy-octadecadienoates derived from linolenic chains under gastrointestinal digestive conditions is evidenced by ^{1}H NMR.

8. SPME-GC/MS technique provided very interesting information about the volatile aldehydes generated during flaxseed oil digestion that could not be detected by ^{1}H NMR because of their occurrence in low amount; among them, potentially reactive aldehydes like *(E)*-2-alkenals, 2,4-alkadienals and alkatrienals, typically arising from ω-3 acyl groups oxidation. In the case of oxidized samples, these compounds were formed in higher abundances than in non-oxidized ones, and the generation of the oxygenated α,β-unsaturated aldehyde 4,5-epoxy-2-heptenal was highlighted.

9. During digestion lipids rich in ω-3 acyl groups (flaxseed oil) undergo a greater oxidation extent than those rich in ω-6 ones (sunflower oil), although the nutritive value loss and the generation of potentially hazardous compounds is greatly dependent on the initial oxidative status of the ingested lipids. Further research is needed on this topic, since the intake of oils rich in ω-3 lipids has been encouraged in recent years due to their potential health benefits.

10. The selection of markers of lipid oxidation occurrence and extent should vary depending on the oxidation process conditions and on food lipid composition. The traditional mechanisms proposed for lipid oxidation might provide a too simplistic view, which does not fit to that taking place along the gastrointestinal tract. Therefore, it is of paramount importance to study each oxidation process in depth in order to properly select the oxidation markers that better

reflect the evolution of each process, and to use techniques that allow the study at once of a broad variety of oxidation markers, including not only conjugated dienes, hydroperoxides, and carbonyl compounds, but also epoxides and hydroxides.

In the **OBJECTIVE 3.3.** (**Manuscript 9**):

11. The food bolus composition influences considerably lipid hydrolysis and oxidation reactions occurring under *in vitro* gastrointestinal conditions.

12. A significantly greater hydrolysis takes place in triglycerides than that occurring in diglycerides and monoglycerides during *in vitro* digestion of slightly oxidized sunflower and flaxseed oil in the presence of proteins (ovalbumin and soy protein isolate) than in their absence.

13. Lipid oxidation occurring during *in vitro* digestion of slightly oxidized sunflower and flaxseed oil occurs to a lesser extent in the presence of ovalbumin and soy protein isolate proteins than in their absence. ^{1}H NMR and SPME-GC/MS study showed a smaller decrease of polyunsaturated acyl groups/ fatty acids and a lower generation of total lipid oxidation products during oils digestion in the presence of proteins.

14. For the first time, the potential antioxidant activity of ovalbumin and soy protein hydrolysates released during *in vitro* digestion is demonstrated by means of spectroscopic techniques, like ^{1}HNMR and SPME-GC/MS, instead of the typical *in vitro* chemical assays.

15. The simultaneous digestion of proteins and lipids not only provokes a decrease of lipid oxidation reactions, but also the occurrence of lipid reduction reactions. ^{1}H NMR study showed that lipid hydroperoxides, in particular *(E,E)*-isomers, were almost totally reduced to more stable hydroxides.

16. Caution must be taken when selecting oxidation compounds target of analysis (markers) to assess lipid oxidation extent, because: i) depending on the oxidative conditions, the kind of lipid involved, and the presence of other food components, the typical mechanism of oxidation process might not properly fit; ii) erroneous conclusions on the oxidative status of a digested sample might be reached when determining the amount of only one or two kinds of lipid oxidation products. Thus, the best option to obtain sound results is to use techniques that allow a global study of multiple lipid oxidation products, in a single run and without chemical transformations of the sample.

In the **OBJECTIVE 3.4.** (**Manuscript 10**):

17. BHT is a synthetic phenolic antioxidant which has been widely used as an additive in the food, animal feed, cosmetic and plastic industries for the last 70 years. Although it is considered safe for human health at authorized levels, ubiquitous presence of BHT, its controversial toxicological data, a lack of information about its true dietary intake can be of great concern for consumers.

18. Increasing knowledge on BHT-derived compounds is needed because some of them (quinone methide derivatives) exert potentially toxic effects.

19. The current extent of human exposure to BHT and its metabolites should be evaluated, not only as a result of their presence in authorized foods, but also as related to other additional sources that reach the food chain, such as carry-over processes from feed to farmed animal products, migration from plastic pipelines and packaging to water and food, and their presence in smoke flavourings and in natural environments.

20. Further research on the possible dual role of BHT as either antioxidant or pro-oxidant is needed, as well as on the conditions and the mechanisms by which BHT acts on foods in these two ways.

In the **OBJECTIVE 3.5.** (**Manuscript 11**):

21. During *in vitro* gastrointestinal digestion cod liver oil oxidation takes place, resulting in the decrease of ω-3 acyl groups/fatty acids and of naturally present vitamin A and the generation of oxidation compounds derived from both.

22. For the first time, the potential formation during cod liver oil digestion of toxic oxygenated α,β-unsaturated aldehydes containing hydroperoxy and hydroxy groups, together with monoepoxides and *(Z,E)*-conjugated dienic systems associated with hydroperoxy groups, has been proved by ^{1}H NMR spectroscopy.

23. SPME-GC/MS confirms the generation of typical reactive aldehydes arising from ω-3 lipids. Moreover, the high sensitivity of this latter technique allowed the identification of other kinds of oxidation compounds not detectable by ^{1}H NMR arising from ω-3 and ω-6 acyl groups/ fatty acids degradation (furan derivatives, alkanals, *(E)*-2-alkenals, alkadienals, alkatrienals and 4,5-epoxy-2-heptenals) and from vitamin A oxidation.

24. The enrichment of cod liver oil with the synthetic antioxidant BHT at 20 or 800 ppm greatly limited the advance of cod liver oil oxidation under gastrointestinal *in vitro* conditions, increasing significantly the bioaccessibility of polyunsaturated lipids and that of vitamin A, and decreasing the generation of the above-mentioned toxic aldehydes.

25. As a result of its antioxidant activity, the oxidation of BHT itself takes place under *in vitro* gastrointestinal digestion, leading to a decrease of its abundance and the occurrence in very low abundances of volatile BHT-oxidation products, among which alcohol (BHT-OH) and quinone (BHT-Q) derivatives can be cited.

26. Caution is needed during cod liver oil supplementation due to the potential intake of oxidized compounds and its further oxidation during digestion. In this sense, the simultaneous intake of other compounds with antioxidant activity would be required in order to increase the bioaccessibility of polyunsaturated acyl groups or fatty acids and of vitamin A, and to decrease or inhibit the formation of toxic oxygenated α,β-unsaturated aldehydes.

AIM 4: ***In vitro*** **gastrointestinal digestion of processed fish. Influence of some technological processes like salting and smoking on the lipids performance under digestive conditions**

In the **OBJECTIVE 4.1.** (**Manuscript 12**):

1. During fish *in vitro* digestion, lipid oxidation occurs to a low extent. ^{1}H NMR evidenced the generation in very low amounts of primary oxidation compounds, such as conjugated dienes supported on chains having also hydroperoxy and hydroxy groups. SPME-GC/MS showed the formation of secondary oxidation compounds of low molecular weight coming from fish unsaturated acyl groups.

2. Moreover, the occurrence of amino acids degradation, Maillard-type reactions between nitrogenated components and lipid oxidation products and esterification reactions is also proved by means of the detection of volatile end-products.

3. The previous salting of fish clearly favours the advance of the above-mentioned chemical reactions during *in vitro* digestion, especially when intense salting processes (dry-salting) are performed. By contrast, no differences are observed between *in vitro* lipolysis of unsalted and salted fish.

4. ^{1}H NMR and SPME-GC/MS provided very useful and complementary information, in a fast way and without any chemical modification of the sample. They enabled a deeper study at a molecular level of the reactions taking place during fish *in vitro* digestion, which is of paramount importance to gain further knowledge concerning the relationships existing between food composition, technological processing and human health. It must be noted that this information about the specific nature of the generated compounds cannot be obtained when traditional methods are used.

In the **OBJECTIVE 4.2.** (**Manuscript 13**):

5. Smoking process does not affect the extent of lipolysis reaction during *in vitro* digestion of sea bass meat.

6. Smoking with liquid smoke flavourings protects fish lipids from oxidative degradation during *in vitro* gastrointestinal digestion. This kind of fish processing inhibits the generation of primary and secondary oxidation compounds arising from polyunsaturated ω-3 and ω-6 lipids that takes place during digestion of unsmoked fish samples.

7. The protective effect of smoking towards fish lipid oxidation under gastrointestinal conditions is also confirmed by the markedly hindrance of the loss of the synthetic antioxidant BHT originally present in farmed sea bass.

8. For the first time, the potential bioaccessibility of smoke flavouring components is proved by means of both ^{1}H NMR and SPME-GC/MS. Among the smoke-derived components remaining bioaccessible after gastrointestinal digestion, there is a great variety of phenolic compounds. This issue is of paramount importance from a health point of view since these compounds could also limit *in vivo* oxidative damage. Further confirmatory *in vivo* studies on this topic would be appropriate.

REFERENCES

Adams, A., Bouckaert, C., Van Lancker, F., De Meulenaer, B., & De Kimpe, N. (2011). Amino acid catalysis of 2-alkylfuran formation from lipid oxidation-derived α,β-unsaturated aldehydes. *Journal of Agricultural and Food Chemistry*, *59*(20), 11058-11062.

Addis, P. B. (1986). Occurrence of lipid oxidation products in foods. *Food and Chemical Toxicology, 24*(10-11), 1021-1030.

Alasalvar, C., Taylor, K. D. A., Zubcov, E., Shahidi, F., & Alexis, M. (2002). Differentiation of cultured and wild sea bass (*Dicentrarchus labrax*): total lipid content, fatty acid and trace mineral composition. *Food Chemistry, 79*(2), 145-150.

Alcicek, Z. (2011). Determination shelf life and PAHs content of smoked anchovy (*Engraulis encrasicolus, Linneaus, 1758*) nugget with different level liquid smoke flavors during chilled storage. *Journal of Animal and Veterinary Advances, 10*(20), 2691-2695.

Allam, S. S. M., & Mohamed, H. M. A. (2002). Thermal stability of some commercial natural and synthetic antioxidants and their mixtures. *Journal of Food Lipids, 9*(4), 277-293.

Allen, J. R. (1976). Long-term antioxidant exposure effects on female primates. *Archives of Environmental & Occupational Health, 31*(1), 47-50.

Al-Akid, Y. F., El-Rahman, A. E. A., Hussein, H. A., & Wassif, G. A. (2001). Nephro- and pneumotoxic response to chronic administration of butylated hydroxytoluene (BHT) in adult albino rats. *Al-Azhar Journal of Pharmaceutical Sciences, 28*, 171-195.

Al-Malaika, S. (2004). Perspectives in stabilisation of polyolefins. In: Albertsson AC, editor. Long-term properties of polyolefins. Berlin: Springer. p 121-50.

Al-Saghir, S., Thurner, K., Wagner, K.-H., Frisch, G., Luf, W., Razzazi-Fazeli, E., & Elmadfa, I. (2004). Effects of different cooking procedures on lipid quality and cholesterol oxidation of farmed salmon fish (*Salmo salar*). *Journal of Agricultural and Food Chemistry*, *52*(16), 5290-5296.

Amato, M. E., Ballistreri, F. P., Pappalardo, A., Tomaselli, G. A., Toscano, R. M., & Sfrazzetto, G. T. (2013). Selective oxidation reactions of natural compounds with hydrogen peroxide mediated by methyltrioxorhenium. *Molecules*, *18*(11), 13754-13768.

Amira, M. B., Hanene, J. H., Madiha, D., Imen, B., Mohamed, H., & Abdelhamid, C. (2010). Effects of frying on the fatty acid composition in farmed and wild gilthead sea bream (*Sparus aurata*). *International Journal of Food Science & Technology*, *45*(1), 113-123.

André, C., Castanheira, I., Cruz, J. M., Paseiro, P., & Sanches-Silva, A. (2010). Analytical strategies to evaluate antioxidants in food: a review. *Trends in Food Science & Technology, 21*(5), 229-246.

Ansorena, D., Gimeno, O., Astiasarán, I., Bello, J. (2001). Analysis of volatile compounds by GC-MS of a dry fermented sausage: chorizo de Pamplona. *Food Research International 34*(1), 67-75.

Armand, M., Pasquier, B., André, M., Borel, P., Senft, M., Peyrot, J., Salducci, J., Portugal, H., Jaussan, V., & Lairon, D. (1999). Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract. *The American Journal of Clinical Nutrition*, *70*(6), 1096-1106.

Aruoma, O. I. (1991). Pro-oxidant properties: an important consideration for food additives and/or nutrient components? In: Aruoma OI, Halliwell B, editors. *Free radicals and food additives*. London: Taylor and Francis Ltd. p 173-94.

Aubourg, S. P., & Ugliano, M. (2002). Effect of brine pre-treatment on lipid stability of frozen horse mackerel (*Trachurus trachurus*). *European Food Research and Technology, 215*(2), 91-95.

Augustin, M. A., & Berry, S. K. (1983). Efficacy of the antioxidants BHA and BHT in palm olein during heating and frying. *Journal of the American Oil Chemists´ Society, 60*(8), 1520-1523.

Awah, F. M., Eyibe, U. N., Ikhelowa, A. O., Anyimigbo, G. C., & Nkuche, B. C. (2012). Protective capabilities of rutin in acute butylated hydroxytoluene-induced oxidative damage in Wistar rats. *International Journal of Pharmaceutical Frontier Research, 2*(2), 12-20.

Babich, H. (1982). Butylated hydroxytoluene (BHT): A review. *Environmental Research, 29*(1), 1-29.

Babu, B., & Wu, J. T. (2008). Production of natural butylated hydroxytoluene as an antioxidant by fresh water phytoplankton. *Journal of Phycology, 44*, 1447-1454.

Bakar, J., Rahimabadi, E. Z., & Che Man, Y. B. (2008). Lipid characteristics in cooked, chill-reheated fillets of Indo-Pacific king mackerel (*Scomberomorous guttatus*). *LWT - Food Science and Technology*, *41*(10), 2144-2150.

Barclay, L. R. C., Xi, F., & Norris, J. Q. (1997). Antioxidant properties of phenolic lignin model compounds. *Journal of Wood Chemistry and Technology*, *17*(1-2), 73-90.

Becker, H. D. (1969). Quinone dehydrogenation. IV. One-electron oxidations with 2,3-dichloro-5,6-dicyanobenzoquinone. *The Journal of Organic Chemistry, 34*(5), 1203-1210.

Belitz, H. D., Grosch, W., & Schieberle, P. Aroma analysis. In *Food Chemistry*, edition no4; Belitz, H. D., Grosch, W., Schieberle, P., Eds; Springer-Verlag: Berlin Heidelberg, Germany, 2009.

Bemrah, N., Leblanc, J. C., & Volatier, J. L. (2008). Assessment of dietary exposure in the French population to 13 selected food colours, preservatives, antioxidants, stabilizers, emulsifiers and sweeteners. *Food Additives & Contaminants. Part B, Surveillance, 1*(1), 2-14.

Bergan, J. G., & Draper, H. H. (1970). Absorption and metabolism of 1-^{14}C-methyl linoleate hydroperoxide. *Lipids*, *5*(12), 976-982.

Bianchi, L., Colivicchi, M. A., Della Corte, L., Valoti, M., Sgaragli, G. P., & Bechi, P. (1997). Measurement of synthetic phenolic antioxidants in human tissues by high-performance liquid chromatography with coulometric electrochemical detection. *Journal of Chromatography B, 694*(2), 359-365.

Björkhem, I., Henriksson-Freyschuss, A., Breuer, O., Diczfalusy, U., Berglund, L., & Henriksson, P. (1991). The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology, 11*, 15-22.

Bläckberg, L., Hernell, O., Bengtsson, G., & Olivecrona, T. (1979). Colipase enhances hydrolysis of dietary triglycerides in the absence of bile salts. *The Journal of Clinical Investigation*, *64*(5), 1303-1308.

Bolton, J. L. , Sevestre, H., Ibe, B. O., & Thompson, J. A. (1990). Formation and reactivity of alternative quinone methides from butylated hydroxytoluene: possible explanation for species-specific pneumotoxicity. *Chemical Research in Toxicology, 3*(1), 65-70.

Bomhard, E. M., Bremmer, J. N., & Herbold, B. A. (1992). Review of the mutagenicity/genotoxicity of butylated hydroxytoluene. *Mutatation Research, 277*(3), 187-200.

Borawska, J., Darewicz, M., Vegarud, G. E., Iwaniak, A., & Minkiewicz, P. (2015). *Ex vivo* digestion of carp muscle tissue--ACE inhibitory and antioxidant activities of the obtained hydrolysates. *Food & Function*, *6*(1), 211-218.

Borgström, B., Tryding, N., & Westöö, G. (1957). On the extent of hydrolysis of triglyceride ester bonds in the lumen of human small intestine during digestion. *Acta Physiologica Scandinava, 40*(2-3), 241-247.

Borgström, B. (1964). Influence of bile salt, pH, and time on the action of pancreatic lipase; physiological implications. *Journal of Lipid Research, 5*, 522-531.

Borgström, B., & Erlanson, C. (1973). Pancreatic lipase and co-lipase. *European Journal of Biochemistry*, *37*(1), 60-68.

Bortolomeazzi, R., Sebastianutto, N., Toniolo, R., & Pizzariello, A. (2007). Comparative evaluation of the antioxidant capacity of smoke flavouring phenols by crocin bleaching inhibition, DPPH radical scavenging and oxidation potential. *Food Chemistry, 100*(4), 1481-1489.

Botterweck, A. A., Verhagen, H., Goldbohm, R. A., Kleinjans, J., & van den Brandt, P. A. (2000). Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands Cohort Study. *Food and Chemical Toxicology, 38*(7), 599-605.

Bower, C. K., Hietala, K. A., Oliveira, A. C. M., & Wu, T. H. (2009). Stabilizing oils from smoked pink salmon (*Oncorhynchus gorbuscha*). *Journal of Food Science, 74*(3), C248-257.

Branen, A. L., Richardson, T., Goel, M. C., & Allen, J. R. (1973). Lipid and enzyme changes in the blood and liver of monkeys given butylated hydroxytoluene and butylated hydroxyanisole. *Food and Cosmetics Toxicology, 11*(5), 797-806.

Branen, A. L. (1975). Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. *Journal of the American Oil Chemists´ Society, 52*(2), 59-63.

Braumann, U., Händel, H., Strohschein, S., Spraul, M., Krack, G., Ecker, R., & Albert, K. (1997). Separation and identification of vitamin A acetate isomers by supercritical fluid chromatography—^{1}H NMR coupling. *Journal of Chromatography A*, *761*(1), 336–340.

Brocca, D., Arvin, E., & Mosbaek, H. (2002). Identification of organic compounds migrating from polyethylene pipelines into drinking water. *Water Research, 36*(15), 3675-3680.

Brogård, M., Troedsson, E., Thuresson, K., & Ljusberg-Wahren, H. (2007). A new standardized lipolysis approach for characterization of emulsions and dispersions. *Journal of Colloid and Interface Science*, *308*(2), 500-507.

Brugh, M. Jr. (1977). Butylated hydroxytoluene protects chickens exposed to Newcastle disease virus. *Science, 197*(4310):1291-1292.

Calvo, P., Lozano, M., Espinosa-Mansilla, A., & Gónzalez-Gómez, D. (2012). *In vitro* evaluation of the availability of ω-3 and ω-6 fatty acids and tocopherols from microencapsulated walnut oil. *Food Research International, 48*, 316-321.

Capolino, P., Guérin, C., Paume, J., Giallo, J., Ballester, J.M., Cavalier, J. F., & Carrière, F. (2011). *In vitro* gastrointestinal lipolysis: Replacement of human digestive lipases by a combination of rabbit gastric and porcine pancreatic extracts. *Food Digestion*, *2*(1-3), 43-51.

Cardenia, V., Olivero, G., & Rodriguez-Estrada, M. T. (2015). Thermal oxidation of cholesterol: Preliminary evaluation of 2-methyl-6-heptanone and 3-methylbutanal as volatile oxidation markers. *Steroids, 99*(Part B), 161-171.

Carriere, F., Barrowman, J., Verger, R., & Laugier, R. (1993). Secretion and contribution to lipolysis of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology*, *105*(3), 876-888.

Chang, S. S., Peterson, R. J., & Ho, C. T. (1978). Chemical reactions involved in the deep-fat frying of foods. *Journal of the American Oil Chemists´ Society, 55*(10), 718-727.

Chevillard, G., Nouhi, Z., Anna, D., Paquet, M., & Blank, V. (2010). Nrf3-deficient mice are not protected against acute lung and adipose tissue damages induced by butylated hydroxytoluene. *FEBS Letters, 584*(5), 923-928.

Choi, Y. H., Kim, H. K., Wilson, E. G., Erkelens, C., Trijzelaar, B., & Verpoorte, R. (2004). Quantitative analysis of retinol and retinol palmitate in vitamin tablets using ^{1}H-nuclear magnetic resonance spectroscopy. *Analytica Chimica Acta, 512*(1), 141-147.

Chou, T. H., Chen, J. J., Lee, S. J., Chiang, M. Y., Yang, C. W., & Chen, I. S. (2010). Cytotoxic Flavonoids from the Leaves of Cryptocarya chinensis. *Journal of Natural Products*, *73*(9), 1470-1475.

Chu, F. L., & Yaylayan, V. A. (2008). Model studies on the oxygen-induced formation of benzaldehyde from phenylacetaldehyde using pyrolysis GC-MS and FTIR. *Journal of Agricultural and Food Chemistry, 56*(22), 10697-10704.

Chung, H., Choi, A., Cho, I. H., & Kim, Y.-S. (2011). Changes in fatty acids and volatile components in mackerel by broiling. *European Journal of Lipid Science and Technology, 113*(12), 1481-1490.

Clapp, N. K., Tyndall, R. L., & Cumming, R. B. (1973). Hyperplasia of hepatic bile ducts in mice following long-term administration of butylated hydroxytoluene. *Food and Cosmetics Toxicology, 11*(5), 847-849.

Clarysse, S., Tack, J., Lammert, F., Duchateau, G., Reppas, C., & Augustijns, P. (2009). Postprandial evolution in composition and characteristics of human duodenal fluids in different nutritional states. *Journal of Pharmaceutical Sciences*, *98*(3), 1177-1192.

Colle, I. J. P., Van Buggenhout, S., Lemmens, L., Van Loey, A. M., & Hendrickx, M. E. (2012). The type and quantity of lipids present during digestion influence the *in vitro* bioaccessibility of lycopene from raw tomato pulp. *Food Research International*, *45*(1), 250-255.

Collings, A. J., & Sharratt, M. (1970). The BHT content of human adipose tissue. *Food and Cosmetics Toxicology, 8*(4), 409-412.

Commission of the EC-Commission of the European Communities. (1989). Reports of the Scientific Committee for Food, Twenty-second Series. Available from: http://ec.europa.eu/food/fs/sc/scf/reports/scf_reports_22.pdf. Accessed 2014 July 02.

Compton, D. L., Vermillion, K. E., & Laszlo, J. A. (2007). Acyl Migration Kinetics of 2-Monoacylglycerols from Soybean Oil via 1H NMR. *Journal of the American Oil Chemists' Society*, *84*(4), 343-348.

Conacher, H. B., Iverson, F., Lau, P. Y., & Page, B. D. (1986). Levels of BHA and BHT in human and animal adipose tissue: interspecies extrapolation. *Food and Chemical Toxicology, 24*(10-11), 1159-1162.

Connell, J. J. (1975). Control of Fish Quality, 1st Edn., Fishing NewBooks, Oxford (UK).

Conning, D. M., & Phillips, J. C. (1986). Comparative metabolism of BHA, BHT and other phenolic antioxidants and its toxicological relevance. *Food and Chemical Toxicology, 24*(10-11), 1145-1148.

Cui, P. H., Duke, R. K., & Duke, C. C. (2008). Monoepoxy octadecadienoates and monoepoxy octadecatrienoates 1: NMR spectral characterization. *Chemistry and Physics of Lipids*, *152*(2), 122-130.

Cui, P. H., Zhang, W. V., Hook, J., Tattam, B. N., Duke, C. C., & Murray, M. (2009). Synthesis and NMR characterization of the methyl esters of eicosapentaenoic acid monoepoxides*. Chemistry and Physics of Lipids*, *159*(1), 30-37.

Dacre, J. C. (1961). The metabolism of 3,5-di-*tert*-butyl-4-hydroxytoluene and 3,5-di-*tert*-butyl-4-hydroxybenzoic acid in the rabbit. *Biochemical Journal, 78*(4), 758-766.

D'Ambrosio, D. N., Clugston, R. D., & Blaner, W. S. (2011). Vitamin A metabolism: an update. *Nutrients*, *3*, 63-103.

Daniel, J. W., & Gage, J. C. (1965). The absorption and excretion of butylated hydroxytoluene (BHT) in the rat. *Food and Cosmetics Toxicology, 3*, 405-415.

Daniel, J. W., Gage, J. C., Jones, D. I., & Stevens, M. A. (1967). Excretion of butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) by man. *Food and Cosmetics Toxicology, 5*, 475-479.

Daniel, J. W., Gage, J. C., & Jones, D. I. (1968). The metabolism of 3,5-di-*tert*-butyl-4-hydroxytoluene in the rat and in man. *Biochemical Journal, 106*(4), 783-790.

Daniel, J. W. (1986). Metabolic aspects of antioxidants and preservatives. *Xenobiotica, 16*(10-11), 1073-1078.

De Groot, W. Th. M. (1972). Acyl migration solid phase isomerization of 1,2-diglycerides to 1,3-isomers. *Lipids, 7*(9), 626-628.

Decker, E. A., Elias, R. J., & McClements, D. J. (2010). Oxidation in foods and beverages and antioxidant applications. Vol 1: Understanding mechanisms of oxidation and antioxidant activity. Edited by Eric A. Decker, Ryan J. Elias and D. Julian McClements. Woodhead Publishing, Abington (Cambridge).

Desnuelle, P., & Savary, P. (1963). Specificity of lipases. *Journal of Lipid Research, 4*(4), 369-384.

Di Maio, S., & Carrier, R. L. (2011). Gastrointestinal contents in fasted states and post-lipid ingestion. *In vivo* measurements and *in vitro* models for studying oral drug delivery. *Journal of Controlled Release, 151*(2), 110-122.

Dobarganes, M. C., & Perez-Camino, M. C. (1988). Fatty acid composition: a useful tool for the determination of alteration level in heated fats. *Revue Française des Corps Gras*, *35*(2), 67-70.

Dopico-García, M. S., López-Vilariñó, J. M., & González-Rodríguez, M. V. (2007). Antioxidant content of and migration from commercial polyethylene, polypropylene, and polyvinyl chloride packages. *Journal of Agricultural and Food Chemistry, 55*(8), 3225-3231.

Echarte, M., Zulet, M. A., & Astiasaran, I. (2001). Oxidation process affecting fatty acids and cholesterol in fried and roasted salmon. *Journal of Agricultural and Food Chemistry*, *49*(11), 5662-5667.

Eder, K. (1995). Gas chromatographic analysis of fatty acid methyl esters. *Journal of Chromatography B: Biomedical Sciences and Applications*, *671*(1+2), 113-131.

EFSA-European Food Safety Authority. Panel on Food Additives and Nutrient Sources added to Food (ANS). (2012). Scientific opinion on the re-evaluation of butylated hydroxytoluene BHT (E 321) as a food additive. *EFSA Journal, 10*(3):2588, 1-43.

Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., Balance, S., Barberá, R., Brodkorb, A., Cattenoz, T., Clemente, A., Comi, I., Dupont, D., Garcia-Llatas, G., Lagarda, J., Le Feunteun, S., JanssenDuijghuijsen, L., Karakaya, S., Lesmes, U., Mackie, A. R., Martins, C., Meynier, A., Miralles, B., Murray, B. S., Pihlanto, A., Picariello, G., Santos, C. N., Simsek, S., Recio, I., Rigby, N., Rioux, L. E., Stoffers, H., Tavares, A., Tavares, L., Turgeon, S., Ulleberg, E. K., Vegarud, G. E., Vergères, G., & Portmann, R. (2016). The harmonized INFOGEST *in vitro* digestion method: From knowledge to action. *Food Research International*, *88*, *Part B*, 217–225.

Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition*, *48*(5), 430-441.

Esterbauer, H. (1993). Cytotoxicity and genotoxicity of lipid-oxidation products. *The American Journal of Clinical Nutrition*, *57*(5), 779S-785S.

European Parliament and Council Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners.

European Parliament and Council Regulation (EC) No. 1831/2003 on additives for use in animal nutrition.

European Parliament and Council Regulation (EC) No 1333/2008 of 16 December 2008 on food additives

Faine, L. A., Rodrigues, H. G., Galhardi, C. M., Ebaid, G. M. X., Diniz, Y. S., Fernandes, A. A. H., & Novelli, E. L. B. (2006). Butyl hydroxytoluene (BHT)-induced oxidative stress: effects on serum lipids and cardiac energy metabolism in rats. *Experimental and Toxicologic Pathology, 57*(3), 221-226.

Fatouros, D. G., Bergenstahl, B., & Mullertz, A. (2007). Morphological observations on a lipid-based drug delivery system during *in vitro* digestion. *European Journal of Pharmaceutical Sciences*, *31*(2), 85-94.

Fernandez-Alvarez, M., Lores, M., Jover, E., Garcia-Jares, C., Bayona, J. M., & Llompart, M. (2009). Photo-solid-phase microextraction of selected indoor air pollutants from office buildings. Identification of their photolysis intermediates. *Journal of Chromatography A, 1216*(51), 8969-8978.

Fontana, A., Antoniazzi, F., Ciavatta, M. L., Trivellone, E., & Cimino, G. (1993). ^{1}H NMR study of cholesterol autooxidation in egg powder and cookies exposed to adverse storage. *Journal of Food Science, 58*(6), 1286-1290.

Frank, D., Poole, S., Kirchhoff, S., & Forde, C. (2009). Investigation of sensory and volatile characteristics of farmed and wild barramundi (*Lates calcarifer*) using gas chromatography-olfactometry mass spectrometry and descriptive sensory analysis. *Journal of Agricultural and Food Chemistry, 57*(21), 10302-10312.

Frankel, E. N., Neff, W. E., & Selke, E. (1981) Analysis of autoxidized fats by gas chromatography-mass spectrometry VII. Volatile thermal decomposition products of pure hydroperoxides from autoxidized and photosensitized oxidized methyl oleate, linoleate and linolenate. *Lipids*, *16*(5), 279-85.

Frankel, E. N., Neff, W. E., & Miyashita, K. (1990). Autoxidation of polyunsaturated triacylglycerols. II. Trilinolenoylglycerol. *Lipids*, *25*(1), 40-47.

Frankel, E. N. (2005). *Lipid Oxidation*, 2nd ed. The oily press, Bridgwater, England.

Frankel, E. N. (2007). *Antioxidants in food and biology: facts and fiction*, 2nd ed. Oily Press Bridgwater, U.K.

Frawley, J. P., Kay, J. H., & Calandra, J. C. (1965). The residue of butylated hydroxytoluene (BHT) and metabolites in tissue and eggs of chickens fed diets containing radioactive BHT. *Food and Cosmetics Toxicology, 3*(3), 471-474.

Fries, E., & Püttmann, W. (2002). Analysis of the antioxidant butylated hydroxytoluene (BHT) in water by means of solid-phase extraction combined with GC/MS. *Water Research, 36*(9), 2319-2327.

Fries, E., & Püttmann, W. (2004). Monitoring of the antioxidant BHT and its metabolite BHT-CHO in German river water and ground water. *Science of the Total Environment, 319*(1-3), 269-282.

Fuentes, A., Fernández-Segovia, I., Serra, J. A., & Barat, J. M. (2010). Comparison of wild and cultured sea bass (*Dicentrarchus labrax*) quality. *Food Chemistry*, *119*(4), 1514-1518.

Fujisawa, S., Kadoma, Y., & Yokoe, I. (2004). Radical-scavenging activity of butylated hydroxytoluene (BHT) and its metabolites. *Chemistry and Physics of Lipids, 130*(2), 189-195.

Gage, J. C. (1966). The metabolism of phenolic antioxidants. *Fett Wissenschaft Technologie, 68*(11), 951-954.

García, R. S., Silva, A. S., Cooper, I., Franz, R., & Losada, P. P. (2006). Revision of analytical strategies to evaluate different migrants from food packaging materials. *Trends in Food Science & Technology, 17*(7), 354-366.

Gardner, H. W., & Weisleder, D. (1972). Hydroperoxides from oxidation of linoleic and linolenic acids by soybean lipoxygenase: Proof of the *trans*-11 double bond. *Lipids*, *7*(3), 191-193.

Gardner, H. W., & Grove, M. J. (1998). Soybean lipoxygenase-1 oxidizes 3*Z*-nonenal. A route to 4S-hydroperoxy-2*E*-nonenal and related products. *Plant Physiology*, *116,* 1359-1366.

Garrett, D. A., Failla, M. L., & Sarama, R. J. (1999). Development of an *in vitro* digestion method to assess carotenoid bioavailability from meals. *Journal of Agricultural and Food Chemistry*, *47*(10), 4301-4309.

Geyer, H., Scheunert, I., & Korte, F. (1986). Bioconcentration potential of organic environmental chemicals in humans. *Regulatory Toxicology and Pharmacology, 6*(4), 313-347.

Gladyshev, M. I., Sushchik, N. N., Gubanenko, G. A., Demirchieva, S. M., & Kalachova, G. S. (2006). Effect of boiling and frying on the content of essential polyunsaturated fatty acids in muscle tissue of four fish species. *Food Chemistry*, *101*, 1694-1700.

Gladyshev, M. I., Sushchik, N. N., Gubanenko, G. A., Demirchieva, S. M., & Kalachova, G. S. (2006). Effect of way of cooking on content of essential polyunsaturated fatty acids in muscle tissue of humpback salmon (*Oncorhynchus gorbuscha*). *Food Chemistry, 96*(3), 446-451.

Gobert, M., Rémond, D., Loonis, M., Buffière, C., Santé-Lhoutellier, V., & Dufour, C. (2014). Fruits, vegetables and their polyphenols protect dietary lipids from oxidation during gastric digestion. *Food & Function*, *5*(9), 2166-2174.

Goicoechea, E., Van Twillert, K., Duits, M., Brandon, E. D. F. A., Kootstra, P. R., Blokland , M. D. (2008). Use of an *in vitro* digestion model to study the bioaccessibility of 4-hydroxy-2-nonenal and related aldehydes present in oxidized oils rich in omega-6 acyl groups. *Journal of Agricultural and Food Chemistry*, *56*(18), 8475-8483.

Goicoechea, E., & Guillén, M. D. (2010). Analysis of hydroperoxides, aldehydes and epoxides by ^{1}H nuclear magnetic resonance in sunflower oil oxidized at 70 and 100º C. *Journal of Agricultural and Food Chemistry*, *58*(10), 6234-6245.

Goicoechea, E., Brandon, E. F. A., Blokland, M. H., & Guillén, M. D. (2011). Fate in digestion *in vitro* of several food components, including some toxic compounds coming from omega-3 and omega-6 lipids. *Food and Chemical Toxicology*, *49*(1), 115-124.

Golding, M., & Wooster, T. J. (2010). The influence of emulsion structure and stability on lipid digestion. *Current Opinion in Colloid & Interface Science*, *15*(1–2), 90-101.

Gorelik, S., Lapidot, T., Shaham, I., Granit, R., Ligumsky, M., Kohen, R., & Kanner, J. (2005). Lipid peroxidation and coupled vitamin oxidation in simulated and human gastric fluid inhibited by dietary polyphenols: health implications. *Journal of Agricultural and Food Chemistry*, *53*(9), 3397-3402.

Gorelik, S., Ligumsky, M., Kohen, R., & Kanner, J. (2008). The stomach as a «bioreactor»: when red meat meets red wine. *Journal of Agricultural and Food Chemistry*, *56*(13), 5002-5007.

Grigorakis, K. (2007).Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it: A review. *Aquaculture, 272*(1–4), 55-75.

Grogan, M. W. (1986). Toxicity from BHT ingestion. *The Western Journal of Medicine, 145*(2), 245-246.

Grootveld, M., Silwood, C. J. L., & Claxson, A. W. D. (1999). Warning: thermally-stressed polyunsaturates are damaging to health. *Food Chemistry, 67*(2), 211-213.

Grosch, W., & Laskawy, G. (1975). Differences in the amount and range of volatile carbonyl compounds formed by lipoxygenase isoenzymes from soybeans. *Journal of Agricultural and Food Chemistry*, *23*(4), 791-794.

Guillén, M. D., & Ibargoitia, M. L. (1998). New components with potential antioxidant and organoleptic properties, detected for the first time in liquid smoke flavoring preparations. *Journal of Agricultural and Food Chemistry, 46*(4), 1276-1285.

Guillén, M. D., & Ibargoitia, M. L. (1999). GC/MS analysis of lignin monomers, dimers and trimers in liquid smoke flavourings. *Journal of the Science of Food and Agriculture, 79*, 1889-1903.

Guillén, M. D., & Manzanos, M. J. (2001). Some compounds detected for the first time in oak wood extracts by GC/MS. *Sciences des Aliments, 21*, 65-70.

Guillén, M. D., & Ruiz, A. (2001). High resolution ^{1}H nuclear magnetic resonance study of edible oils and fats. *Trends in Food Science & Technology*, *12*, 328-338.

Guillén, M. D., & Errecalde, M. C. (2002). Volatile components of raw and smoked black bream (*Brama raii*) and rainbow trout (*Oncorhynchus mykiss*) studied by means of solid-phase microextraction and gas chromatography/mass spectrometry. *Journal of the Science of Food and Agriculture, 82*, 945-952.

Guillén, M. D., & Manzanos, M. J. (2002). Study of the volatile composition of an aqueous oak smoke preparation. *Food Chemistry, 79*(3), 283-292.

Guillén, M. D., & Ruiz, A. (2003). Edible oils: Discrimination by ^{1}H nuclear magnetic resonance. *Journal of the Science of Food and Agriculture*, *83*(4), 338-346.

Guillén, M. D., & Ruiz, A. (2003). Rapid simultaneous determination by proton NMR of unsaturation and composition of acyl groups in vegetable oils. *European Journal of Lipid Science and Technology*, *105*, 688-696.

Guillén, M. D., & Ruiz, A. (2004). Study of the oxidative stability of salted and unsalted salmon fillets by ^{1}H nuclear magnetic resonance. *Food Chemistry*, *86*(2), 297-304.

Guillén, M. D., Ruiz, A., & Cabo, N. (2004). Study of the oxidative degradation of farmed salmon lipids by means of Fourier transform infrared spectroscopy. Influence of salting. *Journal of the Science of Food and Agriculture, 84*(12), 1528-1534.

Guillén, M. D., Cabo, N., Ibargoitia, M. L., & Ruiz, A. (2005). Study of both sunflower oil and its headspace throughout the oxidation process. Occurrence in the headspace of toxic oxygenated aldehydes. *Journal of Agricultural and Food Chemistry*, *53*(4), 1093-1101.

Guillén, M. D., & Ruiz, A. (2005a). Oxidation process of oils with high content of linoleic acyl groups and formation of toxic hydroperoxy- and hydroxyalkenals. A study by ^{1}H nuclear magnetic resonance. *Journal of the Science of Food and Agriculture*, *85*(14), 2413-2420.

Guillén, M. D., & Ruiz, A. (2005b). Monitoring the oxidation of unsaturated oils and formation of oxygenated aldehydes by proton NMR. *European Journal of Lipid Science and Technology*, *107*(1), 36-47.

Guillén, M. D., Errecalde, M. C., Salmerón, J., & Casas, C. (2006). Headspace volatile components of smoked swordfish (*Xiphias gladius*) and cod (*Gadus morhua*) detected by means of solid-phase microextraction and gass chromatography-mass spectrometry. *Food Chemistry, 94*, 151-156.

Guillén, M. D., & Goicoechea, E. (2007). Detection of primary and secondary oxidation products by fourier transform infrared spectroscopy (FTIR) and ^{1}H nuclear magnetic resonance (NMR) in sunflower oil during storage. *Journal of Agricultural and Food Chemistry*, *55*(26), 10729-10736.

Guillén, M., Carton, I., Goicoechea, E., & Uriarte, P. S. (2008). Characterization of cod liver oil by spectroscopic techniques. New approaches for the determination of compositional parameters, acyl groups, and cholesterol from ^{1}H Nuclear Magnetic Resonance and Fourier transform infrared spectral data. *Journal of Agricultural and Food Chemistry*, *56*(19), 9072-9079.

Guillén, M. D., & Goicoechea, E. (2008). Formation of oxygenated α,β-unsaturated aldehydes and other toxic compounds in sunflower oil oxidation at room temperature in closed receptacles. *Food Chemistry*, *111*(1), 157-164.

Guillén, M. D., & Goicoechea, E. (2008). Toxic oxygenated α,β-unsaturated aldehydes and their study in foods: a review. *Critical Reviews in Food Science and Nutrition, 48*(2), 119-136.

Guillén, M. D., Carton, I., Salmerón, J., & Casas, C. (2009). Headspace composition of cod liver oil and its evolution in storage after opening. First evidence of the presence of toxic aldehydes. *Food Chemistry, 114*(4), 1291-1300.

Guillén, M. D., & Goicoechea, E. (2009). Oxidation of corn oil at room temperature: Primary and secondary oxidation products and determination of their concentration in the oil liquid matrix from ^{1}H nuclear magnetic resonance data. *Food Chemistry, 116*(1), 183-192.

Guillén, M. D., & Uriarte, P. S. (2009). Contribution to Further Understanding of the Evolution of Sunflower Oil Submitted to Frying Temperature in a Domestic Fryer: Study by ^{1}H Nuclear Magnetic Resonance. *Journal of Agricultural and Food Chemistry*, *57*(17), 7790-7799.

Guillén, M. D., & Uriarte, P. S. (2012a). Aldehydes contained in edible oils of a very different nature after prolonged heating at frying temperature: Presence of toxic oxygenated α,β unsaturated aldehydes. *Food Chemistry*, *131*(3), 915-926.

Guillén, M. D., & Uriarte, P. S. (2012b). Monitoring by ^{1}H nuclear magnetic resonance of the changes in the composition of virgin linseed oil heated at frying temperature. Comparison with the evolution of other edible oils. *Food Control*, *28*(1), 59-68.

Guillén, M. D., & Uriarte, P. S. (2012c). Simultaneous control of the evolution of the percentage in weight of polar compounds, iodine value, acyl groups proportions and aldehydes concentrations in sunflower oil submitted to frying temperature in an industrial fryer. *Food Control, 24*(2), 50-56

Guillén, M. D., & Uriarte, P. S. (2012d). Study by ^{1}H NMR spectroscopy of the evolution of extra virgin olive oil composition submitted to frying temperature in an industrial fryer for a prolonged period of time. *Food Chemistry, 134*(1), 162-172.

Gunstone, F. D. (1991). ^{13}C-NMR studies of mono-, di-and tri-acylglycerols leading to qualitative and semiquantitative information about mixtures of these glycerol esters. *Chemistry and Physics of Lipids*, *58*(3), 219-224.

Guyton, K. Z., Bhan, P., Kuppusamy, P., Zweier, J. L., Trush, M. A., & Kensler, T. W. (1991). Free radical-derived quinone methide mediates skin tumor promotion by butylated hydroxytoluene hydroperoxide: expanded role for electrophiles in multistage carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(3), 946-950.

Hallier, A., Prost, C., & Serot, T. (2005). Influence of rearing conditions on the volatile compounds of cooked fillets of *Silurus glanis* (European catfish). *Journal of Agricultural and Food Chemistry*, *53*(18), 7204-7211.

Halliwell, B., Zhao, K., & Whiteman, M. (2000). The gastrointestinal tract: a major site of antioxidant action? *Free Radical Research, 33*(6), 819-830.

Halliwell, B. (2011). Free radicals and antioxidants - quo vadis? *Trends in Pharmacological Sciences, 32*(3), 125-130.

Hamama, A. A, & Nawar, W. W. (1991). Thermal decomposition of some phenolic antioxidants. *Journal of Agricultural and Food Chemistry, 39*(6), 1063-1069.

Hammer, M., & Schieberle, P. (2013). Model studies on the key aroma compounds formed by an oxidative degradation of ω-3 fatty acids initiated by either copper(II) ions or lipoxygenase. *Journal of Agricultural and Food Chemistry*, *61*(46), 10891-10900.

Hayes, D. G., Kleiman, R., Weisleder, D., Adlof, R. O., Cuperus, F. P., & Derksen, J. T. P. (1995). Occurrence of estolides in processed *Dimorphotheca pluvialis* seed oil. *Industrial Crops and Products*, *4*(4), 295-301.

Haywood, R. M., Claxson, A. W. D., Hawkes, G. E., Richardson, D. P., Coumbarides, G., Hawkes, J., Lynch, E. J., & Grootveld, M. C. (1995). Detection of aldehydes and their conjugated hydroperoxydiene precursors in thermally-stressed culinary oils and fats: investigations using High Resolution Proton NMR spectroscopy. *Free Radical Research*, 22, 441–482.

Helbig, A., Silletti, E., Timmerman, E., Hamer, R. J., & Gruppen, H. (2012). *In vitro* study of intestinal lipolysis using pH-stat and gas chromatography. *Food Hydrocolloids*, *28*(1), 10-19.

Herland, H., Esaiassen, M., Cooper, M., & Olsen, R. L. (2009).Changes in trimethylamine oxide and trimethylamine in muscle of wild and farmed cod (*Gadus morhua*) during iced storage. *Aquaculture Research, 41,* 95-102.

Hernández, F., Portolés, T., Pitarch, E., & López, F. J. (2009). Searching for anthropogenic contaminants in human breast adipose tissues using gas chromatography-time-of-flight mass spectrometry. *Journal of Mass Spectrometry, 44*(1), 1-11.

Hirose, M., Shibata, M., Hagiwara, A., Imaida, K., & Ito, N. (1981). Chronic toxicity of butylated hydroxytoluene in Wistar rats*. Food and Cosmetics Toxicology, 19*, 147-151.

Hofmann, A. F., Borgström, B. (1963). Hydrolysis of long-chain monoglycerides in micellar solution by pancreatic lipase. *Biochimica et Biophysica Acta, 70*, 317-331.

Holaas, E., Bohne, V. B., Hamre, K., & Arukwe, A. (2008). Hepatic retention and toxicological responses during feeding and depuration periods in Atlantic salmon (*Salmo salar*) fed graded levels of the synthetic antioxidant, butylated hydroxytoluene. *Journal of Agricultural and Food Chemistry, 56*(23), 11540-11549.

Holder, G. M., Ryan, A. J., Watson, T. R., & Wiebe, L. I. (1970). The metabolism of butylated hydroxytoluene, (3,5-di-*t*-butyl-4-hydroxytoluene) in man. *Journal of Pharmacy and Pharmacology, 22*(5), 375-376.

Holman, R. T, & Elmer, O. C. (1947). The rates of oxidation of unsaturated fatty acids and esters. *Journal of the American Oil Chemists´ Society, 24*(4), 127-129.

Horiuchi, M., Umano, K., & Shibamoto, T. (1998). Analysis of volatile compounds formed from fish oil heated with cysteine and trimethylamine oxide. *Journal of Agricultural and Food Chemistry, 46*(12), 5232-5237.

Hsieh, R. J., & Kinsella, J. E. Oxidation of Polyunsaturated Fatty Acids: Mechanisms, Products, and Inhibition with Emphasis on Fish. In J. E. Kinsella (Ed.), *Advances in Food and Nutrition Research* (Vol. 33, pp. 233-341). Academic Press. (1989).

Huang, M. H., Chang, L. W., Sung, W. C., Vong, W. J., & Wang, B. S. (2011). Protective effects of three smoke flavouring phenols on oxidative damage and nitric oxide production. *Food Chemistry, 126*(4), 1655-1661.

Hur, S. J., Decker, E. A., & McClements, D. J. (2009). Influence of initial emulsifier type on microstructural changes occurring in emulsified lipids during *in vitro* digestion. *Food Chemistry*, *114*(1), 253-262.

Hur, S. J., Joo, S. T., Lim, B. O., Decker, E. A., & McClements, J. D. (2011a). Impact of salt and lipid type on *in vitro* digestion of emulsified lipids. *Food Chemistry*, *126*(4), 1559-1564.

Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011b). *In vitro* human digestion models for food applications. *Food Chemistry*, *125*(1), 1-12.

IARC-International Agency for Research on Cancer. (1987). Some naturally occurring and synthetic food components, furocoumarins and ultraviolet radiation summary of data reported and evaluation. IARC Monographs on the evaluation of carcinogenic risks to humans 40. Available from: http://monographs.iarc.fr/ENG/Monographs/vol1-42/mono40.pdf. Accessed 2014 July 02.

Iglesias, J., & Medina, I. (2008). Solid-phase microextraction method for the determination of volatile compounds associated to oxidation of fish muscle. *Journal of Chromatography A, 1192*(1), 9-16.

Inai, K., Kobuke, T., Nambu, S., Takemoto, T., Kou, E., Nishina, H., Fujihara, M., Yonehara, S., Suehiro, S., & Tsuya, T. (1988). Hepatocellular tumorigenicity of butylated hydroxytoluene administered orally to B6C3F1 mice. *Japanese Journal of Cancer Research, 79*(1), 49-58.

JECFA-Joint FAO/WHO Expert Committee on Food Additives. (1996). Butylated hydroxytoluene. Toxicologycal evaluation of certain food additives and contaminants in food. WHO Food additives Series 35. Available from: http://www.inchem.org/documents/jecfa/jecmono/v35je02.htm. Accessed 2014 July 02.

JECFA-Joint FAO/WHO Expert Committee on Food Additives. (2000). Evaluation of certain food additives (Fifty-first report). WHO-World Health Organization Technical Report Series, No. 891. Available from: http://whqlibdoc.who.int/trs/WHO_TRS_891.pdf?ua=1. Accessed 2014 July 02.

Jiang, G., Lin, S., Wen, L., Jiang, Y., Zhao, M., Chen, F., Prasad, K. N., Duan, X., & Yang, B. (2013). Identification of a novel phenolic compound in litchi (*Litchi chinensis* Sonn.) pericarp and bioactivity evaluation. *Food Chemistry, 136*, 563-568.

Jin, F., Kawasaki, K., Kishida, H., Tohji, K., Moriya, T., & Enomoto, H. (2007). NMR spectroscopic study on methanolysis reaction of vegetable oil. *Fuel*, *86*(7-8), 1201-1207.

JMHLW-Japanese Ministry of Health, Labour and Welfare. (2005). The Japan food chemical research foundation: maximum residue limits (MRLs) list: compositional specification for foods. Available from: http://www.m5.ws001.squarestart.ne.jp/foundation/agrdtl.php?a_inq=30500. Accessed 2014 July 02.

JMPR-Joint FAO/WHO. (2005). Pesticide residues in food. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues. Available from: http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/JMPR05 report.pdf. Accessed 2014 July 02.

Josephson, D. B., & Lindsay, R. C. (1987). Retro-aldol degradations of unsaturated aldehydes: Role in the formation of c-4-heptenal from t2,c6-nonadienal in fish, oysters and other flavors. *Journal of American Oil Chemists´ Society, 64*(1), 132-138.

Kagan, V. E., Serbinova, E. A., & Packer, L. (1990). Generation and recycling of radicals from phenolic antioxidants. *Archives of Biochemistry and Biophysics, 280*(1), 33-39.

Kahl, R. (1984). Synthetic antioxidants: biochemical actions and interference with radiation, toxic compounds, chemical mutagens and chemical carcinogens. *Toxicology, 33*(3-4):185-228.

Kalantzi, L., Goumas, K., Kalioras, V., Abrahamsson, B., Dressman, J. B., & Reppas, C. (2006). Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. *Pharmaceutical Research*, *23*(1), 165-176.

Kalogeropoulos, N., Andrikopoulos, N. K., & Hassapidou, M. (2004). Dietary evaluation of Mediterranean fish and molluscs pan-fried in virgin olive oil. *Journal of the Science of Food and Agriculture*, *84*(13), 1750-1758.

Kalogeropoulos, N., Salta, F. N., Chiou, A., & Andrikopoulos, N. K. (2007). Formation and distribution of oxidized fatty acids during deep- and pan-frying of potatoes. *European Journal of Lipid Science and Technology, 109*(11), 1111-1123.

Kanazawa, K., & Ashida, H. (1998). Dietary hydroperoxides of linoleic acid decompose to aldehydes in stomach before being absorbed into the body. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, *1393*(2–3), 349-361.

Kaneko, T., Honda, S., Nakano, S., & Matsuo, M. (1987). Lethal effects of a linoleic acid hydroperoxide and its autoxidation products, unsaturated aliphatic aldehydes, on human diploid fibroblasts. *Chemico-Biological Interactions*, *63*(2), 127-137.

Kanner, J., Harel, S., & Jaffe, R. (1991). Lipid peroxidation of muscle food as affected by sodium chloride. *Journal of Agricultural and Food Chemistry, 39*(6), 1017-1021.

Kanner, J., & Lapidot, T. (2001). The stomach as a bioreactor: dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants. *Free Radical Biology & Medicine*, *31*(11), 1388-1395.

Kanner, J. (2007). Dietary advanced lipid oxidation endproducts are risk factors to human health. *Molecular Nutrition & Food Research*, *51*(9), 1094-1101.

Karovicová, J., & Simko, P. (2000). Determination of synthetic phenolic antioxidants in food by high-performance liquid chromatography. *Journal of Chromatography A, 882*(1-2), 271-281.

Kawai, K., Matsuno, K., & Kasai, H. (2006). Detection of 4-oxo-2-hexenal, a novel mutagenic product of lipid peroxidation, in human diet and cooking vapor. *Mutation Research, 603*(2), 186-192.

Kawai, T. (1996). Fish flavor. *Critical Reviews in Food Science and Nutrition*, *36*(3), 257-298.

Kenmogne-Domguia, H. B., Meynier, A., Boulanger, C., & Genot, C. (2012a). Lipid oxidation in food emulsions under gastrointestinal-simulated conditions: the key role of endogenous tocopherols and initiator. *Food Digestion*, *3*(1-3), 46-52.

Kenmogne-Domguia, H. B., Meynier, A., Viau, M., Llamas, G., & Genot, C. (2012b). Gastric conditions control both the evolution of the organization of protein-stabilized emulsions and the kinetic of lipolysis during *in vitro* digestion. *Food & Function*, *3*(12), 1302-1309.

Kenmogne-Domguia, H. B., Moisan, S., Viau, M., Genot, C., & Meynier, A. (2014). The initial characteristics of marine oil emulsions and the composition of the media inflect lipid oxidation during in vitro gastrointestinal digestion. *Food Chemistry, 152*, 146-154.

Kerem, Z., Chetrit, D., Shoseyov, O., & Regev-Shoshani, G. (2006). Protection of lipids from oxidation by epicatechin, *trans*-resveratrol, and gallic and caffeic acids in intestinal model systems. *Journal of Agricultural and Food Chemistry, 54*(26), 10288-10293.

Kharasch, M. S., & Joshi, B. S. (1957). Reactions of hindered phenols. II. Base-catalyzed oxidations of hindered phenols*. *The Journal of Organic Chemistry*, *22*(11), 1439-1443.

Kikuchi, M., Yaoita, Y., & Kikuchi, M. (2008). Monohydroxy-Substituted Polyunsaturated Fatty Acids from *Swertia japonica*. *Helvetica Chimica Acta*, *91*(10), 1857-1862.

Kim, Y.-S., & Ho, C.-T. (1998). Formation of pyridines from thermal interaction of glutamine or glutamic acid with a mixture of alkadienals in aqueous and oil media. *Journal of Food Lipids*, *5*(3), 173-182.

Kirkpatrick, D. C., & Lauer, B. H. (1986). Intake of phenolic antioxidants from foods in Canada. *Food and Chemical Toxicology, 24*(10-11), 1035-1037.

Klein, P. J., Van Vleet, T. R., Hall, J. O., & Coulombe, R. A. (2003). Effects of dietary butylated hydroxytoluene on aflatoxin B1-relevant metabolic enzymes in turkeys. *Food and Chemical Toxicology, 41*(5), 671-678.

Kong, F., & Singh, R. P. (2010). A human gastric simulator (HGS) to study food digestion in human stomach. *Journal of Food Science*, *75*(9), E627-E635.

Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergères, G., Portmann, R., & Egger, L. (2012). Validation of an *in vitro* digestive system for studying macronutrient decomposition in humans. *The Journal of Nutrition*, *142*(2), 245-250.

Kosaka, K., Suzuki, K., Hayakawa, M., Sugiyama, S., & Ozawa, T. (1994). Leukotoxin, a linoleate epoxide-its implication in the late death of patients with extensive burns. *Molecular and Cellular Biochemistry*, *139*, 141-148.

Kristinova, V., Storrø, I., & Rustad, T. (2013). Influence of human gastric juice on oxidation of marine lipids-*in vitro* study. *Food Chemistry*, *141*(4), 3859-3871.

Kubow, S. (1992). Routes of formation and toxic consequences of lipid oxidation products in foods. *Free Radical Biology & Medicine*, *12*(1), 63-81.

Kuffa, M., Priesbe, T. J., Krueger, C. G., Reed, J. D., & Richards, M. P. (2009). Ability of dietary antioxidants to affect lipid oxidation of cooked turkey meat in a simulated stomach and blood lipids after a meal. *Journal of Functional Foods*, *1*(2), 208-216.

Kumar, R., Bansal, V., Tiwari, A. K., Sharma, M., Puri, S. K., Patel, M. B., & Sarpal, A. S. (2011). Estimation of glycerides and free fatty acid in oils extracted from various seeds from the Indian region by NMR spectroscopy. *Journal of the American Oil Chemists' Society*, *88*(11), 1675-1685.

Kupfer, R., Dwyer-Nield, L. D., Malkinson, A. M., & Thompson, J. A. (2002). Lung toxicity and tumor promotion by hydroxylated derivatives of 2,6-di-*tert*-butyl-4-methylphenol (BHT) and 2-*tert*-butyl-4-methyl-6-iso-propylphenol: correlation with quinone methide reactivity. *Chemical Research in Toxicology, 15*(8), 1106-1112.

Ladomery, L. G., Ryan, A. J., & Wright, S. E. (1967). The biliary metabolites of butylated hydroxytoluene in the rat. *Journal of Pharmacy and Pharmacology, 19*(6), 388-394.

Lambert, C. R., Black, H. S., & Truscott, T. G. (1996). Reactivity of butylated hydroxytoluene. *Free Radical Biology & Medicine, 21*(3), 395-400.

Lamothe, S., Corbeil, M.-M., Turgeon, S. L., & Britten, M. (2012). Influence of cheese matrix on lipid digestion in a simulated gastro-intestinal environment. *Food & Function*, *3*(7), 724-731.

Larsen, D., Quek, S. Y., & Eyres, L. (2010). Effect of cooking method on the fatty acid profile of New Zealand king salmon (*Oncorhynchus tshawytscha*). *Food Chemistry*, *119*(2), 785-790.

Larsson, K., Cavonius, L., Alminger, M., & Undeland, I. (2012). Oxidation of cod liver oil during gastrointestinal *in vitro* digestion. *Journal of Agricultural and Food Chemistry*, *60*(30), 7556-7564.

Leclercq, C., Arcella, D., & Turrini, A. (2000). Estimates of the theoretical maximum daily intake of erythorbic acid, gallates, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in Italy: a stepwise approach. *Food and Chemical Toxicology 38*(12), 1075-1084.

Lee, G. H., Suriyaphan, O., & Cadwallader, K. R. (2001). Aroma components of cooked tail meat of American lobster (*Homarus americanus*). *Journal of Agricultural and Food Chemistry*, *49*(9), 4324-4332.

Lemercier, J. N., Meier, B. W., Gomez, J. D., & Thompson, J. A. (2004). Inhibition of glutathione S-transferase P1-1 in mouse lung epithelial cells by the tumor promoter 2,6-di-*tert*-butyl-4-

methylene-2,5-cyclohexadienone (BHT-quinone methide): protein adducts investigated by electrospray mass spectrometry. *Chemical Research in Toxicology, 17*(12), 1675-1683.

Leventhal, B., Daun, H., & Gilbert, S. G. (1976). Isolation and identification of 3,3′ ,5,5′ -tetra-bis-(*tert*-butyl)-stilbenequinone from edible oil with added BHT. *Journal of Food Science, 41*(2), 467-468.

Li, Y. X., Li, Y., Qian, Z. J., Kim, M. M., & Kim, S. K. (2009). *In vitro* antioxidant activity of 5-HMF isolated from marine red alga Laurencia undulata in free-radical-mediated oxidative systems. *Journal of Microbiology and Biotechnology, 19*(11), 1319-1327.

Li, Y., & McClements, D. J. (2010). New mathematical model for interpreting pH-stat digestion profiles: Impact of lipid droplet characteristics on *in vitro* digestibility. *Journal of Agricultural and Food Chemistry*, *58*(13), 8085-8092.

Li, Y., Hu, M., & McClements, D. J. (2011). Factors affecting lipase digestibility of emulsified lipids using an *in vitro* digestion model: Proposal for a standardised pH-stat method. *Food Chemistry*, *126*(2), 498-505.

Lin, H. M., Yen, F. L., Ng, L. T., & Lin, C. C. (2007). Protective effects of *Ligustrum lucidum* fruit extract on acute butylated hydroxytoluene-induced oxidative stress in rats. *Journal of Ethnopharmacology, 111*(1), 129-136.

Lindahl, A., Ungell, A. L., Knutson, L., & Lennernäs, H. (1997). Characterization of fluids from the stomach and proximal jejunum in men and women. *Pharmaceutical Research*, *14*(4), 497-502.

Lindenschmidt, R. C., Tryka, A. F., Goad, M. E., & Witschi, H. P. (1986). The effects of dietary butylated hydroxytoluene on liver and colon tumor development in mice. *Toxicology 38*(2):151-160.

Liu, J.-K., Zhao, S.-M., Xiong, S.-B., & Zhang, S.-H. (2009). Influence of recooking on volatile and non-volatile compounds found in silver carp *Hypophthalmichthys molitrix*. *Fisheries Sciences, 75*(4), 1067-1075.

Lorrain, B., Dangles, O., Loonis, M., Armand, M., & Dufour, C. (2012). Dietary iron-initiated lipid oxidation and its inhibition by polyphenols in gastric conditions. *Journal of Agricultural and Food Chemistry*, *60*(36), 9074-9081.

Lundebye, A. K., Hove, H., Måge, A., Bohne, V. J. B., & Hamre, K. (2010). Levels of synthetic antioxidants (ethoxyquin, butylated hydroxytoluene and butylated hydroxyanisole) in fish feed and commercially farmed fish. *Food Additives & Contaminants, 27*(12), 1652-1657.

Lützhøft, H. C. H., Waul, C. K., Andersen, H. R., Seredynska-Sobecka, B., Mosbæk, H., Christensen, N., Olsson, M. E., & Arvin, E. (2013). HS-SPME-GC-MS analysis of antioxidant degradation products migrating to drinking water from PE materials and PEX pipes. *International Journal of Environmental Analytical Chemistry, 93*(6), 593-612.

Malkinson, A. M. (1983). Review: putative mutagens and carcinogens in foods. III. Butylated hydroxytoluene (BHT). *Environmental and Molecular Mutagenesis, 5*(3), 353-362.

Malkinson, A. M., Koski, K. M., Evans, W. A., & Festing, M. F. W. (1997). Butylated hydroxytoluene exposure is necessary to induce lung tumors in BALB mice treated with 3-methylcholanthrene. *Cancer Research, 57*(14), 2832-2834.

Mannina, L., Sobolev, A. P., Capitani, D., Iaffaldano, N., Rosato, M. P., Ragni, P., Reale, A., Sorrentino, E., D'Amico, I., & Coppola, R. (2008). NMR metabolic profiling of organic and aqueous sea bass extracts: Implications in the discrimination of wild and cultured sea bass. *Talanta, 77*(1), 433-444.

Marmesat, S., Morales, A., Velasco, J., & Dobarganes, M. C. (2010). Action and fate of natural and synthetic antioxidants during frying. *Grasas Aceites 61*(4), 333-340.

Márquez-Ruiz, G., Pérez-Camino, M. C., & Dobarganes, M. C. (1993). Evaluation of hydrolysis and absorption of thermally oxidized olive oil in non-absorbed lipids in the rat. *Annals of Nutrition & Metabolism*, *37*(3), 121-128.

Márquez-Ruiz, G., Guevel, G., & Dobarganes, M. C. (1998). Applications of chromatographic techniques to evaluate enzymatic hydrolysis of oxidized and polymeric triglycerides by pancreatic lipase *in vitro*. *Journal of the American Oil Chemists' Society*, *75*(2), 119-126.

Márquez-Ruiz, G., Garcia-Martinez, M. C., & Holgado, F. (2008). Changes and effects of dietary oxidized lipids in the gastrointestinal tract. *Lipid Insights*, 2, 11-19.

Martin, D., Nieto-Fuentes, J. A., Señoráns, F. J., Reglero, G., & Soler-Rivas, C. (2010). Intestinal digestion of fish oils and ω-3 concentrates under *in vitro* conditions. *European Journal of Lipid Science and Technology*, *112*(12), 1315–1322.

Martínez-Yusta, A., Goicoechea, E., & Guillén, M. D. (2014). A Review of Thermo-Oxidative Degradation of Food Lipids Studied by ^{1}H NMR Spectroscopy: Influence of Degradative Conditions and Food Lipid Nature. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5), 838-859.

Martínez-Yusta, A., & Guillén, M. D. (2014a). A study by ^{1}H nuclear magnetic resonance of the influence on the frying medium composition of some soybean oil-food combinations in deep-frying. *Food Research International*, *55*, 347-355.

Martínez-Yusta, A., & Guillén, M. D. (2014b). Deep-frying food in extra-virgin olive oil: a study by ^{1}H Nuclear Magnetic Resonance of the influence of food nature on the evolving composition of the frying medium. *Food Chemistry*, *150*, 429-437.

Martínez-Yusta, A., & Guillén, M. D. (2014c). Deep-frying. A study of the influence of the frying medium and the food nature, on the lipidic composition of the fried food, using ^{1}H nuclear magnetic resonance. *Food Research International*, *62*, 998-1007.

Martínez-Yusta, A., & Guillén, M. D. (2016). Monitoring compositional changes in sunflower oil-derived deep-frying media by ^{1}H Nuclear Magnetic Resonance*. European Journal of Lipid Science and Technology, 118*(7), 984-996.

Marze, S., Meynier, A., & Anton, M. (2013). *In vitro* digestion of fish oils rich in n-3 polyunsaturated fatty acids studied in emulsion and at the oil-water interface. *Food & Function*, *4*(2), 231-239.

Matsuo, M., Mihara, K., Okuno, M., Ohkawa, H., & Miyamoto, J. (1984). Comparative metabolism of 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) in mice and rats. *Food and Chemical Toxicology, 22*(5), 345-354.

Matsushita, S. (1975). Specific interactions of linoleic acid hydroperoxides and their secondary degraded products with enzyme proteins. *Journal of Agricultural and Food Chemistry*, *23*(2), 150-154.

Mattson, F. H., & Beck, L. W. (1956). The specificity of pancreatic lipase for the primary hydroxyl groups of glycerides. *The Journal of Biological Chemistry*, *219*(2), 735-740.

Mattson, F. H., & Volpenhein, R. A. (1962). Synthesis and properties of glycerides. *Journal of Lipid Research, 3*(3), 281-295.

Mattson, F. H., & Volpenhein, R. A. (1964). The digestion and absorption of triglycerides. *Journal of Biological Chemistry*, *239*(9), 2772-2777.

Maziero, G. C., Baunwart, C., & Toledo, M. C. (2001). Estimates of the theoretical maximum daily intake of phenolic antioxidants BHA, BHT and TBHQ in Brazil. *Food Additives & Contaminants, 18*(5), 365-373.

McClements, D. J., Decker, E. A., & Park, Y. (2009). Controlling lipid bioavailability through physicochemical and structural approaches. *Critical Reviews in Food Science and Nutrition*, *49*(1), 48-67.

Meier, B. W., Gomez, J. D., Kirichenko, O. V., & Thompson, J. A. (2007). Mechanistic basis for inflammation and tumor promotion in lungs of 2,6-di-*tert*-butyl-4-methylphenol-treated mice: electrophilic metabolites alkylate and inactivate antioxidant enzymes. *Chemical Research in Toxicology, 20*(2), 199-207.

Methven, L., Tsoukka, M., Oruna-Concha, M. J., Parker, J. K., & Mottram, D. S. (2007). Influence of sulfur amino acids on the volatile and nonvolatile components of cooked salmon (*Salmo salar*). *Journal of Agricultural and Food Chemistry*, *55*(4), 1427-1436.

Miettinen, T. A., & Siurala, M. (1971). Bile salts, sterols, sterol esters, glycerides and fatty acids in micellar and oil phases of intestinal contents during fat digestion in man. *Zeitschrift Für Klinische Chemie Und Klinische Biochemie*, *9*(1), 47-52.

Milo, C., & Grosch, W. (1996). Changes in the odorants of boiled salmon and cod as affected by the storage of the raw material. *Journal of Agricultural and Food Chemistry*, *44*(8), 2366-2371.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D. J., Ménard, O., Recio, I., Santos, C. N., Singh, R. P., Vegarud, G. E., Wickham, M. S. J., Weitschies, W., & Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food - an international consensus. *Food & Function*, *5*(6), 1113-1124.

Minekus, M., Marteau, P., Havenaar, R., & Huis in 't Veld, J. H. H. (1995). Multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. *Alternatives to Laboratory Animals, 23*, 197-209.

Mizutani, T., Nomura, H., Nakanishi, K., & Fujita, S. (1987). Hepatotoxicity of butylated hydroxytoluene and its analogs in mice depleted of hepatic glutathione. *Toxicology and Applied Pharmacology, 87*(1), 166-176.

Mjøs, S. A., & Solvang, M. (2006). Patterns in volatile components over heated fish powders. *Food Research International*, *39*(2), 190-202.

Mnari Bhouri, A., Jrah Harzallah, H., Dhibi, M., Bouhlel, I., Hammami, M., & Chaouch, A. (2010). Nutritional fatty acid quality of raw and cooked farmed and wild sea bream (*Sparus aurata*). *Journal of Agricultural and Food Chemistry*, *58*(1), 507-512

Molly, K., Woestyne, M. V., & Verstraete, W. (1993). Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. *Applied Microbiology and Biotechnology*, *39*(2), 254-258.

Moradi, Y., Bakar, J., Motalebi, A. A., Syed Muhamad, S. H., & Che Man, Y. (2011). A review on fish lipid: composition and changes during cooking methods. *Journal of Aquatic Food Product Technology, 20*(4), 379-390.

Moreira, N., Valente, L. M. P., Castro-Cunha, M., Cunha, L. M., & Guedes de Pinho, P. (2013). Effect of storage time and heat processing on the volatile profile of Senegalese sole (*Solea senegalensis* Kaup, 1858) muscle. *Food Chemistry, 138*(4), 2365-2373.

Mu, H., & Høy, C. E. (2004). The digestion of dietary triacylglycerols. *Progress in Lipid Research*, *43*(2), 105-133.

Mun, S., Decker, E. A., & McClements, D. J. (2007). Influence of emulsifier type on *in vitro* digestibility of lipid droplets by pancreatic lipase. *Food Research International*, *40*(6), 770-781.

Murakami, N., Shirahashi, H., Nagatsu, A., & Sakakibara, J. (1992). Two unsaturated 9R-hydroxy fatty acids from the cyanobacterium *Anabaena flos-aquae f. flos-aquae*. *Lipids*, *27*(10), 776-778.

Nagai, F., Ushiyama, K., & Kano, I. (1993). DNA cleavage by metabolites of butylated hydroxytoluene. *Archives of Toxicology, 67*(8), 552-557.

Nakatsugawa, K., & Kaneda, T. (1983). Absorption and metabolism of methyl linoleate hydroperoxides in rats. *Journal of Japan Oil Chemists´Society, 32*, 361-366.

Nanou, K., & Roukas, T. (2010). Oxidative stress response and morphological changes of *Blakeslea trispora* induced by butylated hydroxytoluene during carotene production. *Applied Biochemistry and Biotechnology, 160*(8), 2415-2423.

Neff, W. E., Frankel, E. N., & Weisleder, D. (1981). High pressure liquid chromatography of autoxidized lipids: II. Hydroperoxy-cyclic peroxides and other secondary products from methyl linolenate. *Lipids*, *16*(6), 439-448.

Negroni, M., D'Agostin, A., & Arnoldi, A. (2001). Effects of olive, canola, and sunflower oils on the formation of volatiles from the Maillard reaction of lysine with xylose and glucose. *Journal of Agricultural and Food Chemistry*, *49*(1), 439-445.

Ng, S. (2000). Quantitative analysis of partial acylglycerols and free fatty acids in palm oil by ^{13}C NMR nuclear magnetic resonance spectroscopy. *Journal of the American Oil Chemists´Society, 77*(7), 749-755.

Niki, E. (2014). Biomarkers of lipid peroxidation in clinical material. *Biochimica Et Biophysica Acta*, *1840*(2), 809-817.

Nurhan, U. (2007). Change in proximate, amino acid and fatty acid contents in muscle tissue of rainbow trout (*Oncorhynchus mykiss*) after cooking. *International Journal of Food Science & Technology*, *42*(9), 1087-1093.

Oikawa, S., Nishino, K., Oikawa, S., Inoue, S., Mizutani, T., & Kawanishi, S. (1998). Oxidative DNA damage and apoptosis induced by metabolites of butylated hydroxytoluene. *Biochemical Pharmacology, 56*(3), 361-370.

Olsen, P., Meyer, O., Bille, N., & Würtzen, G. (1986). Carcinogenicity study on butylated hydroxytoluene (BHT) in Wistar rats exposed in utero. *Food and Chemical Toxicology, 24*(1), 1-12.

Orban, E., Nevigato, T., Lena, G. D., Casini, I., & Marzetti, A. (2003). Differentiation in the lipid quality of wild and farmed seabass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*). *Journal of Food Science, 68*(1), 128-132.

Ozogul, Y., Ozyurt, G., & Kuley Boga, E. (2009). Effects of cooking and reheating methods on the fatty acid profile of sea bream treated with rosemary extract. *Journal of the Science of Food and Agriculture*, *89*(9), 1481-1489.

Oztop, M. H., Sahin, S., & Sumnu, G. (2007). Optimization of microwave frying of potato slices by using Taguchi technique. *Journal of Food Engineering*, *79*(1), 83-91.

Özyurt, G., & Polat, A. (2006). Amino acid and fatty acid composition of wild sea bass (*Dicentrarchus labrax*): a seasonal differentiation. *European Food Research and Technology, 222*(3-4), 316-320.

Pafumi, Y., Lairon, D., Porte, P. L., Juhel, C., Storch, J., Hamosh, M., & Armand, M. (2002). Mechanisms of inhibition of triacylglycerol hydrolysis by human gastric lipase. *Journal of Biological Chemistry, 277*(31), 28070-28079.

Pan, X., Ushio, H., & Ohshima, T. (2004). Photo-oxidation of lipids impregnated on the surface of dried seaweed (*Porphyra yezoensis* Ueda). Characterization of volatiles. *Journal of American Oil Chemists´ Society, 81*(8), 773-781.

Pan, X. Q., Ushio, H., & Ohshima, T. (2005). Comparison of volatile compounds formed by autoxidation and photosensitized oxidation of cod liver oil in emulsion systems. *Fisheries Science*, *71*(3), 639-647.

Philip, M., Rowley, D. A., & Schreiber, H. (2004). Inflammation as a tumor promoter in cancer induction. *Seminars in Cancer Biology, 14*(6), 433-439.

Pirini, M., Testi, S., Ventrella, V., Pagliarani, A., & Badiani, A. (2010). Blue-back fish: Fatty acid profile in selected seasons and retention upon baking. *Food Chemistry*, *123*(2), 306-314.

Price, S. C. (1994). The role of hepatocellular injury in the chronic toxicity of BHT: Two-generation Wistar albino rat study. Robens Institute, U. of Surrey, Guilford, Surrey, U. K. Study No: 1/91/Tx. Final Report No: R193/TOX/0020. Vol. 1-8. Submitted to WHO by Robens Institute. Unpublished.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, *53*(10), 4290-4302.

Prost, C., Serot, T., & Demaimay, M. (1998). Identification of the most potent odorants in wild and farmed cooked turbot (*Scophtalamus maximus* L.). *Journal of Agricultural and Food Chemistry*, *46*(8), 3214-3219.

Rao, G. V. S., Parthasarathy, K. R., & Sundararaj, A. (2000). Haemorrhagic syndrome in butylated hydroxy toluene (BHT) toxicity in broiler chicken. *Indian Veterinary Journal, 77*(2), 117-119.

Regulska-Ilow, B., & Ilow, R. (2002). Comparison of the effects of microwave cooking and conventional cooking methods on the composition of fatty acids and fat quality indicators in herring. *Nahrung, 46*(6), 383-388.

Reis, P., Holmberg, K., Watzke, H., Leser, M. E., & Miller, R. (2009). Lipases at interfaces: A review. *Advances in Colloid and Interface Science*, *147–148*, 237-250.

Remanan, M. K., & Wu, J. (2014). Antioxidant activity in cooked and simulated digested eggs. *Food & Function*, *5*(7), 1464-1474.

Rodil, R., Quintana, J. B., Basaglia, G., Pietrogrande, M. C., & Cela, R. (2010). Determination of synthetic phenolic antioxidants and their metabolites in water samples by downscaled solid-

phase extraction, silylation and gas chromatography-mass spectrometry. *Journal of Chromatography A, 1217*(41), 6428-6435.

Rodil, R., Quintana, J. B., & Cela, R. (2012). Oxidation of synthetic phenolic antioxidants during water chlorination. *Journal of Hazardous Materials, 199-200*, 73-81.

Rodriguez, J. A., Mendoza, L. D., Pezzotti, F., Vanthuyne, N., Leclaire, J., Verger, R., Buono, G., Carriere, F., & Fotiadu, F. (2008). Novel chromatographic resolution of chiral diacylglycerols and analysis of the stereoselective hydrolysis of triacylglycerols by lipases. *Analytical Biochemistry*, *375*(2), 196-208.

Roman, M. J., Burri, B. J., & Singh, R. P. (2012). Release and bioaccessibility of β-carotene from fortified almond butter during *in vitro* digestion. *Journal of Agricultural and Food Chemistry*, *60*(38), 9659-9666.

Sacchi, R., Paolillo, L., Giudicianni, I., & Addeo, F. (1991). Rapid ^{1}H-NMR determination of 1,2 and 1,3 diglycerides in virgin olive oils. *Ital. J. Food Sci.*, (4), 253-262.

Saito, H., & Udagawa, M. (1992). Application of NMR to evaluate the oxidative deterioration of brown fish meal. *Journal of the Science of Food and Agriculture*, *58*, 135-137.

Sampels, S. (2015). The effects of processing technologies and preparation on the final quality of fish products. *Trends in Food Science and Technology, 44,* 131-146.

Sánchez-Muñiz, F. J., Viejo, J. M., & Medina, R. (1992). Deep-frying of sardines in different culinary fats. Changes in the fatty acid composition of sardines and frying fats. *Journal of Agricultural and Food Chemistry, 40*(11), 2252-2256.

Sánchez-Muñiz, F. J., Arroyo, R., Sánchez-Montero, J. M., & Cuesta, C. (2000). *In vitro* digestibility study of thermally oxidised palm oleins. *Food Science and Technology International, 6*(6), 449-456.

Santos, N. A., Cordeiro, A. M. T. M., Damasceno, S. S., Aguiar, R. T., Rosenhaim, R., Carvalho Filho, J. R., Santos, I. M. G., Maia, A. S., & Souza, A. G. (2012). Commercial antioxidants and thermal stability evaluations. *Fuel 97*, 638-643.

Satyarthi, J. K., Srinivas, D., & Ratnasamy, P. (2009). Estimation of free fatty acid content in oils, fats, and biodiesel by ^{1}H NMR spectroscopy. *Energy & Fuels*, *23*(4), 2273-2277.

Scalone, G. L., Cucu, T., De kimpe, N., & De Meulaner, B. (2015). Influence of free amino acids, oligopeptides, and polypeptides on the formation of pyrazines in Maillard model systems. *Journal of Agricultural and Food Chemistry, 63*(22), 5364-5372.

Schaich, K. M. Analysis of lipid and protein oxidation in fats, oils, and foods. In *Oxidative Stability and Shelf Life of Foods Containing Oils and Fats,* 1th edition; Hu, M., Jacobsen, C., Eds.; Academic Press and AOCS Press: Oxford, UK, 2016, pp. 1-131.

Schuh, C., & Schieberle, P. (2005). Characterization of *(E,E,Z)*-2,4,6-nonatrienal as a character impact aroma compound of oat flakes. *Journal of Agricultural and Food Chemistry*, *53*(22), 8699-8705.

Sek, L., Porter, C. J. H., & Charman, W. N. (2001). Characterisation and quantification of medium chain and long chain triglycerides and their *in vitro* digestion products, by HPTLC coupled with in situ densitometric analysis. *Journal of Pharmaceutical and Biomedical Analysis*, *25*(3–4), 651-661.

Sek, L., Porter, C. J. H., Kaukonen, A. M., & Charman, W. N. (2002). Evaluation of the *in vitro* digestion profiles of long and medium chain glycerides and the phase behaviour of their lipolytic products. *Journal of Pharmacy and Pharmacology*, *54*(1), 29-41.

Selke, E., & Rohwedder, W. K. (1983). Volatile components from trilinolenin heated in air. *Journal of the American Oil Chemists´ Society, 60*, 1853-1858.

Serra, A., Macia, A., & Estevez, M. (2013). Synthetic phenolic antioxidants. In: Nollet LML, Toldrá F, editors. Food analysis by HPLC. Boca Raton, Florida: CRC Press. p 551-65.

Shaw, Y. S., & Chen, C. (1972). Ring hydroxylation of di-*t*-butylhydroxytoluene by rat liver microsomal preparations. *Biochemical Journal, 128*(5), 1285-1291.

Shearn, C. T., Fritz, K. S., & Thompson, J. A. (2011). Protein damage from electrophiles and oxidants in lungs of mice chronically exposed to the tumor promoter butylated hydroxytoluene. *Chemico-Biological Interactions, 192*(3), 278-286.

Shirai, T., Hagiwara, A., Kurata, Y., Shibata, M., Fukushima, S., & Ito, N. (1982). Lack of carcinogenicity of butylated hydroxytoluene on long-term administration to B6C3F1 mice. *Food and Chemical Toxicology, 20*(6), 861-865.

Shlian, D. M., & Goldstone, J. (1986). More on BHT toxicity. *Western Journal of Medicine, 145*(5), 699.

Siddiqui, N., Sim, J., Silwood, C. J. L., Toms, H., Iles, R. A., & Grootveld, M. (2003). Multicomponent analysis of encapsulated marine oil supplements using high-resolution ^{1}H and ^{13}C NMR techniques. *Journal of Lipid Research, 44*(12), 2406-2427.

Siems, W., Wiswedel, I., Salerno, C., Crifò, C., Augustin, W., Schild, L., Langhans, C. D., & Sommerburg, O. (2005). *Beta*-carotene breakdown products may impair mitochondrial functions--potential side effects of high-dose *beta*-carotene supplementation. *The Journal of Nutritional Biochemistry*, *16*(7), 385-397.

Sioen, I., Haak, L., Raes, K., Hermans, C., De Henauw, S., De Smet, S., & Van Camp, J. (2006). Effects of pan-frying in margarine and olive oil on the fatty acid composition of cod and salmon. *Food Chemistry*, *98*(4), 609-617.

Sissener, N. H., Julshamn, K., Espe, M., Lunestad, B. T., Hemre, G. I., Waagbo, R., & Mage, A. (2013). Surveillance of selected nutrients, additives and undesirables in commercial Norwegian fish feeds in the years 2000-2010. *Aquaculture Nutrition, 19*(4), 555-572.

Skiera, C., Steliopoulos, P., Kuballa, T., Holzgrabe, U., & Diehl, B. (2012). Determination of free fatty acids in edible oils by ^{1}H NMR spectroscopy. *Lipid Technology*, *24*(12), 279–281.

Skjevrak, I., Due, A., Gjerstad, K. O., & Herikstad, H. (2003). Volatile organic components migrating from plastic pipes (HDPE, PEX and PVC) into drinking water. *Water Research, 37*(8), 1912-1920.

Slaga, T. J. (1995). Inhibition of skin tumor initiation, promotion, and progression by antioxidants and related compounds. *Critical Reviews in Food Science and Nutrition, 35*(1-2), 51-57.

Smirnova, E. G., Lyubimov, Y. I., Malinina, T. G., Lyubimova, E. Y., Alexandrushkina, N. I., Vanyushin, B. F., Kolesova, G. M., & Yaguzhinsky, L. S. (2002). Ionol (BHT) produces superoxide anion. *Biochemistry (Moscow), 67*(11), 1271-1275.

Snipes, W., Person, S., Keith, A., & Cupp, J. (1975). Butylated hydroxytoluene inactivated lipid-containing viruses. *Science, 188*(4183):64-66.

Soldera, S., Sebastianutto, N., & Bortolomeazzi, R. (2008). Composition of phenolic compounds and antioxidant activity of commercial aqueous smoke flavorings. *Journal of Agricultural and Food Chemistry, 56*(8), 2727-2734.

Sommerburg, O., Langhans, C.-D., Arnhold, J., Leichsenring, M., Salerno, C., Crifò, C., Hoffmann, G. F., Debatin, K. M., & Siems, W. G. (2003). *Beta*-carotene cleavage products after oxidation mediated by hypochlorous acid--a model for neutrophil-derived degradation. *Free Radical Biology & Medicine*, *35*(11), 1480-1490.

Sopelana, P., Arizabaleta, I., Ibargoitia, M. L., & Guillén, M. D. (2013). Characterisation of the lipidic components of margarines by ^{1}H Nuclear Magnetic Resonance. *Food Chemistry, 141*(4), 3357-3364.

Soubra, L., Sarkis, D., Hilan, C., & Verger, Ph. (2007). Dietary exposure of children and teenagers to benzoates, sulphites, butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) in Beirut (Lebanon). *Regulatory Toxicology and Pharmacology, 47*, 68-77.

Spiteller, G. (1998). Linoleic acid peroxidation - the dominant lipid peroxidation process in low density lipoprotein - and its relationship to chronic diseases. *Chemistry and Physics of Lipids, 95*(2), 105-162.

Spyros, A., & Dais, P. (2000). Application of ^{31}P NMR spectroscopy in food analysis. 1. Quantitative determination of the mono- and diglyceride composition of olive oils. *Journal of Agricultural and Food Chemistry*, *48*(3), 802-805.

Spyros, A., Philippidis, A., & Dais, P. (2004). Kinetics of diglyceride formation and isomerization in virgin olive oils by employing ^{31}P NMR spectroscopy. Formulation of a quantitative measure to assess olive oil storage history. *Journal of Agricultural and Food Chemistry*, *52*(2), 157-164.

Steppeler, C., Haugen, J.-E., Rødbotten, R., & Kirkhus, B. (2016). Formation of malondialdehyde, 4-hydroxynonenal, and 4-hydroxyhexenal during *in vitro* digestion of cooked beef, pork, chicken, and salmon. *Journal of Agricultural and Food Chemistry*, *64*(2), 487-496.

Stokes, J. D., & Scudder, C. L. (1974). The effect of butylated hydroxyanisole and butylated hydroxytoluene on behavioral development of mice. *Developmental Psychobiology, 7*(4), 343-350.

Suh, H. J., Chung, M. S., Cho, Y. H., Kim, J. W., Kim, D. H., Han, K. W., & Kim, C. J. (2005). Estimated daily intakes of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) and *tert*-butyl hydroquinone (TBHQ) antioxidants in Korea. *Food Additives and Contaminants, 22*(12), 1176-1188.

Suman, M. (2001). Identification of hydrocarbon components generated by transformation processing of cast polypropylene employed for combining with other plastic films. *International Journal of Polymer Materials, 49*(1), 91-100.

Sun, Y., Dwyer-Nield, L. D., Malkinson, A. M., Zhang, Y. L., & Thompson, J. A. (2003). Responses of tumorigenic and non-tumorigenic mouse lung epithelial cell lines to electrophilic metabolites of the tumor promoter butylated hydroxytoluene. *Chemico-Biological Interactions, 145*(1), 41-51.

Suyama, K., Yeow, T., & Nakai, S. (1983). Vitamin A oxidation products responsible for haylike flavor production in nonfat dry milk. *Journal of Agricultural and Food Chemistry*, *31*(1), 22-26.

Takahashi, O. (1992). Haemorrhages due to defective blood coagulation do not occur in mice and guinea pigs fed butylated hydroxytoluene, but nephrotoxicity is found in mice. *Food and Chemical Toxicology, 30*(2), 89-97.

Tanaka, T., Oishi, S., & Takahashi, O. (1993). Three generation toxicity study of butylated hydroxytoluene administered to mice. *Toxicology Letters, 66*(3), 295-304.

Tarvainen, M., Phuphusit, A., Suomela, J.-P., Kuksis, A., & Kallio, H. (2012). Effects of antioxidants on rapeseed oil oxidation in an artificial digestion model analyzed by UHPLC–ESI–MS. *Journal of Agricultural and Food Chemistry*, *60*(14), 3564-3579.

Tassignon, P., de Waard, P., de Rijk, T., Tournois, H., de Wit, D., & de Buyck, L. (1994). An efficient countercurrent distribution method for the large-scale isolation of dimorphecolic acid methyl ester. *Chemistry and Physics of Lipids*, *71*(2), 187-196.

Thomas, N., Holm, R., Rades, T., & Müllertz, A. (2012). Characterising lipid lipolysis and its implication in lipid-based formulation development. *The AAPS Journal*, *14*(4), 860-871.

Thompson, D. C., & Trush, M. A. (1986). The toxicological implications of the interaction of butylated hydroxytoluene with other antioxidants and phenolic chemicals. *Food and Chemical Toxicology, 24*(10-11), 1189-1195.

Thompson, J. A., Carlson, T. J., Sun, Y., Dwyer-Nield, L. D., & Malkinson, A. M. (2001). Studies using structural analogs and inbred strain differences to support a role for quinone methide metabolites of butylated hydroxytoluene (BHT) in mouse lung tumor promotion. *Toxicology, 160*(1-3), 197-205.

Tokur, B. (2007). The effect of different cooking methods on proximate composition and lipid quality of rainbow trout (*Oncorhynchus mykiss*). *International Journal of Food Science & Technology*, *42*(7), 874-879.

Tombesi, N. B., & Freije, H. (2002). Application of solid-phase microextraction combined with gas chromatography–mass spectrometry to the determination of butylated hydroxytoluene in bottled drinking water. *Journal of Chromatography A, 963*(1–2), 179-183.

Tsaknis, J., Lalas, S., & Protopapa, E. (2002). Effectiveness of the antioxidants BHA and BHT in selected vegetable oils during intermittent heating. *Grasas Aceites 53*(2), 199-205.

Tullberg, C., Larsson, K., Carlsson, N. G., Corni, I., Scheers, N., Vegarud, G., & Undeland, I. (2016). Formation of reactive aldehydes (MDA, HHE, HNE) during the digestion of cod liver oil: comparison of human and porcine *in vitro* digestion models. *Food & Function, 7*(3), 1401-1412.

Turchini, G. M., Moretti, V. M., Mentasti, T., Orban, E., & Valfrè, F. (2007). Effects of dietary lipid source on fillet chemical composition, flavour volatile compounds and sensory characteristics in the freshwater fish tench (*Tinca tinca* L.). *Food Chemistry*, *102*(4), 1144-1155.

Türkkan, A. U., Cakli, S., & Kilinc, B. (2008). Effects of cooking methods on the proximate composition and fatty acid composition of seabass (*Dicentrarchus labrax*, Linnaeus, 1758). *Food and Bioproducts Processing, 86*(3), 163-166.

Turner, R., McLean, C. H., & Silvers, K. M. (2006). Are the health benefits of fish oils limited by products of oxidation? *Nutrition Research Reviews*, *19*(1), 53-62.

van Aken, G. A., Bomhof, E., Zoet, F. D., Verbeek, M., & Oosterveld, A. (2011). Differences in *in vitro* gastric behaviour between homogenized milk and emulsions stabilised by Tween 80, whey protein, or whey protein and caseinate. *Food Hydrocolloids*, *25*(4), 781-788.

Varlet, V., & Fernandez, X. (2010). Sulfur-containing volatile compounds in seafood: Occurrence, odorant properties and mechanisms of formation. *Food Science and Technology International, 16*, 463-503.

Verhagen, H., Beckers, H. H., Comuth, P. A., Maas, L. M., ten Hoor, F., Henderson, P. T., & Kleinjans, J. C. (1989). Disposition of single oral doses of butylated hydroxytoluene in man and rat. *Food and Chemical Toxicology, 27*(12), 765-772.

Verhagen, H., Deerenberg, I., Marx, A., Ten Hoor, F., Henderson, P. T., & Kleinjans, J. C. (1990). Estimate of the maximal daily dietary intake of butylated hydroxyanisole and butylated hydroxytoluene in The Netherlands. *Food and Chemical Toxicology, 28*(4), 215-220.

Versantvoort, C. H. M., Van de Kamp, E., & Rompelberg, C. (2004). Development and applicability of an *in vitro* digestion model in assessing the bioaccessibility of contaminants from food. Report No. 320102002. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Available from: http://www.rivm.nl/bibliotheek/rapporten/320102002.pdf (Accessed 2015 November 15).

Versantvoort, C. H. M., Oomen, A. G., Van de Kamp, E., Rompelberg, C. J. M., & Sips, A. J. A. M. (2005). Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. *Food and Chemical Toxicology*, *43*(1), 31-40.

Vidal, N. P., Manzanos, M. J., Goicoechea, E., & Guillén, M. D. (2012). Quality of farmed and wild sea bass lipids studied by ^{1}H NMR: usefulness of this technique for differentiation on a qualitative and a quantitative basis. *Food Chemistry*, *135*(3), 1583-1591.

Vidal, N.P., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2014). Fourier transform infrared spectroscopy as a tool to study farmed and wild sea bass lipid composition. *Journal of the Science of Food and Agriculture, 94,* 1340-1348.

Vidal, N. P., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2015). ^{1}H NMR study of the changes in brine- and dry-salted sea bass lipids under thermo-oxidative conditions: Both salting methods reduce oxidative stability. *European Journal of Lipid Science and Technology, 117*(4), 440-449.

Vidal, N. P., Manzanos, M. J., Goicoechea, E., & Guillén, M. D. (2016a). Farmed and wild sea bass (*Dicentrarchus labrax*) volatile metabolites. A comparative study by SPME-GC/MS. *Journal of the Science of Food and Agriculture, 96*(4), 1181-1193.

Vidal, N. P., Manzanos, M. J., Goicoechea, E., & Guillén, M. D. (2016b). Influence of different salting processes on the evolution of the volatile metabolites of vacuum-packed fillets of farmed and wild sea bass (*Dicentrarchus labrax*) stored under refrigeration conditions. A study by SPME-GC/MS. *Journal of the Science of Food and Agriculture, Ahead of print*. DOI: 10.1002/jsfa.7821.

Vidal, N. P., Manzanos, M. J., Goicoechea, E., & Guillén, M. D. (2016c). Influence of smoking with smoke flavourings on the oxidative stability of farmed sea bass fillets monitored by ^{1}H NMR and FTIR. *European Journal Lipid Science and Technology, Ahead of print*. DOI: 10.1002/ejlt.201600023

Vidal, N. P., Picone, G., Goicoechea, E., Laghi, L., Manzanos, M. J., Danesi, F., Bordoni, A., Capozzi, F., & Guillén, M. D. (2016d). Metabolites release and protein hydrolysis during the *in vitro* digestion of cooked sea bass fillets. A study by ^{1}H NMR. *Food Research International, 88 Part B*, 293–301.

Vidal, N. P., Goicoechea, E., Manzanos, M. J., & Guillén, M. D. (2016e). Effect of smoking using smoke flavorings on several characteristics of farmed sea bass (*Dicentrarchus labrax*) fillets and on their evolution during vacuum-packed storage at refrigeration temperature. *Journal of Food Processing and Preservation, Ahead of print*. DOI: 10.1111/jfpp.12800

Vin, K., Connolly, A., McCaffrey, T., McKevitt, A., O'Mahony, C, Prieto, M., Tennant, D., Hearty A, & Volatier, J. L. (2013). Estimation of the dietary intake of 13 priority additives in France, Italy, the UK and Ireland as part of the FACET project. *Food Additives & Contaminants Part A, 30*(12), 2050-2080.

Vinarov, Z., Petkova, Y., Tcholakova, S., Denkov, N., Stoyanov, S., Pelan, E., & Lips, A. (2012a). Effects of Emulsifier Charge and Concentration on Pancreatic Lipolysis. 1. In the Absence of Bile Salts. *Langmuir*, *28*(21), 8127-8139.

Vinarov, Z., Tcholakova, S., Damyanova, B., Atanasov, Y., Denkov, N. D., Stoyanov, S. D., Pelan, E., & Lips, A. (2012b). Effects of Emulsifier Charge and Concentration on Pancreatic Lipolysis: 2. Interplay of Emulsifiers and Biles. *Langmuir*, *28*(33), 12140-12150.

Vinarov, Z., Petrova, L., Tcholakova, S., Denkov, N. D., Stoyanov, S. D., & Lips, A. (2012). *In vitro* study of triglyceride lipolysis and phase distribution of the reaction products and cholesterol: effects of calcium and bicarbonate. *Food & Function*, *3*(11), 1206-1220.

Vlahov, G. (1996). Improved quantitative ^{13}C nuclear magnetic resonance criteria for determination of grades of virgin olive oils. The normal ranges for diglycerides in olive oil. *Journal of the American Oil Chemists' Society, 73*(9), 1201-1203.

Vlahov, G. (2006). ^{13}C Nuclear Magnetic Resonance. Studies of mono-, di- and triacylglycerols. NOE factors and spin-lattice relaxation of acyl chain carboxy carbons. In Linda T. Welson (Ed), *Triglycerides and cholesterol research* (pp. 251-270). New York: Nova Publishers Inc.

Warner, C. R., Brumley, W. C., Daniels, D. H., Joe, Jr. F. L., & Fazio, T. (1986). Reactions of antioxidants in foods. *Food and Chemical Toxicology, 24*(10–11), 1015-1019.

Warner, C. R., Daniels, D. H., Lin, F. S. D., Joe, F. L., & Fazio, T. (1986). Fate of antioxidants and antioxidant-derived products in deep-fat frying and cookie baking. *Journal of Agricultural and Food Chemistry, 34*(1), 1-5.

Weber, J., Bochi, V. C., Ribeiro, C. P., Victório, A. de M., & Emanuelli, T. (2008). Effect of different cooking methods on the oxidation, proximate and fatty acid composition of silver catfish (*Rhamdia quelen*) fillets. *Food Chemistry*, *106*(1), 140-146.

Whitfield, F. B. (1992). Volatiles from interactions of Maillard reactions and lipids. *Critical Reviews in Food Science and Nutrition*, *31*(1-2), 1-58.

Williams, G. M., Tanaka, T., & Maeura, Y. (1986). Dose-related inhibition of aflatoxin B1 induced hepatocarcinogenesis by the phenolic antioxidants, butylated hydroxyanisole and butylated hydroxytoluene. *Carcinogenesis, 7*(7), 1043-1050.

Williams, G. M., Wang, C. X., & Iatropoulos, M. J. (1990). Toxicity studies of butylated hydroxyanisole and butylated hydroxytoluene. II. Chronic feeding studies. *Food and Chemical Toxicology, 28*(12), 799-806.

Williams, G. M., & Iatropoulos, M. J. (1996). Inhibition of the hepatocarcinogenicity of aflatoxin B1 in rats by low levels of the phenolic antioxidants butylated hydroxyanisole and butylated hydroxytoluene. *Cancer Letters, 104*(1), 49-53.

Williams, G. M., Iatropoulos, M. J., & Whysner, J. (1999). Safety assessment of butylated hydroxyanisole and butylated hydroxytoluene as antioxidant food additives. *Food and Chemical Toxicology, 37*(9–10), 1027-1038.

Wilson, R., Fernie, C. E., Scrimgeour, C. M., Lyall, K., Smyth L., & Riemersmaet, R. A. (2002). Dietary epoxy fatty acids are absorbed in healthy women. *European Journal of Clinical Investigation, 32*, 79-83.

Wurzenberger, M., & Grosch, W. (1986). Enzymic oxidation of linolenic acid to 1,*Z*-5-octadien-3-ol, *Z*-2,*Z*-5-octadien-1-ol and 10-oxo-*E*-8-decenoic acid by a protein fraction from mushrooms (*Psalliota bispora*). *Lipids, 21*(4), 261-266.

Xia, W., Budge, S. M., & Lumsden, M. D. (2015). New ^{1}H NMR-based technique to determine epoxide concentrations in oxidized oil. *Journal of Agricultural and Food Chemistry*, *63*(24), 5780-5786.

Yamamoto, K., Tajima, K., Takemura, M., & Mizutani, T. (1991). Further metabolism of 3,5-di-*tert*-butyl-4-hydroxybenzoic acid, a major metabolite of butylated hydroxytoluene, in rats. *Chemical & Pharmaceutical Bulletin, 39*(2), 512-514.

Yamamoto, K., Fukuda, N., Shiroi, S., Shiotsuki, Y., Nagata, Y., Tani, T., & Sakai, T. (1995). Effect of dietary antioxidants on the susceptibility to hepatic microsomal lipid peroxidation in the rat. *Annals of Nutrition and Metabolism, 39*(2), 99-106.

Yanishlieva, N. V., Marinova, E. M., Gordon, M. H., & Raneva, V. G. (1999). Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. *Food Chemistry, 64*(1), 59-66.

Yoon, J. Y., Kim, J. H., Baek, K.-S., Kim, G. S., Lee, S. E., Lee, D. Y., Choi, J. H., Kim, S. Y., Park, H. B., Sung, G. H., Lee, K. R., Cho, J. Y., & Noh, H. J. (2015). A direct protein kinase B-targeted anti-inflammatory activity of cordycepin from artificially cultured fruit body of *Cordyceps militaris*. *Pharmacognosy Magazine*, *11*(43), 477-485.

Zamora, R., & Hidalgo, F. J. (2005). Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Critical Reviews in Food Science and Nutrition*, *45*(1), 49–59.

Zamora, R., & Hidalgo, F. J. (2011). The Maillard reaction and lipid oxidation. *Lipid Technology, 23*(3), 59-62.

Zhang, C. X., Wu, H., & Weng, X. C. (2004). Two novel synthetic antioxidants for deep-frying oils. *Food Chemistry, 84*(2), 219-222.

Zhang, J., Wu, D., Liu, D., Fang, Z., Chen, J., Hu, Y., & Ye, X. (2013). Effect of cooking styles on the lipid oxidation and fatty acid composition of grass carp (*Ctenopharynyodon idellus*) fillet. *Journal of Food Biochemistry*, *37*(2), 212-219.

Zhang, Y. M., Peng, Y., Yin, X. I., Liu, Z. H., & Li, G. (2014). Degradation of lignin to BHT by electrochemical catalysis on Pb/PbO_2 anode in alkaline solutions. *Journal of Chemical Technology and Biotechnology, 89*(12), 1954-1960.

Zhu, X., Ye, A., Verrier, T., & Singh, H. (2013). Free fatty acid profiles of emulsified lipids during *in vitro* digestion with pancreatic lipase. *Food Chemistry*, *139*(1-4), 398-404.

OTHER CONTRIBUTIONS