Journal of Chromatography A, 1360 (2014) 172–187

Contents lists available at ScienceDirect

Journal of Chromatography A

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

High performance characterization of triacylglycerols in milk and milk-related samples by liquid chromatography and mass spectrometry☆

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ARTICLE INFO

Article history:
Received 12 December 2013
Received in revised form 26 June 2014
Accepted 23 July 2014
Available online 1 August 2014

Keywords:
Milk
Triacylglycerols
Mass spectrometry
Serially-coupled columns
Fused-core

ABSTRACT

In this work, ultra high performance liquid chromatography was used for the characterization of non polar lipids (triacylglycerols) in milk samples of different origin, as well as milk-derivatives. For tackling such a task, three core–shell type octadecylsilica columns were serially coupled, reaching a total stationary phase length of 45 cm, using acetonitrile-isopropanol gradient elution allowing triacylglicerol separation according to increasing partition number. The employment of an ion-trap-time-of-flight detection in conjunction with atmospheric-pressure chemical ionization mass spectrometry was carried out to positively identify a number of 243 different triacylglycerols containing up to 22 fatty acids, with 2–22 carbon atom alkyl chain length, and 0–3 double bonds. This work reports an extensive characterization of the triacylglycerol fraction in milk and milk-related samples of different sources.

1. Introduction

Nowadays there is a great deal of interest in the composition of triacylglycerols (TAGs) in dietary fats due to their influence on physiology and nutritional aspects. Milk fat is a very complex source of TAGs, and several factors such as climate, diet, stage of lactation may strongly influence the chemical composition [1].

In general, the extremely high number of fatty acids (FAs) that can be esterified to the three hydroxyl groups of the glycerol backbone, differing in chain length and number of double bonds (DBs), gives rise to a large number of TAG mixtures.

For TAG characterization, both reversed-phase liquid chromatography (RPLC) and silver-ion chromatography (SIC) methods have been employed [2–23]. In SIC, separation occurs on the basis of unsaturation degree, as well as on DB position or configuration in each FA [3–5]; under RPLC conditions, TAG separation occurs on the basis of the partition number (PN), which is equal to the carbon number of the three FAs minus twice the number of DBs (PN = CN-2DBs). The latter technique, performed under non aqueous reversed-phase liquid chromatography conditions (NARP), is by far the most employed technique for attaining a detailed information on TAG composition in natural samples, as witnessed by the several research works on this field [6–25].

Concerning detection, both UV and evaporative light-scattering (ELS) detection have been employed for TAG analysis [6,10,13], even though mass spectrometry (MS) in combination with LC has gained an ever increasing interest for lipid determination [5–9,11,12,14–28].

Knowledge of the composition of milk lipids, in terms of TAGs, has been evaluated in order to improve the quality of infant-fed formula, as most clinical studies have shown *e.g.* human milk is

☆ Presented at the 20th International Symposium on Electro- and Liquid Phase-Separaton Techniques (ITP 2013), 6–9 October 2013, Puerto de la Cruz, Tenerife, Canary Islands, Spain.

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http://dx.doi.org/10.1016/j.chroma.2014.07.073
0021-9673/

better absorbed, particularly in premature and newborn infants, due to its TAG composition [29,30].

Lipid extraction is a critical step in the analysis of total lipids, since contamination or improper extraction of components of interest may occur leading to misinterpretations [31]. During lipid extraction, samples should be prepared and analyzed carefully to prevent oxidation of lipids and hydrolysis, since artefact production can compromise the identification and quantification of the lipid fraction components [32].

A number of works have already dealt with such a topic, however an extensive milk TAG characterization is missing [14–27]. This is partially related to the very high complexity of lipidic mixtures, often exceeding the separation capability afforded by any single column. Two approaches have been developed so far, to face such an issue: the first consisted in the use of serially-coupled columns [19,20,22,28], the second in the implementation of multidimensional LC systems (MD-LC) for TAG characterization [24,25]. In this contribution, a characterization and comparison of an optimized NARP-UHPLC (ultra high pressure liquid chromatography) method, in combination with positive atmospheric-pressure chemical ionization ion trap-time of flight-mass spectrometry (APCI-IT-TOF-MS) detection was developed for TAG separation in milk and milk-derivatives of different origin. This is the only example reported in literature by using an UHPLC–MS technique for TAG analysis in milk samples and milk-derivates with core–shell type C18 columns. Aiming to estimate the gain in efficiency under true UHPLC operating pressures, the performance of the gradient separation, in terms of peak capacity (n_C), by switching from a single C18 to two- and three-serially coupled C18 columns was evaluated by using a standard mixture of six different TAGs spanning over a broad PN range. Furthermore, thanks to the employment of the IT-TOF detection, accurate mass calculations were reported for all identified TAGs in the most complex sample *viz.* goat milk.

2. Experimental

2.1. Samples and chemicals

Acetonitrile (ACN) and isopropanol (IPA), both MS grade, were obtained from Sigma–Aldrich (Milan, Italy). The tested samples were attained as follows: goat and cow milk from the Calabria region, human milk from a sane voluntary, buffalo mozzarella cheese from the Campania region.

2.2. Sample preparation

Exhaustive extraction of the whole lipid content for the milk samples was achieved according to the Folch extraction method [33], whereas the buffalo mozzarella cheese was treated with the Schmid–Bondzynski–Ratzlaff (SBR) method [34].

The extraction of the milk lipid fraction was carried out from 10 mL of each sample using 40 mL of a chloroform/methanol (2:1, v/v) mixture. The sample/solvent mixture was agitated thoroughly using a vortex for 30 min in an ice bath. Afterwards, the resulting content was left to stand for 5 min, and later on centrifuged in a centrifuge tube (15 min). Once the centrifugation step was completed, the lower phase was carefully taken. The aqueous phase was re-extracted with 20 mL of a chloroform/methanol (2:1, v/v) mixture for two times. The total extract was dried over anhydrous sodium sulfate, filtered and evaporated under vacuum; the final dry residue was stored at −18 °C until use.

The extraction of the buffalo mozzarella cheese lipid fraction was accomplished from 10 g of the sample accurately weighed and dissolved in 20 ml of hydrochloric acid and 20 ml of ethyl alcohol. The preparation was done in a volumetric flask inserted in a boiling water-bath and kept gently moved (for 30 min at 50 °C) with constant magnetic stirring until complete dissolution. Afterwards, the flask was cooled down in running water and a mixture of *n*-hexane and ethyl ether (1:2, v/v) was added and the mixture was shaked for additional 15 min. The suspension was let to stand for 10 min to allow phase separation. The extraction protocol was repeated 3 times. The organic extracts were pooled, dried over anhydrous sodium sulfate, filtered and then brought to dryness under vacuum; the final dry residue was stored at −18 °C until use.

All the samples were weighted in a range from 40 to 50 mg, diluted with 10 mL of acetone and afterwards filtered through a 0.22 μm Acrodisc nylon membrane filter (Pall Life Sciences, Ann Arbor, MI, USA) prior to LC–MS analyses.

2.3. (NARP)LC/APCI-MS analyses

Analyses were carried out on a Nexera liquid chromatography system (Shimadzu, Milan, Italy), already described in Ref. [28], coupled to an LCMS-IT-TOF mass spectrometer through an APCI source (Shimadzu, Kyoto, Japan). Data acquisition was performed by means of the LCMSsolution software (Version 3.50.346, Shimadzu).

Chromatographic separation was achieved on Ascentis Express Fused-core C18 columns, 150 mm × 4.6 mm i.d., 2.7 μm d.p., kindly donated by Supelco/Sigma–Aldrich (Bellefonte, PA, USA).

Mobile phases employed consisted of: (A) ACN, and (B) IPA, under the following gradient: 0 min, 0% B; 50 min, 70% B (hold for 5 min); 56 min, 0% B. The mobile phase flow rate was 1 mL/min. When switching from one- to multi-column set-up, the gradient was adapted on the coupled columns accordingly, with the *xL* factor; *i.e.* for the three-column configuration, each gradient step was three-fold increased, *viz.*: 0 min, 0% B; 150 min, 70% B (hold for 15 min); 168 min, 0% B. LCMS-IT-TOF detection was achieved through an APCI interface operated in positive ionization mode under the following conditions: detector voltage, 1.50 kV; interface temperature: 400 °C; CDL temperature, 230 °C; block heater temperature, 230 °C; nebulizing gas flow (N_2), 2.0 L/min; drying gas flow (N_2), 15.0 L/min; ion accumulation time, 50 ms; full scan range, 200–1200 *m*/*z*; event time, 300 ms; repeat, 3; ASC, 70%. Resolving power (FWHM defined at *m*/*z*): 10,000 (*m*/*z* 1000); Resolution ($\Delta m/z$): 0.1.

2.4. GC–FID and GC–MS analyses

Instrumentation and analytical conditions employed for the buffalo mozzarella cheese sample are the same already reported in Ref. [44]

3. Results and discussion

3.1. Evaluation of column performance on serially-coupled Ascentis Express C18 columns for triacylglycerol analysis

As a starting point, the different milk samples were analyzed on an Ascentis Express C18 column, with a linear gradient of increasing IPA in ACN in a run time of roughly 55 min. Such a stationary phase has already been successfully investigated in a number of research works coming from the same and other research groups [12,13,28,35–42]. Fig. 1 shows the (NARP)LC/APCI-MS chromatograms of the analyzed TAGs on the goat (A), cow (B), human (C) milk and mozzarella cheese (D) samples analyzed on an Ascentis Express C18 column. From a visual inspection it can be appreciated that the goat milk was the most complex one among the Folch milk samples investigated. TAGs were eluted according to increasing PN, reflecting the number of CNs and DBs in acyl