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Separation characteristics of fatty acid methyl esters using SLB-IL111, a new ionic liquid coated capillary gas chromatographic column

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ABSTRACT

The ionic liquid SLB-IL111 column, available from Supelco Inc., is a novel fused capillary gas chromatography (GC) column capable of providing enhanced separations of fatty acid methyl esters (FAMEs) compared to the highly polar cyanopropyl siloxane columns currently recommended for the separation of *cis*- and *trans* isomers of fatty acids (FAs), and marketed as SP-2560 and CP-Sil 88. The SLB-IL111 column was operated isothermal at 168 °C, with hydrogen as carrier gas at 1.0 mL/min, and the elution profile was characterized using authentic GC standards and synthetic mono-unsaturated fatty acids (MUFAs) and conjugated linoleic acid (CLA) isomers as test mixtures. The SLB-IL111 column provided an improved separation of *cis*- and *trans*-18:1 and *cis*/*trans* CLA isomers. This is the first direct GC separation of *c*9,*t*11- from *t*7,*c*9-CLA, and *t*15-18:1 from *c*9-18:1, both of which previously required complimentary techniques for their analysis using cyanopropyl siloxane columns. The SLB-IL111 column also provided partial resolution of *t*13/*t*14-18:1, *c*8- from *c*6/*c*7-18:1, and for several *t*,*t*-CLA isomer pairs. This column also provided elution profiles of the geometric and positional isomers of the 16:1, 20:1 and 18:3 FAMEs that were complementary to those obtained using the cyanopropyl siloxane columns. However, on the SLB-IL111 column the saturated FAs eluted between the *cis*- and *trans* MUFAs unlike cyanopropyl siloxane columns that gave a clear separation of most saturated FAs. These differences in elution pattern can be exploited to obtain a more complete analysis of complex lipid mixtures present in ruminant fats.

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1. Introduction

The fatty acid (FA) analysis of fats and oils as their fatty acid methyl esters (FAMEs) has progressed throughout the years largely in response to the development of more efficient gas chromatographic columns [1]. Currently, highly polar cyanopropyl siloxane coated stationary phases are the most effective columns for the separation of geometric and positional isomers of unsaturated FAMEs commonly encountered in partially hydrogenated vegetable oils (PHVOs) and ruminant fats [2–5]. It is for this reason that these columns were recommended to determine the total *trans* fatty acid (*t*-FA) content in foods and dietary supplements as mandated by the new *t*-FA regulations in many countries [6–8]. An official method was approved in 2005 by the American Oil Chemists' Society (AOCS) for the determination of *t*-FA isomers in vegetable or non-ruminant animal oils and fats that required the use of either the 100 m SP-2560 (from Supelco Inc.) or the CP-Sil 88 capillary column (from Varian Inc.) operating isothermally at 180 °C [9]. These conditions maximized the resolution of the 18:1, 18:2 and 18:3 isomers generally encountered in PHVOs and permitted the use of 21:0 as internal standard (IS) for quantitative purposes [1,10].

A number of alternative approaches have been reported to evaluate the 100 m cyanopropyl siloxane columns for the analysis of ruminant fats. In ruminant fats, the presence of short-chain FAs has necessitated the use of temperature programs starting from as low as 45 or 70 °C, and using 23:0 rather than 21:0 as IS, since the latter elutes within the conjugated linoleic acid (CLA) region [3,5,11]. A direct comparison showed an improved separation of the short-chain FAMEs using a temperature program, while use of

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an isothermal condition at 180 °C improved the resolution of the longer chain FAME isomers [12].

Prior silver-ion thin-layer chromatographic (Ag^+-TLC) separation of the geometric FAME isomers followed by GC separation at selected lower temperatures was an option [2,5]. However, that procedure was lengthy and only adequately addressed the separation of *trans*- and *cis*-monounsaturated fatty acid (MUFA) isomers and not the *trans* containing polyunsaturated fatty acids (PUFA). A method was recently proposed by which each test sample was consecutively analyzed twice using the same GC instrument and column but carried out using two different temperature programs [13]. This approach permitted the identification of most FAME isomers because temperature altered the polarity of the cyanopropyl siloxane phase [14] which in turn changed the relative elution of different types of FAMEs. This made possible the resolution of most isomers under either one of the two separation conditions [1,15]. Others evaluated two different types of GC columns, such as a 30 m 'Carbowax-type' column in combination with a 100 m cyanopropyl siloxane column. The former provided a separation of all FAMEs based on chain length and number of double bonds, while the latter yielded a more detailed composition of the 18:1 and 18:2 isomers [2,16,17].

However, despite the success of the 100 m highly polar cyanopropyl siloxane phase columns for the separation of *t*-FAs, there are common limitations pertaining specifically to the 16:1, 18:1, 18:2, 20:1/18:3, and CLA isomers regions. Some of these limitations were overcome by using a prior Ag^+-TLC technique to resolve *t*9-16:1 and iso-17:0 [18], or two temperature programs to resolve many of the *trans*- and *cis*-16:1, 18:1 and 20:1 isomers [13]. There were also several FA isomers that could only be resolved using much lower isothermal GC conditions at 120 °C, such as the two pairs *t*13- and *t*14-18:1 and *t*11- and *t*12-16:1 [13,18]. However, there are some overlapping FAMEs that cannot be resolved under any condition using the cyanopropyl siloxane phase columns, and are considered characteristic of this stationary phases' separation of FAs, such as *t*6-/*t*7-/*t*8-18:1, *c*6-/*c*7-/*c*8-18:1, and *t*7,*c*9- and *c*9,*t*11-18:2, just to name a few FAs. In addition, there are a few coeluting positional and geometric 18:2, 18:3 and 20:1 isomers present in PHVO and ruminant fats that have received little attention and for which only a few reliable standards are available [13,16,19].

In recent years several gas chromatographic capillary columns containing ionic liquid stationary phases of various polarities have been introduced on the market. Of those, the SLB-IL100 column has been successfully used for the separation of selected FAMEs of 18:1, 18:2 and 18:3 geometric and positional isomers [20]. Few details are available regarding the chemical structure of this stationary phase [21,22], and little is known about how the functional groups of this stationary phase interact with double bonds in FAMEs to provide improved/different selectivities, or if steric hindrances affect the elution properties. The ionic phases exhibit a dual nature retention selectivity that enables them to separate both polar and non-polar compounds [23]. Compared to cyanopropyl stationary phase capillary columns of equal length, ionic liquid coatings SLB-IL100 and SLB-IL111 have been reported to have higher polarity based on their McReynolds constants [22,23].

In this study, we present the separation of numerous FAME mixtures and authentic *cis* and *trans* MUFA isomer mixtures from 14:1 to 20:1 to evaluate the chromatographic properties of these FAMEs on the 100 m SLB-IL111 capillary column from Supelco Inc. (Bellefonte, PA). The elution profiles obtained analyzing the same isomeric mixtures with a 100 m SP-2560 capillary column operated isothermally at 180 °C or with a temperature program from 45 °C to 215 °C were reported by Delmonte et al. [12]. Based on the observation that polar stationary phases are temperature sensitive, the new ionic column was also evaluated for changes in the relative elution of FAMEs with variations in operating temperature of the column. Assuming that differences in the chemical composition of the stationary phases will affect the relative elution pattern of FAMEs differently, this study was also meant to investigate the possibility of whether the observed relative retention time data obtained with these two columns (SP-2560 and SLB-IL111) are complementary and would lead to a more complete and accurate identification of FA composition of ruminant fats than either column alone.

2. Materials and methods

Mixtures containing positional and geometric isomers of 14:1, 15:1, 16:1, 17:1, 18:1, 18:2 and 18:3 FAMEs were prepared and characterized as previously reported [12,24]. The CLA isomers from 6,8- to 13,15-18:2 were synthesized as previously reported [25]. Saturated FAMEs (containing branched chain FAMEs) were isolated from a milk fat sample as previously described [12]. GLC 463 reference mixture (for individual FAs in the mixture see p. 65 in the catalog, http://www.nu-chekprep.com/10_11_catalog.pdf), FAME 21:0 and 14-15:1 (U-38-MX), and a mixture containing conjugated linoleic acid isomers (CLA, UC-59-M) were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). The *cis* (Δ6, Δ7, Δ9, Δ11, Δ12, Δ13 and Δ15) and *trans* (Δ6, Δ7, Δ9, Δ11, Δ13 and Δ15) isomers of 18:1 were available from Sigma (St. Louis, MO, USA). The custom made SLB-IL111 gas chromatographic capillary column (100 m × 0.25 mm, 0.2 μm thickness, Supelco, Bellefonte, PA) was kindly provided by Len Sidisky of Supelco Inc.

Separations were achieved using an Agilent 6890N gas chromatograph (Agilent Tech., Wilmington, DE, USA) equipped with a flame ionization detector. Hydrogen was used as carrier gas at 1 mL/min constant flow with the linear velocity of 26 cm/s. The oven was maintained at 168 °C isothermal temperature, the injection port at 250 °C, and the detector at 250 °C. The split ratio was set to 1:100 and the typical injection volume was 1 μl.

3. Results

The chromatographic separations presented in this manuscript were all obtained using the same 100 m Supelco SLB-IL111 capillary column and were verified to be reproducible over several days. The isothermal temperature condition at 168 °C was selected because it provided the best compromise for the separation of 14:1 to 18:1, CLA, 20:1 and 18:3 FAME isomers commonly found in fats and oils. Evidences supporting this choice will be provided when the 18:1, 18:3 vs 20:1 and CLA regions are presented below. The CLA region was also investigated at the lower isothermal temperature of 130 °C to compare the separation of these FAME isomers. In this study, extensive use was made of authentic *cis* and *trans* MUFAs prepared by repeated bromination and debromination reactions of selected MUFAs, isomerized PUFA and CLA obtained by using either para-toluene sulfinic acid (PTSA) or iodine, and Ag^+-HPLC separation of the unsaturated FAME geometric isomers [12,24].

Fig. 1 shows the separation of the *cis* and *trans* FAMEs of 14:1 and 15:1 produced by the isomerization of *c*9-14:1 and *c*10-15:1, respectively, along with the separation of the saturated FAMEs isolated from milk fat that eluted in the same portion of the gas chromatogram. The separation of the GLC 463 mixture from Nu-Chek Prep, Inc. was added as a reference. For each FA chain length the saturated FA eluted first, followed by MUFAs with *trans* double bonds and then those with *cis* double bonds. The increased polarity of the ionic column generally resulted in the elution of saturated FAs in the transition area between the major *trans* and *cis* clusters of FAs with one carbon less. Regardless of chain length and geometric configuration, the positional isomers of MUFAs eluted in the order of increasing Δ values. The identification of the *cis* and

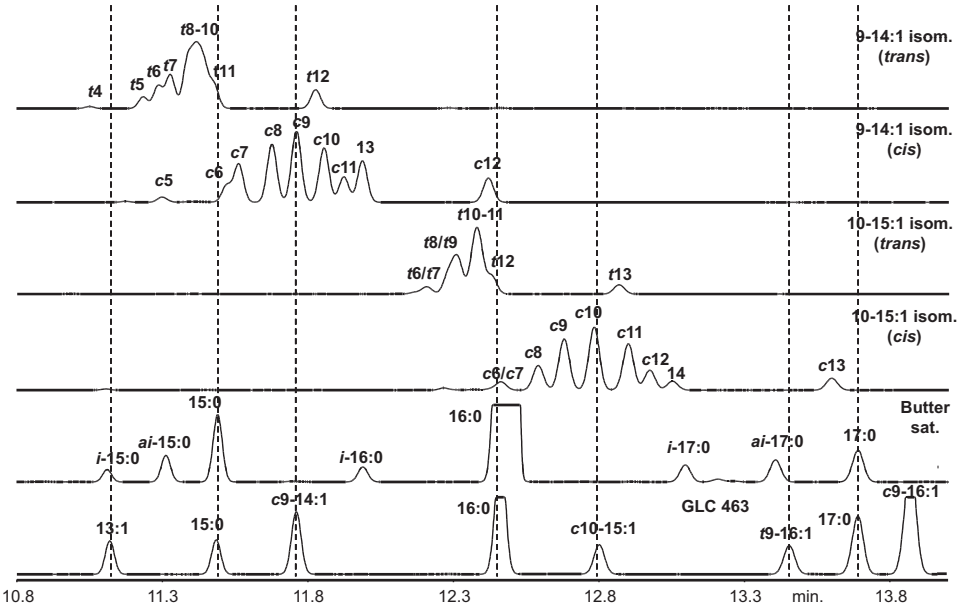

Fig. 1. Partial GC chromatogram of the 14:1 and 15:1 region. From top: *trans* and *cis* fractions obtained from *cis* 9-14:1 and *cis* 10-15:1 FAMEs after five successive brominations and debrominations, saturated FAMEs fractionated from milk fat (as FAMEs), and reference FAME mixture GLC 463. Conditions: Supelco 100 m SLB-IL111 capillary column (100 m × 0.25 mm, 0.2 μm thickness), hydrogen carrier gas at 1 mL/min and 26 cm/s linear velocity, 168 °C isothermal condition. Abbreviations: *i* – iso; *ai* – anteiso.

trans isomers of 14:1 and 15:1 was previously achieved by GC/MS [12], based on comparison to the elution order established using the commercially available *cis*- and *trans*-18:1 isomers from Sigma, and by comparison with published separations [10–13,15,18]. The terminal positional isomer was an exception and showed a reversal in the elution order, i.e., the FA with the terminal double bond ($n-1$) eluted close to the $n-3$ *cis* FA isomer. The terminal double bond position was synthesized after successive bromination and debromination reactions of selected MUFAs, and was found concentrated in the *cis* fraction that had been isolated by prior Ag^+-HPLC. Most of the individual *cis* isomers were baseline resolved, while most of the *trans* isomers were only partially separated. There was minimal overlap of the *cis* and *trans* isomers of 14:1 and 15:1, except for the last *trans* isomer and the minor low Δ-*cis* isomers from *c*4 to *c*7. The saturated FAME fraction from milk fat provided information as to the elution of the *iso* and *anteiso* branched-chain FA of 15:0 and 17:0 and iso-16:0 (Fig. 1).

Fig. 2 shows the separation of the *cis* and *trans* FAMEs produced by the isomerization of *c*9-16:1 and *c*10-17:1, along with the separation of the saturated FAMEs isolated from milk fat and GLC 463. Most of the observations noted above for the separation of 14:1 and 15:1 MUFAs equally apply to the separation of 16:1 and 17:1 MUFAs, with minor exceptions. Increased chain length improved the resolution of the individual *trans* isomers, but the overlap of the *cis* and *trans* isomers became more extensive. All the *trans*-16:1 positional isomers up to *t*11/*t*12-16:1 eluted before the *c*6/*c*7-16:1 FAs. In addition, 17:0 was only partially resolved from the *c*6/*c*7-16:1, *c*8-16-1 and *t*13-16:1, while *anteiso*-17:0 co-eluted with *trans*-16:1 FAs, but *iso*-17:0 eluted just before the major *trans*-16:1 isomers; the identification of the *iso*- and *anteiso*-branched-chain FAs was provided by the saturated FA fraction from milk fat. The MUFA isomers with the terminal double bond are tentatively identified in the *cis* fraction, since their relative abundance is very low.

Fig. 3 shows the separation of the *cis* and *trans* FAMEs produced by the isomerization of an equal mixture of *c*6-/c9-/*c*13-18:1, and of *c*10-19:1, along with the separation of isomerized *c*9,*c*12-18:2, the saturated FAMEs isolated from milk fat, and GLC 463. As previously noted, FAs with the same chain length and geometric configuration eluted in order of increasing Δ position, except for the FA with the double bond in terminal position that eluted just after *c*15 in the case of the 18:1 FAs. All the *trans*-18:1 FAs with double bond positions up to *t*15-18:1 eluted before *c*9-18:1, and *t*16-18:1 eluted between *c*13-18:1 and *c*14-18:1. Several common FAs in this region co-eluted under these conditions, among them 19:0 which co-eluted with *c*9-18:1, *t*9,*t*12-18:2 that co-eluted with *t*8/*t*9-19:1, and 20:0 which co-eluted with *c*9,*t*12-18:2. Linoleic acid (*c*9,*c*12-18:2) was well resolved from all known FAs occurring in fats and oils. The MUFA isomers with the terminal double bond were identified in the *cis* fractions, but their relative abundance was very low.

Differences in column temperature were investigated to maximize the resolution of as many FAMEs as possible. Fig. 4A and B shows the differences in separation achieved when the operating column temperature was lowered to 140 °C or raised to 180 °C, respectively. Even though the resolution of both the *cis* and *trans* 18:1 positional isomers was improved at 140 °C, there was more extensive overlap of the *cis* and *trans* clusters, and 19:0 co-eluted with *c*12-18:1 (Fig. 4A). On the other hand, operation at 180 °C resulted in a near separation of the entire *trans* from the *cis*-18:1 clusters, but this result was accompanied by a loss, particular of the individual *trans* 18:1 isomers, and a co-elution of 19:0 with *c*8-18:1 (Fig. 4B). The column temperature of 168 °C provided a reasonable resolution of all the *trans* 18:1 isomers, resulted in minimal overlap of the geometric isomers, and resulted in a clear identification of *t*15-18:1 before *c*9-18:1.

Fig. 5 shows the separation of the *cis* and *trans* FAMEs produced by a single step bromination and debromination of three selected 20:1 FAMEs, *c*5-, *c*8-, and *c*11-20:1, along with isomerized *c*9,*c*12,*c*15-18:3 (α-linolenic acid; α-LnA) and *c*6,*c*9,*c*12-18:3 (γ-linolenic acid; γ-LnA), and GLC 463; the latter was spiked with 21:0 FAME. The 20:1 and 21:0 FAME peak shapes were asymmetric due to their low solubility in the highly polar stationary phase. α-LnA and γ-LnA were separated from the most common 20:1 isomers and 21:0. Most of the geometric isomers of α-LnA and γ-LnA extensively overlapped with the *cis*- and *trans*-20:1 isomers, but at the isothermal condition of 168 °C reasonable separation was possible.

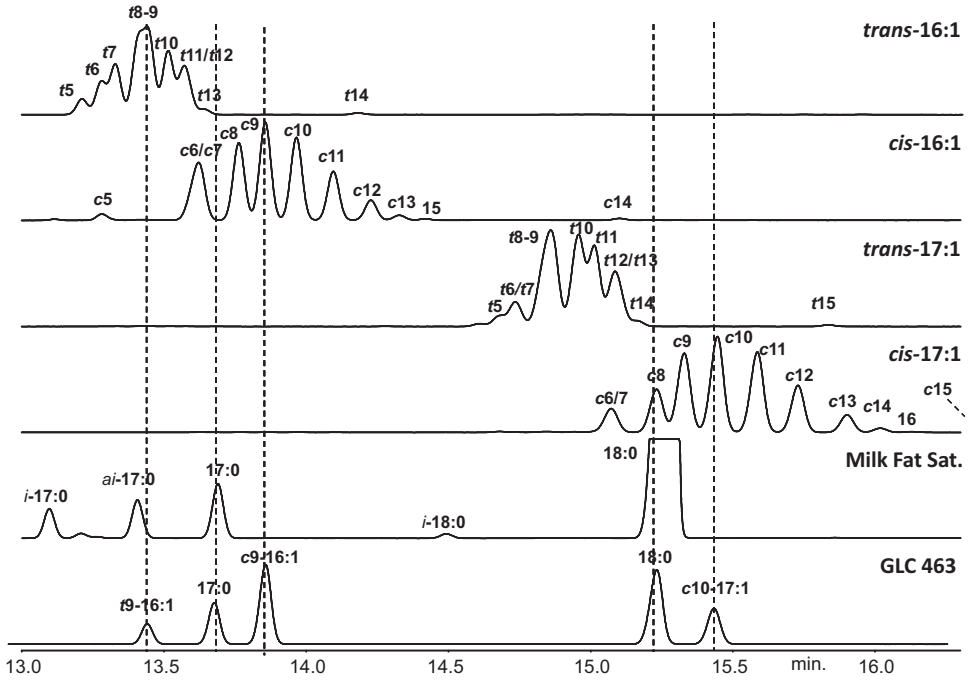

Fig. 2. Partial GC chromatogram of the 16:1 and 17:1 region. From top: *trans* and *cis* fractions obtained from *cis* 9-16:1 and *cis* 10-17:1 FAMEs after five successive brominations and debrominations, saturated FAMEs fractionated from milk fat (as FAMEs), and reference FAME mixture GLC 463. Conditions as in Fig. 1. Abbreviations: *i* – iso; *ai* – anteiso.

Fig. 6 shows the separation of the all synthetic geometric isomers of CLA from double bond positions 6,8- to 13,15-18:2, along with the isomerized mixture UC-59-M from Nu-Chek Prep, Inc., and GLC 463. The elution order of the geometric isomers of CLA with the same double bond position was *c*,*t* < *t*,*c* < *c*,*c* < *t*,*t*; no attempt was made to identify the minor *c*,*c*-CLA isomers. The retention times of the *c*/*t*-CLA isomers increased with increasing Δ values, sufficiently so as to resolve several of the major CLA isomers commonly found in ruminant fats. Of special interest was the separation of *t*7,*c*9-18:2 from *c*9,*t*11-18:2. The elution order among the *t*,*t*-CLA isomers was opposite to that observed with *c*/*t*-CLA, except for the *t*,*t*-CLA isomers of 6,8-, 7,9- and 12,14-CLA which did not fit the trend (Fig. 6). There was also a partial resolution of several *t*,*t*-CLA isomers when a lower isothermal temperature of 130 °C was selected; see below. At the elution temperature of 168 °C, several common FAs in this region co-eluted under these conditions, among them 22:0 with *c*6,*t*8- and *c*7,*t*9-CLA, *c*11,*c*13-20:2 with *t*8,*c*10- and *c*10,*t*12-CLA, and *c*13-22:1 with *t*9,*t*11- and *t*8,*t*10-CLA.

Fig. 7 shows a typical separation of the 18:1 region of an extensively hydrogenated PHVO product, together with its *cis*-

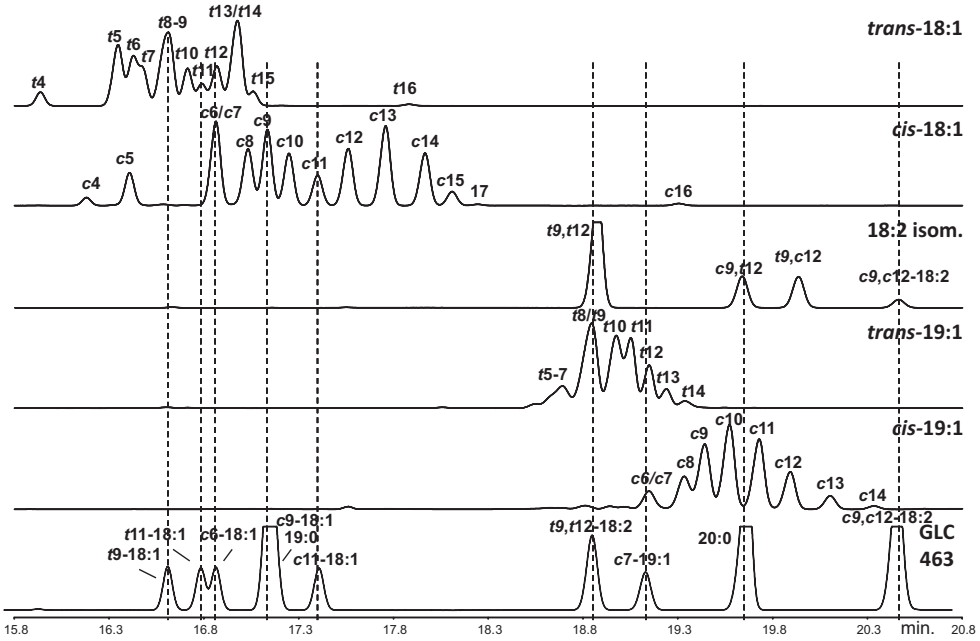

Fig. 3. Partial GC chromatogram of the 18:1, 19:1 and 18:2 region. From top: *trans* and *cis* fractions of 18:1 obtained from a mixture of *cis* 6-, *cis* 9- and *cis* 13-18:1 FAMEs after two successive brominations and debrominations, linoleic acid FAME isomerized by PTSA, *trans* and *cis* fractions of 19:1 obtained by fractionating *cis* 10-19:1 FAME after six reactions, and reference FAME mixture GLC 463. Conditions as in Fig. 1.

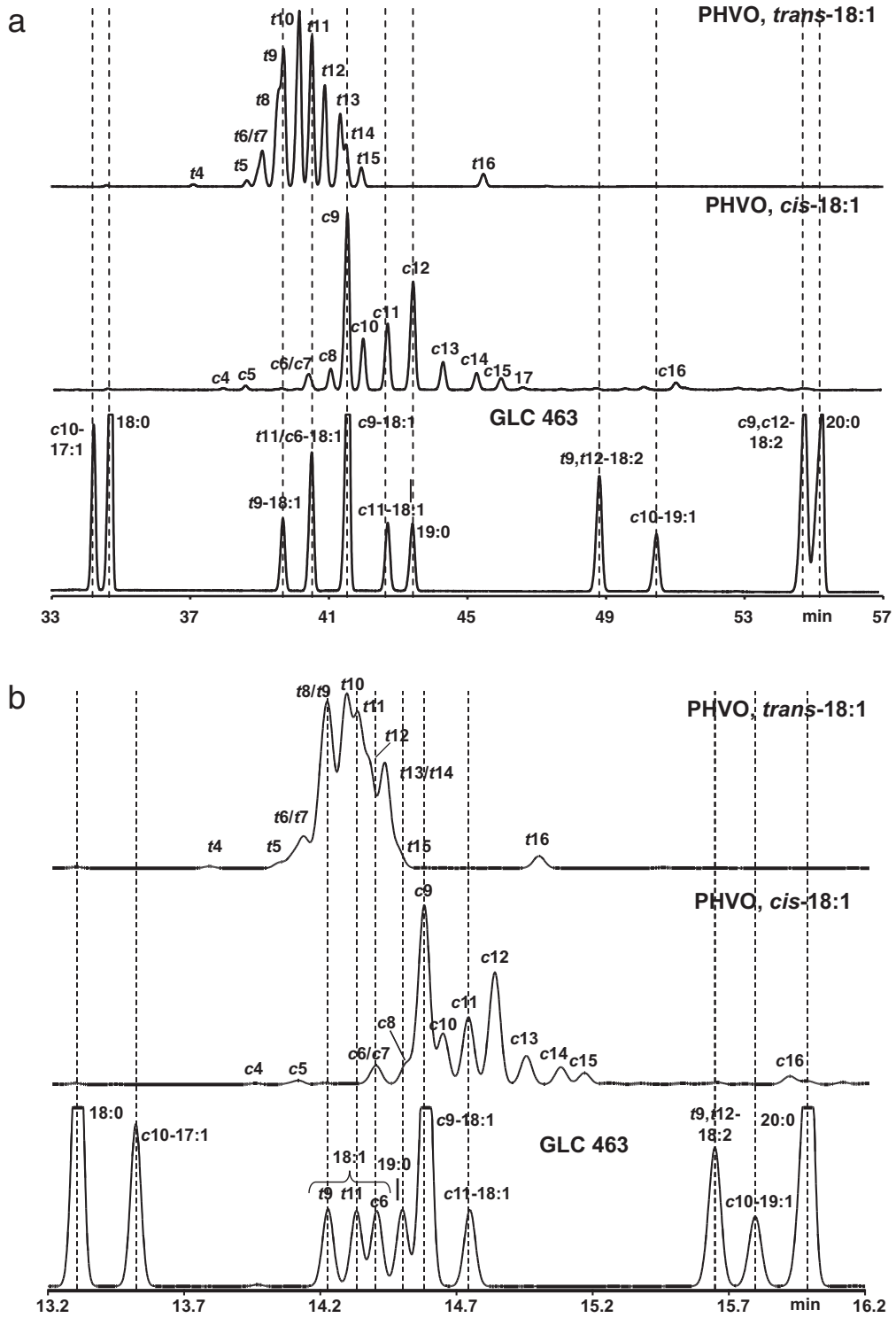

Fig. 4. (A) Partial GC chromatogram of the 18:0 to 20:0 region. From top: *trans* and *cis* fractions of partially hydrogenated vegetable oil (PHVO) and reference FAME mixture GLC 463. Conditions: Supelco 100 m SLB-IL111 capillary column (100 m × 0.25 mm, 0.2 μm thickness), hydrogen carrier gas at 1 mL/min and 26 cm/s linear velocity, 140 °C isothermal condition. (B) Partial GC chromatogram of the 18:0 to 20:0 region. From top: *trans* and *cis* fractions of partially hydrogenated vegetable oil (PHVO) and reference FAME mixture GLC 463. The same as in (A), except for the isothermal condition at 180 °C.

and *trans*-MUFA fractions obtained using Ag^+-HPLC. The relative abundance of all the 18:1 isomers in this unfractionated product was similar, which permitted a better resolution of closely eluting 18:1 isomers. Several isomers remained unresolved including *t*8/*t*9-18:1, *t*13/*t*14-18:1 and *c*6/*c*7-18:1. The overlap of *cis*-18:1 with *trans*-18:1 isomers was limited to *c*4- to *c*8-18:1, while *t*16:1-18:1 was well resolved, eluting between *c*13-18:1 and *c*14-18:1.

Fig. 8 shows the separation of FAMEs in the CLA region with the column operated isothermally at 130 °C. The chromatogram included a milk fat investigated in a previous study [26], our synthetic 7,9-CLA isomerized with iodine, and the CLA mixture (UC-59-M) obtained from Nu-Chek Prep,. Inc. isomerized with iodine. With the exception of a partial co-elution of *t*8,*c*10-18:2 and *c*10,*t*12-18:2, all the other c,*t*- and *t*,*c*-CLA isomers from double

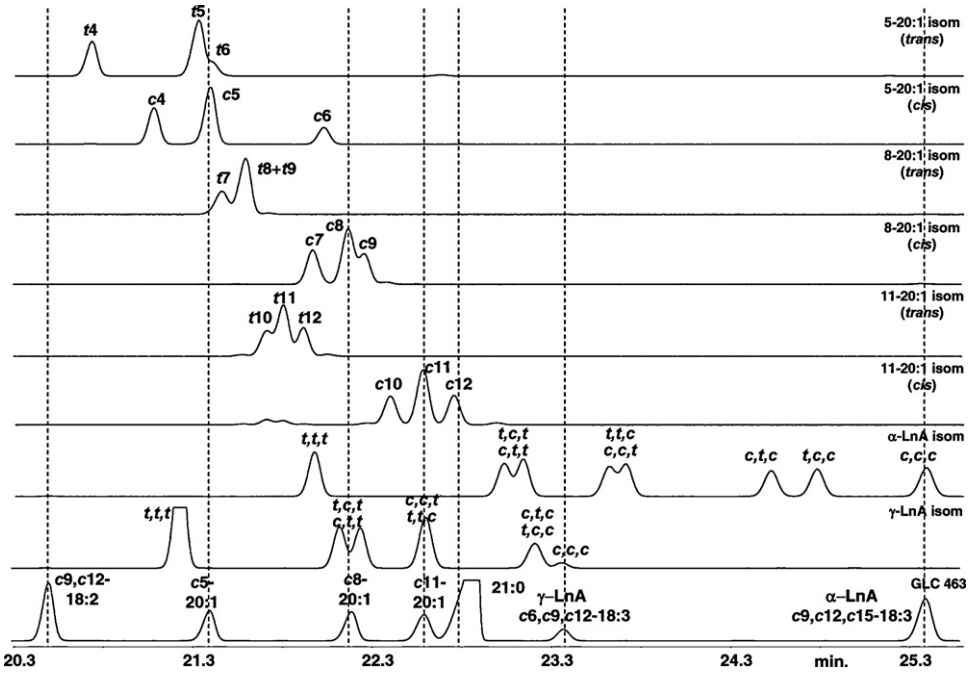

Fig. 5. Partial GC chromatogram of the 20:1 and 18:3 region. From top: *trans* and *cis* fractions of *cis* 5-, *cis* 8- and *cis* 11-20:1 FAMEs after one successive bromination and debromination, α-LnA and γ-LnA isomerized by PTSA, and reference FAME mixture GLC 463. Conditions as in Fig. 1.

bond position 8,10- to 11,13-18:2 were baseline resolved (Fig. 8, bottom chromatogram, and also evident in Fig. 6). The four *t*,*t*-CLA present in the isomerized UC-59-M mixture and *t*7,*t*9-18:2 present in the iodinized synthetic 7,9-18:2 mixture were partially separated on this column. There were three CLA isomers present in the experimental milk fat next in relative abundance after *c*9,*t*11-18:2 (upper graph) identified as *t*7,*c*9-18:2, *t*9,*c*11-18:2, and *t*10,*c*12-18:2. Dairy fats are known to contain 22:0 and *c*11,*c*13-20:2 and both were well separated from the CLA isomers present in this milk fat (Fig. 8).

4. Discussion

The availability of alternative 100 m highly polar ionic liquid capillary GC columns has provided an opportunity to complement the results obtained with the 100 m cyanopropyl siloxane phase coated columns (SP-2560 and CP-Sil 88) currently recommended for the separation of complex mixtures of geometric and positional isomers present in partially hydrogenated fats and ruminant products [15]. To conduct this evaluation, a series of synthetic *cis* and *trans* MUFAs and CLA isomers, a silver-ion separated fraction from

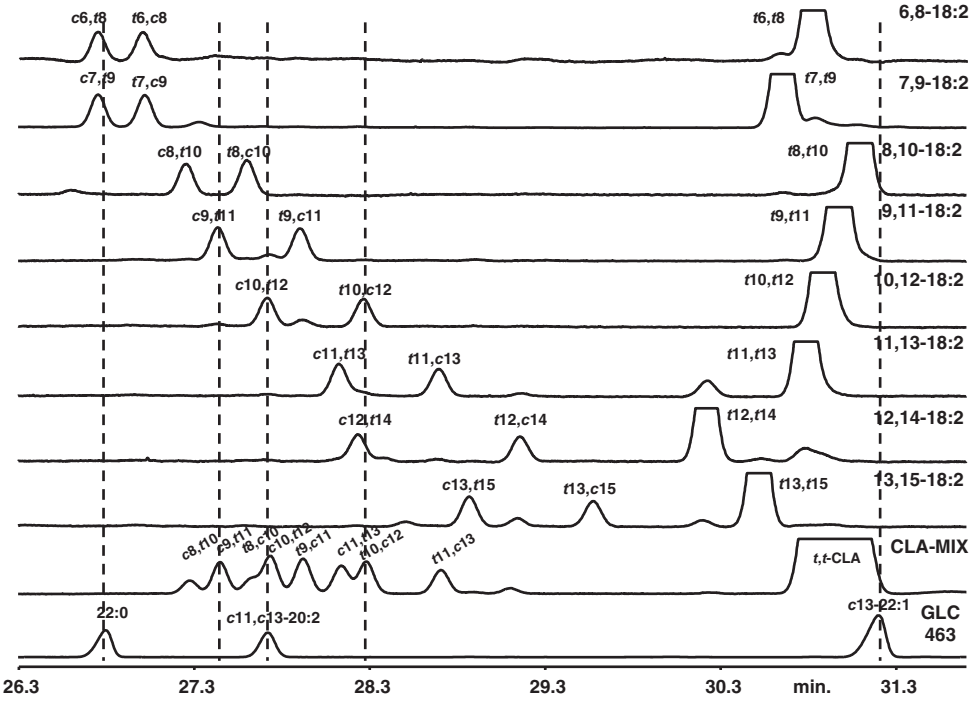

Fig. 6. Partial GC chromatogram of the CLA region. From top: iodine isomerized CLA isomers with double bond position 6,8- to 13,15-18:2, iodine isomerized CLA mixture UC-59-M and reference FAME mixture GLC 463. Conditions as in Fig. 1.

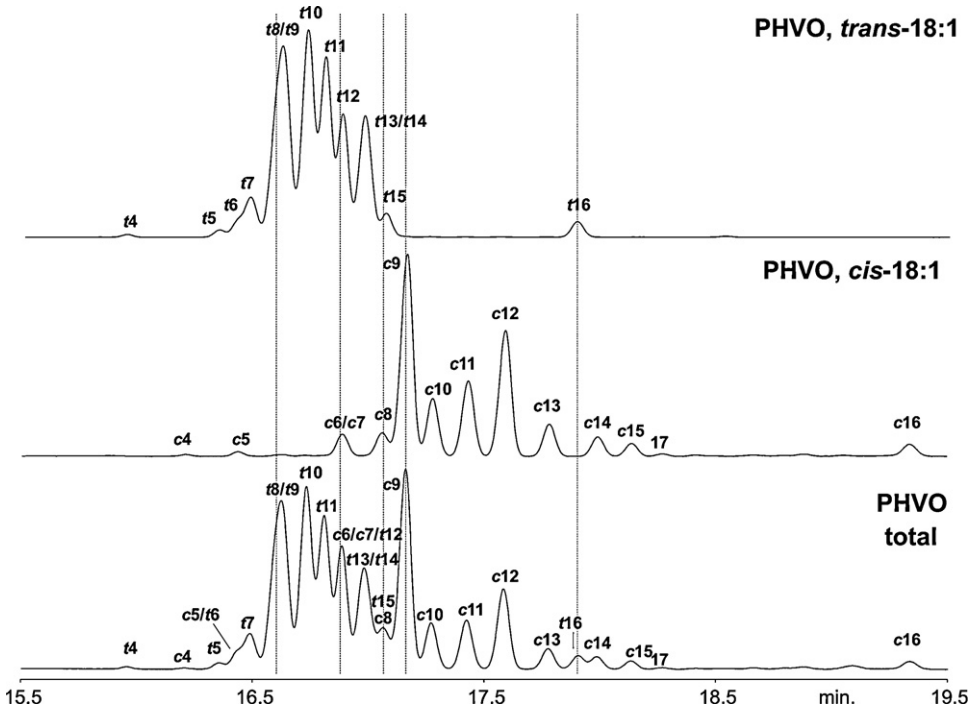

Fig. 7. Partial GC chromatogram of the 18:1 region. From top: *trans* and *cis* fractions of partially hydrogenated vegetable oil (PHVO), and total PHVO. Conditions as in Fig. 1.

milk fat containing saturated FAs, and authentic GC standards were used. The reason for preparing and evaluating the synthetic *cis* and *trans* MUFAs was to aid in the identification of these FA isomers present in ruminant fats. All of these samples had previously been analyzed and reported [12] using a 100 m SP-2560 column operated at isothermal 180 °C condition as recommended in the official AOCS method for *trans* FA analysis [9] or temperature programmed from 45 to 215 °C [5].

The new SLB-IL111 stationary phase is a proprietary polyionic ionic liquid stationary phase that has the highest polarity of the stationary phases that have been commercialized for use in capillary GC – including traditional polysiloxane and polyethylene glycol based stationary phases. The phase is comprised of functionalized cation groups joined as geminal cations by a spacer group. Typical cations based on substituted imidazolium, phosphonium, pyridinium, pyrollidium and ammonium moieties have

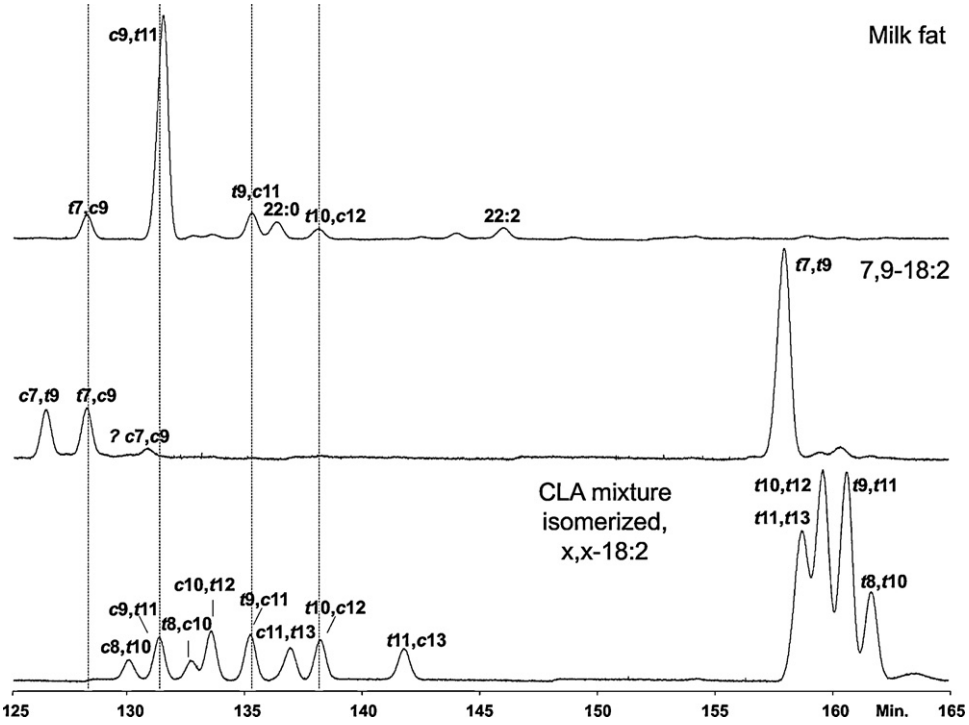

Fig. 8. Partial GC chromatogram of the CLA region. From top: milk fat sample, 7,9-18:2 isomerized with iodine and iodine isomerized CLA mixture UC-59-M. Conditions: SLB-IL111 capillary column (100 m × 0.25 mm, 0.2 μm thickness), hydrogen carrier gas at 1 mL/min, 130 °C isothermal elution.

been the cations of choice. Spacer groups such as various alkyl, polysiloxane or polyethylene glycol groups have been used to join the cations. The anion chain typically used for GC has been a bis(trifluoromethylsulfonyl)imide (NTf2) or the triflate anion since they provide the highest thermal stability for the ionic stationary phase [22,27]. Separations on the ionic liquid column are governed mainly by dipolar interactions and hydrogen bond basicity arising from the anions which produce a column more polar than the 100% cyanopropyl siloxane phases [23]. Based on the McReynolds constants, the SP-2560 column has a polarity number of 81 compared to 111 for the SLB-IL111 column, with each of them normalized to the SLB-IL100 column [22]. The higher polarity of the ionic column compared to that of the SP-2560 column was evident in the elution of all unsaturated FAs relative to saturated FAs. For example, on the SLB-IL111 column, 19:0 eluted before the *c*9-18:1 peak (Fig. 4B), while on the SP-2560 column, 19:0 eluted between *c*13- and *c*14-18:1 at isothermal 180 °C [12]. In addition, 17:0 eluted just after *c*7-16:1 on the SLB-IL111 column at 168 °C (Fig. 2), and just after *c*12-16:1 on the SP-2560 column operated isothermal at 180 °C [12].

The polarity of cyanopropyl siloxane phases is known to be temperature dependent [14,28], which in the case of FAMEs affects their relative elution times and order. Differences in column temperature have been successfully used with cyanopropyl siloxane columns to identify many of the FAME isomers based on changes in the relative elution of FA isomers within and between different groups of FAMEs [13,15]. The ionic liquid columns have also been reported to be temperature sensitive [22,27], and it is also evident by the differences in separations at different temperatures of operation; see Fig. 4A and B. A separation of selected FAMEs was recently reported using the SLB-IL100 column operated at 150 °C isothermal condition [20].

In the present study the temperature of the SLB-IL111 column was raised to 168 °C to maximize the resolution of most of the common FA isomers. We investigated the elution characteristics of the SLB-IL111 column at different isothermal conditions and concluded that isothermal operation at 168 °C provided the best overall resolution of most FAMEs and these separations were compared to those obtained using the cyanopropyl siloxane columns. Just as a note of interest, in this study it became evident that even a change of a few degrees in the operating temperature caused a significant change in the relative elution of FA with a different number of double bonds. For this reason, 168 °C rather than 165 °C or 170 °C was selected for the best overall separation.

A major finding in this study was that the 100 m SLB-IL111 column provided a much improved resolution of the *c*/*t*-CLA isomers than that obtained using the 100 m cyanopropyl siloxane columns. This is the first report of a GC method capable of resolving the two most abundant CLA isomers in ruminant fats, namely *c*9,*t*11- from *t*7,*c*9-CLA at both isothermal 168 °C (Fig. 6) and 130 °C conditions (Fig. 8). These separations were not possible using cyanopropyl siloxane columns at any isothermal temperature [12] or temperature program conditions [5,11,29,30]. Thus the use of this column eliminated the need for a complimentary Ag^+-HPLC technique to resolve these two CLA isomers [5]. The *c*9,*t*11-CLA (or rumenic acid) content in ruminant fats is overestimated unless an additional separation using Ag^+-HPLC is included to resolve these two co-eluting CLA isomers. The SLB-IL111 column will drastically reduce the time and expertise required to obtain this information, since it is capable of resolving these two CLA isomers directly in a single GC analysis.

Use of the SLB-IL111 column also resolved all of the four *t*,*t*-CLA isomers contained in the isomerized CLA mixture (UC-59-M) at 130 °C isothermal condition (Fig. 8). This compares to two peaks obtained by using either the SP-2560 or CP-Sil 88 columns, i.e., separation of *t*11,*t*13- from the remaining 3 *t*,*t*-CLA isomers [5,11,12]. Further work will be necessary to determine whether the *t*,*t*-CLA isomers commonly present in beef fats will require the complimentary Ag^+-HPLC technique or whether a simple GC separation using the 100 m SLB-IL111 column would be sufficient.

At 168 °C, the SLB-IL111 column caused a minimal overlap of the peaks for the *trans* and *cis* isomer of each chain length as judged by analyzing model synthetic FAME mixtures that contained most of the positional isomers of 14:1 to 20:1 (Figs. 1–3 and 5). In each case, the *trans* isomer in the third to last Δ position in a chain eluted before the major *cis* isomers with the same chain length, and the *trans* isomer in second to last Δ position was resolved among the *cis* isomers, which meant that for the 18:1 isomers, *t*15-18:1 eluted before *c*9-18:1, and *t*16-18:1 could be readily identified among the *cis*-18:1 isomers. The separation of the *trans* and *cis* isomer clusters was even more complete when the isothermal temperature of the SLB-IL111 column was increased to 180 °C, which in the case for 18:1 only meant the overlaps of *t*16-18:1 and a few minor *cis*-18:1 isomers (Fig. 4B). Such separations would be useful if only the total *trans*-18:1 content is desired in fat mixtures, but that would not provide a detailed analysis of the isomeric composition. The separation of geometric clusters is not possible using cyanopropyl siloxane columns. Occasionally the total *trans*-18:1 FA content is estimated by summing all of the peaks eluting just before *c*9-18:1, and assuming that the remaining *trans*-18:1 and overlapping *cis*-18:1 isomers are negligible, or cancel each other out. Precht et al. estimated that such determination could underestimate the total *trans* FA content by as much as 35% [31].

Of interest was the identification of MUFAs with the double bond in the terminal position. These MUFAs were expected in the synthetic mixtures after extensive isomerization by bromination and debromination based on theoretical considerations, and based on the decreased relative abundance of the isomers as one moves away from the original double bond position of the starting material. However, their location on the chromatogram could not be confirmed using the SP-2560 columns, since there appeared to be no additional peak in either the *trans* or *cis* fractions after Ag^+-HPLC separation [12]. Using the SLB-IL111 column, an extra peak was observed in each of the *cis* fractions from the synthetic MUFA eluting near the third last double bond ($n-3$); see Figs. 1–3. The extra peak in the *cis*-15:1 fraction was confirmed as 14-15:1 by comparison of the retention time and co-injection with authentic 14-15:1 (U-38-MX) available from Nu-Chek Prep. Inc.; standards for the other FA with terminal double bonds were not available. The reversal in the elution order of the FAMEs with a terminal double bond compared to all the other isomers within the chain was previously observed by Gunstone et al. [32] using a 50 m NPGS (neopentylglycosuccinate) capillary column. This finding indicates that these FAME isomers with terminal double bonds behaved chromatographically on Ag^+-HPLC as *cis* isomers, yet on the SLB-IL111 GC column behaved more like *trans* FAs, eluting time-wise shortly after the $n-2$ *trans* isomer. On the other hand, Gunstone et al. [32] argued that this reversal was due to the uniquely different property of the Δ16 isomers, i.e., eluting later than predicted. The question is why were these isomers not detected when the same mixtures were analyzed using the SP-2560 column [12]. Based on the peak shape and relative abundance of the isomers, it would appear that the MUFAs with the terminal double bond co-eluted with the third last *cis* isomer of each chain length. The ability to detect MUFAs with terminal double bonds in complex mixtures if present is an additional advantage of the SLB-IL111 columns.

The separation of *t*15-18:1 from *c*9-18:1 is a unique feature that distinguishes the two types of columns. On the SLB-IL111 column these two isomers separated both at isothermal 168 °C and 180 °C condition, while such a separation is generally not possible using SP-2560 or CP-Sil 88 columns, unless the relative abundances of the 18:1 isomers are similar. At isothermal 180 °C, *t*15-18:1 co-eluted with *c*9-18:1 [10,24]. A partial separation of *t*15-18:1 from *c*9-18:1 was reported at isothermal 175 °C condition [2,33]. How-

ever, this result was not possible when a temperature program that plateaued at 175 °C was used [5,11,34], or when a continuously increasing temperature program was used [35]. Only when the temperature program was lowered to plateau at 163 °C did *t*15-18:1 elute between *c*9-18:1 and *c*11-18:1, but co-eluted with *c*11-18:1 at 150 °C [13]. It should be noted that a separation of *t*15-18:1 and *c*9-18:1 could be achieved when the concentration of the *c*9-18:1 isomer was not dominant. For instance, when cows were fed fish oil, the *c*9-18:1 content was only 4.84% compared to 18.11% for the control, which resulted in a partial resolution of *t*15-18:1 and *c*9-18:1 [36]. For the same reason, a separation of *t*15-18:1 and *c*9-18:1 was generally observed when highly hydrogenated fats were analyzed at isothermal GC conditions of 180 °C [10], because the content of *c*9-18:1 was not much more than that of the other 18:1 isomers in these fats.

The resolution of the *t*13- and *t*14-18:1 isomers was also a distinguishing feature between these two columns. A near baseline resolution was obtained for most geometric 18:1 isomers in less than 50 min by lowering the elution temperature of the ionic column to 140 °C isothermal condition (Fig. 4A). There was even a partial resolution of the *t*13- and *t*14-18:1 isomers and a separation of *c*8- from unresolved *c*6/*c*7-18:1 (Fig. 4A). Separation of the *t*13- and *t*14-18:1 isomers was not possible using cyanopropyl siloxane columns unless the temperature of the column was lowered to 120 °C isothermal condition, and the isomers required 220 min to elute using either 100 m SP-2560 or CP-Sil 88 columns [3,5,37]. As the 4,4-dimethyl oxazoline (DMOX) derivatives, *c*13- and *c*14-18:1 were resolved at isothermal GC condition at 140 °C [38].

The 20:1/18:3 isomer region has always presented a challenge for the analysis of both PHVO and ruminant fats using cyanopropyl siloxane columns [39–41]. Many of these isomers were identified only by changing the column temperature which also changed the elution pattern of the 20:1 relative to that of the α-LnA isomers [13,40]. By contrast, on the SLB-IL111 column operated at 168 °C, most of the common *cis* and *trans*-20:1 isomers eluted before the common mono-*trans* geometric isomers of α-LnA (Fig. 5). This was not the case for the geometric isomers of γ-LnA that extensively overlapped with the 20:1 isomers (Fig. 5). This is less of a concern since the content of γ-LnA in most natural products is generally low.

The major disadvantage of the SLB-IL111 column appears to be the location of the saturated FAMEs among the MUFA isomers. At 168 °C, the saturated FAs eluted between the *trans* and *cis* clusters of MUFA isomers one carbon less in chain length. The elution characteristics of saturated FAMEs were not previously investigated using the SLB-IL100 column [20], since saturated FAs were not included in the test mixtures analyzed, even though saturated FAs are ubiquitous constituents in all natural products. With the choice of 168 °C isothermal conditions, the coelution of the straight-chain saturated FAs occurred with the minor *trans* MUFA isomer in every chain, while the branched-chain saturated FA eluted among the MUFA isomers. However, the coelution of saturated FAs with the *cis* MUFA isomers depended on the chain length of the FAME. As the chain length of the saturated FA increased, the overlap was with ever increasing Δ-*cis* values of the FAME with one less carbon atom, i.e., 15:0 co-eluted with the *c*6/*c*7-14:1; 16:0 with *c*6/*c*7-15:1; 17:0 between *c*7- and *c*8-16:1; 18:0 with *c*8-17:1; 19:0 with *c*9-18:1; 20:0 between *c*10- and *c*11-19:1, and 21:0 with the *c*12-20:1; see Figs. 1–3 and 5 for the respective chain lengths. Changing the temperature of the SLB-IL111 column affected the elution of saturated FAs relative to the *cis* and *trans* MUFA isomers, and in fact one of the reasons for the choice of isothermal 168 °C was to minimize the interferences of saturated FAs relative to the MUFAs. On the other hand, saturated FAs eluted later relative to MUFAs on cyanopropyl siloxane columns than on the SLB-IL111 column, which was due to the lower polarity of the former. This resulted in generally good separations of most saturated FAs on the SP-2560 or CP-Sil 88 columns, except for 19:0 which eluted among *t*,*t*-18:2 isomers and 21:0 among the CLA isomers [13,34].

The separation of the 17:0 branched-chain FAs presented a unique challenge for both types of GC columns, since they eluted with the *trans*-16:1 isomers on the SLB-IL111 column (Fig. 2) and among the *trans*- and *cis*-16:1 isomers on the cyanopropyl siloxane columns [13,18]. Their identification on cyanopropyl siloxane columns was made possible by conducting a prior silver-ion chromatographic separation [13,18] or by analyzing the same sample using two separate temperature programs [13]. In general, all branched-chain FAs were difficult to resolve from the coeluting *cis*- or *trans*-MUFAs (Figs. 1 and 2), similar to the straight-chain FAs. To identify these branched-chain FAs in dairy products, one could analyze these FAs using two separate GC temperature settings, much the same as was previously accomplished using the 100 m SP 2560 column [13].

The 'dual nature' of the SLB-IL111 column [21] may present an opportunity of co-analyze components other than FAMEs in a mixture, but may also require special attention if they are present. Such constituents may be co-extracted after methylation and injected onto the GC because of similar solubility characteristics to FAMEs. For example, long-chain alcohols could be present in the total FAME mixture after methylation, and if not removed by TLC or silica-SPE columns (methods used to purify FAMEs), would elute on the SLB-IL111 column, but not on a cyanopropyl siloxane column because of their polarity. Therefore, one needs to be aware and cautious of this possibility.

5. Conclusions

In this study, the 100 m ionic liquid capillary column SLB-IL111 proved to have several advantages compared to the cyanopropyl siloxane columns currently recommended for the challenging analysis of mixtures containing geometric and positional isomers of FAMEs. This ionic column gave an improved separation of the many *c*/*t*-CLA isomers when operated isothermally at 168 °C, including the separation of *c*9,*t*11- from *t*7,*c*9-CLA which is not possible using cyanopropyl siloxane columns, but requires the mandatory complementary Ag^+-HPLC method. This new column provided for the first time a GC technique capable of resolving this difficult isomeric pair, and eliminated the concern that in most reports rumenic acid is being overestimated by the contribution of *t*7,*c*9-CLA. This new column also provided a direct GC method to identify *t*15-18:1 which generally co-eluted with *c*9-18:1, and provided a partial resolution of several isomer pairs such as *t*13/*t*14-18:1, *c*8- from *c*6/*c*7-18:1, and several *t*,*t*-CLA isomers. Due to its higher polarity, it caused a significant change in the elution pattern of the 20:1 and 18:3 isomers, of the 16:1 isomers and the branched-chain FAs, and of isomers in the *c*/*t*-18:2 region to provide a valuable complimentary column for the identification of these isomers in complex lipid mixtures. Were it not for the unfortunate elution of saturated FAs (straight and branched-chain FAs) among the geometric and positional isomers of MUFAs, this GC capillary column could be recommended as the most suitable for the analysis of total FAME from ruminant fats. However, the results suggest that there may be merit in combining the results of these two column types to provide a more complete analysis of complex mixtures.

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