Food Chemistry 179 (2015) 182–190

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Usefulness of ^{1}H NMR in assessing the extent of lipid digestion

Bárbara Nieva-Echevarría, Encarnación Goicoechea, María J. Manzanos, María D. Guillén *

Food Technology, Faculty of Pharmacy, Lascaray Research Center, University of the Basque Country (UPV/EHU), Paseo de la Universidad No. 7, 01006 Vitoria, Spain

ARTICLE INFO

Article history:
Received 8 August 2014
Received in revised form 18 December 2014
Accepted 21 January 2015
Available online 30 January 2015

Chemical compounds studied in this article:
Tridocosahexaenoin (PubChem CID: 9546569)
Trieicosapentaenoin (PubChem CID: 9545999)
Trilinolein (PubChem CID: 5322095)
1,2-Diolein (PubChem CID: 5497164)
1,3-Dilinolein (PubChem CID: 45934042)
1-Monolinolein (PubChem CID: 5283469)
2-Monoolein (PubChem CID: 5319879)
Docosahexaenoic acid (PubChem CID: 445580)
Eicosapentaenoic acid (PubChem CID: 446284)
Linoleic acid (PubChem CID: 5280450)

Keywords:
^{1}H NMR
Lipid hydrolysis
Triglycerides
Diglycerides
Monoglycerides
Fatty acids
Glycerol

ABSTRACT

Proton Nuclear Magnetic Resonance (^{1}H NMR) is 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 flesh, as model foods, were subjected to different *in vitro* digestion experiments and the lipolysis levels reached were evaluated using ^{1}H NMR spectral data. Simple observation of the spectra gives very valuable information about the extent of the lipolysis and enables a rapid discrimination among samples having different hydrolysis degree. Equations were developed to quantify all the lipolytic products, and either referred to acyl groups plus fatty acids, or to glyceryl structures. The main hydrolysis products were 1,2-diglycerides, 2-monoglycerides, glycerol and fatty acids, although small proportions of 1,3-diglycerides and of 1-monoglycerides were also found. With this methodology, determination of the extent of lipid digestion in its different definitions can be made. It has been shown that these definitions are not equivalent, which is evidence for the need for a consensus in this regard.

1. Introduction

In recent years a great deal of attention has been paid to food digestion processes and to the estimation, by means of *in vitro* digestion models, of the bioaccessibility and bioavailability of certain compounds which are either toxic or beneficial for human health (Colle, Van Buggenhout, Lemmens, Van Loey, & Hendrickx, 2012; Goicoechea et al., 2008; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005). In fact, 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). However, the extent of the *in vitro* digestion process achieved using the different protocols proposed, has hardly been studied and reported.

Nowadays, lipid digestion is receiving considerable attention from researchers, and the management of lipid release and absorption has become a challenge (McClements, Decker, & Park, 2009). Once ingested, triglycerides (TG) are subjected to hydrolysis which is mainly catalyzed by lipases present in gastric and duodenal digestive juices. Lipolysis reaction is ruled by enzyme regiospecificity (sn-1 and sn-3), yielding 2-monoglyceride (2-MG) and two fatty acids (FA). Complete hydrolysis can also be achieved, after isomerization of 2-MG into 1-monoglyceride (1-MG) (Desnuelle & Savary, 1963; Mattson & Volpenhein, 1964). In turn, MG and

* Corresponding author. Tel./fax: +34 945 013081.
E-mail address: mariadolores.guillen@ehu.es (M.D. Guillén).

http://dx.doi.org/10.1016/j.foodchem.2015.01.104
0308-8146/

FA are solubilized in bile-salt aggregates and then absorbed across the intestinal epithelium after lipolysis (Mu & Høy, 2004). This process continuously removes the products formed, which allows lipolysis to continue.

Different methodologies have been applied to date when quantifying lipid hydrolysis products in *in vitro* digestion studies. The titration of fatty acids released by means of a pH-stat apparatus is the technique which is most commonly employed to estimate the extent of lipid digestion during *in vitro* digestion (Hur, Decker, & McClements, 2009; Li & McClements, 2010; Marze, Meynier, & Anton, 2013; Thomas, Holm, Rades, & Müllertz, 2012). Although this technique allows continuous monitoring of the FA released, limited information on the lipolysis reaction can be obtained since quantification of partial glycerides (DG and MG) is not possible. In other studies chromatographic techniques have also been employed; however, these methodologies are time-consuming and involve many preparation steps (Armand et al., 1999; Capolino et al., 2011; Helbig, Silletti, Timmerman, Hamer, & Gruppen, 2012; Kenmogne-Domguia, Meynier, Viau, Llamas, & Genot, 2012; Sek, Porter, & Charman, 2001; Zhu, Ye, Verrier, & Singh, 2013).

More recently, a new approach based on Proton Nuclear Magnetic Resonance (^{1}H NMR) has been proposed to study qualitatively and quantitatively all the lipolytic products arising from TG hydrolysis (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2014). Due to the proportionality existing between the area of the ^{1}H NMR spectral signals and the number of protons that generate them, different equations were developed and validated to calculate the molar percentages of TG, DG, MG and FA in complex mixtures of standard compounds simulating lipid hydrolysates from vegetable, terrestrial animal and marine origins. This new methodology overcomes the limitations of the above-mentioned alternatives, allowing the quantification of all kinds of glyceryl structures and FA in a simple, fast and accurate way.

The aim of this work is to demonstrate for the first time the usefulness of ^{1}H NMR when studying the extent of lipid hydrolysis reached during *in vitro* digestion of food samples. For this purpose, sunflower oil and minced fish flesh were submitted to *in vitro* digestion under different experimental conditions in order to obtain samples with very different lipid composition and showing different levels of hydrolysis. Afterwards, the lipids of digested samples were extracted and their ^{1}H NMR spectra were studied in detail in order to evaluate the advance of lipolysis during digestion. The different products generated from the hydrolysis of TG were quantified using ^{1}H NMR spectral data and expressed in accordance with previous studies, either as function of acyl groups plus fatty acids, or as function of glyceryl structures. Finally, the extent of lipid digestion reached in digested samples was determined using all the different approaches, usually employed in digestion studies: hydrolysis level, degree of TG transformation, lipid bioaccessibility level and percentage of fatty acids physiologically releasable (Capolino et al., 2011; Pafumi et al., 2002; Vinarov et al., 2012; Zhu et al., 2013).

2. Materials and methods

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 (*Dicentrarchus labrax*) (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 possessing 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 while 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 ground. Sunflower oil and minced fish flesh were submitted to *in vitro* digestion under different experimental conditions in order to obtain samples with 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. In vitro digestion

Digestion experiments were carried out following the *in vitro* digestion model described by Versantvoort et al. (2005), already employed in some previous studies (Goicoechea, Brandon, Blokland, & Guillén, 2011; Goicoechea et al., 2008). This model implies a three-step procedure which simulates digestive processes in the mouth, stomach, and small intestine by sequentially adding 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 juice and bile) were prepared artificially in accordance with Versantvoort et al. (2005), with slight modifications 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 *A. 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.

2.3. 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, St. Louis, MO, USA) as solvent in a proportion of 1:2 (w/v) and assisted by an ultrasonic bath for 1 h. Afterwards, solvent was removed by means of a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. 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 CH_2Cl_2 in a proportion of 2:3 (v/v). As previously described, the solvent was evaporated off and the lipid extracts obtained from *in vitro* digested samples were named SPL, STL, FPL and FTL, just 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 TG hydrolysis.

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. As in previous studies of edible oils carried out in our laboratory (Guillén, Carton, Goicoechea, & Uriarte, 2008; Martínez-Yusta & Guillén, 2014; Nieva-Echevarría et al., 2014; Vidal, Manzanos, Goicoechea, & Guillén, 2012), 200 μl of the lipid samples were mixed with 400 μl deuterated chloroform ($CDCl_3$), which contains 0.2% of non-deuterated chloroform and a small proportion of tetramethylsilane (TMS) used as internal reference (Cortec, Paris, France). The mixture was introduced into a 5 mm diameter tube. 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 used were the following: spectral width 6410 Hz, relaxation delay 3 s, number of scans 64, acquisition time 4.819 s and pulse width 90°. 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. The ^{1}H NMR spectra were plotted at a fixed value of absolute intensity to be valid for comparative purposes. All data derived from ^{1}H NMR spectra are provided as average values together with the standard deviations.

Table 1 shows the assignment of the ^{1}H NMR signals from the spectra of the samples studied, as shown in Figs. 1 and 2; this assignment is in agreement with previous studies (Guillén & Ruiz, 2001, 2003a,b; Guillén et al., 2008; Nieva-Echevarría et al., 2014; Vidal et al., 2012).

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 in the lipid samples can be calculated by the following equations, developed and validated in a previous study (Nieva-Echevarría et al., 2014):

$$N_{2\text{-MG}} = Pc^{*}A_{\text{K}}/4 \quad (1)$$

$$N_{1\text{-MG}} = Pc^{*}A_{\text{L}} \quad (2)$$

$$N_{1,2\text{-DG}} = Pc^{*}(A_{\text{I+J}} - 2A_{\text{L}})/2 \quad (3)$$

$$N_{1,3\text{-DG}} = Pc^{*}(A_{4.04-4.38} - 2A_{4.26-4.38} - 2A_{\text{L}})/5 \quad (4)$$

$$N_{\text{TG}} = Pc^{*}(2A_{4.26-4.38} - A_{\text{I+J}} + 2A_{\text{L}})/4 \quad (5)$$

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

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

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, and $A_{4.04-4.38}$, $A_{4.26-4.38}$, $A_{2.26-2.40}$, $A_{2.26-2.37}$ and $A_{2.37-2.44}$ 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 Eq. (6a) is intended for lipids from vegetable or terrestrial animal origins, and Eq. (6b) for marine lipids. In the case of fish lipid digestion, the hydrolysis of phospholipids was not taken into account in the present study, because they were present in very low proportions in comparison with TG.

These equations were used in the quantification of the products generated during lipolysis, as well as in the assessment of the extent of the lipid digestion.

3. Results and discussion

^{1}H NMR has been shown to be a very useful technique in evaluating the hydrolysis level in complex lipid mixtures of standard compounds (Nieva-Echevarría et al., 2014). The occurrence of certain signals in the ^{1}H NMR spectrum of a digested lipid sample can provide information about the nature of the glycerides (TG, DG and MG) present among the lipid digestion products. In addition, this methodology makes it possible to discriminate between isomers of diglycerides (1,2-DG and 1,3-DG) and monoglycerides (2-MG and 1-MG), which is useful when studying the specificity of digestive lipases. In fact, digestive lipases show positional specificity (Desnuelle & Savary, 1963; Hofmann & Borgström, 1963) and the expected pathway of TG hydrolysis under *in vitro* conditions is the consecutive transformation into 1,2-DG, 2-MG, and G (prior isomerization of 2-MG into 1-MG), unless unspecific lipases are used. Furthermore, since the area of each spectral signal is proportional to the number of protons that generate it, quantification of the molar percentages of the different products generated during the hydrolysis of TG can be accurately carried out by applying the above-mentioned simple equations, which involve signal areas (Nieva-Echevarría et al., 2014). In this context, the present work shows for the first time the usefulness of ^{1}H NMR in studying *in vitro* digested lipids, with different degrees of lipolysis, whose composition of acyl groups is either simple or very complex, such as sunflower oil and fish lipids. Firstly, the information provided directly from the observation of the spectra will be commented on.

3.1. Evaluation of the extent of in vitro lipolysis by simple observation of ^{1}H NMR spectra of digested lipid extracts

3.1.1. In vitro digestion of sunflower oil

Fig. 1 shows the ^{1}H NMR spectra of sunflower oil before (SUL) and after *in vitro* digestion (SPL, STL). In this figure some spectral regions are enlarged, where the most significant differences among the spectra can be observed. The assignment of the signals to the corresponding protons is given in Table 1. As can be observed, the ^{1}H NMR spectrum of unlipolyzed sunflower oil (SUL) presents the typical signals corresponding to the protons supported on the acyl groups (signals **A**, **C**, **D1**, **E**, **F1**, **G**, **T**) and those of the glyceryl backbone of TG (signals **O**, **S**).

When comparing the ^{1}H NMR spectra of the unlipolyzed sample with those of partially (SPL) and totally lipolyzed (STL) ones, noticeable differences can be observed. Certain signals remain almost unchanged (signals **A**, **C**, **E**, **G**, **T**), whereas others disappear gradually (signals **O**, **S**). The intensity in the spectrum of SPL of these latter signals, specific of TG, evidences that a remarkable proportion of TG molecules remained unaltered. By contrast, they

Table 1
Chemical shift assignments and multiplicities of the ^{1}H NMR signals in $CDCl_3$ of the main protons of glycerides and fatty acids present in lipids of vegetable and animal origins, including marine lipids (Nieva-Echevarría et al., 2014). The signal letters agree with those given in Figs. 1 and 2.

Signal	Chemical shift (ppm)	Multiplicity	Functional group Type of protons	Compound
A	0.88	t	$-C\underline{\mathbf{H}}_3$	Saturated, monounsaturated ω-9 and/or ω-7 acyl groups and FA
	0.89	t	$-C\underline{\mathbf{H}}_3$	Unsaturated ω-6 acyl groups and FA
B	0.97	t	$-C\underline{\mathbf{H}}_3$	Unsaturated ω-3 acyl groups and FA
C	1.19–1.42	m*	$-(C\underline{\mathbf{H}}_2)_n-$	Acyl groups and FA
D1	1.61	m	$-OCO-CH_2-C\underline{\mathbf{H}}_2-$	Acyl groups in TG, except for DHA, EPA and ARA acyl groups
	1.62	m	$-OCO-CH_2-C\underline{\mathbf{H}}_2-$	Acyl groups in 1,2-DG, except for DHA, EPA and ARA acyl groups
	1.63	m	$-OCO-CH_2-C\underline{\mathbf{H}}_2-$, $COOH-CH_2-C\underline{\mathbf{H}}_2-$	Acyl groups in 1,3-DG, 1-MG and FA, except for DHA, EPA and ARA acyl groups
	1.64	m	$-OCO-CH_2-C\underline{\mathbf{H}}_2-$	Acyl groups in 2-MG, except for DHA, EPA and ARA acyl groups
D2	1.69	m	$-OCO-CH_2-C\underline{\mathbf{H}}_2-$	EPA and ARA acyl groups in TG
	1.72	m	$COOH-CH_2-C\underline{\mathbf{H}}_2-$	EPA and ARA acids
E	1.92–2.15	m**	$-C\underline{\mathbf{H}}_2-CH{=}CH-$	Acyl groups and FA, except for $-CH_2-$ of DHA acyl group in β-position in relation to carbonyl group
F1	2.26–2.36	dt	$-OCO-C\underline{\mathbf{H}}_2-$	Acyl groups in TG, except for DHA acyl groups
	2.33	m	$-OCO-C\underline{\mathbf{H}}_2-$	Acyl groups in 1,2-DG, except for DHA acyl groups
	2.35	t	$-OCO-C\underline{\mathbf{H}}_2-$, $COOH-C\underline{\mathbf{H}}_2-$	Acyl groups in 1,3-DG, 1-MG and FA, except for DHA acyl groups
	2.38	t	$-OCO-C\underline{\mathbf{H}}_2-$	Acyl groups in 2-MG, except for DHA acyl groups
F2	2.37–2.41	m	$-OCO-C\underline{\mathbf{H}}_2-C\underline{\mathbf{H}}_2-$	DHA acyl groups in TG
	2.39–2.44	m	$COOH-C\underline{\mathbf{H}}_2-C\underline{\mathbf{H}}_2-$	DHA acid
G	2.77	t	$=HC-C\underline{\mathbf{H}}_2-CH=$	Diunsaturated ω-6 acyl groups and FA
H	2.77–2.90	m	$=HC-C\underline{\mathbf{H}}_2-CH=$	Polyunsaturated ω-6 and ω-3 acyl groups and FA
I	3.65	ddd	$ROCH_2-CHOH-C\underline{\mathbf{H}}_2OH$	Glyceryl group in 1-MG
J	3.73	m***	$ROCH_2-CH(OR')-C\underline{\mathbf{H}}_2OH$	Glyceryl group in 1,2-DG
K	3.84	m***	$HOC\underline{\mathbf{H}}_2-CH(OR)-C\underline{\mathbf{H}}_2OH$	Glyceryl group in 2-MG
L	3.94	m	$ROCH_2-C\underline{\mathbf{H}}OH-CH_2OH$	Glyceryl group in 1-MG
M	4.05–4.21	m	$ROC\underline{\mathbf{H}}_2-C\underline{\mathbf{H}}OH-C\underline{\mathbf{H}}_2OR'$	Glyceryl group in 1,3-DG
N	4.18	ddd	$ROC\underline{\mathbf{H}}_2-CHOH-CH_2OH$	Glyceryl group in 1-MG
O	4.22	dd,dd	$ROC\underline{\mathbf{H}}_2-CH(OR')-C\underline{\mathbf{H}}_2OR''$	Glyceryl group in TG
P	4.28	ddd	$ROC\underline{\mathbf{H}}_2-CH(OR')-CH_2OH$	Glyceryl group in 1,2-DG
Q	4.93	m	$HOCH_2-C\underline{\mathbf{H}}(OR)-CH_2OH$	Glyceryl group in 2-MG
R	5.08	m	$ROCH_2-C\underline{\mathbf{H}}(OR')-CH_2OH$	Glyceryl group in 1,2-DG
S	5.27	m	$ROCH_2-C\underline{\mathbf{H}}(OR')-CH_2OR''$	Glyceryl group in TG
T	5.28–5.46	m	$-C\underline{\mathbf{H}}{=}C\underline{\mathbf{H}}-$	Acyl groups and FA

Abbreviations: t: triplet; m: multiplet; TG: triglycerides; DHA: docosahexaenoate; EPA: eicosapentaenoate; ARA: arachidonate; 1,3-DG: 1,3-diglyceride; 1-MG: 1-monoglyceride; FA: fatty acid; 1,2-DG: 1,2-diglyceride; 2-MG: 2-monoglyceride; d: doublet.

* Overlapping of multiplets of methylenic protons in the different acyl groups either in β-position, or further, in relation to double bonds, or in γ-position, or further, in relation to the carbonyl group.

** Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups.

*** This signal shows different multiplicity if the spectrum is acquired from the pure compound or taking part in the mixture.

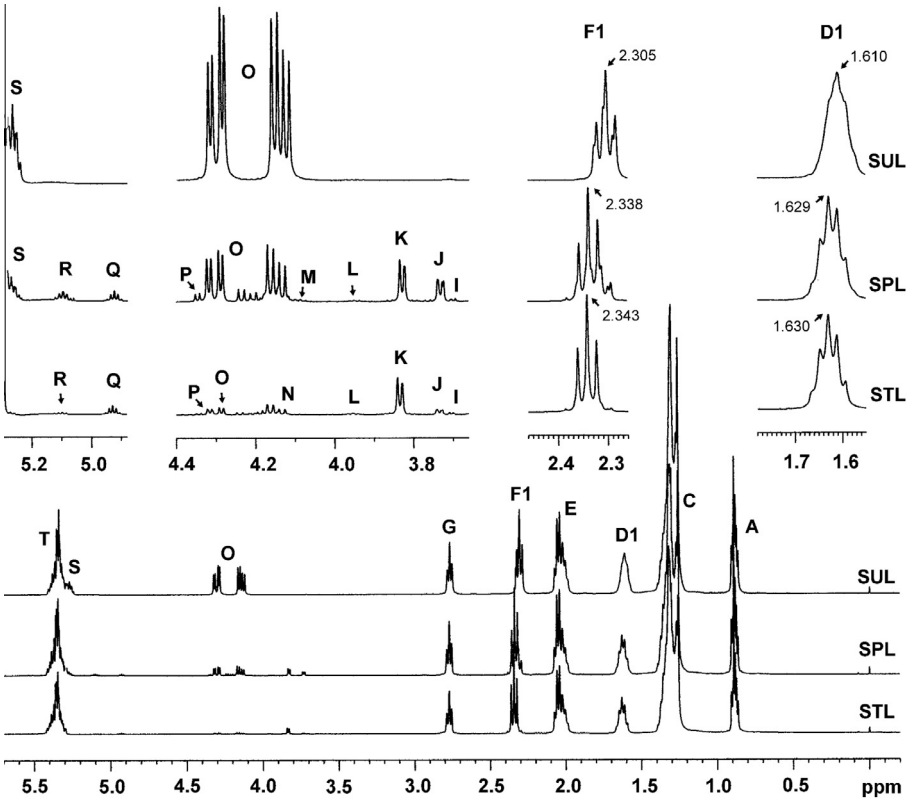

Fig. 1. [1]H NMR spectra of unlipolyzed sunflower oil (SUL) and of partially (SPL) and totally lipolyzed (STL) lipid extracts obtained after *in vitro* digestion. Some spectral regions are enlarged. The assignment of the signals is in agreement with Table 1.

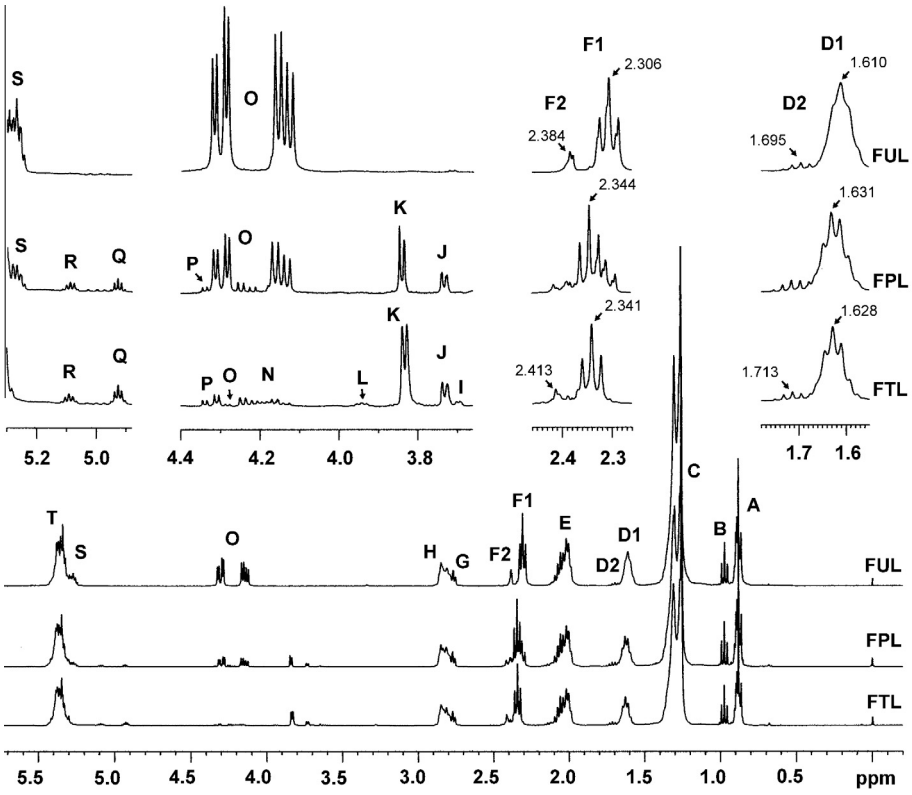

Fig. 2. [1]H NMR spectra of unlipolyzed fish lipids (FUL) and of partially (FPL) and totally lipolyzed (FTL) lipid extracts obtained after *in vitro* digestion. Some spectral regions are enlarged. The assignment of the signals is in agreement with Table 1.

have almost disappeared in the spectrum of STL sample, indicating that nearly all the TG have been hydrolyzed.

In addition to the decrease of the above-mentioned signals, at the same time and as the hydrolysis advances, new signals (**I**, **J**, **K**, **L**, **M**, **N**, **P**, **Q**, **R**) appear in the spectral region ranging from 3.60 to 5.30 ppm. These changes, due to TG hydrolysis during *in vitro* digestion, relate to the appearance of partial glycerides arising from them. Taking into account the assignment of the signals summarized in Table 1, the newly formed glycerides were predominantly 1,2-DG (signals **J**, **P**, **R**) and 2-MG (signals **K**, **Q**), which is in agreement with the lipid hydrolysis reaction occurring within the human tract due to the *in vivo* regiospecificity of digestive lipases (Desnuelle & Savary, 1963; Hofmann & Borgström, 1963). The presence, though in low intensity, of the spectral signals corresponding to 1-MG (signals **I**, **L**, **N**), especially in totally lipolyzed sample (STL), can be explained by the isomerization of 2-MG molecules. In fact, the latter 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 (26–40 °C) (Mattson & Volpenhein, 1962). It is estimated that, *in vivo*, about 28% of 2-MG are rearranged into 1-MG (Mattson & Volpenhein, 1964). 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 SPL, which is in agreement with other digestion studies, either *in vivo* (Miettinen & Siurala, 1971) or *in vitro* (Vlahov, 2006). The low intensity of this signal evidences a negligeable content of this diglyceride in the digested sample. Due to the positional specificity of the lipases used in our *in vitro* digestion protocol, this could be mainly explained by the occurrence of 1,2-DG isomerization (De Groot, 1972; Spyros, Philippidis, & Dais, 2004).

Due to TG hydrolysis, small differences in the chemical shift and multiplicity of signals **D1** and **F1** can also be appreciated in the spectral regions ranging from 1.55–1.75 and 2.25–2.45 ppm, respectively, corresponding to the protons of methylenic groups in β- and α-position in relation to the carbonyl group of acyl groups and to the carboxyl group of FA (see Table 1). As shown in Fig. 1, the intensity of the signals related to TG centered approximately at 1.610 and 2.305 ppm (see spectra of SUL) gradually decrease, whereas the intensity of signals centered at higher chemical shifts (1.630 and 2.343 ppm) increase simultaneously (see spectra of SPL, STL). These new signals are generated by the same protons but supported on DG, MG and FA and are centered at higher chemical shifts than those corresponding to TG (see Table 1). Thus, due to the great degree of overlapping of these signals, the study of both spectral regions can also give information about the extent of lipolysis, but not about the nature of the lipolytic products generated. The advance of lipid hydrolysis can be especially deduced from the observation of signal **F1** because the signal corresponding to TG can be perfectly distinguishable from those due to the same protons but supported on DG, MG and FA (see the enlarged signals **F1** in Fig. 1 and data in Table 1).

3.1.2. In vitro digestion of sea bass

Fig. 2 shows the ^{1}H NMR spectra of sea bass lipid extracts before (FUL) and after *in vitro* digestion (FPL, FTL). As in the previous example, samples submitted to different digestion conditions reached different degrees of lipolysis, which is useful in showing the qualitative changes which occur during the progression of digestion. As previously commented, the ^{1}H NMR spectrum of the unlipolyzed sample (FUL) presents the typical signals corresponding to the protons supported on the acyl groups, (signals **A**, **B**, **C**, **D1**, **D2**, **E**, **F1**, **F2**, **G**, **H**, **T**) and those of the glyceryl backbone of TG (signals **O**, **S**). It has to be noted that signals **D2** and **F2**, related to eicosapentaenoic plus arachidonic and docosahexaenoic acyl groups respectively, are typical of fish lipids, and, unless intentionally added, are absent in the ^{1}H NMR spectra of fats and oils of other origins.

As hydrolysis advances, the same differences already described for sunflower oil samples can be noticed in the ^{1}H NMR spectra of fish lipids submitted to digestion. The intensity of specific signals related to TG (signals **O**, **S**) gradually decreases as the hydrolysis advances, whereas specific signals related to 1,2-DG (signals **J**, **P**, **R**), 2-MG (signals **K**, **Q**) and 1-MG (signals **I**, **L**, **N**) appear. In this case, the presence of signal **M** was not observed. Variation to higher chemical shifts can also be appreciated in signals **D1**, **D2**, **F1**, **F2**, which evidences the occurrence of TG hydrolysis, as has been described before. As observed in Fig. 2, in fish lipid extract before digestion (FUL), the signals **D2** and **F2** related to TG are centered at approximately 1.695 and 2.384 ppm, whereas in the totally lipolyzed sample (FTL) they are centered at 1.713 and 2.413 ppm, respectively.

3.2. Determination of lipolytic products generated during in vitro digestion

In the literature on lipid digestion, two different ways to describe quantitatively the products generated during TG lipolysis are being used. Some authors estimate the molar percentages of FA or acyl groups joined to different glyceryl backbone structures present (TG, DG, MG) in relation to the total number of acyl groups plus fatty acids present in the sample (Helbig et al., 2012; Zhu et al., 2013). However, other authors quantify the lipolytic products in function of the several glyceryl structures (TG, DG, MG, G), determining the molar percentage of each one in relation to the total number of them (Capolino et al., 2011; Rodriguez et al., 2008).

3.2.1. Quantitative description in function of acyl groups plus fatty acids

This method of describing the digestion products quantitatively requires determination of the molar percentages of acyl groups (AG) supported on the different glyceride structures (TG, DG, MG) and also the molar percentage of FA (Helbig et al., 2012; Zhu et al., 2013). By using the equations detailed in the Section 2, this determination can be performed from data obtained from the ^{1}H NMR spectrum by applying the following equations:

$$AG_{TG}\% = 100(3N_{TG})/NT_{AG+FA} \quad (7)$$

$$AG_{1,2\text{-}DG}\% = 100(2N_{1,2\text{-}DG})/NT_{AG+FA} \quad (8)$$

$$AG_{1,3\text{-}DG}\% = 100(2N_{1,3\text{-}DG})/NT_{AG+FA} \quad (9)$$

$$AG_{2\text{-}MG}\% = 100N_{2\text{-}MG}/NT_{AG+FA} \quad (10)$$

$$AG_{1\text{-}MG}\% = 100N_{1\text{-}MG}/NT_{AG+FA} \quad (11)$$

$$FA\% = 100N_{FA}/NT_{AG+FA} \quad (12)$$

In these equations AG is the percentage of acyl groups supported on each kind of glyceride present in the sample, N is the number of moles of each kind of digestion product and NT_{AG+FA} is the total number of moles of acyl groups plus FA present in the sample. The latter can also be determined by using ^{1}H NMR spectral data by means of the following equation:

$$NT_{AG+FA} = 3N_{TG} + 2N_{1,2\text{-}DG} + 2N_{1,3\text{-}DG} + N_{2\text{-}MG} + N_{1\text{-}MG} + N_{FA} \quad (13)$$

It is evident that for each TG there are three acyl groups, for each DG two acyl groups are present and for each MG there is only one; thus these molar percentages cannot be considered as

Table 2
Molar percentages of acyl groups (AG) supported on the different glyceryl backbone structures (TG, 1,2-DG, 1,3-DG, 2-MG, 1-MG) and fatty acids (FA), present in the lipids of both the original samples and the *in vitro* digested samples.

Sample	AG_{TG}%	$AG_{1,2\text{-}DG}$%	$AG_{1,3\text{-}DG}$%	$AG_{2\text{-}MG}$%	$AG_{1\text{-}MG}$%	FA%
SUL	98.9 ± 0.0	0.3 ± 0.0	–	–	–	0.8 ± 0.0
SPL	24.9 ± 0.6	7.0 ± 0.3	1.9 ± 0.3	2.5 ± 0.1	0.7 ± 0.1	62.9 ± 0.7
STL	5.0 ± 1.0	1.7 ± 0.2	–	2.7 ± 0.4	0.7 ± 0.1	89.9 ± 1.5
FUL	97.3 ± 0.0	0.4 ± 0.0	–	–	–	2.3 ± 0.0
FPL	34.1 ± 0.1	7.6 ± 1.6	–	3.1 ± 0.4	0.4 ± 0.1	54.9 ± 1.1
FTL	1.6 ± 0.8	13.2 ± 3.3	–	8.3 ± 0.6	2.3 ± 0.6	74.6 ± 4.0

percentages of whole molecules. Table 2 shows the results obtained when this method of quantifying lipolysis products is used in the study of sunflower oil and fish lipid digestion.

3.2.2. Quantitative description in function of glyceryl structures

As far as the second way of quantifying lipolysis products based on glyceryl structures is concerned, this determines the proportion of TG molecules that remained intact, that were hydrolyzed partially into DG, MG, and totally into G (Capolino et al., 2011; Rodriguez et al., 2008). It must be mentioned that this way of expressing lipid digestion products is also very useful in monitoring the progression of the lipolysis reaction during digestion, allowing a global characterization of the different kinds of products generated considered as whole molecules. Moreover, quantification by this means ensures that the mass balances can be successfully applied, since the proportion of the different lipolytic products reflects the stoichiometry of reaction.

The following equations can be applied to quantify glyceryl structures in relation to the total number of moles of them (NT_{GS}) present in the sample during lipid digestion:

$$TG\% = 100N_{TG}/NT_{GS} \quad (14)$$

$$1,2\text{-}DG\% = 100N_{1,2\text{-}DG}/NT_{GS} \quad (15)$$

$$1,3\text{-}DG\% = 100N_{1,3\text{-}DG}/NT_{GS} \quad (16)$$

$$2\text{-}MG\% = 100N_{2\text{-}MG}/NT_{GS} \quad (17)$$

$$1\text{-}MG\% = 100N_{1\text{-}MG}/NT_{GS} \quad (18)$$

Determination of the molar percentage of glycerol (G) molecules is also possible from ^{1}H NMR data. Although the complete hydrolysis of TG is usually believed to be limited *in vivo*, as previously commented, the generation of G under *in vitro* conditions has been previously reported (Borgström, 1964; Capolino et al., 2011) and should thus be confirmed. After some trials carried out in our laboratory, it was proved that due to its high polarity, G cannot be extracted from the digested samples using dichloromethane. Therefore, it is not possible to determine if complete hydrolysis of TG has occurred by simple observation of ^{1}H NMR spectra of digested lipid extracts. In spite of this, its quantification can be achieved by indirect determination, taking into account the stoichiometry of the hydrolysis reaction, by means of the following equations:

$$N_G = (N_{FA} - N_{1,2\text{-}DG} - N_{1,3\text{-}DG} - 2N_{2\text{-}MG} - 2N_{1\text{-}MG})/3 \quad (19)$$

where N is the number of moles of the corresponding compound determined by the equations detailed in the Section 2. Once the number of glycerol moles (N_G) is determined, its molar percentage can also be calculated by the following equation:

$$G\% = 100N_G/NT_{GS} \quad (20)$$

Finally, the total number of moles of glyceryl structures present in the sample (NT_{GS}) can be determined using the following equation:

$$NT_{GS} = N_{TG} + N_{1,2\text{-}DG} + N_{1,3\text{-}DG} + N_{2\text{-}MG} + N_{1\text{-}MG} + N_G \quad (21)$$

Table 3 shows the results obtained when this second method of describing hydrolysis products quantitatively is applied in the study of sunflower oil and fish lipid digestion. As can be observed, the number of TG molecules that have undergone a total hydrolysis (G%) is considerably high, even in partially hydrolyzed samples; these values are higher than those reported *in vivo* (from 12% to 40%) (Borgström, 1964; Borgström, Tryding, & Westöö, 1957; Mattson & Volpenhein, 1964). This could be explained by the *in vitro* static model used, which hinders the removal of 2-MG. *In vivo*, the generation of G during digestion could be limited by the higher velocity of absorption of 2-MG compared to that of the isomerisation into 1-MG (Borgström, 1964). When comparing these results with those of Table 2, it must be noted that, except for TG, very different values are obtained due to the different meaning of each way of quantifying. However, it has to be pointed that, as they are both related, the values obtained in any sample using one way can be used to determine those that would be determined by the other way.

3.3. Determination from ^{1}H NMR spectral data of the extent of lipid digestion

The hydrolysis of TG implies the cleavage of the ester bonds, resulting in the release of three FA and the formation of G when lipolysis is complete. However, due to several different interpretations of the concept of lipid digestion extent, various approaches have been proposed for its determination: hydrolysis level, degree of TG transformation, lipid bioaccessibility level and percentage of fatty acids physiologically releasable (Capolino et al., 2011; Pafumi et al., 2002; Vinarov et al., 2012; Zhu et al., 2013). In some cases, lipid digestion is considered from the chemical point of view, whereas in others the bioaccessibility of the products generated is taken into account, in an attempt to see matters from a physiological point of view. In spite of this, any one of these approaches can be estimated by applying the methodology developed here.

3.3.1. Hydrolysis in the chemical sense

Several authors evaluate *in vitro* lipid hydrolysis level (H%) by the percentage of FA released in relation to the total number of moles of acyl groups plus fatty acids present in the sample (NT_{AG+FA}), in agreement with the above-mentioned Eq. (12) (Capolino et al., 2011; Helbig et al., 2012; Rodriguez et al., 2008; Zhu et al., 2013). This approach considers that total lipolysis (100%) is achieved when all the glycerides initially present (TG, DG, MG) are converted into FA and G. If the sample only contains TG before digestion, this approach can also be determined using the number of moles of TG initially present in the sample (N_{TGi}).

Table 3
Molar percentages of the different glyceryl structures (TG, 1,2-DG, 1,3-DG, 2-MG, 1-MG and G) present in the lipids of both the original samples and the *in vitro* digested samples.

Sample	TG%	1,2-DG%	1,3-DG%	2-MG%	1-MG%	G%
SUL	98.9 ± 0.0	0.5 ± 0.0	–	–	–	0.6 ± 0.0
SPL	24.9 ± 0.6	10.6 ± 0.4	2.9 ± 0.5	7.6 ± 0.2	2.1 ± 0.2	52.0 ± 1.0
STL	5.0 ± 1.0	2.6 ± 0.3	–	8.1 ± 1.2	2.0 ± 0.4	82.3 ± 2.6
FUL	97.3 ± 0.0	0.6 ± 0.0	–	–	–	2.1 ± 0.0
FPL	34.1 ± 0.1	11.4 ± 1.2	–	9.3 ± 0.6	1.1 ± 0.1	44.1 ± 0.6
FTL	1.7 ± 0.9	19.6 ± 4.7	–	24.9 ± 1.9	6.9 ± 1.9	46.9 ± 6.4

$$H\% = 100N_{FA}/NT_{AG+FA} = 100N_{FA}/(3N_{TGi}) \quad (22)$$

3.3.2. 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., 2012). According to these authors, the lipolysis reached can be calculated by the following equation:

$$T_{TG}\% = 100(N_{TGi} - N_{TG})/N_{TGi} \quad (23)$$

In this case, complete lipolysis (100%) involves the hydrolysis of at least one ester bond in each TG molecule initially present.

3.3.3. Lipid digestion and bioaccessibility

Another more physiological approach is also used to evaluate the extent of lipid digestion in *in vitro* studies. 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 (NT_{AG+FA}) (Capolino et al., 2011; Kenmogne-Domguia et al., 2012). In fact, although further hydrolysis is possible, the complete absorption of a TG only requires its conversion into MG and two FA. Using this approach, the proportion of lipids which are bioaccessible (L_{BA}%) for absorption in the gut lumen can be estimated by means of the following equation:

$$L_{BA}\% = 100(N_{1\text{-}MG} + N_{2\text{-}MG} + N_{FA})/NT_{AG+FA} \quad (24)$$

In this case, a value of 100% involves the transformation of each TG into absorbable molecules, which may be either MG or FA.

3.3.4. Fatty acids versus those which may be released in the conversion of TG into MG

In this context, when using the pH-stat apparatus to monitor an *in vitro* lipolysis reaction, the percentage of FA physiologically releasable (FA_{PR}%) is quantified by taking into account the number of moles of NaOH required to neutralize the FA divided by the number of moles of FA that could be released from the initial TG molecules present (N_{TGi}). This assumes that each TG molecule can generate two FA and one MG and that no further hydrolysis of MG occurs (Lamothe, Corbeil, Turgeon, & Britten, 2012; Li & McClements, 2010; Marze et al., 2013; Pafumi et al., 2002).

$$FA_{PR}\% = 100N_{FA}/(2N_{TGi}) \quad (25)$$

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 G and three molecules of FA, as previously demonstrated. Thus, since hydrolysis of MG into G could occur (Borgström, 1964; Borgström et al., 1957; Mattson & Volpenhein, 1964), this approach would lead to a noticeable overestimation of the extent of lipid digestion if G is generated, which could explain certain percentages above 100% obtained in some studies (Li, Hu, & McClements, 2011).

Table 4 shows the results obtained when these different approaches were applied to estimate the extent of lipolysis reaction in the samples subject of study. Values differ since they deal with different concepts of lipolysis; however, certain similarities can be found between two of the four approaches (H% and L_{BA}%). Regarding samples before digestion, values obtained for H% and L_{BA}% agree because they are composed mainly of TG and, to a lesser extent, of DG and FA. As far as digested samples are concerned, a similar tendency is noticed: L_{BA}% is slightly higher than H%, which is to be expected since the amount of MG is taken into account within the first approach. Thus, it can be deduced that, under the conditions of this study, there is a low proportion of MG among the digested products, which is somewhat more abundant in FTL than in STL sample. This is in agreement with observations made in Figs. 1 and 2 in the signals due to protons in the glycerol backbone of 1-MG (**I**, **L**, **N**) and especially in those of 2-MG (**K**, **Q**). As far as T_{TG}% is concerned, the values obtained are higher than those corresponding to H% and L_{BA}% because this approach only focuses on the conversion of TG into other glycerides, regardless of their nature (DG, MG or G). Finally, in relation to FA_{PR}%, the overestimation obtained is remarkable, as mentioned before. This 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.

Table 4
Hydrolysis level (H%), TG transformation (T_{TG}%), lipid bioaccessibility (L_{BA}%) and percentage of FA physiologically releasable (FA_{PR}%) in the lipids of both the original samples and the *in vitro* digested samples, determined using Eqs. (22–25).

Sample	H%	T_{TG}%	L_{BA}%	FA_{PR}%
SUL	0.8 ± 0.0	1.1 ± 0.0	0.8 ± 0.0	1.1 ± 0.0
SPL	62.9 ± 0.7	75.1 ± 0.6	66.2 ± 0.6	94.4 ± 1.1
STL	89.9 ± 1.5	95.0 ± 1.0	93.3 ± 1.0	134.9 ± 2.3
FUL	2.3 ± 0.0	2.7 ± 0.0	2.3 ± 0.0	3.5 ± 0.0
FPL	54.9 ± 1.1	65.9 ± 0.1	58.3 ± 1.5	82.3 ± 1.7
FTL	74.6 ± 4.0	98.4 ± 0.8	85.1 ± 3.9	111.8 ± 6.0

4. Conclusions

For the first time, a methodology based on ^{1}H NMR spectral data to quantify both lipolytic products and the extent of the lipid digestion has been developed. By means of simple observation of the spectrum, it is possible to obtain information about the nature and proportions of the lipolysis products present, which determines the hydrolysis level of the sample. Equations derived from spectroscopic data are proposed for quantification of all the products arising from triglycerides hydrolysis. Moreover, the present study highlights the versatility of this new methodology in assessing the extent of lipolysis reaction in any of its current definitions. Due to the different interpretations of lipid digestion extent, a definition arrived by consensus is considered necessary. It should be pointed out that this methodology allows us to discriminate between the isomers formed in lipid digestion. Under the conditions of this study, 1,2-diglycerides, 2-monoglycerides, fatty acids and glycerol were the main hydrolysis products arising from triglycerides. However, the presence in very low proportions of 1,3-diglycerides and 1-monoglycerides in digested lipid extracts suggests the possible occurrence of isomerization reactions during *in vitro* digestion. Furthermore, complete hydrolysis of triglycerides into fatty acids and glycerol occurred to a significant extent, which was higher than that reported *in vivo*. In comparison with other methodologies previously employed, ^{1}H NMR allows a global study of the digested sample in a simple and fast way, and without any chemical modification.

Acknowledgements

This work has been supported by the Spanish Ministry of Economy and Competitiveness (MINECO, AGL2012-36466), by the Basque Government (EJ-GV, GIC10/85-IT-463-10) and by the Unit for Education and Research "Food Quality and Safety" (UPV/EHU-UFI-11/21). All authors participate in the COST Action FA1005 INFOGEST. B. N-E. thanks the UPV/EHU for a predoctoral fellowship.

References

Armand, M., Pasquier, B., André, M., Borel, P., Senft, M., Peyrot, J., et al. (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.

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., 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.

Capolino, P., Guérin, C., Paume, J., Giallo, J., Ballester, J. M., Cavalier, J. F., et al. (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.

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.

De Groot, W. Th. M. (1972). Acyl migration solid phase isomerization of 1,2-diglycerides to 1,3-isomers. *Lipids, 7*(9), 626–628.

Desnuelle, P., & Savary, P. (1963). Specificity of lipases. *Journal of Lipid Research, 4*(4), 369–384.

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.

Goicoechea, E., Van Twillert, K., Duits, M., Brandon, E. D. F. A., Kootstra, P. R., Blokland, M. H., et al. (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.

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., & 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., & Ruiz, A. (2003a). 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. (2003b). 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.

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.

Hofmann, A. F., & Borgström, B. (1963). Hydrolysis of long-chain monoglycerides in micellar solution by pancreatic lipase. *Biochimica et Biophysica Acta, 70*, 317–331.

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., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). *In vitro* human digestion models for food applications. *Food Chemistry, 125*(1), 1–12.

Kenmogne-Domguia, H. B., Meynier, A., Viau, M., Llamas, G., & Genot, C. (2012). 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.

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.

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.

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.

Martínez-Yusta, A., & Guillén, M. D. (2014). 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.

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.

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.

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.

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.

Mu, H., & Høy, C. E. (2004). The digestion of dietary triacylglycerols. *Progress in Lipid Research, 43*(2), 105–133.

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.

Pafumi, Y., Lairon, D., Porte, P. L., Juhel, C., Storch, J., Hamosh, M., et al. (2002). Mechanisms of inhibition of triacylglycerol hydrolysis by human gastric lipase. *Journal of Biological Chemistry, 277*(31), 28070–28079.

Rodriguez, J. A., Mendoza, L. D., Pezzotti, F., Vanthuyne, N., Leclaire, J., Verger, R., et al. (2008). Novel chromatographic resolution of chiral diacylglycerols and analysis of the stereoselective hydrolysis of triacylglycerols by lipases. *Analytical Biochemistry, 375*(2), 196–208.

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.

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.

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.

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.

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. (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.

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.