**OPTIMIZATION OF MILK ODD AND BRANCHED-CHAIN FATTY ACIDS
ANALYSIS BY GAS CHROMATOGRAPHY USING AN EXTREMELY POLAR
STATIONARY PHASE**

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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, *aiiso* 13:0, *iso* 15:0, *aiiso* 15:0, 15:0 and *iso* 17:0. Using an initial temperature of 150°C for 1 hour, 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.

Key words: milk, odd fatty acid, branched chain fatty acids, extremely polar column, gas chromatography

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, Shi, Hu, Campos, Rexrode, Orav, 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, *aiiso*-15:0, 17:0, *iso*-17:0 and *aiiso*-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 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, Kia, Kramer, Mossoba, Sidisky, & Rader, 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. Milk fat was derivatized to fatty acid methyl esters (FAME) by base-catalyzed methanolysis of the glycerides (KOH in methanol) according to ISO-IDF procedure (ISO-IDF, 2002). In order to identify different overlaps between OBCFA and other FA, FAME were fractionated by Ag⁺-SPE following the methodology described by Kramer et al. (Kramer, Hernandez, Cruz-Hernandez, Kraft, & Dugan, 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 N₂ 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 x 0.25 mm i.d., 0.20 μm film thickness; Supelco, Bellefonte, PA). Injector and detector temperature was 250 $^{\circ}\text{C}$. 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 former analysis carried out under similar chromatographic conditions (de la Fuente, Rodriguez-Pino, & Juarez, 2015; Delmonte, Kia, Kramer, Mossoba, Sidisky, & Rader, 2011).

3. Results and Discussion

Four initial isothermal GC temperature programs were assayed. Temperatures tested were 150 $^{\circ}\text{C}$, 160 $^{\circ}\text{C}$, 170 $^{\circ}\text{C}$ and 180 $^{\circ}\text{C}$ (Table 1). This temperature range was selected based on previous studies that analyze milk fat with the SLB-IL111 column (Delmonte,

Fardin-Kia, Kramer, Mossoba, Sidisky, Tyburczy, et al., 2012; Delmonte, Kia, Kramer, Mossoba, Sidisky, & Rader, 2011). Leaving aside the oven temperature, all chromatographic parameters remained fixed during the analysis. Figures 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.

Figure 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, Rodriguez-Pino, & Juarez, 2015; Delmonte, et al., 2012). Increasing oven temperature approached 10:1 to 12:0 but, both FA could be easily 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 $(n-1 + 0.7)$ in different GC columns (Woodford & Vangent, 1960). *Aiso* 13:0 was displayed at a CN of 12.7 in the four temperature programs which means that it eluted at 0.7 units when considering the distance between 12:0 and 13:0 as a unit (Figure 1).

Although the elution order and the relative retention times for *iso* and *aiso* isomers were not affected by the isothermal program, such effect was not observed for *cis* and *trans* isomers. *Cis*-9 12:1 and 11-12:1 were resolved with 150 °C temperature program, however, their relative retention times increased and appeared closer to 14:0 when the temperature was raised (Figure 1). It resulted in the overlap of 11-12:1 with *iso* 14:0 at 160 °C which was even more pronounced at higher temperatures. The resolution of *trans*-12:1 from 13:0 was also characteristic of the 150 °C isothermal program as both FA co-eluted at 160, 170 and 180 °C (Figure 1). This would be of special interest as, to our knowledge, *trans*-12:1 has not been reported on SLB-IL111 and it would trigger an over-quantitation of 13:0 when oven temperature is above 150 °C even with 200 m SLB-IL111 capillary columns (Delmonte, et al., 2012).

Figure 2 presents the chromatographic separation from 14:0 to 16:0. *Iso* 15:0, *aiso* 15:0 and 15:0 were baseline resolved in all the temperature programs tested and maintained their relative retention times related to the CN as explained above. The *iso* 16:0 peak was isolated at 150 °C. When the oven temperature rises, there is a shift of *cis*-9 14:1 and 13-14:1 leading to an overlap between 13-14:1 and *iso* 16:0 (160 and 170 °C) or *cis*-9 14:1 and *iso* 16:0 (180 °C) (Figure 2). This co-elution was already reported by Delmonte *et al* (Delmonte, Kia, Kramer, Mossoba, Sidisky, & Rader, 2011) using a 168 °C isothermal temperature program. Thus, in order to properly quantify *iso* 16:0 on target analysis, it would be advisable to decrease the oven temperature to 150 °C.

Iso 17:0, *aiso* 17:0, 17:0, *iso* 18:0 and *cis*-9 17:1 were detected in the chromatographic region delimited by palmitic and stearic acids (Figure 3). The peak *iso* 17:0 showed its best shape at 150 °C eluting just before *trans*-16:1 isomers, while *aiso* 17:0 overlapped with *trans*-16:1 isomers across all temperature programs. This co-elution would be solved either by a complementary analysis using a conventional CP-Sil 88 column (de

la Fuente, Rodriguez-Pino, & Juarez, 2015) or with a 200 m SLB-IL111 column (Delmonte, et al., 2012).

Success in the chromatographic separation of *cis*-16:1 isomers and *iso* 18:0 was accomplished at 160 °C obtaining overlaps between these OBCFA with these 16:1 isomers at higher temperatures. On the other hand, the temperature effect on the relative retention time of *cis*-9 17:1 was very illustrative (Figure 3). This FA was observed just before the peak front of stearic acid at 150 °C. Raising the isothermal to 160 °C provoked a co-elution among both analytes, but at higher temperatures *cis*-9 17:1 appeared in the peak tailing of 18:0. For that reason, well selected temperatures would be desirable to discriminate *cis*-9 17:1 from 18:0 and to allow a proper quantitation of this unsaturated and odd-chain FA.

From stearic acid onwards, the only OBCFA present in milk fat were 19:0 (Figure 4) and 21:0 (Figure 5). Both saturated FA shifted to shorter retention times when oven temperature increased. 19:0 elutes in the complex region of octadecenoic acids where geometrical and positional isomers overlap. It has been sought to improve the chromatographic separation of 18:1 isomers by changing the analytical temperature of SLB-IL 111 columns (Yoshinaga, Asanuma, Mizobe, Kojima, Nagai, Beppu, et al., 2014). However, to our knowledge, 19:0 has not been isolated even with a 200 m column (Delmonte, et al., 2012). In our experimental conditions, 19:0 switched from a co-elution with *cis*-11 18:1 (150 °C) to a multiple overlap with *cis*-9, *cis*-10 and *trans*-15 18:1 (160 and 170 °C) and finally with *trans*-13 and *trans*-14 18:1 at 180 °C (Figure 4). Regarding 21:0, previous studies have reported a co-elution of this FAME with 18:3 n-6 (Zeng, Chin, Nolvachai, Kulsing, Sidisky, & Marriott, 2013) and *cis*-11 20:1 (Delmonte, et al., 2012) using SLB-IL111 stationary phases. Fortunately, the present research showed that the peak of 21:0 can be resolved at 160 °C (Figure 5).

Table 1 gives an overview of the advantages and disadvantages of the four temperature programs assayed. Based on these results, it would be advisable to operate an isothermal 150 °C program in the analysis of OBCFA because most odd (11:0, 13:0, 15:0, *cis*-9 17:1) and branched-chain (*iso* 13:0, *aiso* 13:0, *iso* 14:0, *iso* 15:0, *aiso* 15:0, *iso* 16:0, *iso* 17:0, *iso* 18:0) FA were resolved. Such chromatographic conditions presented overlaps for *aiso* 17:0, 17:0, 19:0 and 21:0 FAME. Running a complementary temperature program at 160 °C would resolve 17:0 and 21:0 peaks, but *aiso* 17:0 and 19:0 separations remain a challenge with 100 m SLB-IL111 columns.

The present research demonstrate that the extremely polar 100 m SLB-IL111 column exhibits its best performance for the analysis of OBCFA when it operates initially isothermal at 150 °C. It facilitates the separation and quantification of most OBCFA detected in milk fat. 17:0 and 21:0 were isolated only when oven initial temperature was elevated at 160°C. *Aiso* 17:0 and minor *trans* 16:1 isomers separation is still pending for future research.

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Figure Captions

Figure 1. Partial gas chromatograms (GC) of the 10:0 to 14:0 region analyzing milk fatty acid methyl esters at four isothermal temperature programs using a 100 m SLB-IL111 column. GC conditions are described in Table 1. Abbreviations: *c*, *cis*; *t*, *trans*

Figure 2. Partial gas chromatograms (GC) of the 14:0 to 16:0 region analyzing milk fatty acid methyl esters at four isothermal temperature programs using a 100 m SLB-IL111 column. GC conditions are described in Table 1. Abbreviations: *c*, *cis*; *t*, *trans*

Figure 3. Partial gas chromatograms (GC) of the 16:0 to 18:0 region analyzing milk fatty acid methyl esters at four isothermal temperature programs using a 100 m SLB-IL111 column. GC conditions are described in Table 1. Abbreviations: *c*, *cis*; *t*, *trans*

Figure 4. Partial gas chromatograms (GC) of the 18:1 region analyzing milk fatty acid methyl esters at four isothermal temperature programs using a 100 m SLB-IL111 column. GC conditions are described in Table 1. Abbreviations: *c*, *cis*; *t*, *trans*

Figure 5. Partial gas chromatograms (GC) of the 18:2 n-6 to 18:3 n-3 region analyzing milk fatty acid methyl esters at four isothermal temperature programs using a 100 m SLB-IL111 column. GC conditions are described in Table 1. Abbreviation: *c*, *cis*