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The challenge of monitoring the hydrolysis of foods lipids during gastrointestinal digestion

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Mini review

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CONFLICTS OF INTEREST

There are no conflicts of interest for any of the authors.

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ABSTRACT

During the last decade obtaining further knowledge on lipid digestion has become a challenging task in the field of Food Science and Nutrition research. However, the great complexity of this process requires the use of sound, accurate and simple analytical techniques which are able to provide as much information as possible; only thus can a global view and therefore a better understanding of the ongoing process be obtained. This review tackles the advantages and drawbacks of the different methodologies currently employed for this purpose, focusing on a new approach, recently developed and based on Proton Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy. This new methodological approach not only provides a great deal of information in a simple, rapid and accurate way, but also overcomes many of the disadvantages of the techniques employed to date. In this sense, ^{1}H NMR can be considered a very promising alternative for research on lipid digestion, contributing to shed more light on the complex digestion process of lipids and the factors that may affect it.

Keywords: lipolysis, digestion, NMR spectroscopy, pH-stat titration, chromatography.

INTRODUCTION

Consumer demand for healthier foods is a general trend, especially in Western countries where concern for maintaining and/or improving health status through diet has grown considerably. Nevertheless, the design of healthier foods requires, among other things, deeper knowledge of the food digestion process and of the fate of the different macro and micronutrients in the gastrointestinal tract until their absorption into the bloodstream. In this context, special attention is nowadays being paid to the study of food

lipids and the digestion process to which they are submitted by digestive enzymes (McClements, Decker, & Park, 2009). This interest can be partially explained because of the nutritional quality of lipids, which provide a high amount of energy in comparison with other nutrients (9 kcal/g) and are source of several bioactive compounds (sterols, vitamins, essential long-chain polyunsaturated fatty acids, etc).

In this review the digestion of food lipids and the techniques used for its monitoring will be discussed. Although several methodologies have been employed in food lipid research, each of them presents different advantages and drawbacks, in such a way that the study of the advance of lipolysis and of the bioaccessibility of lipidic components (release of potentially absorbable molecules in the gastrointestinal lumen) still can be considered as a challenging task.

Lipolysis reaction taking place during digestion

Food lipids mostly consist of triglycerides (TG), which are esters made up from the esterification of three fatty acids (FA) with a molecule of glycerol (Gly). However, as TG cannot be directly absorbed by the intestinal cells, a process of hydrolysis of the ester bonds is required before their incorporation into the bloodstream. For this purpose, several types of lipases (gastric and pancreatic lipases, among them colipase-dependent lipase, carboxyl ester hydrolase or bile salt stimulated lipase and phospholipase A2) are secreted within digestive juices, ensuring the absorption of 95% of ingested lipids in the form of monoglycerides (MG) and FA. Both are the only lipolytic products arising from TG that can be absorbed. In healthy adults, hydrolysis of TG mainly occurs in the first section of the small intestine (duodenum) due to the activity of colipase-dependent lipase at the lipid-water interface (Reis, Holmberg, Watzke, Leser, & Miller, 2009; Golding & Wooster, 2010).

The hydrolysis of TG proceeds as a 2-step reaction, which is directed by the regiospecificity of human lipases. Firstly, the hydrolysis of the ester bond in position 3 of a TG yields a FA and a 1,2-diglyceride (1,2-DG). Secondly, this 1,2-DG is hydrolyzed in position 1 to release a second FA and the corresponding 2-monoglyceride (2-MG) (Desnuelle & Savary, 1963; Mattson & Volpenhein, 1964). Isomerization reactions of 1,2-DG into 1,3-DG and of 2-MG into 1-MG can also occur in the gastrointestinal lumen, making possible the complete hydrolysis of TG into three FA and a molecule of Gly (Miettinen & Siurala, 1971; Borgström, Tryding, & Westöö, 1957; Borgström, 1964).

Therefore, techniques able to identify and quantify all the several kinds of molecular species (including isomers) that may be formed during digestion of TG are needed for a deep study of lipid hydrolysis and for a proper assessment of the advance of lipolysis reaction.

Methodologies usually employed for the study of lipid digestion

As previously commented, several analytical techniques are being used in lipid digestion research, either when performing *in vivo*, *ex vivo* or *in vitro* experiments.

1) Titration of fatty acids

One of the techniques most commonly employed to measure the rate of lipolysis is the titration of released FA. The equipment used for this purpose is named pH -stat titration unit, which records the volume of an alkaline solution (usually NaOH) that is continuously added to the reaction medium in order to maintain the pH at a constant value (Beisson, Tiss, Riviere, & Verger, 2000). In fact, as lipases release FA from the different glycerides (TG, DG, MG), the pH of the medium tends to drop. Then, the molar percentage of FA can be estimated in a very simple way by calculating the number of moles of alkali consumed divided by the number of moles of FA that would arise from TG after being digested. Due to its ease of use, this technique is one of the most commonly employed to estimate the extent of lipid digestion (Fatouros, Bergenstahl, & Mullertz, 2007; Brogård, Troedsson, Thuresson, & Ljusberg-Wahren, 2007; Li & McClements, 2010; Helbig, Silletti, Timmerman, Hamer, & Gruppen, 2012; Lamothe, Corbeil, Turgeon, & Britten, 2012; Marze, Menier, & Anton, 2013; Zhu,

Ye, Verrier, & Singh, 2013).

Nevertheless, this methodology presents some limitations. The first drawback is that pH-stat can only be used in *in vitro* experiments in which lipolysis reactions occur in a closed vessel where conditions can be continuously controlled. Moreover, this methodology is a one-step procedure, generally employed to monitor the activity of pancreatic lipases during the intestinal step. In the case of investigating the activity of gastric lipases, analysis by this methodology becomes more tedious, because a back-titration is also required in order to take into account those FA which are not ionised at low pH values (Beisson et al., 2000).

In addition to this, the quantitative information obtained by means of pH-stat titration technique is very limited because only information regarding FA is obtained, leaving unknown the number of moles of 1- and 2-MG, as well as of 1,2- and 1,3-DG generated during lipolysis. Bearing in mind that MG are also potentially absorbable molecules, pH-stat titration technique offers a very partial view of lipolysis reaction, hampering the proper assessment of lipid bioaccessibility. Several authors even assume in their calculations that complete hydrolysis of TG into three FA and one molecule of Gly does not occur (Pafumi et al., 2002; Li & McClements, 2010; Lamothe et al., 2012; Marze et al., 2013), although it is well known that it does (Borgström, Tryding, & Westöö, 1957; Borgström, 1964; Mattson & Volpenhein, 1964); this inadequate assumption leads to a noticeable overestimation of the advance of lipid digestion.

Furthermore, the results obtained by means of this methodology might not be very accurate. In fact, the drop of pH in the digestion vessel may not always be representative of the FA released, especially when using complex matrices (such as foods) or complex digestion juices (with compositions similar to those of human juices). For instance, the buffering capacity of certain components like proteins, present in either food or digestive juices themselves, can counterbalance the decrease of pH, underestimating the advance of hydrolysis reaction. For this reason, simple solutions whose composition widely differs from *in vivo* digestion juices are employed as buffers (Di Maio & Carrier, 2011). Besides, the accuracy of the results obtained by titration is also dependent on both the ionization of each FA and its availability to be titrated. Indeed, the lipid composition in the several kinds of acyl groups, the pH of the reaction medium and the concentration of bile salts and electrolytes can greatly influence the volume of alkaline solution to be consumed (Sek, Porter, & Charman 2001; Kanicky & Shah, 2003; Thomas, Holm, Rades, & Müllertz, 2012). For example, the amount of alkali consumed to neutralize 1 mol of butyric acid (C4:0) can be 1000-fold higher than that employed to neutralize 1 mol of stearic acid (C18:0); likewise, the pKa of a mixture of several FA might differ from the pKa of the single FA (Zhu et al. 2013; Kanicky & Shah, 2003). Thus, in certain cases, the moles of alkali calculated might not be equivalent to those of the FA released.

Finally, the selection of the end point value of the pH is of primary importance. This latter should be higher than the apparent pKa of the mixture of FA in order to ensure that all the carboxylic groups of FA are in their ionized form, as well as to increase their solubility in water and thus, their availability for neutralization. At pH ranging from 9 to 10, the ionic repulsion between adjacent ionized carboxylic groups is increased, enhancing the solubility of FA (Kanicky & Shah, 2003). Not only would the presence of FA negatively charged and of FA dimmers negatively affect the reliability of titration, but also the potential formation of calcium soaps. Indeed, the complexation of FA with cations would also decrease the volume of alkali consumed, the number of moles of FA released and thus, the advance of hydrolysis reaction could be underestimated. In this regard, the influence of the compositions of digestive juices and of food matrices is considered of paramount importance.

2) Chromatographic techniques

Apart from pH-stat titration, several studies have employed chromatographic techniques to assess the extent of lipid digestion. Among these can be cited High Performance Liquid Chromatography coupled to an Evaporative Light Scattering Detector (HPLC-ELSD) (Martin, Nieto-Fuentes, Señoráns, Reglero, & Soler-Rivas, 2010; Kenmogne-Domguia, Meynier, Viau,

Llamas, & Genot, 2012), Thin Layer Cromatography coupled to Flame Ionisation Detector (TLC-FID) (Capolino et al., 2011) or to video densitometry (Armand et al., 1999; Sek et al., 2001), Gas Chromatography followed by Mass Spectrometry (GC-MS) (Shen, Apriani, Weerakkody, Sanguansri, & Augustin, 2011; Ye, Cui, Zhu, & Singh, 2013; Zhu et al., 2013) or by FID (GC-FID) (Helbig et al., 2012). Other less common methodologies, such as Ultra High Liquid Chromatography-Electrospray Ionization/ Mass Spectrometry (UHPLC-ESI/MS), have been also employed for the study of lipolysis advance during *in vitro* digestion (Tarvainen, Suomela, & Kallio, 2011; Tarvainen, Phuphusit, Suomela, Kuksis, & Kallio, 2013). By means of the above-mentioned methodologies, separation, identification and quantification of the different molecular species that may be generated during the hydrolysis of TG can be carried out.

However, it has to be taken into account that several preparation steps are often needed prior to analysis in order to chemically modify the sample. For example, the estimation of the content of FA in the digested lipid extract by means of GC-MS requires both an acid and an alkaline esterification of the FA and/or transesterification of acyl groups present in the sample and further quantification of the methyl esters obtained. In acid medium FA and acyl groups present in the several kinds of glycerides (TG, DG and MG) are (trans)esterified, whereas in the alkaline one, only the acyl groups are transesterified. Afterwards, the methyl esters obtained (commonly known as Fatty Acid Methyl Esters or FAMEs) are quantified and the content of FA in a digested sample is estimated by difference (Shen et al., 2011; Zhu et al., 2013). Thus, these multi-step chromatographic techniques are quite tedious and laborious.

Likewise, when using these methodologies, reference compounds and calibration curves are needed for quantification purposes. For example, in the case of analysis by means of HPLC-ELSD, TLC-FID or TLC coupled to video densitometry, after separation of TG, MG, DG and FA by chromatography, identification is performed by comparison of retention times with that of pure standard compounds. Then, calibration curves of each of the standard compounds are needed for further quantification.

In comparison with the pH-stat titration method, these methodologies can also be considered less environmentally friendly because of the large amounts of polluting organic solvents used.

Furthermore, taking into account that certain discrepancies among data obtained with the above-mentioned methodologies have been reported (Sek et al., 2001; Helbig et al., 2012; Thomas et al., 2012), sound methodological developments are still needed.

3) Nuclear Magnetic Resonance

Spectroscopic techniques, such as Nuclear Magnetic Resonance (NMR), have been previously applied to identify and quantify DG, MG and FA in fats, oils or other lipidic mixtures. Quantification by NMR is based on the premise that the signal produced by exciting a nucleus from a fully relaxed state is directly proportional to the number of molecules containing the nucleus of interest (Fernandes, de Souza, & de Vasconcellos Azeredo, 2012).

With regard to ^{13}C NMR, signals associated with the glycerol carbon atoms and the first two carbon atoms in the acyl chains have been used to identify and quantify partial glycerides in standard mixtures and naturally present in complex mixtures of several glycerol esters and oils (Gunstone, 1991; Fernandes et al., 2012; Vlahov, 1996, 2006). Although the different isomers of DG and MG can be differentiated, the main disadvantage of this technique is that it involves long relaxation delays and lengthy accumulations to achieve a satisfactory signal to noise ratio necessary for an accurate quantification. Moreover, the use of internal and external reference compounds is also required for calibration curves.

Regarding ^{31}P NMR spectroscopy, Spyros & Dais (2000) developed a methodology to determine the content of MG and DG in vegetable oils. This technique showed an excellent resolution between the chemical shifts of the phosphorylated hydroxyl groups present in 1-MG, 2-MG, 1,2-DG and 1,3-DG, allowing a reliable quantification of these lipolytic products. Nevertheless, a previous derivatization of the labile hydrogens in partial glycerides with 2-chloro-4,4,5,5-tetramethyldioxaphospholane is required, as well as

the introduction of cyclohexanol as internal standard in the reaction mixture for the subsequent quantification of the phosphorylated derivatives.

As far as ^{1}H NMR is concerned, a more extensive overlapping of the spectral signals comparing to ^{13}C NMR and ^{31}P NMR occurs, because of the shorter range of chemical shifts. Nevertheless, in spite of the above-mentioned disadvantage, recent studies have demonstrated the usefulness of this technique to study in detail lipolysis reaction occurring during digestion (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2014, 2015). In fact, the ^{1}H NMR spectra of TG, 1,2-DG, 1,3-DG, 2-MG, 1-MG and FA greatly differ, especially regarding the spectral region ranging from 3.50 to 5.30 ppm, where specific signals related to the protons in the glycerol backbone of glycerides appear, and the spectral region ranging from 2.25 to 2.45 ppm, where protons of methylenic groups in α-position in relation to the carbonyl group of FA and all acyl groups are visible (except those of docosahexaenoic acid/acyl group). Differences among the ^{1}H NMR spectra of TG, partial glycerides and FA can be observed in **Figure 1**.

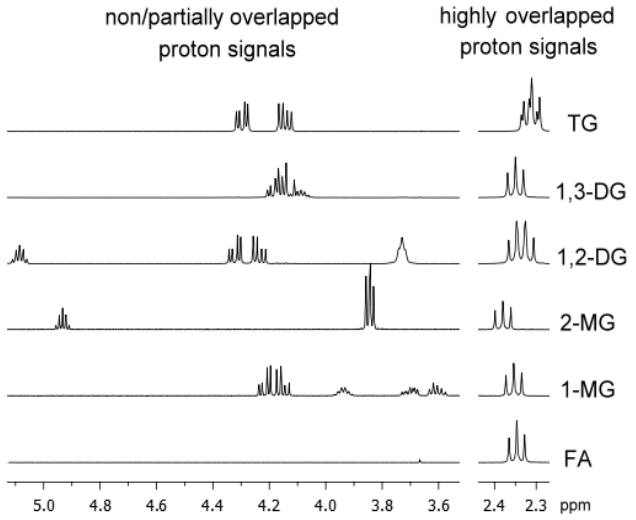

Figure 1. Enlargements of certain spectral regions of the ^{1}H NMR spectra of triolein, 1,3- and 1,2-diolein, 2- and 1-monoolein and oleic acid.

Since most of these ^{1}H NMR signals do not overlap or only do so partially, the identification of the different glycerides present in a lipid hydrolysate can be easily performed by the simple observation of the presence/absence of the corresponding signals in the spectrum. Thus, no chemical modification of the lipid sample is needed.

In addition, due to the proportionality existing between the area of the ^{1}H NMR spectral signals and the number of protons that generate them, quantitative information on the proportions of the several kinds of lipolytic products can be easily obtained just by applying different equations and calculating the intensity of specific spectral signals. In this case, the performance of calibration curves with standards for each one of the compounds under study is not required. The accuracy of the results obtained with ^{1}H NMR spectral data was validated by using mixtures of known composition made up with several standard compounds which simulated lipid hydrolysates from different origins (vegetable or animal). Comparison of the molar percentages of TG, DG, MG and FA obtained by weight and those obtained by applying the new developed equations showed a very high level of agreement, the error in the determination ranging from 0 to 9% (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2014).

In later studies, the application of this new approach to study qualitatively and quantitatively the changes due to the progression of lipolysis under digestive conditions was carried out (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2015, 2016, 2017).

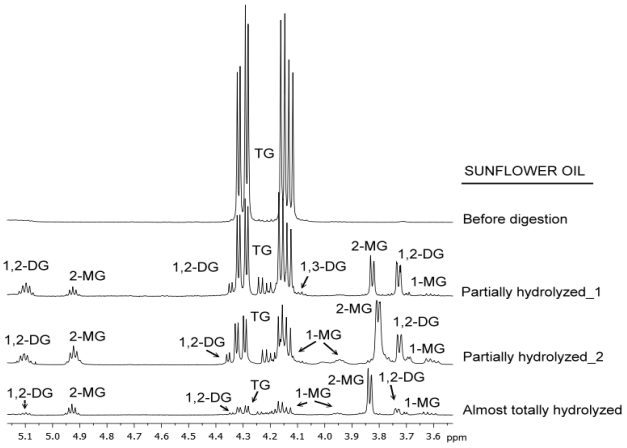

Figure 2. Enlargements of certain spectral regions of the ^{1}H NMR spectra of sunflower oil before and during *in vitro* digestion process.

As shown in **Figure 2**, significant changes in the ^{1}H NMR spectra of lipids occur as lipolysis advances: spectral signals corresponding to TG tend to disappear, whereas other new signals (related with lipolytic products) appear and show higher intensity as digestion advances. Hence, by means of this technique, it is possible to discriminate among samples with different lipolytic levels by the simple observation of their spectra, in a rapid way and without further enlargement.

In addition, the high versatility of ^{1}H NMR enables the assessment of the extent of lipolysis reaction in any of its current meanings (lipid bioaccessibility, percentage of FA physiologically releasable, hydrolysis level or degree of TG transformation), in contrast to chromatographic and pH-stat titration techniques (Nieva-Echevarría et al., 2015).

In summary, ^{1}H NMR allows a global qualitative and quantitative study of lipid digestion in a simple and fast way, and without any chemical modification of the sample. It must be noted that the advantages of this technique for the evaluation of lipolysis degree should not only be considered in the fields of food technology and nutrition, but also in those of enzymology, pharmacology, medicine and petrochemistry, among others.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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