Journal of Chromatography A, 1233 (2012) 137–146

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

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

Evaluation of highly polar ionic liquid gas chromatographic column for the determination of the fatty acids in milk fat

Pierluigi Delmonte[a,*], Ali Reza Fardin-Kia[a], John K.G. Kramer[b], Magdi M. Mossoba[a], Len Sidisky[c], Cynthia Tyburczy[a], Jeanne I. Rader[a]

[a] Office of Regulatory Science, Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, MD, USA
[b] Retired from Guelph Food Research Center, Agriculture and Agri-Food Canada, Guelph, ON, Canada
[c] Supelco Sigma–Aldrich, Bellefonte, PA, USA

ARTICLE INFO

Article history:
Received 28 October 2011
Received in revised form 28 January 2012
Accepted 4 February 2012
Available online 13 February 2012

Keywords:
Ionic liquid
SLB-IL 111
GC
Gas chromatography
trans fatty acid
Conjugated linoleic acid
CLA
SP-2560
Milk fat

ABSTRACT

The SLB-IL111, a new ionic liquid capillary column for gas chromatography available from Supelco Inc., was recently shown to provide enhanced separation of unsaturated geometric and positional isomers of fatty acid (FAs) when it was compared to cyanopropylsiloxane (CPS) columns currently recommended for the analysis of fatty acid methyl esters (FAMEs). A 200 m SLB-IL111 capillary column, operated under a combined temperature and eluent flow gradient, was successfully used to resolve most of the FAs contained in milk fat in a single 80 min chromatographic separation. The selected chromatographic conditions provided a balanced, simultaneous separation of short-chain (from 4:0), long-chain polyunsaturated fatty acids (PUFAs), and most of the unsaturated FA positional/geometric isomers contained in milk fat. Among the monounsaturated fatty acids (MUFAs), these conditions separated *t*11-18:1 and *t*10-18:1 FAs, the two most abundant *trans* fatty acids (*t*-FA) contained in most dairy products. These *t*-FAs reportedly have different biological activities. The conjugated linoleic acid (CLA) isomers commonly found in dairy products were separated from each other, including *t*7,*c*9-18:2 from *c*9,*t*11-18:2, which eliminated the need for their complementary silver ion HPLC analysis. The application of the SLB-IL111 column provided a complementary elution profile of FAMEs to those obtained by CPS columns, allowing for a more comprehensive FA analysis of total milk fat. The FAMEs were identified by the use of available reference materials, previously synthesized and characterized reference mixtures, and prior separations of the milk fat FAMEs by silver ion chromatography based on the number/geometry of double bonds.

Published by Elsevier B.V.

1. Introduction

The FAs contained in a food, oil, or fat test sample are most commonly measured by gas chromatography (GC) after their extraction and conversion into their FAME derivatives by utilizing the most polar and long capillary columns available. Based on this assumption, the CPS coated SP-2560 (Supelco Inc., Bellefonte, PA) and CP-Sil 88 (Varian Inc., Walnut Creek, CA) GC capillary columns have been used for several years as the preferred choice for FA analysis. The commercial introduction of capillary columns coated with highly polar ionic liquids (SLB-IL100, SLB-IL111, Supelco Inc.) [1] provides analysts with GC capillary columns with markedly different separation characteristics for FAMEs [2,3]. These differences are due to their higher polarity [1–3], their selective interaction with the double bonds of FAMEs [4,5], and to the unique chemical structures of these novel dicationic and tricationic stationary phases [6–10]. The capability of separating selected FAMEs using capillary columns coated with a highly polar ionic liquid was first demonstrated by Ragonese et al. [2] using an SLB-IL100 column. Delmonte et al. [3] recently showed the enhanced separation of FAMEs provided by a 100 m SLB-IL111 column, particularly for the *cis* and *trans* isomers of MUFAs and conjugated linoleic acid (CLA) isomers, compared to the separation provided by an SP-2560 column [3,11,12]. Of particular interest was the ability to separate pairs of coeluting FA isomers including selected *trans*-18:1 FAs (*t*15- from *c*9-18:1, and *t*11- from *t*10-18:1) and CLA isomers (*t*7,*c*9- from *c*9,*t*11-CLA) that could not be resolved by the 100 m SP-2560 (or CP-Sil 88) capillary column under chromatographic conditions suitable for routine analysis. The analysis of these FAMEs previously required either (i) prior fractionation of FAs by silver ion thin-layer chromatography (Ag^+-TLC) or silver ion HPLC (Ag^+-HPLC) [13–15], (ii) complementing GC quantification with Ag^+-HPLC data [14,16,17], or (iii) combining results from separate GC analyses

* Corresponding author at: HFS-717, US Food and Drug Administration, 5100 Paint Branch Pkwy, College Park, MD 20740, USA. Tel.: +1 240 402 1779; fax: +1 240 402 2622.
E-mail address: Pierluigi.delmonte@fda.hhs.gov (P. Delmonte).

0021-9673/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.chroma.2012.02.012

on the same column obtained by using different temperature programs [18].

The FAs contained in milk fat are characterized by a wide range of chain lengths from 4:0 to 26:0, up to 6 unsaturations, many geometric and positional combinations of double bond configurations, as well as alkyl branch-chains and other functional groups (hydroxyl, keto, and cyclic). In total, milk fat had been estimated to contain over 400 different FAs [19]. Several chromatographic conditions, primarily consisting of different temperature programs, were proposed to maximize the comprehensive separation of the FAs (or selected groups of them) contained in milk fat by using 100 m CPS columns [20,21]. The American Oil Chemists' Society (AOCS) introduced an Official Method for dairy fats analysis (Ce 1j-07) based on the separation of FAMEs provided by a 100 m SP-2560 (or CP Sil 88) column operated isothermally at 180 °C for 32 min, followed by a temperature ramp to 215 °C to elute the long-chain saturated and unsaturated FAMEs [22]. All the proposed separations, however, showed numerous overlaps of FAMEs [11,21,23], even when complementary results were obtained by applying different temperature programs [18].

A critical limitation of CPS coated capillary columns is the inability to separate several *cis* and *trans* positional isomers of 16:1 and 18:1 from each other and from other saturated FAs eluting in the same chromatographic region when utilizing conditions that are suitable for routine analysis [22,24–26]. Of particular interest is the separation of *t*11-18:1 FAME (vaccenic acid) from *t*10-18:1, the other often predominant *t*-18:1 isomer present in ruminant-derived food products [21,23,27]. Of these only vaccenic acid has been associated with potential human health benefits [28]. The CPS coated capillary columns can separate *t*11-18:1 FAME from other occurring *t*-18:1 FAMEs only when the elution temperature is significantly reduced, causing the loss of separation of other FAMEs and a longer time of analysis [13,18,29,30].

Delmonte et al. [3] utilized authentic reference materials and previously characterized FAME mixtures to show that, relative to CPS capillary columns, a 100 m SLB-IL111 can provide an enhanced separation of most geometric and positional isomers of the methyl esters of MUFAs often present in partially hydrogenated oils and ruminant products. The elution temperature of 168 °C provided the most balanced separation of *c*-/*t*-MUFAs (from 14:1 to 20:1), of 18:2 and 18:3 FAs, and of CLAs. The chromatographic conditions described [3] were optimized for the analysis of processed (refined, deodorized and bleached) or partially hydrogenated vegetable oils not containing short-chain FAs or long-chain PUFAs, and would need to be modified for the analysis of milk fat which contains these FAs. Also, the 100 m SLB-IL111 capillary column operated at the elution temperature of 168 °C appeared to be capable of separating certain closely eluting FAMEs such as *t*10-/*t*11-/*t*12-18:1 only when these are present in similar and low amounts.

The current study reports the separation of milk fat FAs after their conversion to FAMEs by using a 200 m SLB-IL111 capillary column and a combined gradient of elution temperature and eluent gas flow rate. The 200 m length of the column was selected to further maximize the separation of the *cis*/*trans* 16:1 and 18:1 FA isomers, to ensure the resolution of short-chain FAs and PUFAs, and to evaluate conditions that can be applied to routine analysis. The aim of this study was to evaluate whether an enhanced separation of the FAMEs found in milk fat could be obtained using a 200 m SLB-IL111 column to serve as a single analysis for milk fat. In addition, milk fat was selected as the test matrix to further extend our knowledge of the separation characteristics of this ionic liquid column because of its complexity and its content of all FAs that would be encountered in most fats and oils of non-marine origin.

2. Materials and methods

Mixtures containing positional and geometric isomers of 16:1, 17:1, 18:1, 19:1, 20:1, 23:1, 24:1, 18:2 and 18:3 FAMEs where prepared and characterized as previously reported [11,31]. The GLC 463 reference mixture, the FAME 21:0, 12–13:1, 14–15:1, and a mixture containing conjugated linoleic acid isomers (CLA, UC-59-M), were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). The 25:0 and 26:0 FAs where purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Methyl phytanate and pristanate were purchased from Larodan (Malmo, Sweden). Butter (milk fat) was purchased locally and FAMEs were prepared as previously described [14]. The 200 m SLB-IL111 gas chromatographic capillary column was made by coupling two 100 m SLB-IL111 columns (100 m × 0.25 mm, 0.2 μm thickness, Supelco Inc., Bellefonte, PA, USA) with an Agilent Ultimate Union (Agilent Tech., Wilmington, DE).

Separations were achieved using an Agilent 7890 N gas chromatograph (Agilent Tech., Wilmington, DE, USA) equipped with a flame ionization detector. The temperature gradient was as follows: 170 °C for 50 min, then 6 °C/min to 185 °C and then maintained for 35 min. Hydrogen was used as carrier gas with the following flow program: 1.6 ml/min for 35 min, then increased 0.3 ml/min/min to 3.0 ml/min which was then maintained until the end of the separation. Total run time was 87.5 min, with 5 min post injection re-equilibration. The detector was maintained at 250 °C and the injection port at 300 °C. Detector constant make up gas plus column flow was set to 30 ml/min, hydrogen to 30 ml/min, and air to 400 ml/min. Injector split ratio was set to 1:100, which was changed to 1:50 (gas saver) 2 min after injection. A typical injection volume was 1 μl.

Silver ion HPLC fractionation of milk fat FAMEs was carried out with an Alliance 2695 separation module (Waters, Milford, USA) equipped with a Waters 2996 PDA detector and a Waters Fraction Collector II. A semi-preparative Chromspher 5 Lipids column (10 × 250 mm, 5 μm particle size, Varian Inc. Walnut Creek, CA) was operated at 5 ml/min with a gradient of acetonitrile (MeCN) saturated isooctane solution (A) and isooctane (B) as follows: 10% A from 0 to about 15 min, then immediately switched to 100% A that was maintained until the elution of all PUFAs. The re-equilibration time was 20 min. The switching from 10 to 100% A mobile phase composition at about 15 min and the timing of the collection windows shown in Fig. 1 were adjusted as needed to compensate for small changes occurring during separation. The sample was dissolved in the mobile phase at the concentration of approximatively 50 mg/ml, and a typical injection volume was 100 μl. The MeCN saturated solution in isooctane was prepared by adding an excess of MeCN (about 100 ml) to one gallon of isooctane, stirring the solution overnight, and leaving the two-phase system to equilibrate for one day.

3. Results

The chromatographic separations presented in this manuscript were verified to be reproducible by using two different 200 SLB-IL111 columns and by conducting replicate analyses over several days. The capillary columns were conditioned according to the manufacturer's recommendations and equilibrated for several hours prior to acquiring the separations shown in Figs. 2–7. The initial elution temperature plateau of 170 °C was selected based on the observation that increasing the temperature by 2 °C compared to the previous condition of 168 °C with the 100 m SLB-IL111 column [3] optimized the separation of 16:1, 18:1 and 18:2 FAMEs. The second temperature plateau of 185 °C was selected as the best compromise between the elution of all PUFAs and the total time of

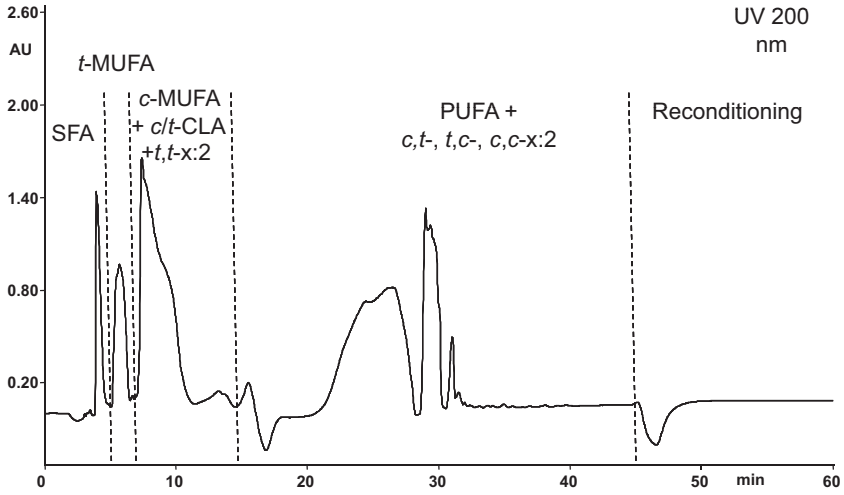

Fig. 1. Milk fat FAME Ag^{+}-HPLC separation used to collect the fractions subsequently analyzed by gas chromatography. One semipreparative Chromshper 5 lipid column (10 mm × 250 mm, 5 μm particle size, Varian Inc.), flow rate 5 ml/min., 10:90 saturated MeCN in isooctane solution/isooctane until about 15 min, then 100% MeCN saturated solution in isooctane. *X*:2 represents any FAME having 2 double bonds.

analysis. The flow rate was increased from 1.6 to 3.0 ml/min after the elution of the 18:2 FAMEs to reduce the total separation time and improve the peak shape of the late eluting FAMEs. The flow rate of 1.6 ml/min at 170 °C provided an average linear velocity of 26.1 cm/min, about the same value obtained using 1.0 ml/min of hydrogen carrier with a 100 m column of the same internal diameter. The initial elution required 50.03 psi of pressure on the injection port, increasing to 74.24 psi after the combined flow and temperature gradient. The sum of the column carrier and detector makeup gases was set constant at 30 ml/min.

Identifications were made by comparison with separations obtained using available pure reference materials, commercial reference mixtures, previously synthesized and characterized FAME mixtures, and published separations [3,11,18].

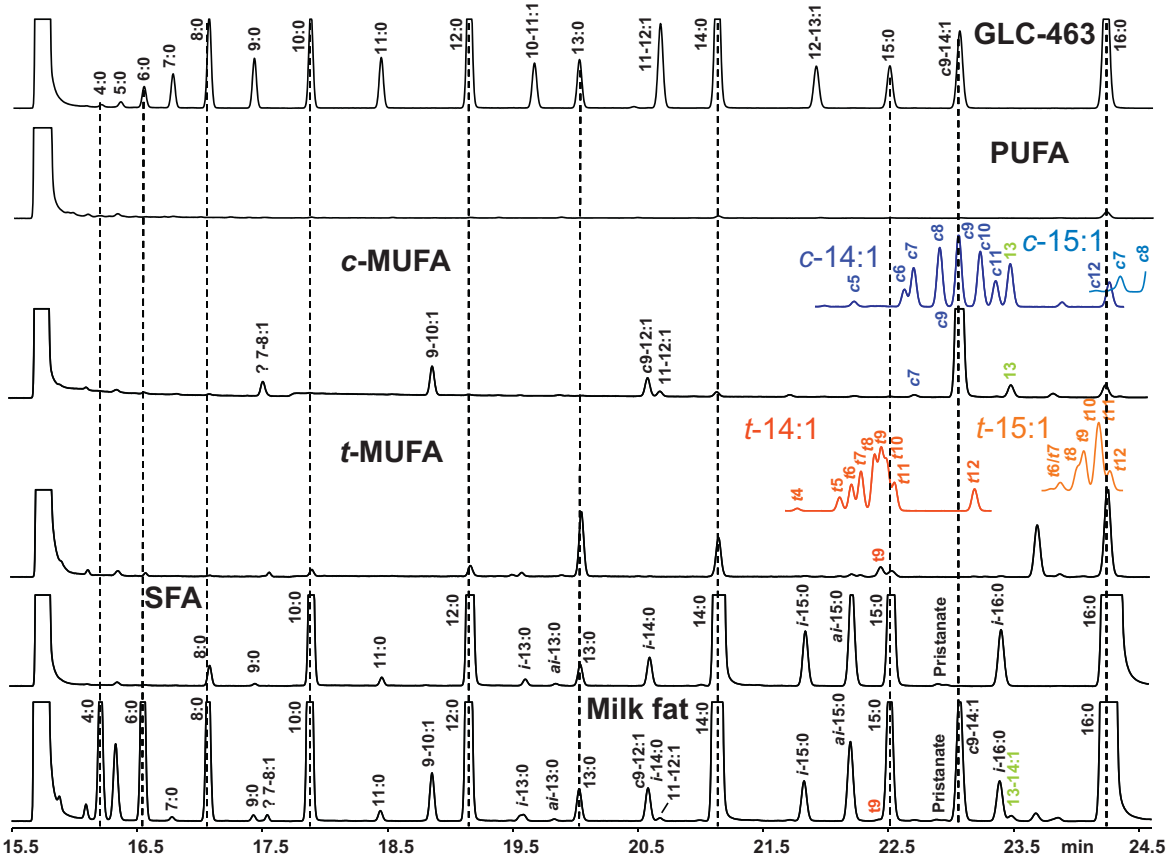

Fig. 2. Partial GC chromatogram of the 4:0 to 16:0 elution profile region. From the bottom: non fractionated milk fat FAMEs, milk fat fractions containing SFAs, *t*-MUFAs, *c*-MUFAs, PUFAs, and reference FAME mixture GLC 463, conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 mm, 0.2 μm thickness), temperature/flow gradients as described in Section 2. Colored insets are shown for the synthetic *cis* (blue) and *trans* (red) 14:1 and 15:1 positional isomer mixtures. FAMEs of the n-1 series are labeled in green.

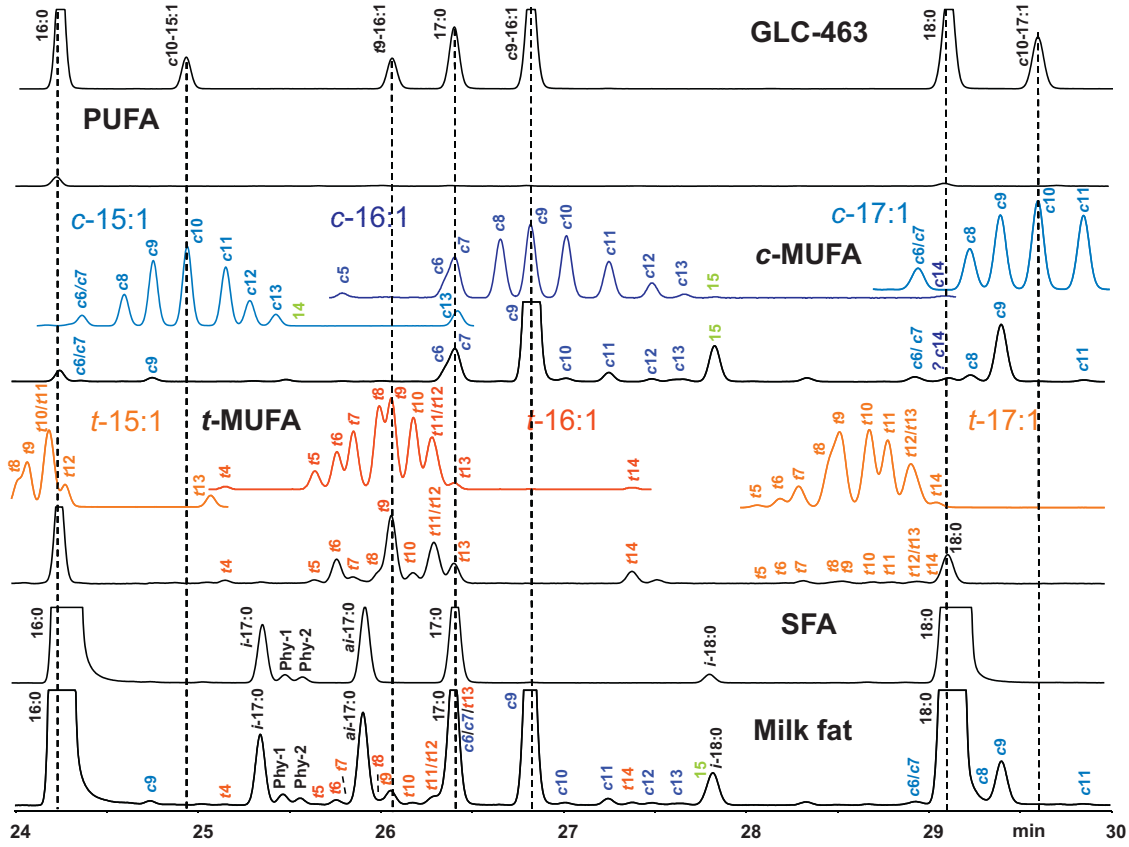

Fig. 3. Partial GC chromatogram of the 16:0 to 18:0 elution profile region. From the bottom: non fractionated milk fat FAMEs, milk fat fractions containing SFAs, *t*-MUFAs, *c*-MUFAs, PUFAs, and reference FAME mixture GLC 463, conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 mm, 0.2 μm thickness), temperature/flow gradients as described in Section 2. Abbreviations: *i*- = *iso*; *ai*- = *anteiso*; Phy-1 = methyl 3*S*,7*R*,11*R* phytanate; Phy-2 = methyl 3*R*,7*R*,11*R* phytanate. Colored insets are shown for the synthetic *cis* (blue) and *trans* (red) 15:1; 16:1 and 17:1 positional isomers mixtures. FAMEs of the n-1 series are labeled in green.

Fig. 1 shows the Ag^+-HPLC semi-preparative separation of the milk fat FAMEs. Consecutive separations showed reproducible retention times and the saturated MeCN in isooctane solution was able to elute all the PUFAs including DHA with minimum carryover of most unsaturated FAs to the following separation. If the carryover of PUFA is a problem, it can be reduced or eliminated by increasing the elution time of the MeCN saturated solution. Retention times were observed to be significantly shortened when the column was overloaded, which also led to a lack of resolution of geometric isomers of MUFAs. The fractionation of milk fat FAMEs by Ag^+-HPLC separated them into groups with a specific number of double bonds and geometric configuration, simplyfing their identification by GC. The first three fractions contained, respectively, saturated FAs (SFAs), *t*-MUFAs, and *c*-MUFAs plus *c*,*t*-/*t*,*c*-CLA and non-conjugated *t*,*t* dienoic FAMEs such as *t*9,*t*12-18:2. The fourth fraction consisted of the non-conjugated *c*,*t*/*t*,*c*/*c*,*c* dienoic FAMEs and all the FAMEs with more than 2 double bonds. The *t*,*t*-CLA eluted between the *t*-MUFA and the *c*-MUFA fractions, and could be monitored by UV at 233 nm. These FAMEs could then be collected partially or completely in fraction 2 or 3.

Figs. 2–6 show six GC chromatograms each. Starting from the bottom, every figure exhibits the GC separation of the unfractionated FAMEs prepared from the milk fat sample, the Ag^+-HPLC fractions in order of elution from the HPLC column (namely, SFA, *trans*-MUFA, *cis*-MUFA, and PUFA, respectively), and finally the GLC 463 reference mixture from Nu-Chek Prep at the top. Fig. 2 shows the separation of milk FAMEs with the shorter chain lengths from 4:0 up to 16:0. The 4:0 is clearly separated from the solvent peak and elutes slightly before toluene if the latter is added during the preparation of the sample. The branched-chain FAMEs coeluted with the saturated FAMEs and were identified based on the principle that even chain length FAs are present in milk only as the *iso* isomer, while odd chain length FAs are present as the *iso* and *anteiso* isomers. The predominant SFAs in milk fat were 12:0, 14:0 and 16:0 and they showed minor carryover into the subsequently eluting silver ion fractions. Their presence was helpful in verifying the correct alignment of the GC chromatograms. The separations of the synthetically isomerized 14:1 and 15:1 FAME standards (shown in the *cis* and *trans* fractions) were included as inserts to help identify the MUFAs and to provide a more complete FAME elution profile.

Fig. 3 shows the separation of the milk FAMEs from 16:0 and 18:0. The partial separations of the *cis*/*trans* 16:1 and 17:1 FAME synthetic mixtures were inserted to facilitate the identification of MUFAs contained in the milk sample. Methyl phytanate, present as two isomers (Phy-1 and Phy-2), was identified by comparison with the reference material. The three 17:0 FA isomers (*n*-, *iso*- and *anteiso*-) were partially separated from the most abundant *c*- and *t*-16:1 FAs. *Trans*-16:1 FA isomers were well resolved from each other with the exception of the *t*8/*t*9 and *t*11/*t*12 pairs, while the overlap of the *t*-17:1 FAs was limited to the *t*8/*t*9 and the *t*12/*t*13 isomer pairs. Of special interest was the 15–16:1 isomer in the *cis*-MUFA fraction which happened to coelute with *iso*-18:1 at 170 °C.

Fig. 4 shows the separation of the FAMEs from 18:0 to linoleic acid (LA; *c*9,*c*12-18:2). The separations of the synthetic *cis*/*trans* 18:1 and 19:1 FAME mixtures, and of LA isomerized with *p*-toluene sulfinic acid (PTSA) [11] are also included. Milk fat contained all possible positional and geometric isomers of 18:1 FAME and there was minimal overlap except for *c*5- with *t*6-, *c*6/*c*7- with *t*12-, and *c*8- with *t*15-18:1. The *t*16-18:1 FAME was instead clearly separated from the *c*13- and *c*14-18:1. *Trans* 11–18:1 is clearly separated from

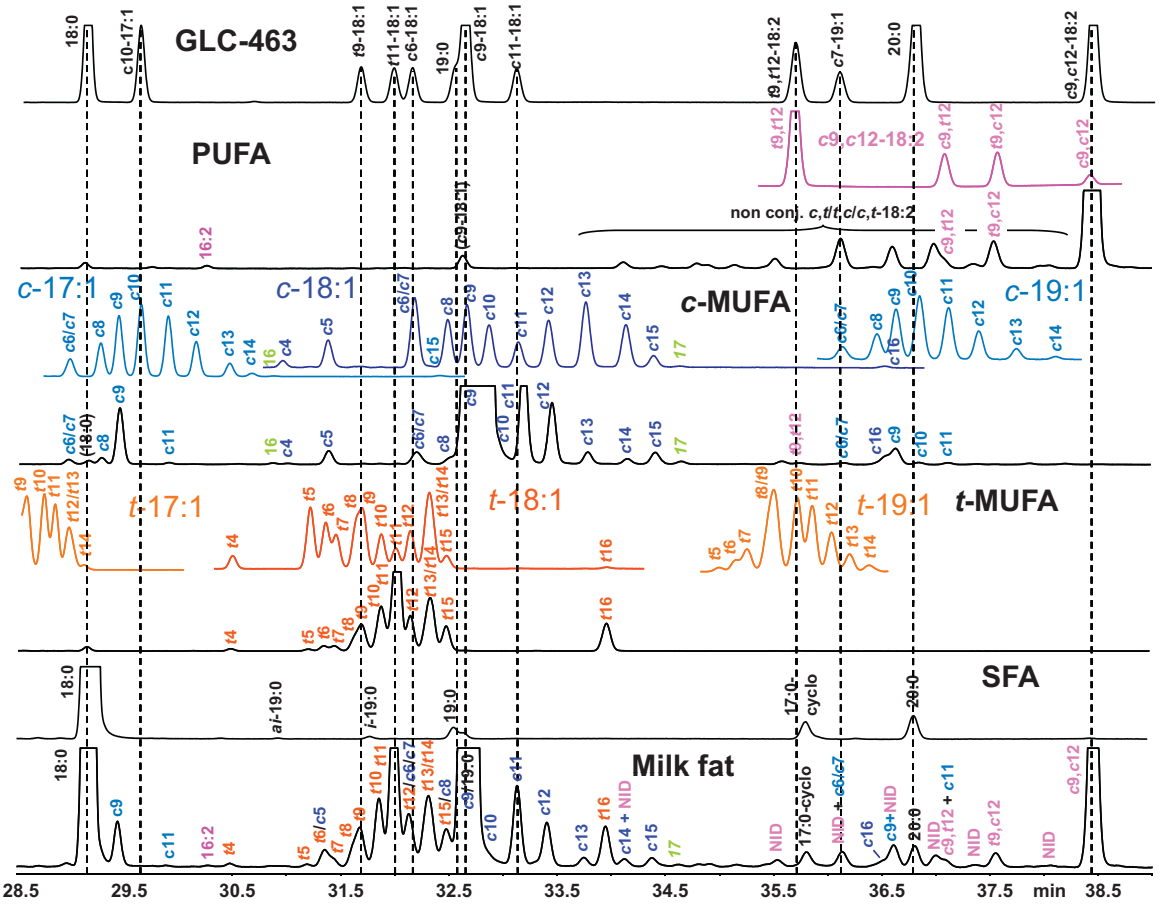

Fig. 4. Partial GC chromatogram of the 18:0 to *c*9,*c*12-18:2 elution profile region. From the bottom: non fractionated milk fat FAMEs, milk fat fractions containing SFAs, *t*-MUFAs, *c*-MUFAs, PUFAs, and reference FAME mixture GLC 463, conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 mm, 0.2 μm thickness), temperature/flow gradients as described in Section 2. 17:0 cyclo, methyl 11-cyclohexylundecanoate. NID, methylene interrupted dienes that were not identified. Colored insets are shown for the synthetic *cis* (blue) and *trans* (red) 17:1, 18:1 and 19:1 positional isomer mixtures, and isomerized linoleic acid (*c*9,*c*12-18:2, pink). FAMEs of the n-1 series are labeled in green.

*t*10- and *t*12-18:1, which allows the independent quantification of these *t*-18:1 isomers. The predominant *c*9-18:1 isomer (oleic acid) completely masked the identification of the minor 19:0 FA peak that was seen to elute slightly ahead of *c*9-18:1 in the reference standard GLC-463, but *t*15-18:1 was well separated from *c*9-18:1. On the other hand, 20:0, being a minor FAME in milk fat showed little interference with other FAMEs eluting in the same region. Several non-conjugated *c*,*t*- *t*,*c*-18:2 FAs were separated in fraction 4, but no attempt was made to identify them in this study. The cyclo-17:0 FA was tentatively identified based on the fact that it eluted by Ag^+-HPLC with the saturated FAMEs, and on previous observations [18,32].

Fig. 5 shows the separation of FAMEs from LA to 20:3n-6. The separations of selected *c*-/*t*-20:1 FAMEs with double bonds in positions 4 to 12, α- and γ-linolenic acid (LnA) isomerized with PTSA and of the mixture of CLA isomers obtained after iodine isomerization of the reference mixture (Nu-Chek Prep, Inc.) are also included. Of the SFAs in this region, 21:0 is only partially separated from *c*11-20:1, and 23:0 will interfere with the *t*,*t*-CLA isomers, but 22:0 does not interfere with any other FAME. Several *t*-20:1 and *c*-20:1 isomers were observed to be present in milk fat at levels above the limit of detection after fractionation and concentration, of which *c*9- and *c*11-20:1 were the predominant components. The two most abundant CLA isomers, *t*7,*c*9-CLA and *c*9,*t*11-CLA were clearly resolved, and no other FAME was observed to interfere with the quantitation of *t*7,*c*9-CLA. However, there was only a partial separation of *c*9,*t*11-CLA and *c*11,*c*14-20:2, which could be improved by lowering the elution temperature to 168 °C. The other minor *c*/*t*-CLA isomers identified in this milk sample included *t*9,*c*11-, *t*10,*c*12- and *t*11,*c*13-CLA, all of which were well separated, and did not interfere with other FAMEs. The *t*,*t*-CLA eluted in both the *t*-MUFA and the *c*-MUFA HPLC fractions, but the individual isomers could not be definitively identified. All the major PUFAs in the PUFA fraction were separated from other FAMEs and could be identified.

Fig. 6 shows the separation of the long-chain FAMEs, from 20:3n-6 to 22:6n-3. The 22:6n-3, which was considered the last eluting FAME of the milk sample, eluted in about 80 min. Identifications were based on comparison with the available reference materials. Two long-chain MUFAs eluting in the *c*-MUFA fraction, 23:1 and 24:1, were identified in milk fat. Their identities were confirmed by overlaying their chromatographic profile with those of the reference mixtures prepared by repeated bromination and debromination of *c*14-23:1 and *c*15-24:1 FAME according to previously published procedures [11]. Two unknown peaks were observed in this region of the chromatogram. The unknown GC peak at 65.5 min in total milk fat also eluted in the *t*-MUFA fraction. The GC peak at 79.0 min that occurred in several Ag^+-HPLC fractions was considered an artifact, since it was not present in total milk fat.

Fig. 7 shows the separation of the FAMEs from 18:0 to LA prepared from a beef fat, a partially hydrogenated canola oil, and a shortening sample. In these test samples all the *t*-18:1 FAMEs were resolved, with the exception of the two pairs *t*8/*t*9 and *t*13/*t*14. In addition, there were overlaps of some minor 18:1 isomers, *c*5- with *t*6-, *c*6/*c*7- with *t*12-, and *c*8- with *t*15-18:1. *Trans* 10–18:1

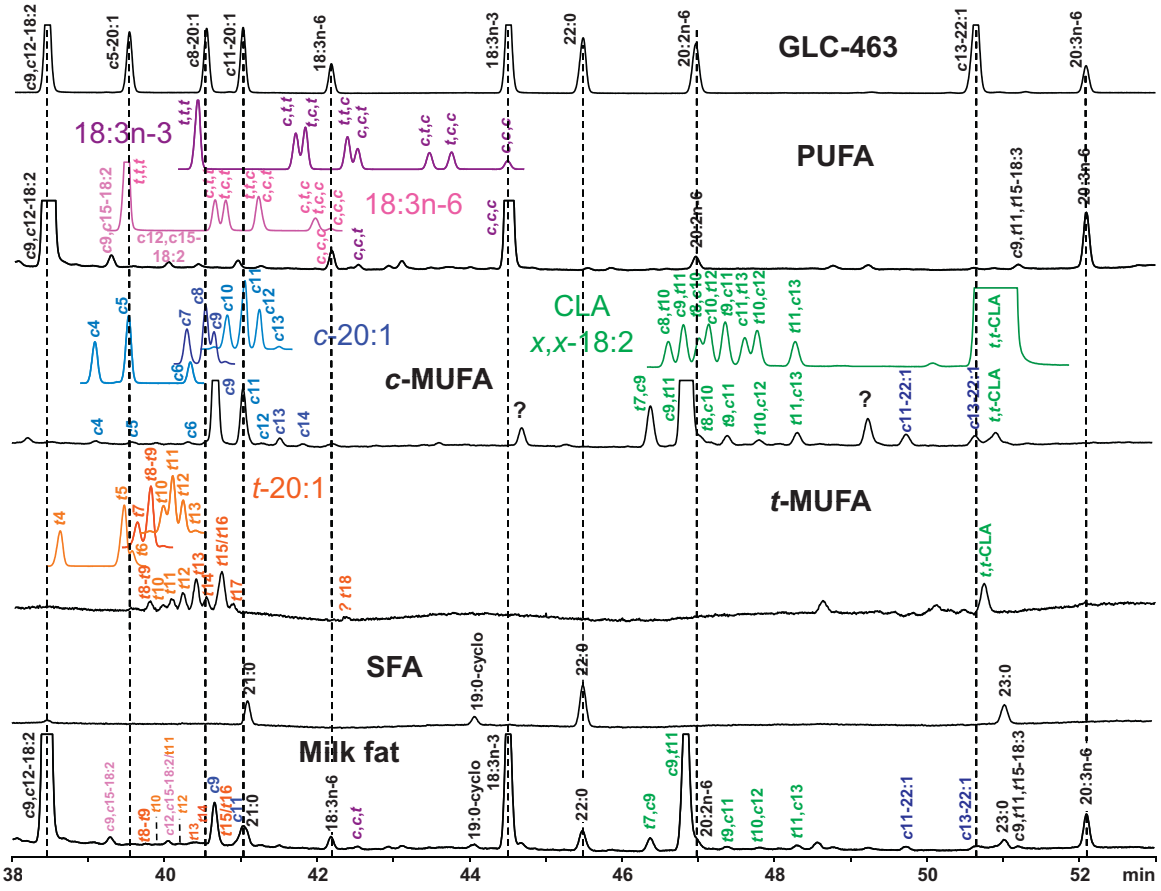

Fig. 5. Partial GC chromatogram of the *c*9,*c*12-18:2 to 20:3 elution profile region. From the bottom: non fractionated milk fat FAMEs, milk fat fractions containing SFAs, *t*-MUFAs, *c*-MUFAs, PUFAs, and reference FAME mixture GLC 463, conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 mm, 0.2 μm thickness), temperature/flow gradients as described in Section 2. CLA = conjugated linoleic acid. Colored insets are shown for the synthetic *cis* (blue) and *trans* (red) 20:1 positional isomer mixtures, isomerized CLA (green), γ-linolenic acid (18:3n-6, pink), and α-linolenic acid (18:3n-3, violet).

was the major *t*-18:1 isomer in the beef fat sample. Partially hydrogenated canola oil contained small amounts of *c*9,*t*12- and *t*9,*c*12-18:2, while beef fat contained other *c*/*t*-18:2 FAMEs that were not identified. In these separations, 19:0 coeluted with *c*9-18:1, while *c*10-18:1 was masked in the beef sample because of the predominant *c*9-18:1 peak.

4. Discussion

We elected to use a 200 m instead of a 100 m SLB-IL111 capillary column because the latter column provided only a partial separation of the individual *t*-18:1 isomers from *t*5- to *t*15-18:1, with the partial overlap of a few *c*-18:1 isomers [3]. Relative to the 100 m, the 200 m SLB-IL111 would provide twice the number of theoretical plates, and the gain in resolution is proportional to the square route of the gain in the total number of theoretical plates. It is well recognized that the elution temperature affects the selectivity of CPS columns, affecting the relative elution of FAMEs with a different number of double bonds and geometric configuration [18,21,33–35]. The selectivity of the ionic liquid columns was shown to be similarly affected by temperature [3,36]. Therefore, to evaluate these separations, we analyzed total milk fat, the milk fat fractions obtained by silver ion HPLC, and the reference and synthetic standards at several elution temperatures in the range from 150 °C to 180 °C. However, in the present study, we report only the GC results observed at 170 °C. The current separations were compared with the ones we recently published using a 100 m SLB-IL111. Changing the initial elution temperature from 168 °C to 170 °C improved the resolution of several closely eluting FAME in the 18:1, 18:2/19:1, 20:1/18:3 and the CLA region, but not in all cases, which will be discussed in the appropriate sections below. The age of the GC column is also known to reduce the polarity of the stationary phase [23]. This was evident in a number of closely eluting FAMEs with different numbers of double bonds, such as 19:0 *vs.* *c*9-18:1, 22:0 *vs.* *t*7,*c*9-CLA, and 21:0 *vs.* *c*11-20:1. In each case, the retention times of the more unsaturated FAMEs progressively decreased with column age relative to those of the more saturated FAMEs. This can be partially corrected by increasing the elution temperature by 1–2 °C, which will provide an opposite effect.

The results of analyzing the FAMEs prepared from total milk fat using a 200 m SLB-IL111 capillary column show that despite the relatively high initial temperature of 170 °C at the beginning of the analysis, the 4:0 FAME was well resolved from the solvent peak and eluted slightly before toluene when the latter is included in the methylation procedure (Fig. 2). The separation of 4:0 from the solvent front and other solvents often used during sample preparation had been a concern, since similar high initial temperature conditions proved to be a challenge for the quantiation of 4:0 FAME using 100 m CPS columns [21–23]. For this reason, temperature programs were preferred to improve the resolution of the short-chain FAMEs in milk fats [20,21].

The higher polarity of the SLB-IL111 column compared to the SP-2560 (or CP-Sil 88) column provided an advantage in separating the shorter-chain SFA from the n-1 MUFA present in milk fats, while these FAME pairs in reference mixture GLC 463 coeluted using the

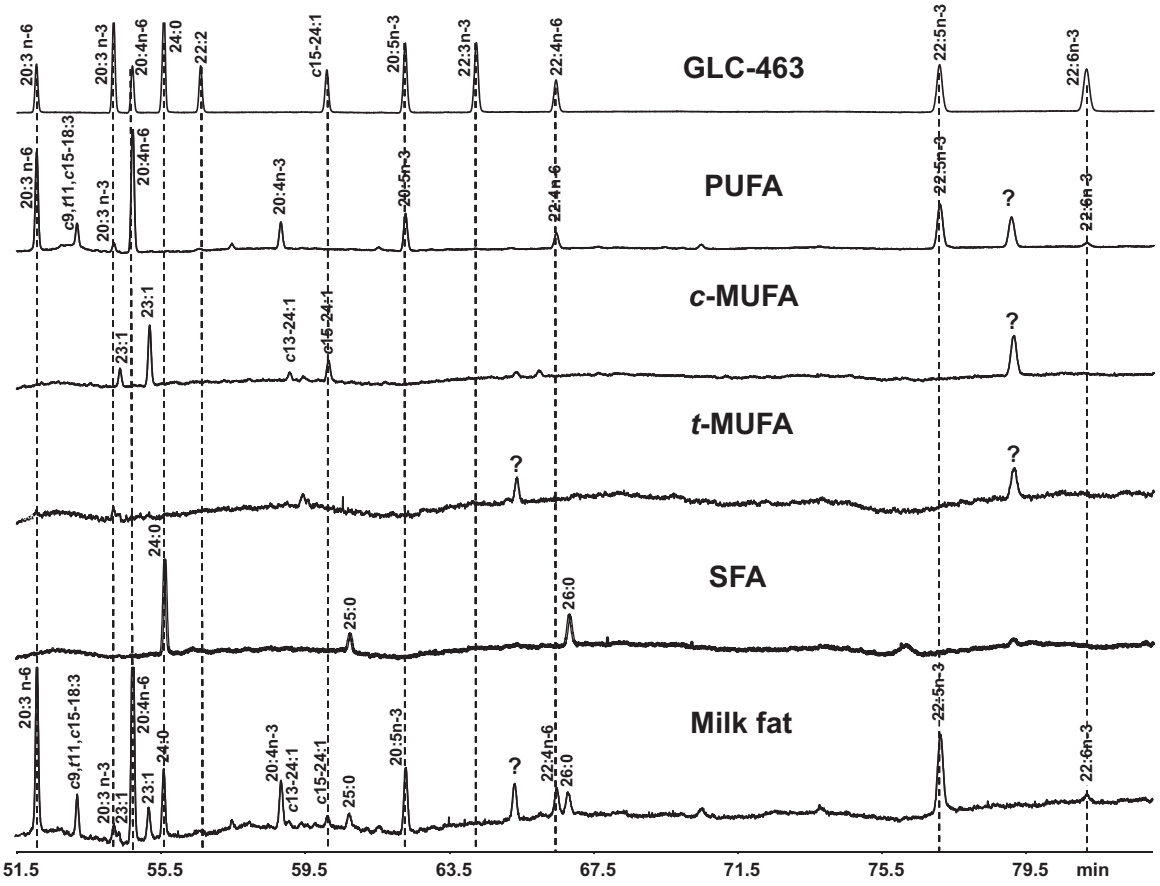

Fig. 6. Partial GC chromatogram of the 20:3 to 22:6 elution profile region. From the bottom: non fractionated milk fat FAMEs, milk fat fractions containing SFAs, *t*-MUFAs, *c*-MUFAs, PUFAs, and reference FAME mixture GLC 463, conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 mm, 0.2 μm thickness), temperature/flow gradients as described in Section 2.

100 m CP-Sil 88 columns, *i.e.*, 10–11:1 and 12:0, 11–12:1 and 13:0, 12–13:1 and 14:0 [37,38]. It was of special interest to confirm the presence of previously reported 9–10:1 and two 12:1 isomers in milk fats [39,40] using the 200 m SLB-IL111 column. The two 12:1 isomers were identified as *c*9-12:1 and 11–12:1 (Fig. 2, *c*-MUFA panel). This enabled us to demonstrate for the first time the presence of an entire series of even numbered n-1 MUFA from 9–10:1 to 17–18:1. In addition, milk fat was shown to contain the *c*7-, *c*9- and *t*9-14:1 FAs (Fig. 2). Trace amounts of methyl pristanate were also detected eluting just before *c*9-14:1, which was confirmed by using an authentic standard. Only a small amount of 13:0 FAME was present in milk fat, which would make this FA suitable for use as an internal standard (IS) for quantifying milk fat FAs.

The 16:1 region of milk fats (Fig. 3) has not been extensively investigated. The complexity of this region was only resolved by prior Ag^+-TLC separation of the total FAMEs of milk fat followed by isothermal GC analysis of the fractions at 120 °C [13,39]. However, no single GC separation condition including an isothermal separation at 120 °C [13,39], or by any temperature program, adequately resolved these isomers [11,18]. Partial success in analyzing the geometric and positional isomers of 16:1 in the presence of the branched-chain 17:0 FAs and *n*-17:0 was obtained by comparing the results from two different GC separations using the same 100 m CP Sil 88 column operated at two temperature settings [18]. The 100 m SLB-IL111 column operated at 168 °C provided a different separation pattern compared to that of the 100 m CPS columns, but several FAs still overlapped, *i.e.*, *anteiso*-17:0 with *t*8/*t*9-16:1, and *c*6/*c*7- with *t*11/*t*12/*t*13-16:1 [3]. The current method of analysis using a 200 m SLB-IL111 column operated at 170 °C showed an improved separation of the 17:0 (*iso*-, *anteiso*-, and *n*-) from the 16:1 isomers. *Anteiso*-17:0 coeluted only with two minor *t*-16:1 isomers (*t*7- and *t*8-16:1), and 17:0 eluted with the isomers *c*6/*c*7- and *t*13-16:1 (Fig. 3). The 16:1n-1 FAME, 15–16:1, was also identified for the first time in milk fat. This was confirmed using the synthetic *c*-16:1 mixture. Unfortunately, this FA partially overlapped with *iso*-18:0 at 170 °C (Fig. 3), but these two FAs could be clearly resolved by lowering the elution temperature to 168 °C with 15–16:1 eluting before *iso*-18:0 (unpublished data). In addition, two peaks associated with methyl phytanate (methyl 3,7,11,15-tetramethylhexadecanoate) were identified in the milk lipids that were confirmed using an authentic synthetic standard of methyl phytanate from Larodan. The two peaks were similar to the 3*S*,7*R*,11*R* and 3*R*,7*R*,11*R* isomers previously reported [40,41].

The separation of the FA isomers in the 18:1 region (Fig. 4) has been the subject of many investigations since they are common to both partially hydrogenated fats or oils, and ruminant fats. The 100 m CPS columns have failed to resolve all the 18:1 isomers whether the column was operated isothermally [11,26,42] or by using temperature programs [11,14,18,23,43]. The literature reports show that the 18:1 FAME isomers from *t*6- to *t*8- and from *c*6- to *c*8- could not be resolved under any set of chromatographic conditions. The *t*13- and *t*14-18:1 could instead only be resolved if the CPS GC column was operated at the temperature of about 120 °C or lower [13,20,29], the *t*15- and *c*9-18:1 generally coelute unless the eluton temperature plateau during the temperature program was set at 163 °C [18,23], and in most cases *t*16- coeluted with *c*14-18:1. Limited separations of some 18:1 isomers were reported using a 200 m CP-Select for FAME column (Varian Inc., Walnut Creek, CA), but not all the isomers were investigated [23,30,44]. On the other hand, the 100 m SLB-IL111 column operated at 168 °C

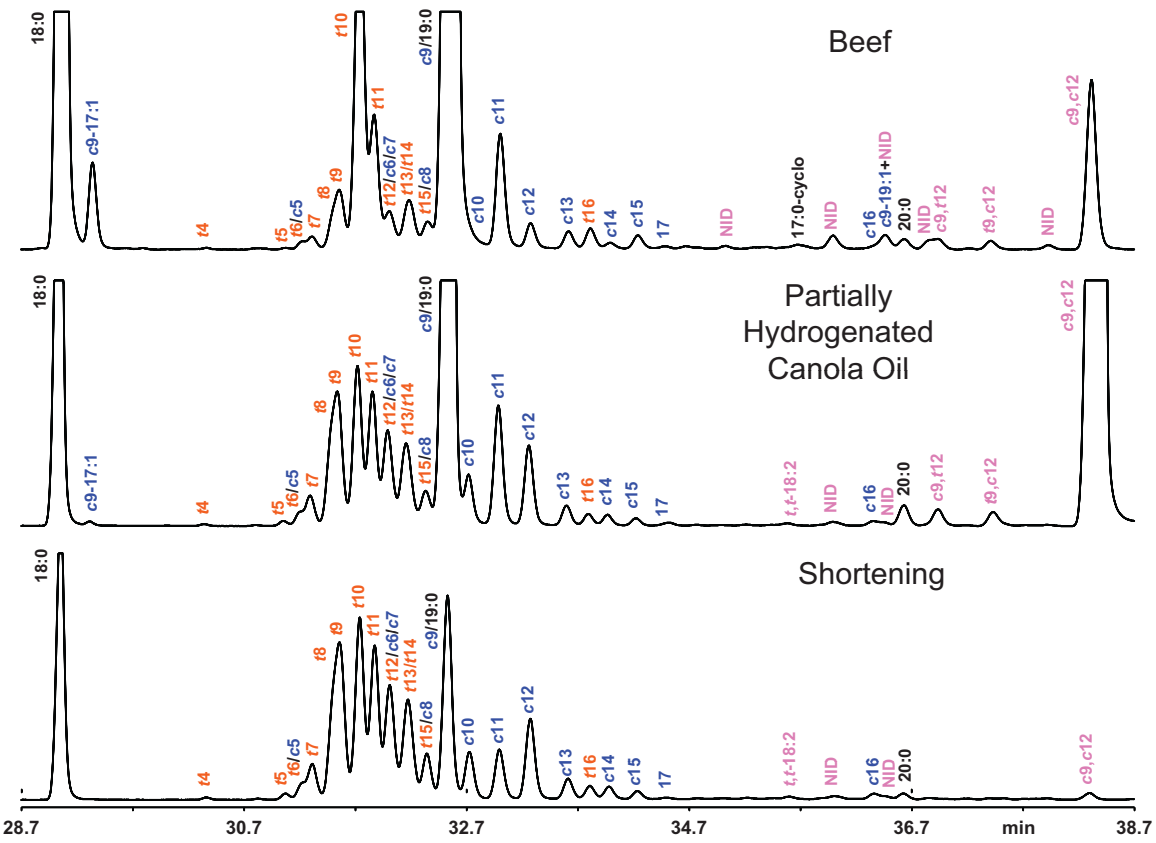

Fig. 7. Partial GC chromatogram of the 18:0 to *c*9,*c*12-18:2 elution profile region. From top: FAMEs prepared from a beef fat, a hydrogenated canola oil, and a shortening sample. Conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 mm, 0.2 μm thickness), temperature/flow gradients as described in Section 2. 17:0 cyclo = methyl 11-cyclohexylundecanoate.

provided for the first time the separation of *t*15-18:1 from *c*9-18:1, *t*6-/*t*7- from *t*8-18:1, and *c*6-/*c*7- from *c*8-18:1, but *t*8- still coeluted with *t*9-18:1, *c*6-/*c*7- with *t*12-18:1, and *t*13- still remained unresolved from *t*14-18:1, and 19:0 overlapped with *c*9-18:1.

The results of the 200 m SLB-IL111 column operated at 170 °C showed a marked improvement in the separation of all the *t*-18:1 isomers from *t*5- to *t*16-18:1, particularly the *t*10- and *t*11-18:1 isomers that often occur as the two major isomers in milk fats. However, two minor *c*-18:1 isomers (*c*6-/*c*7-18:1) still coeluted with *t*12-18:1, *c*8- coeluted with *t*15-18:1, and *t*13- still remained unresolved from *t*14-18:1. There was a good separation of *t*16-18:1 and *c*14-18:1, however, 19:0 remained a problem because it eluted just ahead of the large *c*9-18:1 peak, while *c*10-18:1 eluted at the tail end of the *c*9-18:1 peak. Attempts to further resolve all the 18:1 isomers by changing the elution temperature using the 200 m SLB-IL111 column may not be successful, which is a reflection of the complexity of FAs of milk fats. A reduction in the elution temperature progressively improved the separation among the positional isomers of *t*- or *c*-MUFAs, but it also progressively increased the overlap between the *t*- and *c*-MUFA clusters, while an increase in elution temperature yielded the opposite result. Typical separations of the 18:1 region of commonly analyzed test samples containing *trans* fatty acids are shown in Fig. 7 and will be discussed later.

The region between the 18:1 isomers and linoleic acid showed several peaks consisting of methylene- and non-methylene interrupted 18:2, and 19:1 isomers when analyzed using the 200 m SLB-IL111 column (Fig. 4). The FAs 20:0 and cyclo-17:0 (methyl 11-cyclohexylundecanoate) were well resolved under these conditions, largely because these and adjacent FAMEs eluting in the same region were similar in relative concentration. The 100 m CPS coated columns also showed a number of peaks in this region that were tentatively identified [32]. However, it would appear that the FAMEs of milk fats are more complex than first anticipated, since analyses using different GC temperature conditions show different FA profiles [18,32]. Studies are currently in progress to determine the structure of these 18:2 isomers by GC/MS and to compare the separations on both the SLB-IL111 and the SP-2560 columns.

The geometric isomers of α-LnA and γ-LnA eluted in the same chromatographic region as the *c*- and *t*-20:1 isomers using the 100 m SLB-IL111 column [3], very similar to that observed using the CPS columns [11,18]. However, there were differences that provided valuable complementary comparisons. For example, the major *c*-20:1 isomers in milk fat, *c*9- and *c*11-20:1, were better resolved from the major geometric isomers of α-LnA on the 100 m and 200 m SLB-IL111 columns [3; this study], than on the CPS columns, where these two 20:1 isomers generally coeluted with the *c*,*t*,*c* and *t*,*c*,*c* isomers of α-LnA [11,18]. Using the 200 m SLB-IL111 column, there was limited overlap of the *t*- and *c*-20:1 isomers, and the *t*-20:1 were free of interfering FAs, except for *c*12,*c*15-18:2, which coeluted with *t*11-20:1 (Fig. 5). The two major *c*-20:1 isomers elute in the vicinity of the *t*/*t*/*c*-18:3 isomers, but the more significant overlap was between *c*11-20:1 and 21:0 (Fig. 5). However, the analysis at 168 °C resulted in a clear separation of these two FAs with 21:0 eluting after *c*11-20:1 (data not shown). The use of 21:0 as internal standard (IS) was not recommended with CPS columns since 21:0 was found to elute anywhere between *t*9,*c*11- and *t*11,*c*13-CLA depending on the column used and its age [14,18,23,45]. It would appear that 21:0 is equally undesirable as IS when SLB-IL111 columns are used, since a relatively large addition of 21:0 would interfere with *c*11-20:1 and/or di *t*/*t*/*c*-18:3 isomers.

Using CPS capillary columns, all possible CLA isomers elute after α-LnA and before *c*11,*c*14-20:2 [14,17]. Only minor amounts of some 20:2 isomers and 21:0 were observed to elute in the CLA region [46]. Moreover, not all the CLA isomers were resolved using the CPS GC columns, particularly the two most abundant CLA isomers in ruminant fats, *c*9,*t*11- (rumenic acid) and *t*7,*c*9-CLA coeluted [16]. A complementary analysis for the CLA isomers using Ag^+-HPLC columns was required to resolve these two main CLA isomers as well as several other isomers [14,16]. It was therefore a real breakthrough when we found that the new 100 m SLB-IL111 column made it possible for the first time to baseline resolve these two major CLA isomers [3]. The 200 m SLB-IL111 column also separated most of the other CLA isomers, with the exception of a few *t*,*t*-CLA isomers, and three other FAs eluting in the CLA region should be closely monitored, *i.e.*, 22:0 (close eluting to *t*7,*c*9-CLA), *c*11,*c*14-20:2 (parlially overlapping with *c*9,*t*11-CLA), and *c*13-22:1 (partially overlapping with *t*8,*t*10-, *t*9,*t*11-CLA). If the main interest was to quantify the main CLA isomers, including *t*7,*c*9- and *c*9,*t*11-CLA, it can be achieved by direct analysis using this GC column. However, if minor CLA isomers are of interest, Ag^+-HPLC analysis may be preferred because of its lower limit of quantification and good resolution between most of the *t*,*t*-CLA isomers.

Milk fats contain minor amounts of n-6 and n-3 PUFA. All the long-chain FAs up to 26:0 and PUFAs up to 22:6n-3 separated within 80 min using the 200 m SLB-IL111 column and the chromatographic conditions described in Section 2 (Fig. 6). The separation could be shortened further by increasing the temperature of the second elution plateau. Alternatively, the flow of the hydrogen carrier gas could be further increased. Two 23:1 and 24:1 isomers were identified in the milk fat. The position of the double bonds in 23:1 could not be confirmed since standards are unavailable. The isomers of 24:1 were identified as *c*15-24:1 present in reference standard GLC-463 from Nu-Chek Prep Inc. and *c*13-24:1 that is also found in fish oil. The first isomerization product of α-LnA is *c*9,*t*11,*c*15-18:3 [47] and it eluted between 20:3n-6 and 20:3n-3 on this column (Fig. 6), which is much later than on the CPS columns where it elutes between 20:2n-6 and 22:0 [48]. In addition, *c*9,*t*11,*t*15-18:3, an isomer of *c*9,*t*11,*c*15-18:3 reported by Gómez-Cortés et al., was detected in milk fat [49] and it eluted just after 23:0 on the 200 m SLB-IL111 column (Fig. 5).

The question of whether a 200 m SLB-IL111 column could be used to analyze in a single GC separation total milk fat or a partially hydrogenated fat depends largely on how well the column is able to resolve most of the 16:1, 18:1, 18:2, 20:1 and CLA FAME isomers. To test this hypothesis, three typical test samples that differed in the amount and proportions of 18:1 and 18:2 isomers were selected. Fig. 7 (upper) shows the separation of a beef fat test sample in which the predominant *t*-18:1 isomer was *t*10-18:1 rather than *t*11-18:1. The other two test samples were two partially hydrogenated vegetable oils that had been hydrogenated to different extents. It is clearly evident that the resolution of most isomers improved when their relative abundance was similar, as in the sample of shortening (Fig. 7, lower). In this case, the relative concentration of *c*9-18:1 was not predominant and most 18:1 and 18:2 isomers were baseline resolved and could be identified by other techniques unless they coeluted. In fact, a small change in the elution temperature could be used to achieve specific separations if desired, such as that of 19:0 from *c*9-18:1. However, this was not the same with the other two test samples that contained predominant peaks. In each case, the separation of smaller adjacent FAMEs was either masked or resulted in a shoulder, which has remained the challenge in the analysis of ruminant fats.

5. Conclusions

Compared to the 100 m CPS columns recommendend for the analysis of milk fat FAMEs, the 200 m SLB-IL111 column provided an enhanced separation of many FAMEs and also provided alternative separation patterns that can be used to separate and quantify most FAs in complex lipid mixtures. Milk fat was choosen because it provided a matrix in which to evaluate the separation of most of the FAMEs found in foods, except fish or marine components. The selected experimental conditions provided a balanced separation within 80 min of all FAMEs found in ruminant fats from the short-chain FAs to PUFAs, while maximizing the separation of the 16:1, 18:1, 18:2, 20:1 and CLA FAME isomers. These conditions were shown to be applicable to the separation of the FAMEs contained in other *trans* fat containing fats and oils, excluding marine or fish oil products. Most *t*-18:1 were well separated from each other and from the *c*-18:1 isomers, including the *t*10-, *t*11- and *t*12-18:1 FA isomers. As shown in Fig. 7, this method can be applied to the separation of the 18:1 and 18:2 FAs commonly found in many fats and oils and it provides an enhanced separation of the *t*-18:1 FAMEs, thus allowing the independent quantification of both *t*10- and *t*11-18:1. which is of particular interest because not all the ruminant fats contain *t*11-18:1 as the main *trans* fat component (Fig. 7, upper panel) [27]. The 200 m SLB-IL111 column also provided a separation of all CLA isomers present in dairy products, including of the separation of *t*7,*c*9- from *c*9,*t*11-CLA. This eliminated the need for a complementary Ag^+-HPLC separation of the CLA isomers unless lower limits of quantification are needed to determine minor CLA components. The results of this study suggest that the 200 m SLB-IL111 column might be a preferred choice to analyze more completely all the FAs in ruminant products using a single GC analysis instead of the 100 m CPS column recommended by the official method Ce 1j-07 [22] for dairy products by the AOCS. Very few FAMEs remain unresolved, and several new FAs were separated and identified for the first time using this column. This is encouraging since most other procedures involving CPS columns require additional or prior separations to achieve similar results.

References

[1] Supelco Ionic Liquid GC Columns, Introduction to the Technology, 2010, Updated 09.08.10. Available from: http://sigma-aldrich.com/il-gc.
[2] C. Ragonese, P.Q. Tranchida, P. Dugo, G. Dugo, L.M. Sidisky, M.V. Robillard, L. Mondello, Anal. Chem. 81 (2009) 5561.
[3] P. Delmonte, A.-R. Fardin Kia, J.K.G. Kramer, M.M. Mossoba, L. Sidisky, J.I. Rader, J. Chromatogr. A 1218 (2011) 545.
[4] J.L. Anderson, J. Ding, T. Welton, D.W. Armstrong, J. Am. Chem. Soc. 124 (2002) 14247.
[5] J.L. Anderson, D.W. Armstrong, Anal. Chem. 77 (2005) 6453.
[6] T. Payagala, Y. Zhang, E. Wanigasekara, K. Huang, Z.S. Breitbach, P.S. Sharma, L.M. Sidisky, D.W. Armstrong, Anal. Chem. 81 (2009) 160.
[7] J.L. Anderson, D.W. Armstrong, Anal. Chem. 75 (2003) 4851.
[8] J.L. Anderson, R. Ding, A. Ellern, D.W. Armstrong, J. Am. Chem. Soc. 127 (2005) 593.
[9] D.W. Armstrong, L. He, Y.S. Liu, Anal. Chem. 71 (1999) 3873.
[10] Z.S. Breitbach, D.W. Armstrong, Anal. Bioanal. Chem. 390 (2008) 1605.
[11] P. Delmonte, A.R. Fardin Kia, Q. Hu, J.I. Rader, J. AOAC Int. 92 (2009) 1310.
[12] P. Delmonte, J.A. Roach, M.M. Mossoba, G. Losi, M.P. Yurawecz, Lipids 39 (2004) 185.
[13] D. Precht, J. Molketin, Eur. J. Lipid Sci. Technol. 102 (2000) 102.
[14] C. Cruz-Hernandez, Z. Deng, J. Zhou, A.R. Hill, M.P. Yurawecz, P. Delmonte, M.M. Mossoba, M.E.R. Dugan, J.K.G. Kramer, J. AOAC Int. 87 (2004) 545.
[15] P. Delmonte, J.K.G. Kramer, M.P. Yurawecz, in: M.M. Mossoba, J.K.G. Kramer, J.T. Brenna, R.E. McDonald (Eds.), Lipid Analysis and Lipodomics: New Techniques and Applications, AOCS Press, Champaign, IL, 2006, p. 191.
[16] M.P. Yurawecz, J.A.G. Roach, N. Sehat, M.M. Mossoba, J.K.G. Kramer, J. Fritsche, H. Steinhart, Y. Ku, Lipids 33 (1998) 803.
[17] N. Sehat, R. Rickert, M.M. Mossoba, J.K.G. Kramer, M.P. Yurawecz, J.A.G. Roach, R.O. Adlof, K.M. Morehouse, J. Fritsche, K. Eulitz, H. Steinhart, Y. Ku, Lipids 34 (1999) 407.
[18] J.K.G. Kramer, M. Hernandez, C. Cruz-Hernandez, J. Kraft, M.E.R. Dugan, Lipids 43 (2008) 259.
[19] R.G. Jensen, J. Dairy Sci. 85 (2002) 295.

[20] J.K.G. Kramer, C. Cruz-Hernandez, J. Zhou, Eur. J. Lipid Sci. Technol. 103 (2001) 600.
[21] M.M. Mossoba, J. Moss, J.K.G. Kramer, J. AOAC Int. 92 (2009) 1284.
[22] AOCS Official Method Ce 1j-07, Determination of *cis*-, *trans*-, Saturated, Monounsaturated, and Polyunsaturated Fatty Acids in Dairy and Ruminant Fats by Capillary GLC, AOCS, Urbana, IL, 2007.
[23] M.M. Mossoba, J.K.G. Kramer, Official Methods for the Determination of Trans Fat, 2nd Ed., AOCS Press, Urbana, IL, 2009.
[24] AOCS Official Method Ce 1h-05, Determination of cis-, trans-, Saturated, Monounsaturated and Polyunsaturated Fatty Acids in Vegetable or Non-ruminant Animal Oils and Fats by Capillary GLC, 2005.
[25] W.M.N. Ratnayake, L.J. Plouffe, E. Pasquier, C. Gagnon, J. AOAC Int. 85 (2002) 1112.
[26] W.M.N. Ratnayake, S.L. Hansen, M.P. Kennedy, J. Am. Oil Chem. Soc. 83 (2006) 475.
[27] N. Aldai, M.E.R. Dugan, D.C. Roland, J.K.G. Kramer, Can. J. Anim. Sci. 89 (2009) 315.
[28] C.J. Field, H.H. Blewett, S. Proctor, D. Vine, Appl. Physiol. Nutr. Metab. 34 (2009) 979.
[29] D. Precht, J. Molketin, Nahrung 43 (1999) 233.
[30] P.A. Golay, F. Giuffrida, F. Dionisi, F. Destaillats, J. AOAC Int. 92 (2009) 1301.
[31] P. Delmonte, Q. Hu, A.R. Fardin Kia, J.I. Rader, J. Chromatogr. A 1214 (2008) 30.
[32] D. Precht, J. Molkentin, Milchwissenschaft 58 (2003) 30.
[33] G. Castello, S. Vezzani, G. D'Amato, J. Chromatogr. A 779 (1997) 275.
[34] S.A. Mjøs, J. Chromatogr. A 1015 (2003) 151.
[35] L.M. Sidisky, H.J. Ridley, J. High Resolut. Chrom. 3 (1991) 191.
[36] K. Huang, X. Han, X. Zhang, D.W. Armstrong, Anal. Bioanal. Chem. 389 (2007) 2265.
[37] J.K.G. Kramer, C.B. Blackadar, J. Zhou, Lipids 37 (2002) 823.
[38] S.F. Herb, P. Magidman, F.E. Luddy, R.W. Riemenschneider, J. Am. Oil Chem. Soc. 39 (1962) 142.
[39] D. Precht, J. Molkentin, Int Dairy J. 6 (1996) 791.
[40] R.G. Ackman, R.P. Hansen, Lipids 2 (1967) 357.
[41] S. Ferdinandusse, H. Rusch, A.E.M. van Lint, G. Dacremont, R.J.A. Wanders, P. Vreken, J. Lipid Res. 43 (2002) 438.
[42] J. Molkentin, D. Precht, Chromatographia 41 (1995) 267.
[43] S.P. Alves, R.J.B. Bessa, J. Chromatogr. A 1216 (2009) 5139.
[44] K. Kunt, M. Baehr, C. Rohrer, G. Jahreis, Eur. J. Lipid Sci. Technol. 113 (2011) 1281.
[45] C. Cruz-Hernandez, J.K.G. Kramer, J. Kraft, V. Santercole, M. Or-Rashid, Z. Deng, M.E.R. Dugan, P. Delmonte, M.P. Yurawecz, in: M.P. Yurawecz, J.K.G. Kramer, O. Gudmundsen, O.M.W. Pariza, S. Banni (Eds.), Advances in Conjugated Linoleic Acid Research, Vol. 3, AOCS Press, Champaign, IL, 2006, p. 45.
[46] J.A.G. Roach, M.P. Yurawecz, J.K.G. Kramer, M.M. Mossoba, K. Eulitz, Y. Ku, Lipids 35 (2000) 797.
[47] C.G. Harfoot, G.P. Hazlewood, in: P.N. Hobson, C.S. Stewart (Eds.), The Rumen Microbial Ecosystem, Blackie Academic and Professional, London, UK, 1997, p. 382.
[48] P. Kairenius, V. Toivonen, K.J. Shingfield, Lipids 46 (2011) 587.
[49] P. Gómez-Cortés, C. Tyburczy, J.T. Brenna, M. Juárez, M.A. de la Fuente, J. Lipid Res. 50 (2009) 2412.