



Behaviour of non-oxidized and oxidized flaxseed oils, as models of omega-3 rich lipids, during *in vitro* digestion. Occurrence of epoxidation reactions



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ABSTRACT

Fresh and partially oxidized flaxseed oil, as models of omega-3 rich lipids, were submitted to *in vitro* gastrointestinal digestion. Hydrolysis level, lipid composition and oxidative status of the samples before and after digestion were studied by Proton Nuclear Magnetic Resonance (¹H NMR) and Solid Phase Microextraction–Gas Chromatography/Mass Spectrometry (SPME–GC/MS). Although a great degree of lipolysis was reached in both kinds of samples after digestion, it was somewhat lower in the digests of oxidized flaxseed oil. The occurrence of lipid oxidation during digestion was evidenced by decreased unsaturated lipids and increased primary and secondary oxidation products, especially in oxidized samples. In these latter, linolenic-derived monoepoxy-octadecadienoates were the main oxidation products generated. SPME–GC/MS study showed the highest abundances of highly reactive alkadienals (C5–C10), alkatrienals (C9–C10) and linolenic-derived 4,5-epoxy-2-heptenals in the headspace of oxidized flaxseed oil digests. Volatile markers of Maillard-type reactions were also detected.

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1. Introduction

The occurrence of lipid oxidation in foods has been subject of a great concern for a long time, not only because of its economic implications, but also due to the potential adverse health effects that certain oxidation products may have (Kubow, 1992). To date, scientific research has mainly focused on the impact of food processing and storage conditions on the occurrence of this reaction and on the nature of the compounds derived from it (Frankel, 2005; Martínez-Yusta, Goicoechea, & Guillén, 2014). However, a further oxidative degradation of unsaturated lipids might also take place during the digestion process, especially in the gastric step where lipids can be exposed to pro-oxidant conditions, like the acid pH of gastric fluid, the presence of oxygen incorporated to food during mastication, as well as of heme groups in certain proteins and of food-released transition metals, among others (Halliwell, Zhao, & Whiteman, 2000; Kanner & Lapidot, 2001).

Oxidation reactions occurring during digestion have been studied very little, especially under *in vivo* conditions (Gobert et al., 2014; Gorelik, Ligumsky, Kohen, & Kanner, 2008) because of ethical and economical issues. As a result *in vitro* methodologies simulating gastric and intestinal environment have widely been employed in recent years, demonstrating their usefulness as an initial approach to

investigate lipid oxidation during digestion and to identify factors affecting it (Kenmogne-Domguia, Meynier, Boulanger, & Genot, 2012; Larsson, Cavinus, Alminger, & Undeland, 2012; Steppeler, Haugen, Rødbotten, & Kirkhus, 2016). Thus, it has been reported that food lipids can oxidize under the physiological conditions of stomach and/or small intestine, and it has been shown that the advance of lipid oxidation can be greatly influenced by several factors. Among these may be cited: the presence of heme proteins, free iron or minor dietary compounds showing antioxidant properties (Gorelik et al., 2008; Kanner & Lapidot, 2001; Kenmogne-Domguia et al., 2012; Larsson et al., 2012), as well as lipid composition, initial oxidative status and food fat content (Kenmogne-Domguia, Moisan, Viau, Genot, & Meynier, 2014; Larsson et al., 2012; Steppeler et al., 2016). It must be noted that in most of these studies complex food matrices such as emulsions or cooked meat products already containing oxidation initiators or showing a certain degree of oxidation were digested. This may complicate the interpretation and comparison of the results obtained due to the interactions, often unknown, of other sample components with the lipid oxidation process (Schaich, 2016). In this sense, additional systematic studies addressing the *in vitro* digestion of simpler lipid matrixes, such as bulk oils, could better clarify the susceptibility of lipids to oxidation under gastrointestinal conditions and the extent to which this reaction can be influenced by several factors.

Furthermore, the lipid oxidation level of the samples in the above-mentioned studies was mainly assessed by indirect measurements

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(oxygen uptake and loss of “antioxidant” compounds) and by classical techniques, which might offer limited accuracy and specificity (Frankel, 2005; Schaich, 2016), such as: determination of lipid hydroperoxides by iodometric titration or by ferrous ion oxidation in the presence of xylenol orange or thiocyanate, and measurement of conjugated dienes absorbance or of Thiobarbituric Acid Reactive Substances (TBARS) test. It must be noted that in two previous studies the determination of two specific oxidation markers (4-hydroxy-(*E*)-2-nonenal and 4-hydroxy-(*E*)-2-hexenal) by chromatographic techniques, after their extraction and derivatization, was also carried out (Kenmogne-Domguia et al., 2014; Steppeler et al., 2016). Nonetheless, these techniques are very laborious, require chemical transformation of the sample, involve the use of large amounts of solvents, and provide a very partial view of the lipid oxidation process, during which a high number of compounds of very different molecular weight and nature can be generated (Frankel, 2005; Schaich, 2016).

Recently, the *in vitro* digestion of fresh and slightly oxidized sunflower oil, as a model of omega-6 rich lipids, was studied by means of Proton Nuclear Magnetic Resonance (^1H NMR) and Solid Phase Microextraction followed by Gas Chromatography/ Mass Spectrometry (SPME-GC/MS) (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2017). Both techniques provided a great deal of information on several ongoing reactions, among which lipid hydrolysis and oxidation can be highlighted. It was observed that during the *in vitro* digestion of non-oxidized sunflower oil small amounts of (*Z,E*)-hydroperoxy-octadecadienoates and typical secondary oxidation aldehydes were generated, whereas the digestion of slightly oxidized sunflower oil gave rise to not only (*Z,E*)-hydroperoxy-octadecadienoates but also to (*Z,E*)- and (*E,E*)-hydroxy-octadecadienoates and large amounts of volatile α,β -unsaturated aldehydes. Bearing in mind these results, a greater degree of lipid oxidation could be expected during the digestion of oils which are rich in polyunsaturated omega-3 acyl groups. As the intake of these latter has been encouraged in recent years, because of the health benefits which derive from this, a deeper knowledge of their potential chemical transformation during digestion can be considered of great relevance from a nutritional and food safety point of view. Indeed, the specific nature of the compounds that may arise from the oxidation under digestive conditions of oils rich in polyunsaturated omega-3 acyl groups still remains unknown.

In this context, the aim of this work is to investigate, for the first time, the chemical reactions taking place during *in vitro* digestion of flaxseed oil, as a model of omega-3 rich lipids, paying special attention to lipid oxidation. For this purpose, fresh and slightly oxidized flaxseed oil will be submitted to *in vitro* gastrointestinal digestion and the samples before and after digestion will be studied by means of ^1H NMR and SPME-GC/MS. It must be noted that the latter technique, which is the more sensitive, was not employed with the aim of quantifying concentrations of volatile compounds, but with that of obtaining valuable data for comparative purposes among samples. Information extracted from both techniques will be analyzed and compared to that obtained previously from the *in vitro* digestion of fresh and slightly oxidized sunflower oil (Nieva-Echevarría et al., 2017).

2. Materials and methods

2.1. Fresh and oxidized flaxseed oil samples

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

compounds (hydroperoxides also supporting conjugated dienes), in accordance with previous studies in which flaxseed oil was submitted to the same accelerated storage conditions (Guillén & Ruiz, 2005).

2.2. *In vitro* gastrointestinal digestion

Flaxseed oil samples (0.5 g), either non-oxidized or slightly oxidized, were digested following the *in vitro* gastrointestinal protocol developed by Versantvoort, Oomen, Van de Kamp, Rompelberg, and Sips (2005), which was slightly modified as described in detail previously to reach a similar lipolysis extent to that reported *in vivo* (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2016). Detailed additional information about this protocol is provided as Supplementary Material. In short, it involves a three-step procedure which simulates digestive processes in the mouth, stomach and small intestine by adding sequentially the corresponding digestive juices. The transit times employed for oral, gastric, and intestinal *in vitro* digestion were 5 min, 2 h and 4 h, respectively. Digestive juices (saliva, gastric, duodenal and bile juices) were prepared in accordance with the original protocol, although some modifications were performed in order to reach a higher level of lipolysis, namely: addition of *Aspergillus niger* lipase to the gastric juice at 100 U/mL and use of bovine bile extract at 18.75 g/L instead of 30.00 g/L in the bile juice. All the reagents for the preparation of the digestive juices were acquired from Sigma-Aldrich (St. Louis, MO, USA). Blank samples, corresponding to the mixture of juices submitted to digestive conditions in the absence of oil sample (**J**), were also undertaken in each experiment for further analysis. For consistency of results the experiment was carried out in quadruplicate. The digested samples obtained from non-oxidized (fresh) flaxseed oils were named **DF** ($n = 4$), and from the digestion of slightly oxidized samples **DFx** ($n = 4$).

2.3. Lipid extraction and ^1H NMR spectra acquisition

Lipids of the digests (**DF**, **DFx**) were extracted using dichloromethane as solvent (CH_2Cl_2 , HPLC grade, Sigma-Aldrich) and following the same methodology as in a previous study (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2015). Digested samples were submitted to a liquid-liquid extraction, using the above-mentioned solvent in a proportion of 2:3 (v/v). Afterwards, to ensure a complete protonation of fatty acids and the dissociation of the potential salts formed, the remaining water phase was acidified to $\text{pH} \approx 2$ with HCl (37%) and a second extraction was carried out. Both CH_2Cl_2 extracts of each sample were mixed and solvent was eliminated using a rotary evaporator under reduced pressure at room temperature, in order to avoid lipid oxidation. With the aim of evaluating if the performed extraction was complete, the water phase was freeze-dried and later extracted with CH_2Cl_2 for subsequent analysis by ^1H NMR spectroscopy, which confirmed the absence of proton signals of lipidic components in this water phase.

The ^1H NMR spectra of the starting oils and of the corresponding digested lipid extracts were acquired using a Bruker Avance 400 spectrometer operating at 400 MHz. To do this, the sample preparation to acquire the corresponding spectra, the acquisition conditions and the study of the spectral data were the same as those used in previous studies (Guillén & Ruiz, 2003, 2005; Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2014; Nieva-Echevarría et al., 2015, 2016). 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 possible their use for quantitative purposes. Each spectrum was acquired in duplicate. The ^1H NMR spectra shown in Fig.1 and Fig.2 were plotted at a fixed value of absolute intensity to be valid for comparative purposes using MNova program (Mestrelab Research, Santiago de Compostela, Spain).

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