Food Research International 66 (2014) 379–387

Contents lists available at ScienceDirect

Food Research International

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

A method based on ^{1}H NMR spectral data useful to evaluate the hydrolysis level in complex lipid mixtures

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 n° 7, 01006 Vitoria, Spain

ARTICLE INFO

Article history:
Received 3 July 2014
Accepted 21 September 2014
Available online 12 October 2014

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

Chemical compounds studied in this article:
Tridocosahexaenoin (PubChem CID: 9546569)
Trieicosapentaenoin (PubChem CID: 9545999)
Trilinolenin (PubChem CID: 5462874)
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)

ABSTRACT

A new approach is reported, based on Proton Nuclear Magnetic Resonance (^{1}H NMR) spectral data, to study qualitatively and quantitatively the hydrolysis level in complex lipid mixtures. It is based on a detailed study of the spectra of tri-, di-, mono-glycerides and fatty acids. It has been validated by using mixtures of standard compounds which simulate the complex mixtures formed in lipid digestion. To this aim the ^{1}H NMR spectra of pure compounds, as well as of mixtures containing different known proportions of triglycerides, 1,2-diglycerides, 1,3-diglycerides, 1-monoglycerides, 2-monoglycerides and fatty acids, were acquired. These mixtures cover a very broad range of concentrations and simulate edible oils and fats of vegetable and animal origins, including fish, with different levels of hydrolysis. Equations based on spectral data are proposed to determine the molar percentage of the different kinds of compounds that may be formed in lipid digestion, thus allowing quantification of the advance of lipolysis. To the best of our knowledge, this is the first time that a method based on ^{1}H NMR spectral data to quantify triglycerides, as well as partial glycerides and fatty acids in complex lipid mixtures coming from lipolysis is proposed. This methodology offers many advantages and is useful not only in the fields of food technology and nutrition, but also in others like enzymology, pharmacology, medicine and petrochemistry, among others.

1. Introduction

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 and other glycerides, 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, among which lipids are very important (McClements, Decker, & Park, 2009).

Triglycerides are the major components of dietary fats and oils, and once ingested, they are submitted to a hydrolytic process catalyzed by lipases present in gastric, and especially in duodenal digestive juices. The evolution of the triglycerides during digestion and the role of the antioxidants present in the system, if any, are subjects of great interest nowadays.

Monitoring hydrolysis advance is an important task in lipid digestion research, and in consequence, the development of methodologies which are able to quantify accurately all the products arising from this process is needed. The technique most commonly employed to estimate hydrolysis level during digestion is the titration of fatty acids released by means of a pH-stat apparatus, in which titration with NaOH is carried out (Brogård, Troedsson, Thuresson, & Ljusberg-Wahren, 2007; Fatouros, Bergenstahl, & Mullertz, 2007; Helbig, Silletti, Timmerman, Hamer, & Gruppen, 2012; Li & McClements, 2010; Zhu, Ye, Verrier, & Singh, 2013). However, it has been pointed out that the accuracy of the pH-stat titration technique in quantifying the fatty acids released during lipid digestion is highly dependent on the ionization of each fatty acid 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, Holm, Rades, & Müllertz, 2012; Zhu et al.,

* Corresponding author. Tel.: +34 945 013081; fax: +34 945 013014.
E-mail address: mariadolores.guillen@ehu.es (M.D. Guillén).

http://dx.doi.org/10.1016/j.foodres.2014.09.031

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).

Chromatographic techniques have also been applied to quantify the different lipolytic products generated, such as High Performance Liquid Chromatography (HPLC), High Performance Thin Layer Chromatography (HPTLC) or Gas Chromatography (GC) followed by Mass Spectrometry (MS) (Armand et al., 1999; Capolino et al., 2011; Helbig et al., 2012; Hur, Joo, Lim, Decker, & McClements, 2011; Kenmogne-Domguia, Meynier, Viau, Llamas, & Genot, 2012; Sek, Porter, & Charman, 2001; Zhu et al., 2013). Nevertheless, these methodologies are time-consuming, usually implying many preparation steps, including calibration with standard compounds, and also involving 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 (Helbig et al., 2012; Sek et al., 2002; Thomas et al., 2012). Therefore, the need for further research on methodological developments to study lipid hydrolysis is evident.

Previous studies have used ^{13}C and ^{31}P Nuclear Magnetic Resonance (^{13}C and ^{31}P NMR) to study di-, mono-glycerides and fatty acids in lipid mixtures (Gusntone, 1991; Ng, 2000; Spyros & Dais, 2000; Vlahov, 1996). However, ^{1}H NMR has been scarcely used to this aim (Martínez-Yusta & Guillén, 2014; Sopelana, Arizabaleta, Ibargoitia, & Guillén, 2013). In this context, the aim of this study is to analyze the usefulness of ^{1}H NMR in characterizing and quantifying all the different products arising from the hydrolysis of triglycerides during lipolysis, and thus evaluate the advance of this process. For this purpose, the ^{1}H NMR spectra of the different kinds of compounds which may form during lipolysis were studied in order to develop an approach which was as general as possible. This approach was validated by using several standard mixtures of known composition. This ^{1}H NMR methodology has many advantages over the above-mentioned ones, because it is fast and simple and does not involve any chemical modification of the sample.

2. Materials and methods

2.1. 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 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.

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.

2.2. ^{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. In order to select the most appropriate values to obtain accurate quantitative results in the smallest 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. As a result of these tests, the acquisition parameters used were: spectral width 6410 Hz, relaxation delay 3 s, number of scans 64, acquisition time 4.819 s and pulse width 90°, as in previous studies on edible oils and fats carried out in our laboratory (Guillén, Carton, Goicoechea, & Uriarte, 2008; Martínez-Yusta & Guillén, 2014; Vidal, Manzanos, Goicoechea, & Guillén, 2012). 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. Each spectrum was recorded in duplicate and data provided are average values together with the standard deviations obtained from the integration in triplicate of the corresponding signals. The assignment of the spectral signals to the several protons of the different standard compounds is given in Table 2.

^{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.

3. Results and discussion

Lipid digestion implies the formation of small structures as consequence of the hydrolytic activity of enzymes. In a first step, the hydrolysis of the ester bond in position 3 of a triglyceride (TG) yields a 1,2-diglyceride (1,2-DG) and the corresponding fatty acid (FA) molecule. Due to the regiospecificity of digestive lipases, the hydrolysis of the ester bond in position 2 to yield 1,3-diglycerides (1,3-DG) does not occur (Mu & Høy, 2004). In turn, 1,2-DG is hydrolyzed in position 1 producing a second FA and the corresponding 2-monoglyceride (2-MG). The hydrolysis of 2-MG can also be achieved, resulting in the generation

Table 2
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. The signal letters agree with those given in Figs. 1–3.

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[a]	$-(C\underline{\mathbf{H}}_2)_n-$	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[b]	$-C\underline{\mathbf{H}}_2-CH{=}CH-$	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[c]	$ROCH_2-CH(OR')-C\underline{\mathbf{H}}_2OH$	Glyceryl group in 1,2-DG
K	3.84	m[c]	$HOC\underline{\mathbf{H}}_2-CH(OR)-C\underline{\mathbf{H}}_2OH$	Glyceryl group in 2-MG
L	3.94	m	$ROC\underline{\mathbf{H}}_2-C\underline{\mathbf{H}}OH-CH_2OH$	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. [a] 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; 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. [b] Overlapping of multiplets of the α-methylenic protons in relation to a single double bond of the different unsaturated acyl groups; d: doublet. [c] This signal shows different multiplicity if the spectrum is acquired from the pure compound or taking part in the mixture.

of two new molecules, one FA and one glycerol (G). In fact, isomerization from 2-MG to yield 1-monoglycerides (1-MG) can also take place during digestion, although this process is generally believed to be limited *in vivo* (Mattson & Volpenhein, 1964). In short, all these kinds of species can be found in the lipid extract of a digested product, and all of them can give signals in the ^{1}H NMR spectrum. For this reason, a detailed study of the spectra of each one of these kinds of compounds is required in order to evaluate if sound quantitative information about the extent of the lipolysis in digestion can be obtained from ^{1}H NMR spectral data.

3.1. Characteristics of the ^{1}H NMR spectra of components that may be present among the lipid digestion products

The ^{1}H NMR spectra of triglycerides (TGs) have been subject of many studies due to the fact that they are the main components in edible oils and fats. Fig. 1 shows the spectra of two mixtures of TG (Mx1 and Mx2) which differ, as Table 1 shows, in the unsaturation degree of their acyl group chains. Mx1 consists of trilinolenin, trilinolein, triolein and triestearin, and Mx2 of the first three TGs mentioned, plus tridocosahexaenoin and trieicosapentaenoin. The assignment of their signals, shown in Fig. 1, to the corresponding protons, either of the glyceryl backbone (signals O and S) or of the different acyl chains (signals A, B, C, D1, D2, E, F1, F2, G, H and T) has been very broadly commented on in previous papers and is indicated in Table 2 (Guillén & Ruiz, 2001, 2003a,b; Guillén et al., 2008; Vidal et al., 2012). It is important to note that TG has specific signals (O and S), due to the protons present in the glyceryl backbone. It is also remarkable that signals D2 and F2 are typical of marine lipids, because they are produced by protons of highly polyunsaturated ω-3 acyl groups such as eicosapentaenoic (EPA) and by docosahexaenoic (DHA) groups respectively. Arachidonic acyl groups, which can also generate signal D2, are present in animal lipids, either of terrestrial or marine origin. It should be pointed out that the signal F2, due to the DHA groups, is produced by protons of this group supported on carbon atoms in *alpha* and *beta* positions in relation to the carbonyl group. However, signal F1 is produced by protons of any other acyl group supported exclusively on carbon atoms in *alpha* position in relation the carbonyl group.

As commented on above, the hydrolysis of TG yields 1,2-diglycerides and fatty acids in a first step. In relation to 1,2-diglycerides (1,2-DG), the ^{1}H NMR spectral signals of the protons of acyl group chains are similar to those found in the spectra of TG. However, small differences in the chemical shifts of protons supported on carbon atoms in *alpha* and *beta* positions in relation to the carbonyl group (signals D1 and F1) occur, as can be observed in the ^{1}H NMR spectrum of 1,2-diolein, shown in Fig. 1 and Table 2. It should be noticed that, to the best of our knowledge this is the first time that these small differences in the chemical shifts and multiplicities of these kinds of protons are shown.

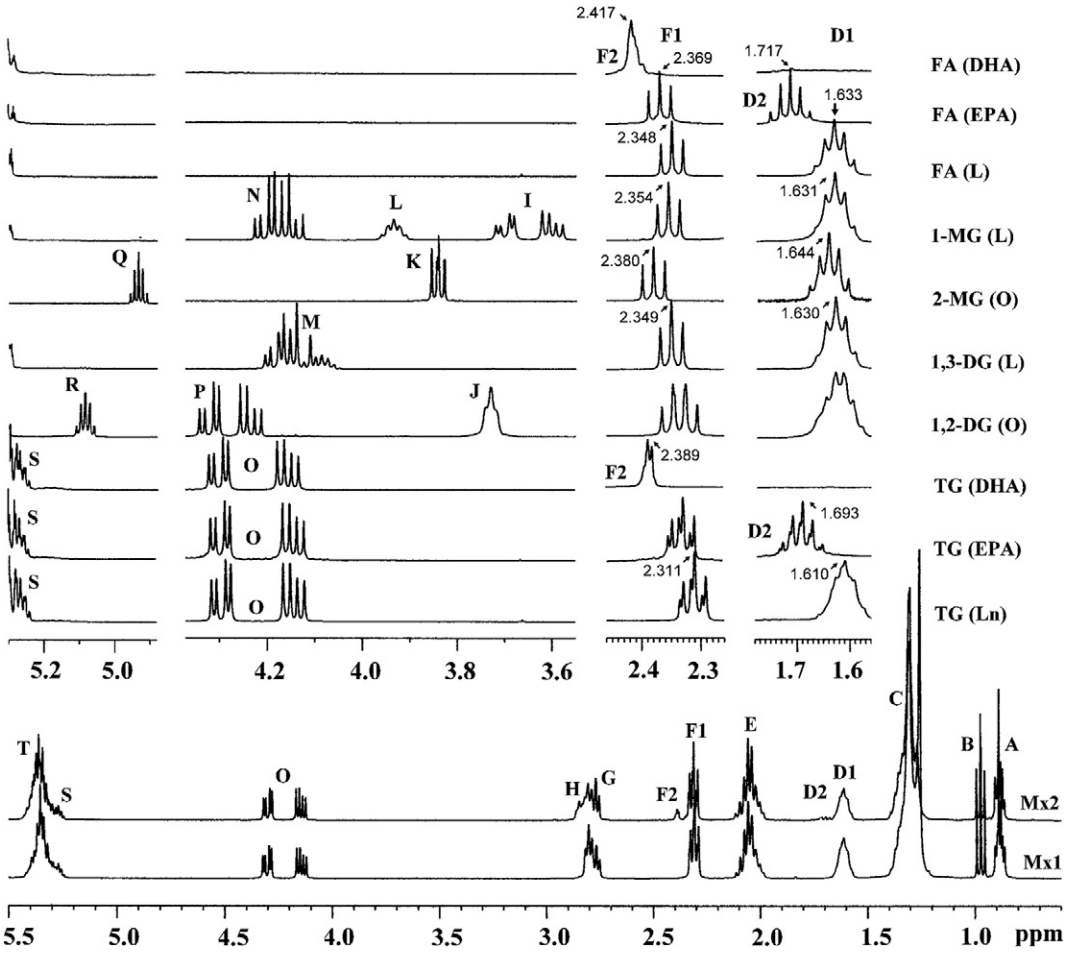

Fig. 1. [1]H NMR spectra of standard mixtures (Mx1 and Mx2) containing exclusively triglycerides, together with the enlargement of regions between 1.55–1.80 ppm, 2.25–2.45 ppm, 3.55–4.40 ppm and 5.05–5.25 ppm of the spectra of some standard compounds: trilinolenin TG(Ln), trieicosapentaenoin TG(EPA), tridocosahexaenoin TG(DHA), 1,2-diolein 1,2-DG(O), 1,3-dilinolein 1,3-DG(L), 2-monoolein 2-MG(O), 1-monolinolein 1-MG(L), linoleic acid FA(L), eicosapentaenoic acid FA(EPA) and docosahexaenoic acid FA(DHA). The signal letters agree with those in Table 2.

Nevertheless, the protons of the glyceryl backbone of 1,2-DG produce specific signals (J, P and R) clearly different from those of TG, as the spectrum of 1,2-diolein in Fig. 1 shows and as indicated in Table 2.

Although hydrolysis of TG does not produce the formation of 1,3-diglycerides (1,3-DG) during lipid digestion, these could have been present in the ingested meal, making their occurrence in the digested product also possible. As can be observed in Fig. 1 and Table 2, the [1]H NMR spectrum of 1,3-dilinolein shows the same signals produced by the protons supported on the acyl group chains as those of 1,2-DG and TG above mentioned, with certain small differences in chemical shifts and multiplicities in the signals D1 and F1. Furthermore, it can be observed in this figure that the spectrum of 1,3-DG has a specific signal (M) produced by the protons of its glyceryl backbone, which is useful in detecting the presence of this kind of compounds. It has to be pointed out that this signal M overlaps partially with signal O of TG, which will have to be taken into account for quantitative purposes if both kind of compounds are present in the sample.

The subsequent hydrolysis of 1,2-DG in digestion yields 2-monoglycerides (2-MG). Taking as an example the spectrum of 2-monoolein in Fig. 1, their [1]H NMR spectra show the above-mentioned typical signals of the protons of the acyl group chain. These signals are very similar to those found in the spectra of TG and DG. There are only small differences in the chemical shifts and multiplicities of the signals generated by protons supported on carbon atoms in *alpha* and *beta* positions in relation to the carbonyl group (signals D1 and F1). Other signals (K and Q) generated by the protons of the glyceryl backbone of 2-MG can also be observed. These latter do not overlap with any other, thus making them useful for indicating the presence of 2-MG in the sample subject of study and for quantitative purposes.

As mentioned before, the presence of 1-monoglycerides (1-MG) among the digestion products could be due to their occurrence in the original ingested lipids, to their formation after isomerization of 2-MG (Mattson & Volpenhein, 1964) or to the hydrolysis of 1,3-DG if present in the ingested meal. The protons supported on their acyl group chains generate signals in the spectra which are similar to those commented for the other lipidic components (see Fig. 1, spectrum of 1-monolinolein). In addition, the protons of the glyceryl backbone of 1-MG provide specific signals (I, L, and N).

It is noteworthy that the above-mentioned specific signals of glycerides are generated by the protons located in the glyceryl backbone, next either to the ester bond or to the alcohol group. Thus, these protons in the glyceryl backbone are greatly influenced by the chemical environment. In addition, in a limited extent, this different chemical environment in glycerides produces small differences in the chemical shift of signals due to protons in *alpha* and *beta* positions in relation to the carbonyl group.

Finally, the release of fatty acids (FA) occurs in any of the hydrolysis steps of the digestion process. Their [1]H NMR spectra, as Fig. 1 shows for linoleic, EPA and DHA acids, present the typical signals of the protons of the acyl group chains, with small differences in multiplicity and chemical shifts in the signals generated by the protons supported on *alpha* and *beta* carbon atoms in relation to the carboxyl group (D1, D2, F1 and F2) due to small differences in the chemical environment. It should also be

commented on that, under the conditions of this study, the spectra of FA do not show any specific signals which are different from those already mentioned.

3.2. Information about lipolysis advance which may be obtained directly from the simple observation of the ^{1}H NMR spectrum of lipid mixtures

Bearing in mind all the above-mentioned, it is evident that simple observation of the ^{1}H NMR spectrum of lipid mixtures provides important information about the lipolytic products present. This observation can also give quantitative information about the progression of lipolysis reaction, if the intensity of certain signals of the spectrum is considered.

Thus, the presence in the spectrum of signals O and S would indicate that a certain amount of TG remains intact and that lipid digestion has not been completed. The higher the advance of the lipolysis, the lower the intensity of these signals will be. The spectra of Mx3 and Mx4 in Fig. 2 and of Mx7, Mx8 and Mx9 in Fig. 3 could be considered examples of samples whose TG have undergone different degrees of hydrolysis. By contrast, the absence of these signals in the spectrum of digested lipids would indicate that all TGs have undergone total hydrolysis. Spectra of Mx5 in Fig. 2 and Mx10 in Fig. 3 could be considered examples of samples in which all TGs have undergone a hydrolysis reaction.

In the same way, the presence in the spectrum of signals J, P and R would indicate the occurrence of 1,2-DG in the digested lipids, and that of signal M would indicate the occurrence of 1,3-DG. Spectra of Mx3-5 in Fig. 2 and of Mx7-10 in Fig. 3 can be considered examples of samples containing variable concentrations of 1,2-DG, and those of Mx5, Mx9 and Mx10 in Fig. 3, examples of samples containing 1,3-DG. Furthermore, if the ^{1}H NMR spectrum contains signals K and Q, the presence of 2-MG in the sample is evidenced (see spectra of Mx3 and Mx7 in Figs. 2 and 3), as is that of 1-MG by means of the presence of signals I, L, N in the spectrum (see spectra of Mx4-5 and Mx8-10 in Figs. 2 and 3). Bearing in mind that the intensity of these signals is proportional to the concentration in the mixture, the higher the intensity of these signals higher is the abundance of the compound responsible.

Finally, the absence in the ^{1}H NMR spectrum of digested lipids of the signals due to protons of any of the possible glyceryl backbone structures (from I to S) would indicate that hydrolysis has been totally completed, yielding only FA. In this case, the only signals present in the spectrum would correspond to protons of the FA chain.

3.3. Approach based on ^{1}H NMR spectral data to determine quantitatively the advance of lipid hydrolysis

In addition to the information which can be extracted directly from the observation of the ^{1}H NMR spectrum, accurate quantitative data about the molar percentage of the different kinds of compounds constituting a complex lipid mixture can also be obtained.

This approach, as mentioned in the experimental section, is based on the proportionality existing between the area of the ^{1}H NMR signals and the number of protons that generate the signal. Therefore, the molar

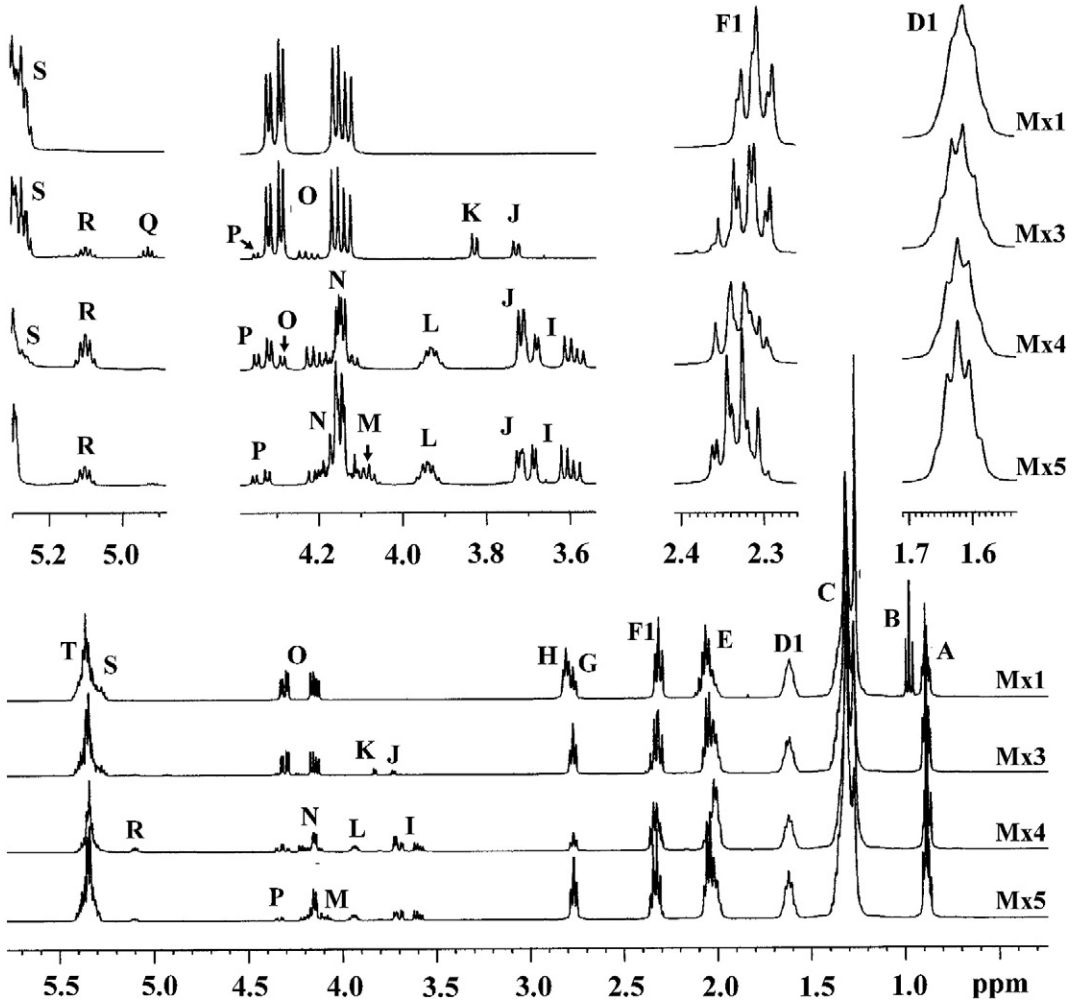

Fig. 2. ^{1}H NMR spectra of standard mixtures (Mx1 and Mx3–5) containing glycerides and fatty acids present in oils and fats of vegetable and terrestrial animal origins. Some spectral regions are properly enlarged. The signal letters agree with those in Table 2.

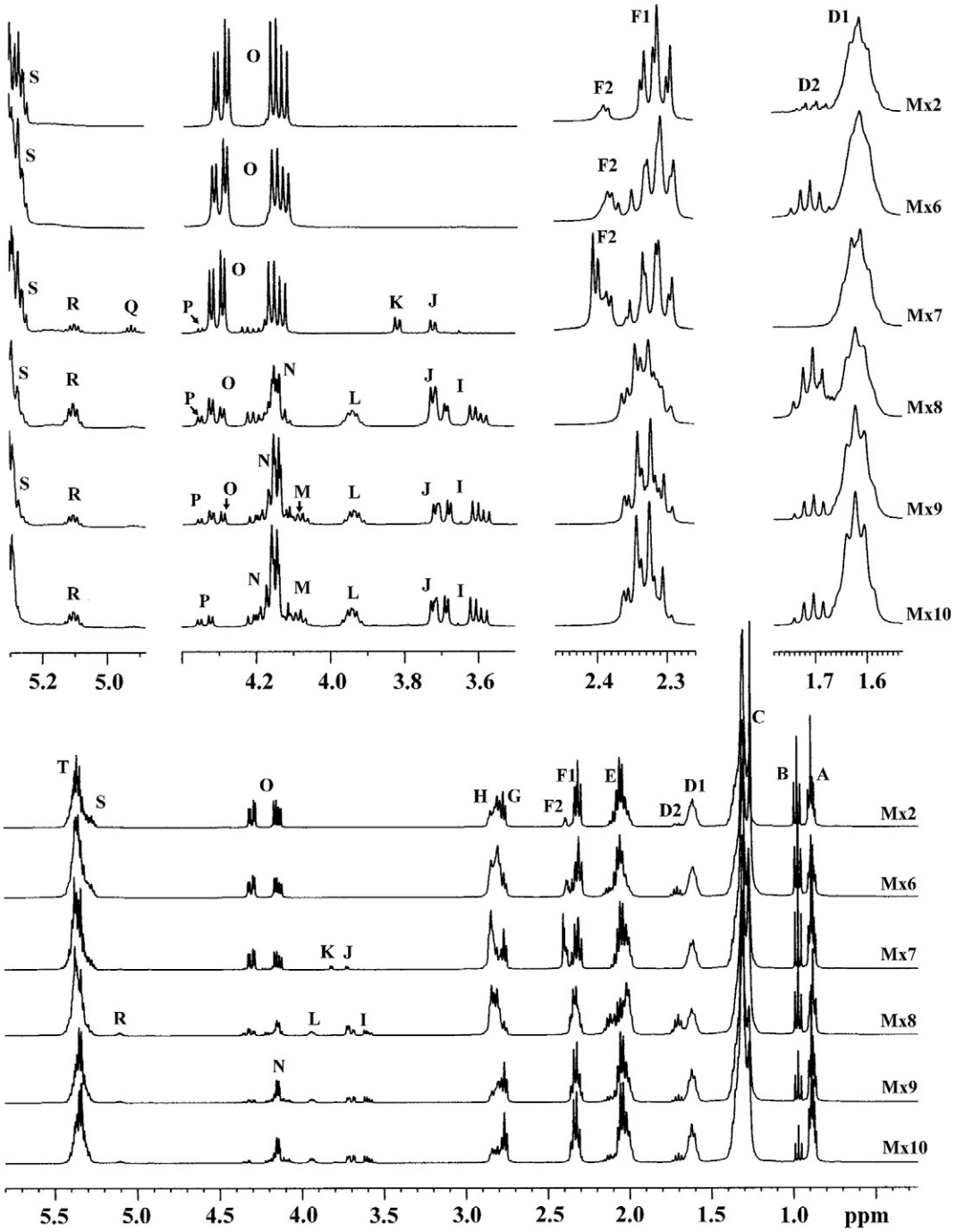

Fig. 3. [1]H NMR spectra of standard mixtures (Mx2 and Mx6–10) containing typical glycerides and fatty acids present in marine lipids, which include polyunsaturated ω-3 acyl groups, like docosahexaenoate and eicosapentaenoate. Some spectral regions are properly enlarged. The signal letters agree with those in Table 2.

percentages of the different kinds of compounds present in a complex mixture of glycerides and FA, can be calculated by using different equations in which the areas of different spectral signals are involved. It has to be noted that depending on the presence or absence of the different kinds of lipolytic products in the sample subject of study and on the signals selected for quantification, different equations can be developed for quantification. The equations proposed in the following section can be employed when all kinds of compounds are present in the sample, being simplified in the case of absence of any of them. Moreover, 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 can be determined by using the following equations:

$$N_{2\text{-MG}} = Pc * A_Q \quad (1a)$$

$$N_{2\text{-MG}} = Pc * A_K/4 \quad (1b)$$

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

$$N_{1,2\text{-DG}} = Pc * A_R. \quad (3a)$$

In these equations Pc is the proportionality constant before mentioned; A_Q, A_K, A_L and A_R are the area of the corresponding signals indicated in Figs. 1–3 and in Table 2.

The determination of the number of moles of 1,2-DG in the sample, in the absence of 1-MG, can also be determined by the equation:

$$N_{1,2\text{-DG}} = Pc * A_J/2 \tag{3b}$$

where A_J is the area of signal J indicated in Figs. 1–3 and in Table 2.

However, if 1-MG are also present in the sample, signal J of 1,2-DG totally overlaps with signal I of 1-MG and this fact should be taken into account for the quantification. In this case, the determination of the number of moles of 1,2-DG can be alternatively made using the following equation:

$$N_{1,2\text{-DG}} = \left(Pc * A_{I+J} - 2N_{1\text{-MG}}\right)/2 \tag{3b'}$$

where A_{I+J} represents the areas of overlapped signals I and J ranging from 3.54 to 3.75 ppm.

Furthermore, the quantification of TG requires one to consider that half of signal O overlaps with half of the signal P of 1,2-DG.

$$N_{TG} = \left(Pc * 2A_{4.26\text{–}4.38} - 2N_{1,2\text{-DG}}\right)/4 \tag{4}$$

where $A_{4.26–4.38}$ represents the area of the spectrum signals comprised between 4.26 and 4.38 ppm

It should be commented on that alternative equations can be used to determine the number of moles of TG depending on the nature of the other glycerides present in the digested sample. In this way, Eq. (5) can be used in the absence of 1-MG and 1,3-DG, and Eq. (6) in the absence of only 1,3-DG:

$$N_{TG} = \left(Pc * A_{O+P} - 2N_{1,2\text{-DG}}\right)/4 \tag{5}$$

$$N_{TG} = \left(Pc * A_{N+O+P} - 2N_{1,2\text{-DG}} - 2N_{1\text{-MG}}\right)/4 \tag{6}$$

where A_{O+P} and A_{N+O+P} represent the areas of overlapped signals O and P, and signals N, O and P respectively, ranging both from 4.10 to 4.38 ppm.

Likewise, the determination of the number of moles of 1,3-DG also requires bearing in mind that there is an overlapping between signal M and signals N, O and P of 1-MG, TG and 1,2-DG respectively. In spite of this, the following equations can be applied:

$$N_{1,3\text{-DG}} = \left(Pc * A_{M+N+O+P} - 4N_{TG} - 2N_{1\text{-MG}} - 2N_{1,2\text{-DG}}\right)/5 \tag{7}$$

where $A_{M+N+O+P}$ represents the areas of overlapped signals M, N, O and P ranging from 4.04 to 4.38 ppm, if all of them are present in the spectrum. If any of these signals is not present, the integration range is adjusted.

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 *alpha* 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:

$$N_{FA} = \left(Pc * A_{2.26\text{–}2.40} - 6N_{TG} - 4N_{1,2\text{-DG}} - 4N_{1,3\text{-DG}} - 2N_{1\text{-MG}} - 2N_{2\text{-MG}}\right)/2 \tag{8}$$

where $A_{2.26–2.40}$ is the area of the spectrum signals comprised between 2.26 and 2.40 ppm interval. This equation is simplified considerably in the absence of 1,3-DG and 1-MG in digested lipid samples.

It has to be noted that in other ^{1}H NMR studies on biodiesel, edible oils and fats with a low degree of hydrolysis, the area of the signal centered at near 2.344 ppm is used to determine exclusively the content of FA (Kumar et al., 2011; Satyarthi, Srinivas, & Ratnasamy, 2009). However, due to the overlapping in this spectral region among signals of TG, DG, MG and FA, this quantification of FA would lead to an overestimation if the formers are also present in the sample.

When fish lipids are involved, the determination of the number of moles of FA requires the introduction of some correction factors based on the area of standard compounds of 1-MG and 2-MG. The purpose of these corrections is to eliminate the slight overlapping between the signals of the protons supported on both carbon atoms in *alpha* and *beta* positions in relation to the carboxyl/carbonyl group of DHA acid and acyl group and that of the protons supported on carbon atoms in *alpha* position in 1-MG, 2-MG and EPA fatty acid. In this case the equation proposed for this determination is the following:

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

where $A_{2.26–2.37}$ and $A_{2.37–2.44}$ are the areas of the spectrum signals at 2.26–2.37 and 2.37–2.44 ppm respectively.

As can be observed in Fig. 1, the high chemical shift of the signal due to the protons of EPA acid in *alpha* position in relation to the carboxyl group must be noted, in comparison with the corresponding signal related to other FA, like linoleic. Thus, this signal also overlaps slightly with the signal due to the protons of DHA acyl groups in *alpha* and *beta* positions to the carbonyl group. Nevertheless, as it is not possible to estimate by means of ^{1}H NMR the number of moles corresponding exclusively to EPA fatty acid, this overlapping was not considered in the equations. Anyway, as the content of EPA acyl groups in marine lipids is not high, and in consequence low concentrations of EPA acid will be formed during hydrolysis, the error is minimal. This fact will be proved later with the results of mixture Mx6.

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:

$$N_T = N_{TG} + N_{1,2\text{-DG}} + N_{1,3\text{-DG}} + N_{2\text{-MG}} + N_{1\text{-MG}} + N_{FA} \tag{10}$$

Finally, once the number of moles of all the species present in the sample are known, it is possible to determine the molar percentage of any of them (X) using the following general equation:

$$X(\%) = 100(N_X/N_T) \tag{11}$$

Taking into account all the above mentioned, the integration of the spectrum signals and the use of these equations allow a determination of the molar percentage of the different kinds of compounds generated in lipid digestion, which permits one to know in this way the advance of the hydrolysis of lipids in the process.

3.4. Validation of the approach

As was mentioned before, in order to test the accuracy of the approach developed several mixtures having very different molar proportions of the different compounds potentially present during lipid digestion were prepared by weight and their ^{1}H NMR spectrum were recorded. Table 3 shows the results obtained using the ^{1}H NMR equations proposed to determine the molar percentages of the standard compounds of the 10 mixtures, together with those obtained by weight. It can be observed that the level of agreement between both series of data is very high, which confirms 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 samples subject of study ranged

Table 3
Molar percentages (%) of the different glycerides and fatty acids present in the standard mixtures (Mx1–10), determined by weight and by ^{1}H NMR with two different approaches using the area of the spectra signals generated by the a) lowest and b) the highest number of protons.

Mixtures			TG %	1,2-DG %	1,3-DG %	2-MG %	1-MG %	FA %
Mx1	Weight		100.0	–	–	–	–	–
	^{1}H NMR	a	100.0 ± 0.0	–	–	–	–	–
	^{1}H NMR	b	100.0 ± 0.0	–	–	–	–	–
Mx2	Weight		100.0	–	–		–	–
	^{1}H NMR	a	100.0 ± 0.0	–	–	–	–	–
	^{1}H NMR	b	100.0 ± 0.0	–	–	–	–	–
Mx3	Weight		46.2	6.5		4.0	–	43.3
	^{1}H NMR	a	46.0 ± 0.0	6.5 ± 0.1	–	3.8 ± 0.0	–	43.7 ± 0.1
	^{1}H NMR	b	46.0 ± 0.0	6.4 ± 0.0	–	3.9 ± 0.0	–	43.7 ± 0.0
Mx4	Weight		6.0	19.0	–		30.9	44.1
	^{1}H NMR	a	7.0 ± 0.0	18.9 ± 0.0	–	–	31.3 ± 0.1	42.8 ± 0.2
	^{1}H NMR	b	6.3 ± 0.1	20.2 ± 0.0	–	–	31.3 ± 0.1	42.2 ± 0.1
Mx5	Weight		–	7.0	13.0	–	22.1	57.9
	^{1}H NMR	a	–	7.3 ± 0.1	13.0 ± 0.0	–	21.0 ± 0.0	58.7 ± 0.1
	^{1}H NMR	b	–	6.7 ± 0.0	13.2 ± 0.0	–	20.9 ± 0.0	59.2 ± 0.0
Mx6	Weight		71.0	–	–	–	–	29.0
	^{1}H NMR	a	69.9 ± 0.2	–	–	–	–	30.1 ± 0.2
	^{1}H NMR	b	69.9 ± 0.2	–	–	–	–	30.1 ± 0.2
Mx7	Weight		36.6	4.4	–	2.7	–	56.3
	^{1}H NMR	a	35.5 ± 0.0	4.8 ± 0.4	–	2.8 ± 0.6	–	56.9 ± 1.1
	^{1}H NMR	b	35.9 ± 0.2	4.1 ± 0.1	–	2.5 ± 0.0	–	57.5 ± 0.4
Mx8	Weight		9.1	12.2	–		19.9	58.8
	^{1}H NMR	a	8.9 ± 0.1	12.0 ± 0.0	–	–	18.7 ± 0.1	60.4 ± 0.1
	^{1}H NMR	b	8.9 ± 0.1	11.8 ± 0.1	–	–	18.7 ± 0.1	60.6 ± 0.0
Mx9	Weight		5.1	5.9	10.8	–	18.5	59.7
	^{1}H NMR	a	4.7 ± 0.2	5.9 ± 0.4	11.0 ± 0.1	–	17.9 ± 0.3	60.5 ± 0.0
	^{1}H NMR	b	4.9 ± 0.0	5.5 ± 0.1	10.9 ± 0.1	–	17.9 ± 0.3	60.8 ± 0.1
Mx10	Weight		–	6.2	11.4	–	19.5	62.9
	^{1}H NMR	a	–	5.9 ± 0.1	11.4 ± 0.1	–	19.2 ± 0.2	63.5 ± 0.2
	^{1}H NMR	b	–	5.7 ± 0.0	11.5 ± 0.1	–	19.2 ± 0.2	63.6 ± 0.3

Abbreviations: TG: triglyceride; 1,2-DG: 1,2-diglyceride; 1,3-DG: 1,3-diglyceride; 2-MG: 2-monoglyceride; 1-MG: 1-monoglyceride; FA: fatty acid.

from 0 to 9%, and only in one case (TG % in Mx4 using the first approach) did it reach 17%. Nevertheless, it has to be noted that these results are highly dependent on the quality of the ^{1}H NMR spectrum, the base line correction and the spectral signal integration. Moreover, as commented previously, depending on the proton signal selected to quantify 2-MG and 1,2-DG, two approaches can be carried out, either using the signals generated by the lowest (approach "a") or by the highest (approach "b") number of protons, obtaining similar results.

4. Conclusions

In short, this study evidences the usefulness of ^{1}H NMR to study qualitatively and quantitatively the extent of lipolysis reaction. As far as we know, this is the first time that equations are proposed in order to determine, by means of ^{1}H NMR, the molar percentages of the different kinds of compounds which may be present during lipid digestion. In comparison with other methodologies previously applied to study lipolysis, this approach allows a global study of the sample, providing simultaneous detailed information on all kinds of compounds present, in a simple and fast way, and without any chemical modification of the sample. This technique offers many advantages for the evaluation of lipolysis degree, not only in the field of food technology and nutrition, but also in those of enzymology, pharmacology, medicine and petrochemistry, among others.

Acknowledgments

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). B. N-E. thanks to the UPV/EHU for a predoctoral fellowship.

References

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.

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.

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.

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.

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.

Guillén, M. D., 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.

Gusntone, 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.

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.

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. (2011). Impact of salt and lipid type on *in vitro* digestion of emulsified lipids. *Food Chemistry*, *126*(4), 1559–1564.

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.

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.

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). 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*(5), 429–437.

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.

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.

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

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.

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.

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.

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.

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.

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.

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.

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.

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.