Food Chemistry 231 (2017) 11–18

Contents lists available at ScienceDirect

Food Chemistry

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

Short communication

Optimization of milk odd and branched-chain fatty acids analysis by gas chromatography using an extremely polar stationary phase

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ARTICLE INFO

Article history:
Received 28 October 2016
Received in revised form 8 February 2017
Accepted 9 March 2017
Available online 18 March 2017

Keywords:
Milk
Odd fatty acid
Branched chain fatty acids
Extremely polar column
Gas chromatography

ABSTRACT

Odd and branched-chain fatty acids (OBCFA) are of interest, since they have bioactive properties and could be regarded biomarkers of ruminant fat intake. An accurate analysis of the individual OBCFA in milk by gas chromatography (GC) is not easy due to milk fat complexity. The availability of ionic liquid stationary phases as SLB-IL111 can be a useful tool to discriminate OBCFA from other milk FA eluting in the same chromatographic regions. The elution behavior of OBCFA on SLB-IL111 was evaluated based on different GC oven temperature programs. All programs assayed discriminated 11:0, *iso* 13:0, *anteiso* 13:0, *iso* 15:0, *anteiso* 15:0, 15:0 and *iso* 17:0. Using an initial temperature of 150 °C for 1 h, 13:0 and *iso* 16:0 were separated from *trans*-12:1 and 13-14:1, respectively, whereas *iso* 18:0 was discriminated from *cis*-16:1 isomers. 17:0 and 21:0 were well resolved only when an initial GC temperature of 160 °C was applied.

1. Introduction

Odd and branched-chain fatty acids (OBCFA) are major lipids of bacterial membranes and related to ruminal processes (Fievez, Colman, Castro-Montoya, Stefanov, & Vlaeminck, 2012). They are also primarily components of ruminant food products, but are absent or in very low amounts in other foodstuffs. Furthermore, some studies have regarded individual OBCFA (15:0 and 17:0) as indicators of dairy fat intake (Yakoob et al., 2014). In milk fat OBCFA constitute about 2% of total fatty acids (FA) and are important bioactive components considering their essential role in the gut and potential activity against human breast cancer cells (Astrup, 2014; Ran-Ressler, Bae, Lawrence, Wang, & Brenna, 2014). The most abundant OBCFA in milk fat are 15:0, *iso*-15:0, *anteiso* (*aiso*) -15:0, 17:0, *iso*-17:0 and *aiso*-17:0, although other minor OBCFA can also be found in lower amounts.

More than 400 FA have been estimated to be present in milk fat (Schroeder & Vetter, 2013), and due to this high complexity, determination of minor compounds as OBCFA is not an easy task. The most common methodology to determine milk FA profiling is gas chromatography (GC) using 100-m polar cyanoalkyl polysiloxane (CP-Sil 88) stationary phase columns (Kramer, Blackadar, & Zhou, 2002). Nevertheless, despite the success of these columns for the separation of FA, there remain limitations to achieve an entire separation of all individual OBCFA in milk fat. Such limitations could be overcome with a previous fractionation of FA according to their degree of unsaturation and geometric configuration by argentation thin layer chromatography (Ag^+-TLC) (Precht & Molkentin, 1995), silver ion solid phase extraction (Ag^+-SPE) (Kramer, Hernandez, Cruz-Hernandez, Kraft, & Dugan, 2008) or silver ion high performance liquid chromatography (Ag^+-HPLC) (Delmonte, Hu, Kia, & Rader, 2008) prior GC analysis.

More recently, the availability of capillary columns coated with ionic liquids of extreme polarity as SLB-IL111 has shown new selectivity for milk fat FA. This column provides very unique elution patterns as well as enhanced chromatographic separations of FA isomers (Delmonte et al., 2011). However, milk FA co-elutions are frequent and the available information related to OBCFA resolution in this type of columns is still scarce. The objective of this study was to identify and resolve these minor components in milk fat under different GC temperature programs using an extremely polar 100 m SLB-IL111 capillary column. A further aim was to describe overlaps of OBCFA with other milk FA which are detected in dependence on the GC conditions.

2. Materials and methods

2.1. Milk fat sample derivatization and fractionation

This work was carried out with a butter sample from dairy cows. 25 mg of milk fat were derivatized to fatty acid methyl esters (FAME) by base-catalyzed methanolysis of the glycerides with KOH in methanol according to ISO-IDF procedure (ISO-IDF, 2002). In

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http://dx.doi.org/10.1016/j.foodchem.2017.03.052
0308-8146/

order to identify different overlaps between OBCFA and other FA, FAME were fractioned by Ag^+-SPE following the methodology described by Kramer et al. (2008). Ag^+-SPE cartridges were purchased from Supelco (Bellefonte, PA, USA). FAME were applied to the device and eluted with hexane containing increasing amounts of acetone to obtain different fractions: 99:1 (v/v) eluted saturated FAME; 96:4 (v/v) eluted mono-*trans* FAME; 90:10 (v/v) eluted mono-*cis* and *trans*/*trans* dienes. Finally, 0:100 (v/v) eluted *cis*/*trans* and *cis*/*cis* dienoic FAME. All fractions were taken to dryness in a N_2 stream and then reconstituted in an appropriate volume of hexane before GC analysis.

2.2. Gas chromatography analysis

An Agilent gas chromatograph, model 7820A GC System equipped with auto-injector and FID, was fitted with a SLB-IL111 capillary column (100 m × 0.25 mm i.d., 0.20 μm film thickness; Supelco, Bellefonte, PA). Injector and detector temperature was 250 °C. The column inlet pressure was set at 241 kPa, resulting in helium gas flow rates of 0.86 mL min^{-1}. 1 μL of sample was injected with a split ratio of 1:100. These conditions were identical for the 4 different GC programs assayed. The oven temperature programs are described in detail in Table 1.

2.3. Identification of fatty acid methyl esters

FA identification was accomplished by comparing sample peak retention times with standard mixtures. GLC-409, GLC-411, GLC-423, GLC-461 and GLC-481B analytical standards were purchased from Nu-Chek Prep. Inc. (Elysian, MN, USA). When no commercial standards were available, FAME were investigated by mass spectrometry (MS) on an Agilent chromatograph (model 7890A) with a MS detector (5975C inert MSD). The filament trap current was 400 μA at 70 eV. Chromatographic conditions were similar to those described in the previous paragraph but with a split ratio of 1:20. NIST 05 and Wiley 275 libraries were used to identify the mass spectra. When previous approaches were not enough, FA identification was based on previous analysis carried out under similar chromatographic conditions (de la Fuente, Rodríguez-Pino, & Juárez, 2015; Delmonte et al., 2011).

3. Results and discussion

Four initial isothermal GC temperature programs were assayed. Temperatures tested were 150 °C, 160 °C, 170 °C and 180 °C (Table 1). This temperature range was selected based on previous studies that analyze milk fat with the SLB-IL111 column (Delmonte et al., 2011, 2012). Leaving aside the oven temperature, all chromatographic parameters remained fixed during the analysis. Figs. 1–5 show the chromatographic separations obtained at different initial isothermal temperatures demonstrating a marked influence of oven temperature on the retention time and resolution of most OBCFA.

Fig. 1 shows the separation of FAME from 10:0 to 14:0. OBCFA present in this region of the chromatogram were 11:0, *iso* 13:0, *aiso* 13:0, 13:0 and *iso* 14:0. In all isothermal programs 11:0 and 10:1 eluted separately between 10:0 and 12:0, confirming the efficiency of this stationary phase to discriminate both minor compounds in milk fat (de la Fuente et al., 2015; Delmonte et al., 2012). Increasing oven temperature approached 10:1 to 12:0 but, both FA could be easy and rightly quantified under all GC conditions tested.

It is well known that branched-chain FA elute before the corresponding straight-chain FA on polar and non-polar columns. The SLB-IL111 column displayed *iso* FA with n C-atoms, both odd and even, at a carbon number (CN) of (n-1 + 0.5) following the same principle as other stationary phases (Woodford & Vangent, 1960). As a result, *iso* 13:0 and *iso* 14:0 presented a CN of 12.5 (i.e. eluting right in the middle between 12:0 and 13:0) and 13.5 (i.e. eluting between 13:0 and 14:0) respectively, regardless the temperature program. On the other hand, *aiso* FA would be present at a CN of

Table 1
Temperature programs used for the separation of fatty acids methyl esters onto a SLB-IL111 capillary column and their relative advantages and disadvantages to determine odd and branched-chain fatty acids in milk fat.

Initial temperature	Temperature program	Advantages	Disadvantages
150 °C	150 °C for 60 min Ramp at 10 °C min^{-1} to 210 °C, maintained for 15 min Total time: 81 min	13:0 is resolved *iso* 14:0 is resolved *iso* 16:0 is resolved *iso* 18:0 is resolved *cis*-9 17:1 is resolved	*aiso* 17:0 overlapped with *trans*-9 16:1 17:0 is not well resolved from *cis*-9 16:1 19:0 overlapped with *cis*-11 18:1 21:0 overlapped with 18:3 n-6
160 °C	160 °C for 60 min Ramp at 10 °C min^{-1} to 210 °C, maintained for 15 min Total time: 80 min	17:0 is resolved *iso* 18:0 is resolved 21:0 is resolved	13:0 overlapped with *trans*-12:1 *iso* 14:0 overlapped with 11-12:1 *iso* 16:0 overlapped with 13-14:1 *aiso* 17:0 overlapped with *trans*-9 16:1 *cis*-9 17:1 overlapped with 18:0 19:0 overlapped with *cis* (9 + 10) 18:1 and *trans*-15 18:1
170 °C	170 °C for 60 min Ramp at 10 °C min^{-1} to 210 °C, maintained for 15 min Total time: 79 min		13:0 overlapped with *trans*-12:1 *iso* 14:0 overlapped with *cis*-9 12:1 *iso* 16:0 overlapped with 13-14:1 *aiso* 17:0 overlapped with *trans*-16:1 17:0 overlapped with *cis*-7 16:1 and *trans*-16:1 *iso* 18:0 overlapped with *cis*-15 16:1 *cis*-9 17:1 is not well resolved from 18:0 19:0 overlapped with *cis* (9 + 10) 18:1 and *trans*-15 18:1 21:0 overlapped with *cis*-11 20:1
180 °C	180 °C for 60 min Ramp at 10 °C min^{-1} to 210 °C, maintained for 15 min Total time: 78 min		13:0 overlapped with *trans*-12:1 *iso* 14:0 is not well resolved from *cis*-9 12:1 *iso* 16:0 overlapped with *cis*-9 14:1 17:0 overlapped with *cis*-7 16:1 and *trans*-16:1 *iso* 18:0 overlapped with *cis*-16:1 19:0 overlapped with *trans* (13 + 14) 18:1 21:0 overlapped with *cis*-9 20:1