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Analytical Methods

Analysis of regioisomers of polyunsaturated triacylglycerols in marine matrices by HPLC/HRMS

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ABSTRACT

Natural sources of triacylglycerols containing ω-3 fatty acids are of particular interest due to their protective role against several human diseases. However, as it has been well ascertained, the position of the ω-3 fatty acid on the triacylglycerol backbone influences how digestion occurs. In particular, occurrence at the *sn-2* position allows optimal intestinal absorption conditions. The analytical protocol for regioisomer characterisation of fatty acids in a triacylglycerol usually requires the use of stereospecific lipases before instrumental identification. In this paper, we propose a more direct instrumental determination of triacylglycerol composition along with *sn-2* positional identification of the fatty acids constituents by Liquid Chromatography–High Resolution Mass Spectrometry. Different intensities of product signals obtained in MS^2 and MS^3 experiments were used to define an interpretative scheme able to rationalise the stereochemistry of the TAGs. Marine matrices like tuna and algae oils have been studied in detail, their triacylglycerols identified and *sn-2* positional arrangement of fatty acid constituents assessed.

1. Introduction

For almost two decades, studies on lipid lowering diets examined the role of saturated, monounsaturated and polyunsaturated fatty acids on plasma low density lipoprotein-cholesterol concentrations, since the foundation for atherosclerotic plaques lays on foam cell formation triggered by elevated low density lipoprotein-cholesterol concentrations (Belitz, Grosch, & Schieberle, 2009; Nagao et al., 2011; Russo, 2009; Wijesundera, 2005). However in addition to overall fatty acid composition, their regiospecific distribution in triacylglycerol structure should be considered.

Fatty acids can exist at any of three positions on the glycerol backbone, designated as *sn-1*, *sn-2* and *sn-3*. The regiospecific position of fatty acids is important because it determines how triacylglycerols (TAGs) are digested and absorbed. In fact the fatty acids released from the *sn*-1 and *sn*-3 positions (or α-position) have different metabolic fates than that one retained in the *sn-2* position (or β-position, Decker, 1996).

Lipoprotein lipase has shown positional specificity for the primary ester bonds of triacylglycerols leading to accumulation of *sn-2*-monoacylglycerol. From this and other metabolic studies, it is possible to conclude that the intestinal absorption is influenced by TAGs structure and the absorption is enhanced for fatty acid located in the *sn-2* position (Decker, 1996; Hunter, 2001; Karupaiah & Sundram, 2007; Mu & Porsgaard 2005; Ramirez, Amate, & Gil, 2001; Zampelas, Williams, Morgan, Wright, & Quinlan, 1994).

Several published methods are used in fatty acids analysis. They can be divided into four broad categories: enzymatic, chemical, spectroscopic and spectrometric methods.

The enzymatic approach is based on release of the FAs attached to the *sn-1* and *sn-3* positions of glycerol by partial or complete hydrolysis in the presence of a 1,3-specific lipase followed by determination of the FA profile of the residual 2-monoacylgrycerol (Shen & Wijesundera, 2006).

The chemical method involves partial deacylation of the TAG with a Grignard reagent such as allyl magnesium bromide or ethyl

Abbreviations: FA, fatty acid; La, lauric acid; M, myristic acid; Mo, myristoleic acid; Pd, pentadecanoic acid; P, palmitic acid; Po, palmitoleic acid; Ma, margaric acid; S, stearic acid; O, oleic acid; L, linoleic acid; S4, octadecatetraenoic acid; A1, eicosamonoenoic acid; A3, eicosatrienoic acid; A4, eicosatetraenoic acid; EPA, eicosapentaenoic acid; D1, docosamonoenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; Li, tetracosanoic acid; TAG, triacylglycerol; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectrometry; LC-PUFA, long chain polyunsaturated fatty acid; PUFA, polyunsaturated fatty acid.

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magnesium bromide (Ando, Kobayashi, Sugimoto, & Takamaru, 2004; Blasi et al., 2008; Haddad, Mozzon, Strabbioli, & Freg, 2010; Straarup, Lauritzen, Faerk, Høy, & Michaelsen, 2006) and determination of the FA profile of the resulting 1,3 or 1,2-(2,3)-diacylglycerol.

Nuclear Magnetic Resonance (NMR) is the basic spectroscopic way to characterise regiospecifically FAs (Lie Ken Jie, Lam, & Pasha, 1996; Vlahov, 1998). Characteristic signals in the olefinic and carbonyl regions of the ^{13}C NMR spectrum allow definite determination of the positional distribution of FA between the *sn-2* and *sn-1(3)* positions.

HPLC coupled with mass spectrometry is the fourth approach to determine the position of FAs into TAG backbone. Many articles illustrate the characterisation of different oils (Brydwell & Neff, 2002; Mottram & Evershed, 1996, 2001; Dugo, Beccaria, Fawzy, Donato, & Cacciola, 2012; Gotoh et al., 2011; Lévêque, Héron, & Tchapla, 2010; Mottram, Woodbury, & Evershed, 1997; Mottram, Crossman, & Evershed, 2001; Nagy, Sandoz, Destaillats, & Schafer, 2013; Nagy et al., 2013; Neff, Brydwell, & List, 2001).

However, every one of the cited approaches presents some disadvantages.

The enzymatic method is time consuming and complex (Mottram et al., 2001). In the Grignard method the analysed diacylglycerol may contain some contaminants due to acyl migration.

Both enzymatic and chemical ways present the FA profile at the *sn-1(3)* position as difference from the total FA profile (Mottram et al., 2001; Shen et al., 2006).

^{13}C NMR is an excellent technique for determining the positional distribution of PUFA. However it is unable to distinguish EPA from arachidonic acid and other PUFA with Δ5 unsaturation (Shen et al., 2006).

Finally, the HPLC-MS methods were developed to characterise TAGs with structurally homogeneous FAs constituents typical of vegetable and animal oils (usually less complex than marine ones). A distinct exception is the work of Lévêque et al. (2010) where the success of recognition rested on a post-column system allowing Ag^+-double bond complex formation and MS^5 experiments. The results are not easily transferable to more simple instrumental equipment and anyway long chain polyunsaturated fatty acids were not taken into consideration.

In the present work, we investigated the total TAGs profile of tuna and algae oils. Many of these TAGs have eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) in their constitution. These two polyunsaturated fatty acids, mainly located in *sn*-1,3 positions of TAGs when coming from marine mammals and in *sn-2* position from fish oil (Mu et al., 2005), play important physiological roles (Wijesundera, 2005).

In order to achieve a qualitative pattern as complete as possible and elucidate the stereochemical aspect connected to the *sn-2* position, we used HPLC coupled with hybrid LTQ-Orbitrap as separation and detection technique. Mass spectral data obtained in MS^2 and MS^3 mode were elaborated with the aim to correlate them to structural features of TAGs. Particular attention was paid to regiospecificity of DHA and EPA positions. Quantitative evaluation was not possible due to the absence of pure standards of the majority of TAGs.

2. Materials and methods

Tuna and algae oils were purchased from Bioriginal, Lonza Ltd, Muenchensteinerstrasse 38, CH-4002 Basel, Switzerland.

HPLC grade acetonitrile and dichloromethane were obtained from Carlo Erba (Milan, Italy).

LC-$HRMS^n$ analyses were accomplished with an Ultimate 3000 HPLC instrument (Dionex, Milan, Italy) coupled to an LTQ Orbitrap instrument (Thermo Scientific, Rodano, Italy) with an APCI interface.

2.1. Chromatographic conditions

The separation was achieved by combining two Luna C18(2) (Phenomenex) columns (150, 2.1 mm, 3 μm particle size) in series.

Gradient conditions: acetonitrile (solvent A) and dichloromethane (solvent B) as eluents are used in a program which was initially isocratic at 80:20 (A:B) for 70 min, increased to 70:30 in 35 min, run up to 55:45 at the 120th minute, to 35:65 at the 140th minute, changed to 0:100 after 5 min followed by an isocratic hold for 10 min, and finally reconditioned for 20 min.

The injection volume was 5 μL and the flow rate 200 μL min^{-1}.

2.2. Sample preparation

Samples of tuna and algae oil were simply diluted 1:100 in dichloromethane.

2.3. Mass spectrometric settings

The LC column effluent entered the APCI source with nitrogen as the sheath and auxiliary gas. The source voltage was set to 4.1 kV. The heated capillary temperature was maintained at 250 °C. The instrument was tuned (capillary, magnetic lenses and collimating octapoles voltages) for maximum sensitivity using the parent compound. The parameters adopted were: vapouriser temperature 450 °C, discharge current 5.00 μA, capillary voltage 10.00 V, tube lens 40 V, and all other parameters were optimised for maximum sensitivity. Full scan spectra were acquired in the range of *m*/*z* 200–1500. MS^n spectra were acquired in the range between the ion trap cut-off and precursor ion *m*/*z* values. CID collision energy was selected for each analyte in order to allow the survival of 5–10% of the chosen precursor ion. High resolution was used to reliably identify precursor ions of different TAGs and to characterise neutral losses. High resolution spectra were acquired with a resolution of 60,000 (FWHM) and the mass accuracy of recorded ions (vs. calculated) was ±2 millimass units (without internal calibration).

3. Results and discussion

The high complexity of triacylglycerols content in tuna oil made the chromatographic separation a difficult task. A complete resolution of all of the lipids present in the real samples was unfeasible with satisfactory separation times and efficiency. Separation of the main constituents was achieved by using two columns in series and a long isocratic period in the first part of the chromatographic program (see in the experimental section, paragraph 2.1 chromatographic conditions). The algae oil sample was much less complex but for uniformity was analysed using the same chromatographic conditions. Chromatograms shown in Fig. 1 illustrate the elution profile.

A characterisation of the monitored compounds ($[M_{TAG}+H]^+$) would require the identification of FA constituents and the determination of their regiopositions in the triacylglycerol structure. The first goal is attainable by mass spectrometric detection. The second one is possible with certainty only by using stereospecific lipases (Shen et al., 2006).

Actually silver ion chromatography may be, in principle, a separation technique able to separate regioisomers. However in the case of triacylglycerols containing polyunsaturated fatty acids the retention times are so long to be at the threshold of irreversible retention. Taking into account that gradient separation is very