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Two methods for the separation of monounsaturated octadecenoic acid isomers

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ABSTRACT

The identification and quantification of complex mixtures of *cis* and *trans* octadecenoic (18:1) fatty acid isomers presents a major challenge for conventional one-dimensional GC/FID analysis of their methyl esters. We have compared the use of two methods to achieve optimized separations of positional and geometrical octadecenoic fatty acid isomers—comprehensive two-dimensional gas chromatography (GC × GC), and silver ion high performance liquid chromatography interfaced to atmospheric pressure photoionization (APPI) mass spectrometry. Nine isomers of octadecenoic acid methyl ester were well separated on a single silver ion column with a mobile phase of 0.018% acetonitrile and 0.18% isopropanol in hexane. Reproducible retention times were obtained with relative standard deviations of around 1% over 5 injections. The extra selectivity and reproducibility afforded by APPI-MS, together with the wide separation of *cis* and *trans* isomers by silver ion chromatography, resulted in a promising method for measurement of octadecenoic acid FAME. The GC × GC separation was performed using various column combinations, and optimal separation was obtained by coupling an ionic liquid column (Supelco SLB-IL100 [1,9-di(3-vinyl-imidazolium) nonane bis(trifluoromethyl) sulfonyl imidate]) in the first dimension with a SGE BPX50 (50% phenyl polysilphenylene-siloxane) in the second dimension. These methods have been applied to the analysis of octadecenoic acid in milk and beef fat.

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1. Introduction

Fatty acid chains with 18 carbons (C18) are of special interest due to their importance in nutrition and health. Epidemiological studies have demonstrated positive correlations between *trans* fatty acid (TFA) intake, cholesterol level and coronary heart disease (CHD) death rates [1–3]. As a consequence, the U.S. FDA requires manufacturers to list the *trans* fat content in the nutritional facts panel of food products and there are general recommendations to reduce intake. However, some *trans* fatty acids may have beneficial effects on health. Recent studies [4,5] have revealed that some C18 monounsaturated (C18:1) *trans* fatty acids, specifically *trans*-vaccenic acid (tVA, 11t-C18:1), could be involved in the production of conjugated linoleic acids (CLA), which are thought to have anti-carcinogenic properties [5–7]. During lactation, dietary tVA has been shown to increase CLA levels in mice and humans [6,8]. Dietary tVA has been shown to contribute to reductions in cancer incidence [5,6] and this appears to depend upon enzymatic desaturation to CLA [3,6]. The hypolipodemic effect of tVA has been demonstrated by dietary supplementation of tVA, which reduced triglyceride concentration in rats with lipid metabolic syndrome [9].

Thus, there are a variety of reasons for interest in the tVA content of foods and tissue which may relate to valuable benefits to health. A number of methods have been developed for analysis of octadecenoic fatty acids (FA) in food, such as infrared spectroscopy (FTIR) [10,11], gas chromatography (GC) combined with flame ionization detection (FID) or mass spectrometry (MS) [12–18] and silver ion high performance liquid chromatography (Ag^+-HPLC) [19–22] or reverse phase HPLC [23,24]. Of these methods, GC-FID is by far the most common method to determine fatty acid composition. The preparation of volatile ester derivatives of fatty acids via well-known procedures is necessary prior to GC analysis in order to reduce analysis time and optimize the separation. GC provides high resolution separations which make it possible to determine many individual fatty acids and isomers in a complicated matrix. However, some geometrical isomers overlap or co-elute even when using long columns (100 m) under optimal conditions making accurate analysis problematic. To overcome these drawbacks, silver ion thin layer chromatography (Ag^+-TLC) has been used prior to GC/FID analysis. This works by separating *cis* and *trans* FAME isomers into separate fractions since the former result in more stable complex formation with the bound silver ions and hence elute later [25–29]. Precht and Molkentin used this approach [30] to analyze *trans* positional isomers of octadecenoic acid in butter and milk samples using a 100 m cyanopropyl polysiloxane column (CP Sil 88) operated isothermally at 175 °C. They identified *trans*-vaccenic acid as the most abundant *trans*-C18:1 isomer in milk and also suggested the presence of traces of 4t-C18:1. The

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aim of their study was to develop equations to rapidly estimate the content of *trans* positional C18:1 isomers in milk based on GC measurements of the triacylglycerides. However, triacylglyceride profile was shown to be strongly dependent on the type of animal feed [30]. Similarly, Kramer and co-workers applied the combination of Ag^+-TLC and GC to analyze C18 isomers in milk fat [26,31]. However, in all cases the isomers were still not completely resolved and this method requires considerable sample manipulation. More recently, Kramer et al. described several temperature programs [25] achieving remarkable separation of C18:1 isomers using a 100 m CP-Sil 88 column, but even with such elegant chromatography, quantitation is complicated and several isomers are not fully resolved.

1.1. GC × GC

An alternative method with a high potential to improve separation and quantification of C18:1 geometrical isomers is comprehensive two-dimensional gas chromatography (GC × GC), since such a system has more peak capacity and hence potential resolving power than conventional one-dimensional gas chromatography (1DGC). In the comprehensive GC × GC system used here, a conventional 1DGC system is connected in series to a second column housed in a separate oven *via* a cryogenic modulator. The sample is separated in the first dimension (first column) and then each fraction enters the modulator composed of two stages, each alternately cooling and heating for fixed times (the modulation period). In this way, a component is retained by the cooling pulse then released in a focused band by the hot pulse to the second column while the next fraction is trapped in the first stage [32–37]. All of the first column flow passes into the short, and often microbore, second column which cycles with a period equal to the modulation period. Finally, a single FID detector records the signal eluting from the second column.

Studies of the separation of fatty acids methyl esters (FAMEs) have been published because of the versatility of GC × GC to separate mixture components according to carbon length and number of double bonds. Mondello et al. found that “FAMEs with the same number of carbon atoms elute as clusters with gradual increase of the first-dimension retention time as carbon number increases”. Second dimension retention times increase from saturated, then mono- to hexa-unsaturated FAs with the “homologous series showing up as parallel lines in the 2D plane” [38]. Harynuk et al. described the separation of compound mixtures according to molecular characteristics, which they defined as the “dimensionality of a sample”. They observed various multidimensional separations including the length of the carbon chain, number of double bonds, geometrical isomers, functional groups etc. [39]. Hyoetylaeinen et al. analyzed C18 fatty acids in milk fat using four sets of columns by employing GC × GC equipped with a semi-rotating cryogenic modulator and identified around 14 C18:1 isomers [13]. A further successful separation of geometrical C18:1 FAME isomers in vegetable oil was achieved by de Koning et al. on CP-WAX–VF-23 column combination at a constant temperature of 165 °C without second dimension oven offset [40].

One of the objectives of this study was to assess the potential of two-dimensional gas chromatography (GC × GC) to separate and quantify C18:1 isomers, especially t-VA, in bovine fats (beef fat and milk fat) since there is considerable interest from the agricultural sector to produce CLA (and tVA) enhanced products.

1.2. Ag^+-HPLC/APPI-MS

Silver ion HPLC (Ag^+-HPLC) has become a widely used technique for the separation and detection of C18:1 fatty acid isomers since developed by Christie in 1987 [19,41–45]. *Cis* and *trans* unsaturated fatty acid isomers are readily separated with a silver ion column while the separation of most positional isomers is typically only partial. In Ag^+-HPLC, mobile phases of hexane or dichloromethane with various modifiers including acetonitrile, methanol and isopropanol are used to separate isomeric octadecenoic fatty acids often in the form of methyl esters [43]. Acetonitrile, often used as a modifier in hexane, is believed to compete with double bonds for interaction with silver ions. However, only isocratic elution has proved reliable because of the poor solubility of acetonitrile in hexane. Much attention has also been paid to the effect of the ester moiety of fatty acid on the separation of positional isomeric octadecenoic acids [44].

Studies have demonstrated that conversion of *cis*- and *trans*-11 and 9-18:1 fatty acids into phenacyl esters improved the resolution and sensitivity with UV detection. To date, the best separation by Ag^+-HPLC of the positional isomeric fatty acids *trans* 6, 9 and 11-18:1 as *p*-methoxyphenacyl esters was achieved with a mobile phase of hexane–dichloromethane–isopropanol (70:30:0.2, v/v/v) or dichloromethane-acetonitrile (or methanol) (100:0.025, v/v) [43].

There are obvious benefits to coupling Ag^+-HPLC to mass spectrometry (Ag^+-HPLC/MS) compared to the UV or evaporative light-scattering detectors (ELSD) widely used in literature. This is true even in the analysis of isomers since interfering species of other molecular weights can be separated. Atmospheric pressure photoionisation (APPI) is a novel ionization technique developed for low polarity compounds, which is especially useful when coupled with non-aqueous reverse phase HPLC and normal phase HPLC [46]. It provides a useful alternative to the more widely used ionization techniques such as electrospray (ESI) or atomospheric pressure chemical ionization (APCI). ESI is less compatible with non-polar compounds in non-aqueous solvents and requires the use of post-column make-up flows for use with such systems. APCI can be used directly with non-aqueous solvents but high concentrations of hexane or similar hydrocarbon solvents quickly result in source instability due to contamination of the discharge needle. In our experience, given an appropriate choice of dopant, APPI overcomes these problems and results in extremely stable signals of non-polar compounds. To our knowledge, the use of Ag^+-HPLC-APPI mass spectrometry for analysis of isomeric octadecenoic acids has not been reported to date. Thus, an objective of this study is to develop silver ion HPLC coupled photoionization mass spectrometry (Ag^+-HPLC/APPI-MS) for the separation and detection of isomeric C18:1 fatty acids in the methyl ester form, which are easily prepared compared to other aromatic esters [10]. This required further optimization of the chromatographic conditions for C18:1 FAMEs compared to literature reports [43].

2. Materials and methods

2.1. 1DGC

A Hewlett Packard model 5890 series II GC (Agilent Technologies; Palo Alto, CA, USA) equipped with a flame ionization detector, autosampler and split/splitless injector was used to run samples and standards on a SLB-IL100 column (60 m × 0.2 mm i.d. × 0.2 μm, stationary phase 1,9-di(3-vinyl-imidazolium) nonane bis(trifluoromethyl) sulfonyl imidate, Supelco; Bellefonte, PA, USA). All data was collected by Leco ChromaTOF-GC software v3.34. A Varian 3400 GC (Palo Alto, CA, USA) equipped with autosampler and on-column injector was used to run samples and standards on a SP-2560 column (100 m × 0.25 mm i.d., 0.2 μm nonbonded; bis cyanopropyl polysiloxane phase, Supelco; Bellefonte, PA, USA). All data was collected by Galaxie software v1.19. Other details and chromatographic conditions are listed in Table 1.

Table 1
Columns and experimental conditions used. Retention times refer to tVA.

Column combination	First dimension	Second dimension	Chromatographic conditions
CC #1 (1DGC)	SLB-IL 100[a] 60 m × 0.2 mm × 0.2 μm 1,9-di(3-vinyl-imidazolium) nonane bis(trifluoromethyl) sulfonylimidate	Not applicable	Injector and detector were kept to 210 °C and 220 °C correspondingly. Split ratio 200:1. Carrier gas: He, flow rate: 0.5 mL/min. Make-up gas: He. Injection volume: 1 μL. Temp. program: 120 °C (40 min), 7 °C/min 220 °C (2 min). Retention time: 50 min. Run time: 56 min
CC #2 (1DGC)	SP-2560[a] 100 m × 0.25 mm × 0.2 μm bis-cyanopropyl polysiloxane	Not applicable	On-column injection at 50 °C and detector were kept to 230 °C. Carrier gas: He. Make-up gas: He. Injection volume: 0.5 μL. Temp. program: 45 °C (4 min), 13 °C/min 150 °C (47 min), 4 °C/min 215 °C (35 min). Retention time: 45 min. Run time: 110 min
CC #3 (GC × GC)	DB-Petro[b] 50 m × 0.2 mm × 0.5 μm 100% DMPS	BPX-50[d] 2 m × 0.1 mm × 0.1 μm 50% phenyl polysilphenylene-siloxane	Injector and detector were kept to 220 °C and 315 °C correspondingly. Split ratio 50:1. Injection volume: 1 μL. Carrier gas: He, flow rate: 1 mL/min. Make-up gas: He. Temp. program: first column 190 °C (0.2 min), 1 °C/min 240 °C (0.2 min); second column 200 °C (0.2 min), 1 °C/min 250 °C (0.2 min). Second dimension separation time 4 s, modulator temperature offset 50 °C, hot pulse 1 s, cool time 0.8 s. Retention time: 29 min. Run time: 50 min
CC #4 (GC × GC)	Famewax[c] 30 m × 0.25 mm × 0.25 μm 100% carbowax	BPX-50[d] 2 m × 0.1 mm × 0.1 μm 50% phenyl polysilphenylene-siloxane	Injector and detector were kept to 220 °C and 245 °C correspondingly. Split ratio 50:1. Injection volume: 1 μL. Carrier gas: He, flow rate: 1 mL/min. Make-up gas: He. Temp. program: first column 160 °C (0.2 s), 0.5 °C/min 165 °C (20 min); second column 165 °C (20 min). Second dimension separation time 6 s, modulator temperature offset 15 °C, hot pulse 1 s, cool time 0.8 s. Retention time: 24 min. Run time: 30 min
CC #5 (GC × GC)	BPX90[d] 60 m × 0.25 mm × 0.25 μm (90% cyanopropyl polysilphenylene-siloxane)	BPX-50[d] 3 m × 0.1 mm × 0.1 μm 50% phenyl polysilphenylene-siloxane	Injector and detector were kept to 210 °C and 230 °C correspondingly. Split ratio 100:1. Injection volume: 1 μL. Carrier gas: He, flow rate: 0.5 mL/min. Make-up gas: He. Temp. program: first column 45 °C (1 min), 2 °C/min 220 °C (30 min); second column 51 °C (0.2 min), 2 °C/min 225 °C (30 min). Second dimension separation time 5 s, modulator temperature offset 30 °C, hot pulse 1 s, cool time 0.8 s. Retention time: 75 min. Run time: 118 min
CC #6 (GC × GC)	Rxi-5[c] 30 m × 0.25 mm × 0.25 μm 5% diphenyl 95% dimethylpolysiloxane	BPX-50[d] 1.5 m × 0.1 mm × 0.1 μm 50% phenyl polysilphenylene-siloxane	Injector and detector were kept to 250 °C. Split ratio 50:1. Injection volume: 1 μL. Carrier gas: He, flow rate: 1 mL/min. Make-up gas: He. Temp. program: first column 40 °C (2 min), 30 °C/min 160 °C (0 min), 2 °C/min 300 °C (5 min); second column 45 °C (2 min), 30 °C/min 165 °C (0 min), 2 °C/min 305 °C (5 min). Second dimension separation time 5 s, modulator temperature offset 30 °C, hot pulse 0.6 s, cool time 1.9 s. Retention time: 33 min. Run time: 81 min
CC #7 (GC × GC)	SLB-IL 100[a] 60 m × 0.2 mm × 0.2 μm 1,9-di(3-vinyl-imidazolium) nonane bis(trifluoromethyl) sulfonylimidate	BPX-50[d] 3 m × 0.1 mm × 0.1 μm 50% phenyl polysilphenylene-siloxane	Injector and detector were kept to 210 °C and 230 °C correspondingly. Split ratio 100:1. Injection volume: 1 μL. Carrier gas: H_2, flow rate: 0.5 mL/min. Make-up gas: He. Temp. program: first column 45 °C (1 min), 2 °C/min 220 °C (30 min); second column 50 °C (1 min), 2 °C/min 225 °C (30 min). Second dimension separation time 5 s, modulator temperature offset 30 °C, hot pulse 1 s, cool time 1.5 s. Retention time: 69 min. Run time: 118 min

[a] Supelco; Bellefonte, PA, USA.
[b] J&W Scientific; Folsom, CA, USA.
[c] Restek; Bellefonte, PA, USA.
[d] SGE Analytical Science; Ringwood, VIC, Australia.

2.2. GC × GC

All GC × GC experiments were conducted using a LECO comprehensive GC × GC/FID system (LECO; St. Joseph, MI, USA) consisting of a dual oven gas chromatograph equipped with flame ionization detector and cryogenic modulator (quadjet) cooled with liquid nitrogen and split–splitless injector. All data was collected by Leco ChromaTOF-GC software v 3.34 optimized for GC × GC/FID.

Five column combinations were tested in GC × GC. Details and chromatographic conditions are listed in Table 1.

2.3. Ag$^+$-HPLC/APPI-MS

All Ag$^+$-HPLC/APPI-MS separations were carried out on an Agilent 1200 liquid chromatograph (Agilent Technologies; Palo Alto, CA, USA) coupled to a QStar Elite mass spectrometer (Applied Biosystems/MDS Sciex; Concord, ON, Canada) with a Photospray^{TM} photoionization source. Dopants were delivered with a Harvard Model '11' Plus syringe pump (Holliston, MA, USA). Analyst QS 2.0 software was used for data acquisition and analysis. A ChromSpher 5 Lipids column (250 mm × 2 mm i.d., 5 μm) (Varian, Lake Forest, CA, USA) was used for separation. The mobile phase was composed of solvent A hexane: isopropanol: acetonitrile (100:1:0.1, v/v/v) and solvent B hexane. The separations were performed using isocratic elution of 18% solvent A at a flow rate of 200 μL/min. The injection volume was 1 μL.

The Photospray ion source was used in positive ion mode. All mass spectrometric detection parameters were optimized by infusion of 5 mg/L of methyl cis-9 octadecenoate. The protonated molecular ion at *m*/*z* 297 of target compound was used for tuning. The optimal conditions were as follows: curtain gas (N_2) at 25, gas 1 at 20, nebulizing gas 2 at 70, ion source temperature at 400 °C and ionspray voltage at 1300 V. The declustering potential (DP), focus potential (FP), and DP2 were 35, 130, and 5 V, respectively. Toluene was used as dopant at a flow rate of 20 μL/min. The flow rate of lamp gas was 4 L/min.

2.4. Samples and standards

A mixture of 52 components (Nuchek 463) and individual reference standards of 6t-C18:1, 9t-C18:1, 11t-C18:1, 6c-C18:1, 7c-C18:1, 9c-C18:1, 11c-C18:1, and C17:0 were purchased from-Nuchek Prep Inc. (Elysian, MN, USA). The samples tested were local commercial milk fat and freeze-dried beef fat supplied by the Bovine Genomics Group, Department of Agricultural, Food and Nutritional Sciences, University of Alberta. Lipids were extracted with a mixture of chloroform: methanol according to the Bligh & Dyer procedure cited in reference [47,48]. FAMEs were prepared by acid-catalysed esterification using methanolic hydrochloric acid (Supelco; Bellefonte, PA, USA, cat 33050-U) according to a procedure described by Christie [48]. HPLC-grade hexanes, methanol, acetonitrile and isopropanol were purchased from Fisher Scientific Company (Ottawa, Ontario) and were used without further purification. All other solvents are of analytical grade unless specified.

3. Results and discussion

3.1. One dimension gas chromatography (1DGC)

The use of a recently commercialized ionic liquid GC column (SLB-IL100, 60 m) in the separation of FAME isomers was explored using a standard mixture of nine C18:1 isomers (Fig. 1A). It was found that four of the nine isomers were fully resolved but overlap of the 6-*cis*, 9-*trans* and 7-*cis* isomers as well as overlap of 9-*cis* and 13-*trans* isomers occurred. An important finding was that considerable separation of *cis* and *trans* isomers was observed consistent with recent findings of Ragonese et al. [49]. For example, complete separation of 11-*cis* and 11-*trans* C18:1 isomers was obtained as can be seen in Fig. 1A. For comparison, Fig. 2 shows the separation of a mixture of 9 isomers run isothermally on a 100 m SP-2560 column which is typical of the current best practice for separation of milk fat [25,27]. Three of the nine isomers elute together which indicates a comparable level of isomer separation. Similar results were published recently by Ragonese et al. [49]. However, although

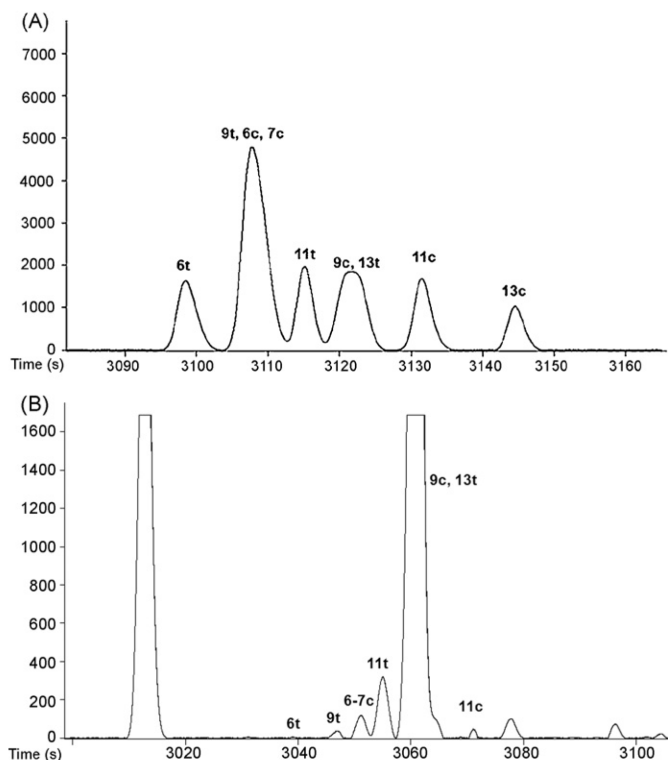

Fig. 1. (A) Chromatogram of nine C18:1 isomers (6t, 9t, 11t, 13t, 6c, 7c, 9c, 11c, 13c-C18:1) in 1DGC using SLB-IL100 column, CC #1 (see Table 1 for conditions). (B) Chromatogram of milk fat sample in 1DGC using SLB-IL100 column, CC #1 (see Table 1 for conditions).

useful for retention time matching and method development, such standard mixtures do not properly reflect the distribution of FAME isomers in real-world samples where the difference in concentration between isomers can be enormous resulting in conflict between overloading for some components versus detection limits for others. This is illustrated by a milk fat sample run on the 60 m SLB-IL100 column as shown in Fig. 1B. Although the major isomers can be identified (Fig. 1B), coelutions are evident. In addition, in attempting to avoid overloading the dominant oleic acid peak (9c-C18:1) minor components are lost and the width of the oleic acid peak is indicative of coelutions or the inability to completely avoid overloading.

Therefore, due to the challenges in achieving the desired level of separation using one-dimensional GC methods on complex FAME mixtures with disparate concentrations, we investigated two alternative two-dimensional methods, namely GC × GC/FID and Ag$^+$-HPLC/APPI-MS.

3.2. Method development

3.2.1. GC × GC

Standard mixtures of six and nine C18:1 FAME isomers were run in five different column systems (see Table 1). The polarity in the first dimension ranges from non-polar such as DB-Petro to highly polar BPX90 and SLB-IL100. However, in all the GC × GC experiments we report here the 2nd dimension is a 0.1 mm ID BPX-50 phase tested at various column lengths. BPX50, an intermediate polarity (50% phenyl polysilphenylene-siloxane), low-bleed phase, has been successfully used in the second dimension in previous work for the separation of fatty acids methyl esters, natural components, essential oils, drug analysis and oil [50–53].

The separation obtained with low polar–semipolar arrangements (Fig. 3A) seems to be poorer than the separation obtained

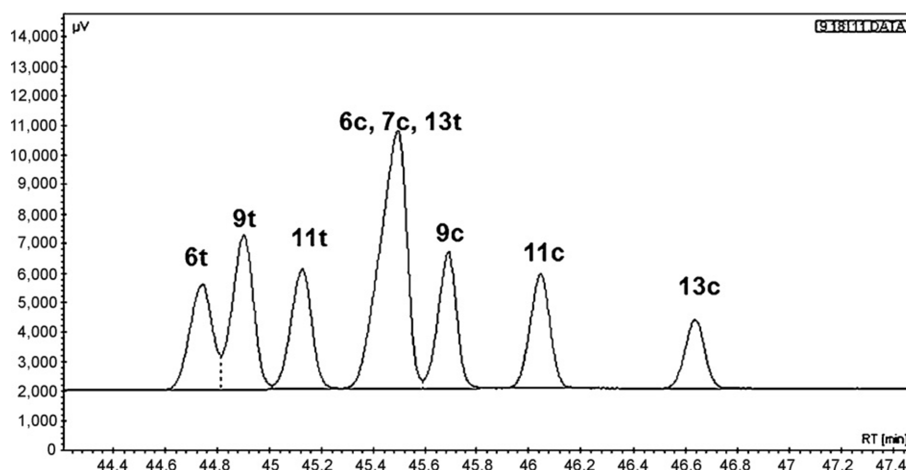

Fig. 2. Chromatogram of nine C18:1 isomers (6t, 9t, 11t, 13t, 6c, 7c, 9c, 11c, 13c-C18:1) using SP-2560 column, CC#2 (see Table 1 for conditions).

with polar–semipolar arrangements (Fig. 3B and C). Thus, as the polarity of the stationary phase in the first dimension is increased more separation space is available in two dimensions. This can clearly be seen in Fig. 4 which compares using the general purpose 5% phenylpolysiloxane phase with the ionic liquid SLB-IL100 phase in the first dimension. The SLB-IL100 column [1,9-di(3-vinylimidazolium) nonane bis(trifluoromethyl) sulfonyl imidate] is a new generation of highly polar stationary phases [54] recently available commercially. When highly polar columns of the same length are compared, it is noticeable that the ionic liquid column generates better separation between the nine C18:1 isomers (Figs. 3C and D and 5).

Despite the fact that previous work focused on separating FAMEs using a lower polarity column in the first dimension rather than in the second [32–35,38], the arrangement of a highly polar column in the first dimension may be more effective. Hyoetylaeinen et al. compared the performance of two column sets—one combination of polar/non-polar and the other non-polar/polar [13]. They claimed a mixed mechanism (volatility and polarity) of separation in both columns, thereby reducing the independence between the separations in both dimensions. Here, we observed that as the polarity of the first dimension is increased, whilst keeping the second dimension constant as the intermediate polarity BPX50, better resolution is achieved between the isomers. Thus, the SLB-IL100 which is more polar than BPX90 was more effective in separating C18:1 isomers in GC × GC. The dominant mechanism of isomer separation between them appears to be controlled by interaction with the polar stationary phase since the isomer boiling points are virtu-

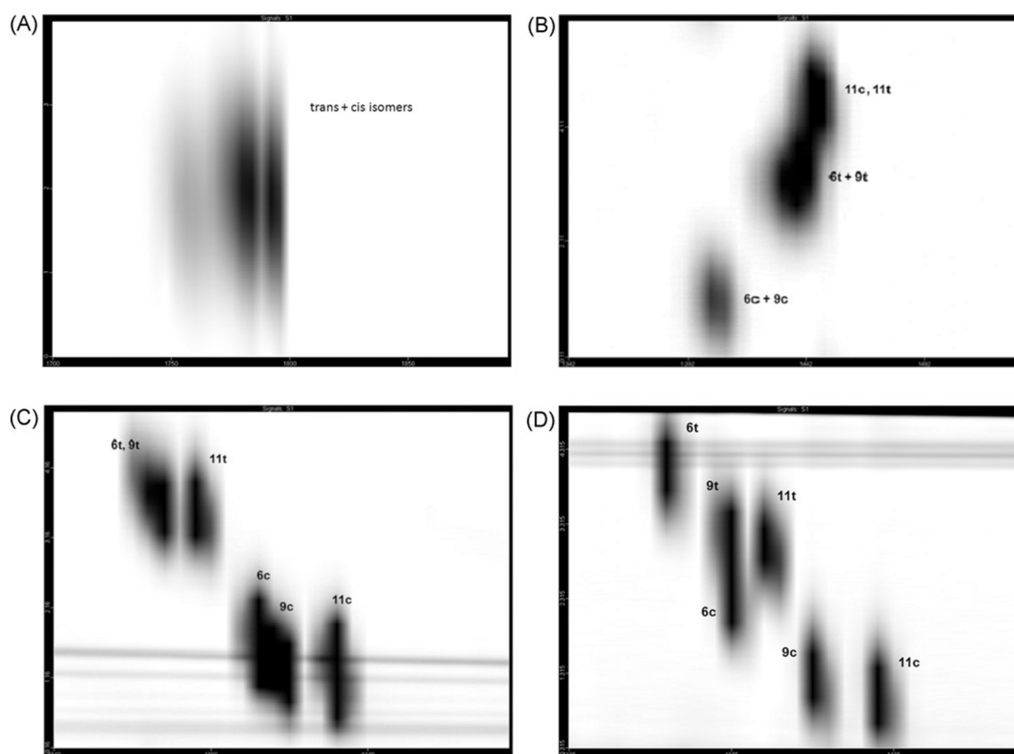

Fig. 3. (A) GC × GC contour plot of six C18:1 isomers (6t, 9t, 11t, 6c, 9c, 11c-C18:1) using DB-Petro–BPX50 columns, CC#3 (see Table 1 for conditions). (B) GC × GC contour plot of six C18:1 isomers (6t, 9t, 11t, 6c, 9c, 11c-C18:1) using Famewax–BPX50 columns, CC#4 (see Table 1 for conditions). (C) GC × GC contour plot of six C18:1 isomers (6t, 9t, 11t, 6c, 9c, 11c-C18:1) using BPX90–BPX50 columns, CC#5 (see Table 1 for conditions). D. GC × GC contour plot of six C18:1 isomers (6t, 9t, 11t, 6c, 9c, 11c-C18:1) using SLB-IL100–BPX50 columns, CC#7 (see Table 1 for conditions).

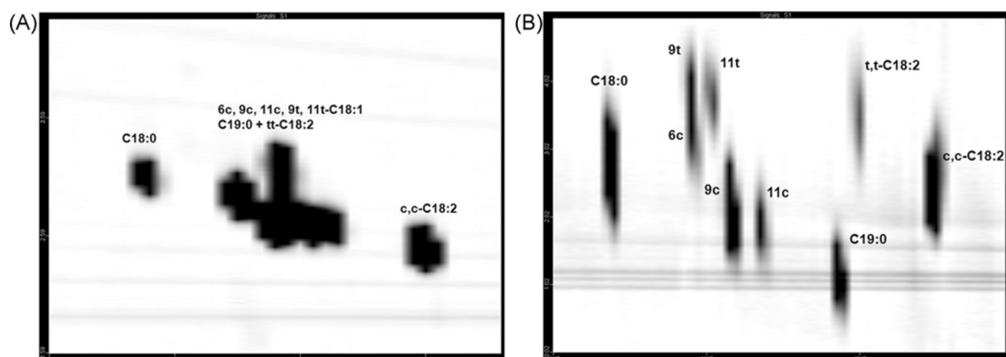

Fig. 4. (A) GC × GC contour plot of C18:1 region of mixture Nuchek 463 using Rxi-5–BPX50 columns, CC#6 (see Table 1 for conditions). (B) GC × GC contour plot of C18:1 region of mixture Nuchek 463 using SLB-IL100–BPX50 columns, CC#7 (see Table 1 for conditions).

ally the same. Hence, maximum isomer separation is not achieved by having a low polarity column (which separates by carbon chain length) in the first dimension.

When the second dimension is added to the 60 m ionic liquid column, seven of the nine C18:1 geometrical and positional isomers in the test mixture are completely separated (Fig. 5). Each *trans* isomer elutes ahead of its homologous *cis* isomer and this results in separation in both dimensions, as evident in Fig. 5. Overall, CC#7 resulted in a separation which is superior to the best achievable in 1DGC and was selected as the best combination tested in this work. In fact, for quantitative purposes better separation between 6c, 7c and 9t C18:1 isomers is achieved when the reconstructed one-dimensional chromatogram is viewed (see below) than is apparent in the contour plot in Fig. 5.

A particular objective of the work is to quantify *trans* vaccenic acid using a chromatographic system that completely resolves this isomer. A preliminary demonstration of the possibility of doing so by GC × GC, using the selected CC#7, is shown in Fig. 6. A linear calibration curve was constructed using tVA standards in the range of 100–400 pg on column using a C17:0 FAME internal standard with a correlation coefficient of 0.991. Fig. 6 shows a one-dimensional view of the GC × GC region around tVA. One feature typical of GC × GC is that multiple peaks (2 are shown) correspond to the single compound tVA FAME. This situation arises because the modulation period (in this case 5 s—the cycle time for the second column) is such that a single component may occur in two or more adjacent periods. Thus, two peaks are seen for tVA separated by 5 s. Of course, in constructing the two-dimensional contour plot, extrapolation between adjacent modulation periods results in a single peak. However, the availability of 2 peaks for use in quantification can be an advantage since both can be independently quantified and any discrepancy could be indicative of a partial coelution. A drawback of the ionic liquid column is the continuous bleed that is particularly evident in GC × GC and seen as horizontal lines across the contour plots in Fig. 5. In this particular case, the column bleed line does not interfere with the *trans* isomer peaks although it might potentially interfere in the quantification of *cis* isomers. Hyoetylaeinen et al. observed similar column bleed interferences using a polar–nonpolar arrangement (100 m CP7420 with 1.5 m HP-5MS) [13].

Overall, it was concluded that quantification of tVA in complex mixtures including both *cis* and *trans* isomers using the GC × GC method is feasible without the need for prior silver ion prefractionation. In this preliminary work, a detection limit of *ca.* 30 pg on column was readily achieved which is quite adequate to quantify levels of tVA at levels of <0.1% total fat.

3.2.2. Ag^+-HPLC/APPI-MS

It is well known that silver ions rapidly form complexes with unsaturated centres in organic molecules [55]. Such charge-transfer complexes are unstable and are formed reversibly which is favorable for use in a chromatographic system. The relative strength of the bonding involved in the complex is a function of the accessibility of participating orbitals so that for example methylene-interrupted dienes are held more strongly than conjugated dienes and *cis*-monenes are held more strongly than *trans*-monenes [55]. In this study, Ag^+-HPLC coupled to atmospheric pressure photoionization mass spectrometry was used for the separation of positionally and geometrically isomeric methyl octadecenoate.

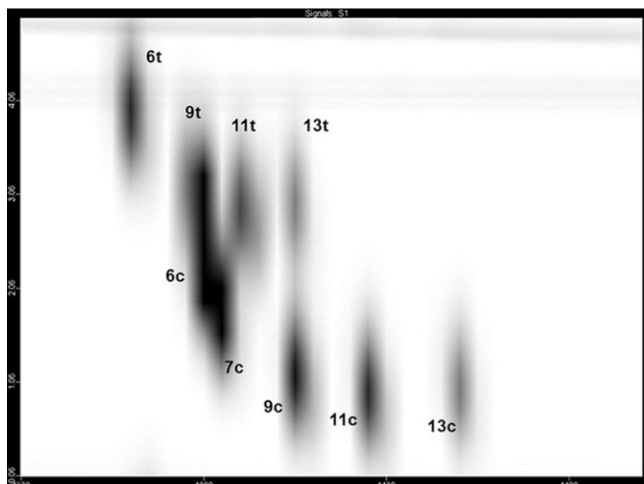

Fig. 5. GC × GC contour plot of nine C18:1 isomers (6t, 9t, 11t, 13t, 6c, 7c, 9c, 11c, 13c) using SLB-IL 100–BPX50 columns, CC#7 (see Table 1 for conditions).

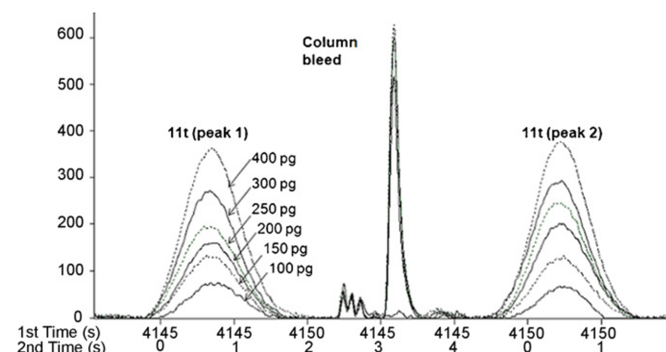

Fig. 6. One-dimensional reconstructed chromatograms for the calibration curve of tVA methyl ester. Overlaid chromatograms of 6 standards in the range of 100–400 pg.

Prior to optimizing the HPLC separation, several parameters that affect APPI-MS, such as the type of dopant, mobile phase flow rate and photoionisation lamp gas flow, were examined. Dopants of low ionization potential are routinely used to enhance ionization in APPI [56]. Using a syringe pump, a 5 mg/L solution of *cis* 9-methyl octadecenoate was infused into a mobile phase of hexane. Toluene was evaluated as the dopant in this study at a ratio of ~10:1 mobile phase to dopant. This experiment showed that toluene dopant complicated the mass spectrum of methyl octadecenoate, since in addition to the protonated molecular ion at nominal *m*/*z* 297, an ion at *m*/*z* 295 was observed at comparable intensity along with a weak ion at *m*/*z* 296. Thus, it seems probable that the toluene dopant promotes the formation of the odd-electron radical cation $M^{\bullet+}$, which subsequently loses a hydrogen atom to form the even electron fragment ion $[M-H]^+$. In addition, abundant fragment ions at *m*/*z* 263 (the even-electron fragment ion $[M-H-CH_3OH]^+$), *m*/*z* 119, and *m*/*z* 135 were observed likely due to the subsequent fragmentation of the reactive $M^{\bullet+}$. In contrast, when no dopant was added, the protonated molecule at *m*/*z* 297 was the base peak and little or no *m*/*z* 295 or other fragmentation was observed. Therefore, we chose not to use dopant in the following experiment. The use of hexane without the need for dopant for APPI is possible because the ionization energy of hexane lies below the 10.6 eV photon energy of the Kr lamp. The ability of various solvents commonly used in normal phase chromatography to be photoionized and hence useable in APPI without dopant has been described previously [57].

The flow rate of the make-up flow was found to affect significantly the intensity of the MH^+ ion of the infused *cis* 9-methyl octadecenoate (*m*/*z* 297). The make-up flow rate was increased over the range of 100–500 μL/min. Above the optimal value of *ca.* 200 μL/min, the response declined to about 50% of its maximum value when the flow rate reached 500 μL/min. A similar phenomenon has been reported in the literature [58,59]. A possible mechanism was presented by Robb and Blades [56] in which the higher flow rate resulted in the growth of ion–solvent clusters with greater solvation energies and less reactivity toward the target analyte. Another explanation proposed by Kauppila et al. [59] is that the loss of dopant radical cations at a higher flow rate was responsible for the sensitivity decrease in observed analyte ions formed through charge exchange, while greater photon absorption by the solvent at a higher flow rate was responsible for the loss in sensitivity of analyte ions formed through proton transfer.

Mobile phase composition is another important factor that can affect ionization efficiency in APPI. However, in the present experiment, the mobile phase is hexane with only a minor percentage of modifiers. Considering this and the resolution required to separate C18:1 FAME isomers, the mobile phase composition was optimized solely on the basis of achieving the best chromatographic performance on the silver ion column, as discussed below. In the design of the APPI source used here in addition to the nebulizer gas, a second flow of nitrogen (the lamp gas) passes through the heated region of the APPI probe past the lamp, which was found to affect significantly the response. Thus, the intensity of the *cis* 9-methyl octadecenoate MH^+ ion increased with an increase in the lamp gas flow rate (optimum *ca.* 4 L/min), likely due to more efficient desolvation of the cluster ions formed in this region.

A mixture of 9 methyl octadecenoate isomers including the *trans* 13-, 11-, 9-, 6- and *cis* 13-, 11-, 9-, 7-, and 6- isomers was prepared in hexane at a concentration of 6 μg/mL for each isomer. Then using the optimized APPI parameters and a single silver ion LC column, various mobile phase compositions were tested to achieve the best separation. Initially, a binary system of solvent A, 0.1% acetonitrile in hexane (v/v) and solvent B, 100% hexane was used. Thus, by changing the percentage of solvent A, the effect of acetonitrile concentration in hexane was examined. It was found that the *trans*-9/*trans*-6 isomers could not be separated when the concentration

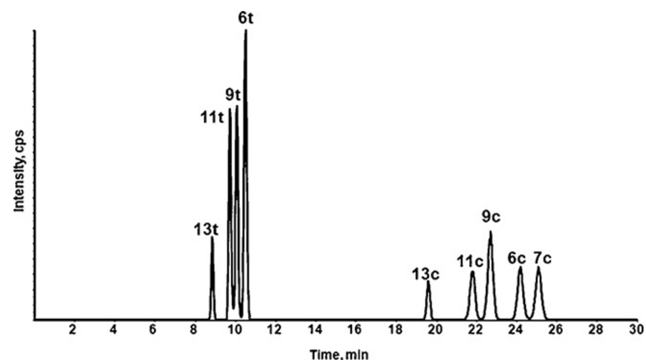

Fig. 7. Extracted ion chromatogram (XIC) of nominal *m*/*z* 297 of nine C18:1 isomers using optimized conditions using ChromSpher 5 Lipids column (250 mm × 2 mm i.d., 5 μm).

of acetonitrile was greater than 0.085% in hexane (i.e. 85% of A). With the concentration of acetonitrile at 0.08%, a good separation of all 9 isomers was obtained, but the retention time tended to become longer after each run. This may be explained by the very limited solubility of acetonitrile in hexane resulting in a decrease in the acetonitrile concentration and hence a change in retention behaviour over time. The instability of hexane–acetonitrile mobile phases was previously observed when a silver ion column was used to analyze linoleic acid isomers [60] and triacylglycerides [61]. Müller et al. [60] found that hexane–propionitrile mixtures showed a higher stability of retention time due to the better solubility of propionitrile in hexane compared to acetonitrile. Harfmann et al. [61] concluded that the loss of acetonitrile in hexane–acetonitrile mixtures due to evaporation was responsible for the inconsistent retention times observed. They further suggested that acetonitrile be replaced by butyronitrile as modifier in hexane.

In this study, in order to achieve baseline separation with reproducible retention times, we further added isopropanol to the mobile phase system. Isopropanol has a polarity in-between hexane and acetonitrile and is miscible in many polar and non-polar solvents. Thus, isopropanol may effectively increase the solubility of acetonitrile in hexane ultimately leading to more reproducible retention times in silver ion chromatography. It has been added to hexane–acetonitrile mixtures in the separation of triacylglycerols containing polyunsaturated fatty acids [62] and to hexane–dichlomethane as a modifier for the separation of *trans* 6-, 9- and 11- octadecenoic acid isomers as their *p*-methoxyphenacyl derivatives [43]. In the present study, mixtures containing *ca.* 0.1–0.4% isopropanol in hexane with various levels of acetonitrile were investigated. It was found that mixtures with more than ~0.1% isopropanol are required to get reproducible retention times. On the other hand, when the concentration of isopropanol exceeded ~0.3%, resolution between the *trans* 9/*trans* 6 and *cis* 9/*cis* 7 isomers was lost. Chromatographic resolution was then optimized using a binary system comprised of solvent A [hexane: acetonitrile: isopropanol (100:0.1:1.0, v/v/v)] and solvent B [100% hexane]. All 9 isomers were well separated with 18% of solvent A as mobile phase (Fig. 7) i.e. a final mobile phase composition of 0.018% acetonitrile and 0.18% isopropanol in hexane.

The mass analyzer in the combined Ag^+-HPLC/APPI-MS system used was a time-of-flight mass analyzer and hence full-scan mass spectra were always recorded. The extracted ion chromatogram of nominal *m*/*z* 297, the MH^+ ion for methyl octadecenoate isomers, is shown in Fig. 7. A great advantage of silver ion chromatography is the wide separation between *cis* and *trans* isomers as clearly seen in Fig. 7. The same elution order for *trans* 13-, 11-, 9-, 6- C18:1 as reported in the literature [42] was found in this study. However, the relative retention time of the *cis* 7-C18:1 isomer was not reported.

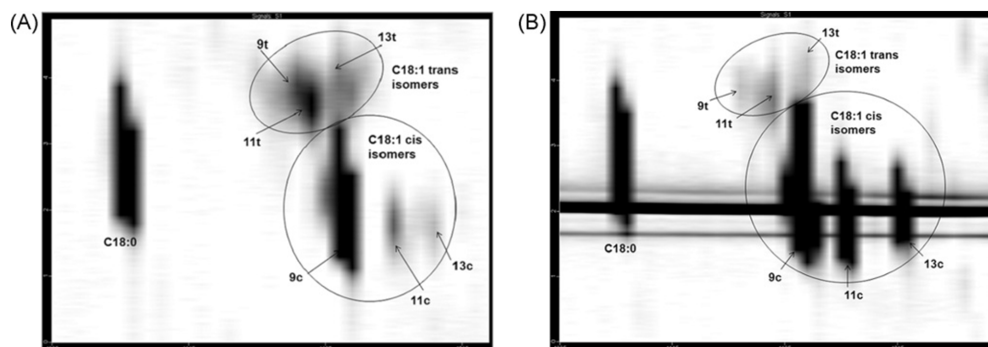

Fig. 8. (A) GC × GC contour plot of milk fat sample using SLB-IL100–BPX50 columns, CC#7 (see Table 1 for conditions). (B) GC × GC contour plot of beef fat sample using SLB-IL100–BPX50 columns, CC#7 (see Table 1 for conditions).

Here, it was found that the elution order in increasing retention time is *cis* 13-, 11-, 9-, 6- and 7- C18:1 methyl ester. Five replicate injections of a solution of the mixture of nine isomers were made in order to test the retention time stability. The relative standard deviation (RSD) of all retention times were around 1%, which indicated that the optimized solvent system containing isopropanol did achieve sufficiently reproducible chromatography.

3.3. Sample analysis

Lipid extracted from samples (see Section 2 for details) of milk fat and beef fat were converted to FAMEs and analyzed by GC × GC in order to quantify *trans*-vaccenic acid (Fig. 8). By using column combination #7 (Table 1), in both of these complex FAME mixtures adequate separation of tVA was achieved in order to allow for reliable quantification even in the presence of the much more abundant *cis* isomers. In both milk and beef fat *trans* -9, -11, -13 and *cis* -9, -11, -13 C18:1 isomers were positively identified (Fig. 8). In addition, other FAME C18:1 isomers were resolved but not definitively identified due to the lack of commercially available standards.

The Ag$^+$-HPLC/APPI-MS method was also used to identify and quantify tVA in the same milk and beef fat FAME samples. The extracted ion chromatogram of the ion at m/z 297 is presented in Fig. 9A and 9B. Note that in this experiment other monounsaturated compounds such as C20:1 FAME isomers may coelute with C18:1 isomers, and hence it is essential to not only extract the m/z of the compound of interest but also to ensure that this is actually the molecular ion rather than a fragment ion of a coeluting species. However, FAMEs with more than one double bond elute later than the C18:1 isomers and hence are not interferences. For example, m/z 295 arising from 18:2 FAMEs cannot be observed in the retention time range shown in Fig. 9.

In this experiment, two criteria can be used to identify target compounds: (1) a retention match with authentic standards and (2) a low deviation between the measured exact mass and calculated mass. These two criteria enabled us to identify the isomers with a high degree of confidence. For both beef and milk fat samples, *trans* 13-, 11-, 9- and 6- C18:1 FAME isomers were identified. A peak eluting at ~9.2 min, between *trans*-13 and *trans*-11 C18:1 FAME was also observed and assumed to be *trans* 12-C18:1 FAME although this cannot be confirmed by the present experiment in the absence of an authentic standard. Other significant peaks belonging to *trans* isomers of C18:1 FAMEs are evident in Fig. 9. For example the peak at 8.26 min in the milk fat sample (Fig. 9A) is identified as a *trans* C18:1 FAME and although a probable identification can be made by extrapolation of the elution order from known isomers, additional standards (or complementary experiments) are needed to confirm the position of unsaturation. *cis* isomers of C18:1 FAMEs were found in milk and beef fat in high concentrations as expected.

The concentration of the *trans*-9 C18:1 isomer is comparable to the *trans*-11 isomer in beef, while the *trans*-11 isomer is the dominant component in milk. However, we cannot rule out the possibility of coelution between *trans*-9 and *trans*-10 isomers in the absence of a standard of the *trans*-10 isomer. These results are consistent with GC/FID data. Overall, this preliminary study indicates that the Ag$^+$-HPLC/APPI-MS method has good potential for the qualitative determination of individual positional and geometrical isomers of C18:1 FAMEs in food samples. A quantitative method validation for this approach is underway and will be presented in a future communication.

A comparison between the established 1DGC and the two methods described here is shown in Table 2. Clearly, GC × GC is advantageous compared to 1DGC since it allows better separation of geometrical isomers thus avoiding ambiguity in quantitation. It also retains the well-known advantages of the FID detector which is

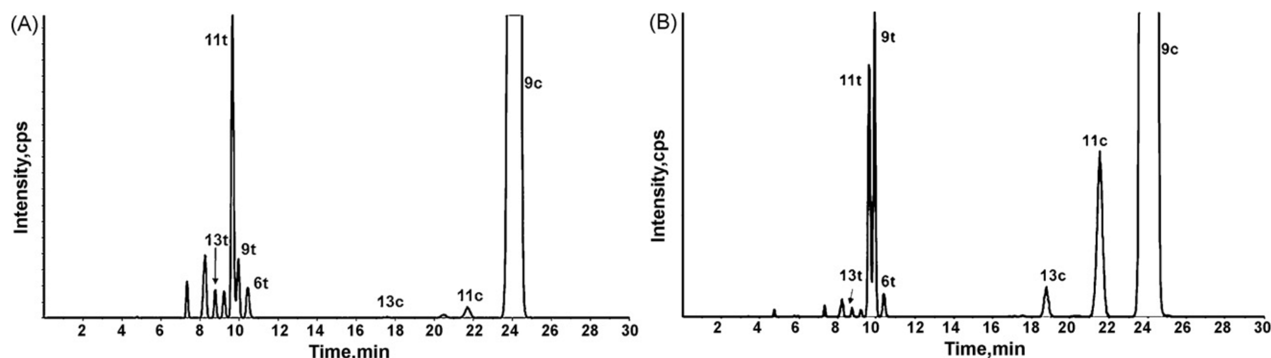

Fig. 9. (A) Extracted ion chromatogram (XIC) of nominal m/z 297 for milk fat. (B) Extracted ion chromatogram (XIC) of nominal m/z 297 for beef fat.

Table 2
Comparison between separation systems.

	1DGC	GC × GC	Ag+-HPLC-APPI-MS
Separation of *cis*/*trans* isomers	–	++	+++
Retention time reproducibility	+++	+++	+
Ease of quantititation	+++	++	+[a]
Separation of positional and geometrical isomers in complex matrices	+	++	+++
Chromatographic resolution of *cis* -6 and -7 C18:1 isomers	–	++	+++
Identification of possible co-elutions	–	+	+++[b]
Typical run time	40–60 min	40–60 min	30 min
Range tested	200–500 ng[c]	5–40 ng[c]	5–100 ng

[a] Good for C18:1 isomers but response factors vary greatly for different unsaturation degree.
[b] Advantage of mass spectrometry for identification.
[c] Before a split ratio of 100:1.

widely available, reliable, and has a wide dynamic range and predictable response. However, care must be taken not to overload the microbore second dimension column, which effectively limits the available dynamic range for quantification. The coupling of GC × GC with a fast-scanning mass spectrometer (not available for the present work) would have obvious advantages for compound identification. However, since molecular ions of FAMEs are often weak in electron impact (EI) mass spectra, chemical ionization (CI) techniques would likely be required.

Ag^+-HPLC/APPI-MS has advantages including shorter run times, total separation of geometrical isomers and the benefit of mass spectrometry to identify co-elutions. However, obtaining stable retention times requires careful attention to the solvent composition, and without making use of the additional dimension of separation afforded by mass spectrometry, identification of C18:1 isomers would not be possible.

4. Conclusions

Two alternative methods for the identification and quantification of tVA and other isomeric *cis* and *trans* C18:1 FAMEs have been demonstrated. Both have the potential to overcome the limitations of the 1DGC method without resorting to fractionation of FAME mixtures. For a definitive quantification of tVA or other FAME isomers, such as in the development of a natural matrix reference material, the combination of results from these two orthogonal techniques could produce reference values with a high degree of confidence not possible by one-dimensional techniques. However, further work is required to validate these techniques for quantitative purposes. To illustrate their potential to determine individual positional and geometrical FAME isomers in food samples, the tVA content of milk and beef fat FAME was investigated.

Ag^+-HPLC/APPI-MS has been shown to be a rapid, specific and reproducible method to determine positional and geometrical isomers of octadecenoic acid FAME. Using a single commercially available silver ion column, nine C18:1 FAME isomers were resolved to baseline separation in less than 30 min. Higher chromatographic resolution could have been achieved by using multiple columns, as has been demonstrated in separations of CLA isomers [63]. Careful control of mobile phase composition allows acceptable retention time stability, which is essential for isomer identification. APPI was found to be a very stable and reliable method for ionization of FAMEs and the combination with accurate mass determinations results in high confidence in peak assignments.

GC × GC experiments were found to allow great versatility in separating complex mixtures in a single run and were very reproducible in retention times in both dimensions. When a standard mixture of C18:1 FAME isomers at similar concentrations was analyzed by an optimized GC × GC method complete separation between *cis* and *trans* C18:1 isomers was achieved along with good separation between positional isomers. The arrangement of a highly polar column in the first dimension and a semi-polar column in the second dimension was found to be more effective in separating C18:1 isomers than nonpolar–polar arrangements. The successful use of a recently commercialized ionic liquid column for FAME analysis was demonstrated.

Acknowledgements

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