Friedrich-Schiller-Universität Jena

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Development of novel methods for separation of trans fatty acids and other nutritionally relevant or related fatty acids

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INDEX OF CONTENTS

PERMEAMBLE

LIST OF ABBREVIATIONS

AOCS	American Oil Chemists' Society
Ag^{+}-HPLC	silver ion-high performance liquid chromatography
c	cis
CI	chemical ionization
CLA	conjugated linoleic acid
CPS	cyanopropylsiloxane
1D	first dimension
2D	second dimension
2D-GC	two-dimensional gas chromatography
DHA	docosahexaenoic acid
DMOX	4,4-dimethyloxazoline
DMA	dimethylacetals
ECL	equivalent chain length
EI	electron impact ionization
EPA	eicosapentaenoic acid
FA	fatty acid(s)
FCL	fractional chain length
FD	furane dimethyl
FDA	Food and Drug Administration
FAME	fatty acid methyl ester(s)
FFA	free fatty acid
FID	flame ionization detector
FM	furan mono-methyl
FT-IR	Fourier transform infrared spectroscopy
GC	gas chromatography
HPLC	high performance liquid chromatography
IR	infrared
MDGC	Multidimensional gas chromatography
MI	methylene interrupted
MS	mass spectrometry
MUFA	monounsaturated fatty acid
NMI	non-methylene interrupted
PEG	polyethylene glycol
PUFA	polyunsaturated fatty acid
RT	retention time
RTIL	room temperature ionic liquid
SFA	saturated fatty acid
SLB	Supelco low bleed (commercial name)
t	trans
TMTD	tetramethyltridecanoate
TOF	time of flight
TSM	Thermal-Sweeper-Modulator
US	United States
*c*9,*t*11-18:2	*cis*9,*trans*11-18:2

LIST OF MANUSCRIPTS

CHAPTER 2

Preparation, chromatographic separation and relative retention times of *cis/trans* heptadecaenoic (17:1) fatty acids

Pierluigi Delmonte, Qing Hu, Ali Reza Fardin-Kia, Jeanne I. Rader
Journal of Chromatography A 1214, 30-36 (2008)

Content

In this study, a simple procedure for obtaining, both geometric and positional isomers pure or in mixtures of a monounsaturated fatty acid was developed. *cis* 10–17:1 Fatty acid methyl ester (FAME) was isomerized into its positional/geometrical isomers by repeated hydrobromination/dehydrobromination of its double bond. Reaction products were fractionated into *cis* and *trans* geometric isomers by silver ion HPLC. Pure *cis*-17:1 FAME positional isomers were obtained by reversed-phase HPLC fractionation and identified by gas chromatography – covalent adduct chemical ionization MS/MS using acetonitrile as the reacting gas. The isomerization with *p*-toluenesulfinic acid of the purified FAME yielded the corresponding *trans* isomers. These products were analyzed by GC with flame ionization detection using a Supelco 2560 capillary column in order to determine their elution order and retention times.

WORK	CONTRIBUTION
Study accomplishment	70%
Analyses (synthesis; GC; GC/MS; HPLC)	50%
Sample preparation	60%
Preparation of the manuscript	60%

CHAPTER 3

Review of Methods for Preparation and Gas Chromatographic Separation of *trans* and *cis* Reference Fatty Acids

Pierluigi Delmonte, Ali Reza Fardin-Kia, Qing Hu, Jeanne I. Rader
Journal of AOAC INTERNATIONAL 92, 1310-1326 (2009)

Content

Fatty acid (FA) analysis is affected by the limited availability of reference materials. Identifications are frequently made simply by comparison with separations reported in the literature. This review described the preparation of mixtures containing fatty acid methyl esters (FAMEs) that are not available as reference materials. These mixtures were used for FAMEs identifications. The prepared mixtures were analyzed under the experimental conditions of the American Oil Chemists' Society (AOCS) Official Method Ce 1h-05 and AOCS Recommended Practice Ce 1j-07.

WORK	CONTRIBUTION
Study accomplishment	80%
Analyses (synthesis; GC; GC/MS; HPLC)	80%
Sample preparation	90%
Preparation of the manuscript	80%

CHAPTER 4

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

Pierluigi Delmonte, Ali Reza Fardin-Kia, John K.G. Kramer, Magdi M. Mossoba,
Len Sidisky, Jeanne I. Rader
Journal of Chromatography A 1218, 545-554 (2011)

Content

In this study, a new gas chromatographic method was developed and a novel highly polar ionic liquid capillary gas chromatography column (200 m SLB-IL111) was evaluated. This method, using a 200 m SLB-IL111, provided an improved separation of *cis*- and *trans*-18:1 and *cis/trans* conjugated linoleic acid (CLA) isomers. The isomers *c*9,*t*11- from *t*7,*c*9-CLA, and *t*15-18:1 from *c*9-18:1 were separated in a single GC run without the need for complimentary separation techniques such as silver ion HPLC. 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 GC method also contributed elution profiles of the geometric and positional isomers of the 16:1, 20:1 and 18:3 fatty acid methyl esters that were complementary to those obtained using the cyanopropyl siloxane columns.

WORK	CONTRIBUTION
Study accomplishment	90%
Analyses (GC; GC/MS; HPLC)	80%
Sample preparation	90%
Preparation of the manuscript	70%

CHAPTER 5

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

Pierluigi Delmonte, Ali Reza Fardin-Kia, John K.G. Kramer, Magdi M. Mossoba, Len Sidisky, Cynthia Tyburczy, Jeanne I. Rader

Journal of Chromatography A 1218, 545-554 (2012)

Content

A 200 m SLB-IL111 capillary column, operated under a combined temperature and eluent flow gradient, was successfully used to resolve most of the fatty acids (FA) 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 (PUFA), and most of the unsaturated FA positional/geometric isomers contained in milk fat. Among the monounsaturated fatty acids (MUFA), these conditions separated *t*11-18:1 and *t*10-18:1 FA, the two most abundant *trans* fatty acids (TFA) contained in most dairy products. 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.

WORK	CONTRIBUTION
Study accomplishment	60%
Analyses (GC; GC/MS; HPLC)	90%
Sample preparation	50%
Preparation of the manuscript	60%

CHAPTER 6

Separation of Fatty Acid Methyl Esters by GC-Online Hydrogenation × GC

Pierluigi Delmonte, Ali Reza Fardin-Kia, and Jeanne I. Rader
Analytical Chemistry 1218, 545-554 (2013)

Content

The separation of fatty acid methyl esters (FAMEs) provided by a 200 m x 0.25 mm SLB-IL111 capillary column was enhanced by adding a second dimension of separation (2D) in a GC×GC design. A novel capillary reducer containing palladium was developed and added between the two dimensions. The two dimensional separation was easily interpreted based on the principle that all the saturated FAMEs were located on a straight diagonal line bisecting the separation plane, while the FAMEs with the same carbon skeleton but differing in the number, geometric configuration or position of double bonds were on lines parallel to the 1D time axis. This technique allowed the separation of *trans* fatty acids and polyunsaturated FA (PUFA) in a single experiment and eliminated the overlap between PUFA with different chain lengths.

WORK	CONTRIBUTION
Study accomplishment	70%
Analyses (GCxGC; GC/MS; HPLC)	90%
Sample preparation	100%
Preparation of the manuscript	60%

CHAPTER 7

Separation of the fatty acids in menhaden oil as methyl esters with a highly polar ionic liquid gas chromatographic column, and identification by time of flight mass spectrometry

Ali Reza Fardin-Kia, Pierluigi Delmonte, John K.G. Kramer, Gerhard Jahreis, Katrin Kuhnt, Viviana Santercole, Jeanne I. Rader

Lipids, just accepted (July/27/ 2013)

Content

The identification and separation of poly unsaturated fatty acid methyl esters prepared from menhaden oil was achieved by GC-FID, GC-TOF/MS and use of a 200 m SLB-IL-111 ionic liquid gas chromatography capillary column. The presence of furan fatty acids and multi-branched chain fatty acids was confirmed by high resolution GC-TOF/MS in chemical ionization mode with isobutane as the mild ionization reagent gas. For the first time, in a single GC run more than 100 different fatty acids were separated and determined in < 100 min.

WORK	CONTRIBUTION
Study accomplishment	100%
Analyses (GC-FID; GC-MS)	90%
Sample preparation	100%
Preparation of the manuscript	70%

CHAPTER 8

Characterization of Pine Nuts in the U.S. Market, Including Those Associated With "Pine Mouth", by GC-FID

Ali Reza Fardin-Kia, Sara M. Handy, and Jeanne I. Rader

Journal of Agricultural and Food Chemistry 60, 2701-2711 (2012)

Content

The fatty acid (FA) composition of 15 reference pine nuts, including their Δ5 fatty acid content, was determined by use of a novel GC capillary ionic liquid column. Furthermore the applicability of a diagnostic index (DI), a ratio of the Δ5-unsaturated FA relative to that of their fatty acid precursors, for the identification of different pine species was examined. More accurate DIs were calculated and the limitation of DI for identification purposes was shown.

WORK	CONTRIBUTION
Study accomplishment	100%
Analyses (GC-FID/HPLC)	90%
Sample preparation	80%
Preparation of the manuscript	80%

CHAPTER 1

INTRODUCTION AND OBJECTIVES

CHAPTER 1

1 INTRODUCTION AND OBJECTIVES

Scientific research over the last two decades has demonstrated relationships between dietary intake of certain fatty acids (FA) with double bonds in the *trans* configuration in their alkyl chain (*trans* fatty acids, TFA), and the development of severe metabolic or cardiovascular diseases such as arteriosclerosis, hypertension and diabetes mellitus type-2 (Mozaffarian et. al 2006 & 2009). In response to such findings, several countries including Denmark, Canada and the United States have implemented regulations regarding limitation and labelling of TFA content of fats, oils, foods and dietary supplements.

Recent studies have shown that intake of TFA with different chemical structures results in different metabolic and health effects. Dietary intake of *c*9,*t*11-18:2 and its biological precursor *t*11-18:1 (vaccenic acid) has been credited with positive health effects [Field et al., 2009]. In contrast, consumption of dietary *t*10,*c*12-18:2 has been associated with hyperinsulinemia and fatty liver in mouse studies [Clément et. al 2002].

However, a critical common limitation of these studies has been the availability of authentic reference materials and appropriate analytical methods to quantify the subject TFA and their metabolites. Organic synthesis of the possible FA isomers involved can play a key role in the correct identification and analysis of these TFA. Misidentification of analytes can lead to an incorrect evaluation of the health effects associated with specific FA, and therefore pure and well-characterized reference materials are essential. A classic case was reported when an overestimation of *trans*-16:1 isomers due to inadequate separation techniques resulted in an association of these isomers with coronary heart disease [Precht et al., 2000a]. It is interesting to note that *trans*-16:1n-7 has also been associated with lower insulin resistance [Mozaffarian et al., 2010]. A clear identification of the *trans*-16:1 isomers is definitely required in order to understand its biological significance.

Furthermore, due to rapid developments in the field of food technology, the compositions of many foods, including infant formulas and adult nutritional products, are becoming increasingly complex. Improved analyses are necessary to provide accurate results to label these products. More accurate labelling will contribute to the information available to consumers for use in choosing healthier diets and reducing the risk of chronic disease.

The main objectives of this dissertation are:

1) Development and Description of novel and powerful analytical methodologies for the analysis of FA from multiple food sources.

2) The synthesis of numerous FA reference materials for the identification of FA present in common foods.

This work will provide critical support for future studies in the field of lipid chemistry.

1.1 TRIGLYCEROLES AND FATTY ACIDS

1.2 TRIGLYCEROLES

Triacylglycerols (TAG) are the most abundant lipid components of plants and animals, and they perform critical functions in their metabolism. They consist of glycerol, a tri-functional alcohol with one fatty acid esterified to each of the alcoholic groups (FIGURE 1).

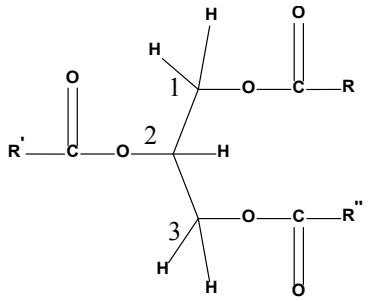


FIGURE 1 A typical mixed triglyceride structure with three different fatty acids (R, R' and R'').

More than 90% of commercial oils and fats used for human consumption are plant-derived and are extracted from various seeds. With few exceptions, in order to reach the organoleptic and stability properties needed for commercialization, crude oils are subjected to various processing steps including degumming, bleaching and deodorization. The refining of crude oils increases the TAG content to about 99% [Kodali, 2005].

Triacylglycerols are synthesized by the human body and stored in the cytoplasm of adipocytes and are then used to produce energy when needed. The oxidation of the fatty acids (FA) contained in TAG produces a large amount of energy, making the production and storage of energy the key functionalities of TAG in human metabolism. Furthermore, TAG are important components of cell membranes and provide the precursors for the synthesis of eicosanoids, and other biologically active molecules. As FA constitute the building blocks of TAG, the FA composition of TAG plays an important role in defining their biological, chemical and physical properties.

If the three FA constituting the TAG are identical, the product is a *simple* TAG. Otherwise, the TAG is defined as a *mixed* TAG. The esterification of only one alcoholic moiety leads to the formation of mono-acylglycerols (MAG), and the esterification of two alcoholic moieties leads to the formation of di-acylglycerols (DAG).

1.3 FATTY ACIDS

Fatty acids are defined as carboxylic acids with long straight aliphatic chains. The term “fatty acids” includes the entire series of straight chain carboxylic acids beginning with formic acid (C1), acetic acid (C2), propionic acid (C3), butyric acid (C4) and continuing to longer chain FA such as stearic acid (C18) or arachidic acid (C20). The lower four members of the series (C1-C4) are hydrophilic substances and are completely miscible with water.

The FA composing TAG may differ in their chain lengths, the presence of double bonds (unsaturations) on the aliphatic chain, additional functional groups such as hydroxyl, ketones and branched chains (*iso*- and *anteiso*- FA), and occasionally cyclic structures. The double bonds located on the aliphatic chains of the naturally occurring FA are predominantly in the *cis* configuration. With a few exceptions described later, *trans* double bonds are produced during processing or handling of fats and oils. The double bond configuration is a flat rigid

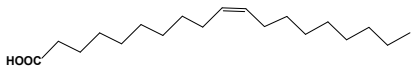
structure. In *cis* double bonds, the two aliphatic chains lie on the same side of the bond, while in *trans* double bonds, the two aliphatic chains are attached across the double bond (FIGURE 2).

cis configuration *trans* configuration

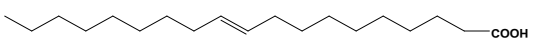
FIGURE 2 *Cis* and *trans* double bond configurations.

Fatty acids with *trans* double bonds are characterized by a more linear structure than FA in which the double bonds occur in the *cis* configuration, reflecting a greater ability of their aliphatic chains and a greater ease of crystal formation. Fatty acids with double bonds in the *trans* configuration are characterized by higher densities and higher melting points compared to the same fatty acids with their double bonds in the *cis* configuration. The lower steric hindrance of *trans* double bonds results in higher thermodynamic stability that leads to the preferential conversion of *cis* double bonds to *trans* double bonds. The inter-conversion of double bonds between the *cis* and *trans* configurations requires breaking and rebuilding of the double bond, a process that requires an activation energy of about 65 kcal/mole. The activation energy barrier needed for the *cis*/*trans* isomerization can be reached by providing energy in the form of heat, as is the case of the deodorization of oils. When the isomerization of double bonds from the *cis* to *trans* configuration is desired, the introduction of a catalyst reduces the inter-conversion energy barrier.

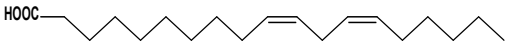
Almost all FA with multiple unsaturation sites (poly-unsaturated FA, PUFA) synthesized by plants and animals have the double bonds separated by one or more methylene groups. These FA are defined as methylene-interrupted (MI) FA (FIGURE 3), and the location of double bonds is commonly indicated using the "Δ" nomenclature, indicating the position of each of them [Christie, 2003]. Alternatively, the n-x nomenclature is used which indicates the distance of the last methylene interrupted (MI) double bond from the terminal end of the aliphatic chain. Linoleic acid is an MI FA with 18 carbons and two *cis* double bonds between carbons C9-C10 and C12-C13 that can be labelled as *c*9,*c*12-18:2 or 18:2n-6. According to the "n" nomenclature, naturally occurring polyunsaturated fatty acids (PUFA) are designated by the chain length followed by a colon and the number of double bond starting from the methyl end of the molecule. In the most common PUFA, the first double bond occurs naturally either at the 3rd (i.e., 18:3 n-3 or *c*9,*c*12,*c*15-18:3), 6th or 9th position from the methyl end of the molecule (FIGURE 3). The term "positional isomers" is commonly used to indicate FA with the same chain length and number of double bonds, but with the latter are in different positions. The term "geometric isomers" indicates FA with the same chain length, same number of double bonds in the same position, but with at least one of the double bonds is in the opposite geometric configuration. There are additional common FA, known as conjugated, in which the double bonds are separated by a single C-C bond. This will be dicussed later.



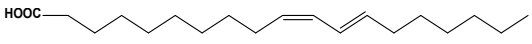
cis 9-18:1
18:1 n-9
Oleic acid



trans 9-18:1
trans 18:1 n-9
Eleidic acid



cis 9, *cis* 12-18:2
18:2 n-6
Linoleic acid



cis 9, *trans* 11-18:2
conjugated linoleic acid (CLA)

FIGURE 3 Molecular structures of common fatty acids.

1.4 ESSENTIAL FATTY ACIDS

Essential FA are those mammals are unable to synthesize. Mammals are only able to synthesize saturated and monounsaturated FA from other precursors and are unable to synthesize linoleic- (*c*9,*c*12-18:2, LA) and γ-linolenic (*c*9,*c*12,*c*15-18:3. γ-LA) acids.

Burr et al. demonstrated that when immature rats were fed a fat-free diet, they grew poorly, lost hair and ultimately died, unless a curative amount of fat was fed [Burr et al. 1929]. Burr et al. showed in another study that none of the saturated fatty acids occurring in hydrogenated coconut oil were effective in curing the disease [Burr et al. 1930]. The animals recovered when pure methyl linolate was administered. The most abundant essential FA in mammals is LA, which makes up10-20 % of the total FA of their TAG and phosphoglycerols [Lehninger, 1978]. As mentioned above LA and γ-LA cannot be synthesized by mammals and must be obtained from plant sources, in which they are abundant. LA is a necessary precursor in mammals for the biosynthesis of arachidonic acid (c5,c8,c11,c14-20:4), which is available in plants. The essential FA are important precursors in the biosynthesis of a group of bioactive fatty acid derivatives called prostaglandins [Grish et al. 1972]. These hormonelike compounds have profound effects on numerous physiological activities.

1.5 TRANS FATTY ACIDS

Trans fatty acids are defined as FA with at least one non-conjugated double bond in the *trans* configuration. Fatty acids with a *trans* double bond are normally designated using the systematic "Δ" nomenclature (i.e., *t*9-18:1). Some examples of PUFA containing *trans* double bonds are *c*9,*t*12-18:2 and *t*9,*c*12,*c*15-18:3 as shown in Figure 3.

The naturally occurring unsaturated FA in vegetable oils contain double bonds in the *cis* configuration. The unsaturated *cis* FA are relatively asymmetric due to the presence of the rigid *cis* "U" shaped double bonds in the middle of the hydrocarbon chain (FIGURE 4). The presence of the *cis* double bonds reduces the inter-chain interactions of the FA, causing a reduction in the melting temperature. Despite their high molecular weights, at room temperature, triacylglycerols containing "all *cis*" PUFA are in the liquid state.
In comparison, FA with *trans* double bonds on the aliphatic chain are more symmetric and the inter-chain interactions are more consistent. These stronger interactions increase the melting temperature of FA with *trans* double bonds and TAG composed of FA with *trans* double bonds are likely to be in the solid state at room temperature.

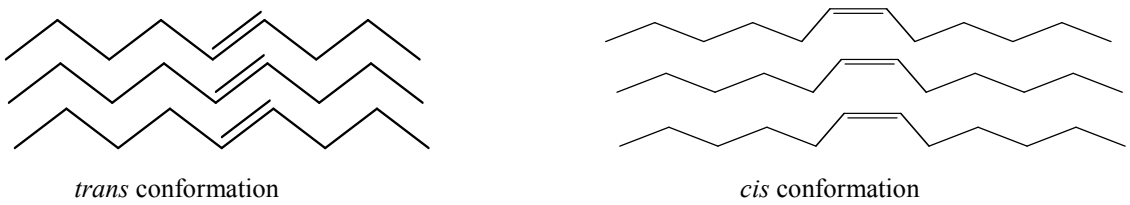


FIGURE 4 The molecular conformation and chain packing of lipid molecules containing *cis* and *trans* isomers.

Trans double bonds also occur in conjugated form in certain unsaturated FA. The major conjugated FA in ruminant fats is *c*9,*t*11-18:2 or conjugated linoleic acid (CLA) [Parodi et al., 1977; Kramer et al., 1998]. The CLA structures in natural products can include positions from 6,8 to 12,14, and most of them with all possible geometric configurations of *cis/cis*,

cis/trans, trans/cis, and trans/trans [Sehat et al., 1999]. Ruminant fats can also contain C18:3 structures in which any two adjacent double bonds are conjugated, such as *c*9,*t*11,*c*15-18:3 [Hobson and Stewart, 1997].

Certain vegetable oils contain a relatively high amount of PUFA which can react with oxygen to form hydroperoxides and other oxidation products [Farmer et. al, 1943; Gardner, 1989]. To improve their oxidative stability or to obtain specific physical functionalities (i.e., melting point near human body temperature), these oils are often subjected to partial or full hydrogenation. The process of hydrogenation reduces some of the double bonds and isomerizes some of the remaining ones from their *cis* to *trans* configurations. The hydrogenation of oils and fats improves their oxidative stability, extends their shelf life, and increases their solid fat content, thereby producing the margarines and shortenings used for baking applications [Singh et al., 2009; Dijkstra, 2006].

1.5.1 INDUSTRIALLY PRODUCED *TRANS* FATTY ACIDS

The process of partial hydrogenation consists of a three phase reaction in which hydrogen gas is introduced into an oil in the liquid form, and a catalyst consisting of a slurry of solid particles is added into the oil [Singh et al. 2009]. Partial hydrogenation is carried out in a special autoclave by dispersing nickel catalyst into the oil at a temperature of 110 °C or more, and providing hydrogen at pressures of 30-70 psi. The most commonly used catalysts are nickel based and are added in the quantities of 0.01-0.15% wt/wt [Veldsink et al. 1997]. The selection of the specific reaction conditions (e.g., hydrogen pressure, catalyst type, and processing temperature) can favor or minimize the formation of TFA. Previously, partial hydrogenation represented the major source of industrially produced TFA (IP-TFA) and certain fats used for bakery products contained more than 40% TFA (% of total fat).

The deodorization of oils and fats during the refining process is also known to produce up to 3% TFA [Ackman et al. 1974, Wolff, 1992]. The extended heating of oils and fats during deep frying of foods can also produce TFA, causing predominantly the isomerization of linoleic acid and linolenic acid present in most frying oils [Sebedio et al. 1996]. Frying of fish products can result in the formation of *trans* containing highly unsaturated PUFA present in fish [Fournier et al., 2006a; Mjos 2006a].

1.5.2 *TRANS* FATTY ACIDS PRESENT IN RUMINANT FATS

Other sources of TFA are dairy products and the meat of ruminant animals. The microorganisms present in the rumen subject PUFA to isomerization/biohydrogenation [Hobson and Stewart, 1997]. The process, catalyzed by microorganisms, converts linoleic acid (*c*9,*c*12-18:2) to *c*9,*t*11-18:2 and vaccenic acid (*t*11-18:1). Vaccenic acid is the most abundant TFA present in the meat and milk of pasture fed ruminant animals [Precht and Molkentin 1996; Kraft et al., 2003; Leheska et al., 2008]. The feeding of concentrates rich in digestible starch and PUFA causes a shift in the isomerization/biohydrogenation process in the bacteria to produce more *t*10-18:1 than *t*11-18:1 that accumulate in ruminant products [Grinarri et al., 1998; Cruz-Hernandez et al., 2007]. The supplementation of the animal's diet with partially hydrogenated vegetable oils (PHVO) causes the increase in the content of other TFA such as *t*9- and *t*10-18:1 compared to vaccenic acid [Wonsil et al., 1994].

The total *trans* mono-unsaturated FA content in milk lipids may reach as high as 23% as percent of total milk fat depending on the diet used for feeding the dairy cows [Cruz-Hernandez et al., 2007]. These include several *trans* positional isomers of 16:1, 18:1, 20:1, 22:1 and 24:1 FA [Precht and Molkentin, 1996; Wolff, 1998; Kramer, 2008].
The TFA found in milk of pasture-fed cows consist predominantly of vaccenic acid [Precht, Molkentin 1997], whereas increasing amounts of *t*10-18:1 are evident in the milk fat of cows fed forages rich in nonstructural carbohydrates (e.g. starches, sugars, fructans) [Piperova et al., 2000], oils rich in PUFA [Griinari et al., 1998; Shingfield et al., 2005] and ionophore antibiotics, such as monensin [Eifert et al., 2005; Cruz-Hernandez et al., 2006].
The fats derived from ruminants (e.g., milk or meat) are the main source of CLA in human nutrition. *c*9,*t*11-18:2 is the most abundant CLA isomer contained in these fats [Sehat et al., 1999; Parodi, 2003,], followed by *t*11,*c*13-18:2 [Kraft et al., 2003 ; Kramer et al., 2004] when ruminant are pasture fed. Other CLA isomers found in the milk fat or meat of ruminants include *t*7,*c*9-18:2 [Yurawecz et al.,1998], whose content generally increases when ruminants are fed forages rich in fiber [Aldai et al., 2008]. The milk fat content in CLA can reach 1.89% (of total fat) when the diet of the cows is supplemented with sunflower oil and 4% when the diet is supplemented with safflower oil [Bauman, 2000]. Fritsche et al. showed that the total CLA content in subcutaneous fat of beef was higher than in the intramuscular fat, and lowest in the phospholipid fraction [Fritsche et al., 2001] The content of *c*9,*t*11-18:2 relative to the total CLA content was above 80% in lambs, 65-78% in grass-fed and concentrate-fed cattle

[Dannenberger et al. 2004], and above 60% in beef commercialized in the Canadian market [Aldai et al., 2009].

1.6 NUTRITIONAL ASPECTS OF *TRANS* FATTY ACIDS

In the last two decades, the health impact of TFA in foods has received significant attention in the scientific literature. Many scientific publications have shown a positive correlation between coronary heart disease (CHD) and dietary intake in TFA, especially those formed during the processing of oils and foods [Gebauer et al., 2011; Willett et al., 1993]. Among others, margarines, cookies and FAt food meals are products that were observed to contain relatively higher amounts of IP-TFA. Furthermore, IP-TFA have been shown to increase the risk in cancer and cardiovascular diseases (CVD), increase plasma concentration in lipids and lipoproteins and LDL-cholesterol. Mozaffarian et al. have shown that if dietary PHVOs are replaced with alternative fats and oils, the risk of CHD may be reduced by 50% [Gebauer et al., 2011; Mozaffarian and Clarke, 2009]. Typical contents in TFA in FAt food meals, beef and dairy products are shown in table 1.

Food	TFA (% total fat)
Hamburger	3-5
Pizza	4-6
French fries	1-13
Shortenings	0-30
Beef and dairy fat	2-9

Table 1 Content of *trans* Fat (% Total Fat) in selected FAt food meals, foods and fats.
Source of data [Tyburczy, 2012; Aro 1998,]

There is also evidence that TFA promote inflammation. In a study using overweight women, high dietary intake in TFA was associated with increased activity of the tumor necrosis factor (TNF) system, and increased content of interleukin-6 and C-reactive proteins [Mozaffarian et al., 2006] In another study utilizing patients with hypercholesterolemia, the production of interleukin-6 and TNF-α by cultured mononuclear cells increased after one month of diet supplementation with soybean-margarine compared with a diet supplemented with soybean oil [Han et al. 2002]. In different studies, the higher dietary intake in TFA was associated with endothelial dysfunction [De Roos, 2001]. Other possible negative effects of TFA consumption are the reduction in the activity of certain enzymes, such as serum paraoxonases and insulin resistance [Christiansen et al., 1997; Lichtenstein et al. 1999].

The functions of FA in metabolism are not limited to energy storage/production. They also perform other molecular activities such as modulation of cell functions, e.g., fluidity of membranes [Stubbs and Smith, 1984] and activation of cell membrane receptors [Clandinin et al., 1991; Feller et al. 2005]. FA may also modulate metabolic and inflammatory responses of the endoplasmic reticulum [Hotamisligil, 2005]. On the basis of these observations, TFA may affect the function and responses of many types of cells [Mozaffarian et al., 2006]. In humans, the consumption of TFA increases the plasma activity of cholesteryl ester transfer protein [van Tol et al., 1995], the main enzyme that performs the transfer of cholesterol esters from HDL to LDL and to very low-density lipoprotein (VLDL) cholesterol.

Conjugated *trans* fatty acids

The major source of TFA in oils and fats commercialized in the US market is partial hydrogenation of vegetable oils. However, milk fat and meat of ruminants are also dietary sources of TFA. Several recent studies have shown that different CLA isomers provide unique and different biological responses in mammalian systems including humans [Pariza et al. 2001]. Some of them have shown negative effects [Belury, 2002]. Vaccenic acid, the most abundant TFA in most dairy products, was observed to be the main biologic precursor of CLA [Mozaffarian, 2006]. Studies involving humans regarding the beneficial effects of CLA consumption have proven inconclusive and apparently dependent upon the specific CLA isomers used for the feeding study. While *c*9,*t*11-18:2 showed possible positive health effects such as decreasing the LDL:HDL cholesterol ratio and total HDL cholesterol content, by contrast *t*10,*c*12-18:2 showed the opposite effect on the blood lipid profile of

healthy humans [Tricon et al., 2004]. Rajakangas et al. have shown, in a Min mice model, that *t*10,*c*12-18:2 acts as a cancer promoter in colon carcinogenesis of mice [Rajakangas et al. 2003].

Non-conjugated *trans* fatty acids

Non-conjugated geometric/positional isomers of linoleic acid (LA) (*c*9,*c*12-18:2), a dienoic FA, are often present in processed foods and oils. Mono-*trans* and di-*trans* dienoic TFA have also been discussed in connection with health risks. The *t*9,*t*12-18:2 and other non-conjugated 18:2 positional/geometric isomers have been reported to inhibit the conversion of LA into arachidonic acid (20:4n-6) [Precht, 2003; Beyers, 1991]. Although several non-conjugated mono-*trans* 18:2 FA have been reported in foods, especially in dairy products, to date little is known regarding the biological activities of these FA. A limiting factor for the development of these studies has been the lack of availability of these FA in pure form. Delmonte et al. reported the presence of at least 10 non-conjugated 18:2 FA in milk fat [Delmonte et al., 2012] and Ratnayake reported the presence of 12 non-conjugated 18:2 TFA in PHVOs.

***Trans* fat dietary intake**

Several studies investigated the consumption of TFA by the US population from 1990 to date. In 2003, Doell and co-workers reported that the current dietary intake of IP-TFA in the US population was 4.6 g/person/day. The same authors estimated in their 2012 publication that the current dietary IP-TFA intake of the US population is 1.3 and 2.6 g/capita/day as mean and 90th percentile [Doell et al, 2012]. Fritsche and Steinhart reported in 1997 an estimated dietary intake in TFA for German women and men between 1.9 - 2.3 g/person/day [Fritsche and Steinhart, 1997].

1.7 ANALYTICAL METHODS FOR THE DETERMINATION OF TRANS FATTY ACIDS

1.7.1 INTRODUCTION

The evidence regarding the negative health effects of dietary TFA has prompted recommendations and regulatory actions in several countries to limit the TFA content. [Duhem 2009; Mossoba et al. 2009]. Therefore, there is an increased need for more refined

and accurate analytical methods of analysis for TFA. Several analytical and spectroscopic methodologies for TFA and FA measurements have been updated to meet the new regulatory requirements.

The TFA content of a fat or oil can be measured by gas chromatography (GC), Fourier-Transform Infrared (FT-IR) or FT-NIR spectroscopy. The analysis of the TFA content of a food requires a prior extraction of the lipid fraction from the matrix for both GC and IR spectroscopic measurements. However, TFA can be determined directly on neat fats and oils using spectroscopic techniques. On the other hand, GC analysis requires the preparation and separation of volatile FA derivatives, most commonly methyl esters (FAME), and the calculation of the total TFA content by adding the content in all the FA meeting the TFA definition. To quantify TFA using the GC method requires the addition of an internal standard to the sample before the extraction of fat. The measurement of the TFA content of a fat or oil by FT-IR requires the simple measurement of the CH out-of-plane deformation band observed at 966 cm^{-1} that is uniquely characteristic of isolated double bonds with *trans* configuration [Mossoba et al., 2009]. FT-IR provides only the measurement of the total TFA content of fats and oils, and not of individual FA or other FA groups. As an example, it does not provide the measurement of total saturated FA (SFA) content which is also required for food labelling purposes [Tyburczy et al., 2012]. In contrast, FT-NIR is not affected by this limitation and it provides the measurement of the most abundant FA contained in fats, oils or lipid extracts [Mossoba et al., 2013 (in press)].

1.7.2 OFFICIAL METHODS

Recent official methods of analysis based on IR spectroscopy [American Oil Chemists' Society (AOCS) Cd 14d-99, AOAC 2000.10], which measure the intensity of the IR absorption band at 966 cm^{-1} by attenuated total reflection FT-IR (ATR-FT-IR), can measure the TFA content of fats and oils at the level of 5% (w/w of total fat) or higher [AOCS Official Method Cd 14d-99; Milosevic, 2004]. The most recent ATR-FTIR official method adopted in 2009 allows the accurate measurement of the height of the negative second derivative of the IR absorption band at 966 cm^{-1} (AOCS Official Method Cd 14e-09) at levels as low as 2% of total fat. The most frequently used and more versatile official GC method for FA analysis [AOAC Official Method 996.06] is applicable to the determination of total, saturated, and

unsaturated fat in food. This GC method was developed and validated in 1996 before TFA regulations were established [AOAC 996.06].

In 2005, the AOCS approved Official Method Ce 1h-05 for the determination of *cis*-, *trans*-, saturated, monounsaturated, and polyunsaturated fatty acids (PUFA) in vegetable or nonruminant animal oils and fats by capillary GLC [AOCS Ce 1h-05]. Official Method AOCS Ce 1h-05 includes labelled chromatograms of common fats and oils, which can to be used for FAME identification. Official Method Ce 1h-05 was designed for the analysis of only pure fats and oils, and must be coupled with a FAME preparation procedure such as AOCS Official Method Ce 2-66 or ISO 5509. Ce 1h-05 is generally coupled with the extraction procedures described in AOAC 996.06 when used for the determination of the TFA content of foods. Ce 1h-05 is not suitable for the analysis of samples that contains short-chain and conjugated FAME, such as dairy fats, or that contain long-chain PUFA. This method, designed for the analysis of hydrogenated and processed vegetable oils which contain mostly 18:1, 18:2 and 18:3 FA, was developed to optimize the separation of 18:1 and 18:2 FAME positional/geometrical isomers. In 2007, Official Method Ce 1h-05 was supplemented with the AOCS Recommended Practice Ce 1j-07 for the determination of *cis*-, *trans*-, saturated, monounsaturated, and PUFA in dairy and ruminant fats by capillary gas liquid chromatography (GLC) [AOCS Ce 1j-07]. AOCS Ce 1j-07 also contains reference chromatograms for FAME identification, but does not include validation data. AOCS Ce 1j-07 must also be coupled with a FAME preparation procedure, such as AOCS Official Method Ce 2-66 or ISO 5509, and if needed, with a fat extraction procedure. Concern has been expressed that the recommended methods used in the methylation (Ce 2-66 or ISO 5509) may isomerize conjugated FA during the procedure since acid catalysts are used [Kramer et al., 1997; Mossoba and Kramer 2009]. The identification of FAME in both methods relies on the comparison with the FAME separation of selected oils and fats, and with available reference materials. AOCS Ce 1j-07 also includes a table of FAME retention times, but these values are not related to the retention time of an internal standard. Unknown samples might contain FA which are not contained in the oils and fats selected for the reference chromatography and which are not commercially available as reference materials. Other FAME, not considered in the reference chromatographic separations, might also elute at the same retention times. The accurate identification of the FAME contained in unknown samples requires skilled analysts, careful evaluation of all available information, and complimentary techniques, such as GC/MS and GCxGC.

1.7.3 GAS CHROMATOGRAPHIC DETERMINATIONS

The separation of analytes in GC is based on differences in their partitioning between a gas phase, the carrier gas, and the thin layer of liquid phase covering the internal wall of the capillary column. In order to be analyzed by GC, low-volatility analytes such as TAG and free FA are preferably transformed into volatile FAME derivatives. The analytes, dissolved in a highly volatile solvent such as hexane, are injected into the injection port of the gas chromatograph where they are vaporized, and a small portion (~1:100) is passed unto the separation column in the stream of carrier gas. The analytes are separated based on their different partitioning in the liquid stationary phase, and then measured using a flame ionization detector (FID). The carrier gas should not interact with the analytes, and the retention is based solely on the interaction with the liquid stationary phase. The main parameters affecting the retention of analytes are the polarity of the separation column and the elution temperature. The double bonds of FAME show a polar character in GC separation. The retention of unsaturated FAME relative to their saturated equivalents progressively increases with increasing polarity of the stationary phase. The stronger interaction between the double bonds of FAME with higher polarity liquid phases results in increased selectivity toward the number, geometric configuration and position of double bonds. After selecting the most appropriate separation column, the elution temperature becomes the almost important parameter governing the separation. The retention times of analytes are quickly reduced by increasing the elution temperature. The relative retention of FAME with different numbers of double bonds, or bonds with different geometric configurations, is affected by the elution temperature. At any given temperature, FAME with more double bonds tend to be more retained then their less unsaturated equivalents.

GC COLUMN SELECTION

Selecting the right stationary phase and column length are the most important steps in selecting a column for GC separations. The stationary phase should be selected based on the application to be performed. The analyses of the FA contained in foods and biological samples are commonly achieved by gas chromatography of their FAME derivatives. Capillary columns commonly used for FAME analysis are coated with stationary phases with polar attributes. Non-polar phases provide the separation of saturated FAME from unsaturated FAME, and the order of elution is the reverse of that achieved using polar columns. Columns coated with polyethylene glycol (PEG) separate FAME according to their carbon number, and secondarily on the number of unsaturations. Columns of higher polarities such as BPX 90 (poly (biscyanopropylsiloxane) type phase), SP-2560, HP-88 (cyanopropyl moiety content about 90%), CP-Sil 88, SLB-IL100 or SLB-IL111 are usually utilized to increase the selectivity toward the number, geometric configuration and position of the FAME double bonds. Very long (100 m) and highly polar capillary columns that provide the separation of *cis* and *trans* unsaturated FA isomers and, with some limitations, of their positional isomers, are generally recommended

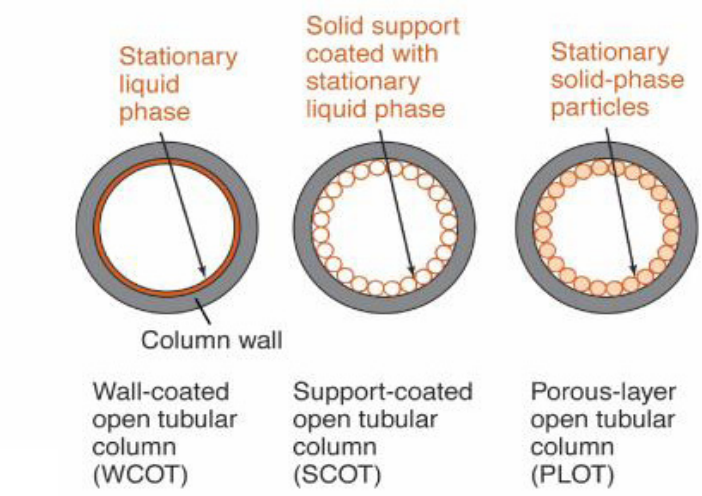


Figure 6 Capillary columns. (source, Minnesota State University, http://web.mnstate.edu)

Ionic Liquid Columns

About a decade ago, Armstrong and co-workers showed that at room temperature ionic liquids (RTIL) possess the volatility, viscosity, solubility and polarity characteristics suitable for use as stationary phases in capillary GC [Anderson and Armstrong, 2003]. As stationary phases in GC, they showed the dual nature of retention selectivity of these columns, separating polar molecules as a polar stationary phase and non-polar molecules as a non-polar stationary phase. The development of dicationic, trigonal tricationic, and cross-linked RTILs led to the novel IL capillary columns currently used for the separation of FAME. Capillary columns coated with RTILs are increasingly used for GC×GC separations due to their unique selectivity and thermal stability. The use of a column coated with an RTIL (trihexyl(tetradecyl) phosphonium bis (trifluoromethane)sulfonamide) for GC×GC separations was first tested by Seeley et al. using standard mixtures of organic compounds with a wide range of functional groups [Seeley et al., 2008].

1.7.4 CAPILLARY GC OF FATTY ACID DERIVATIVES

PREPARATION OF FAME, AND OTHER DERIVATIVES FOR GC/MS ANALYSIS.
Methyl esters are the most common derivatives used in the analysis of the FA prepared from different lipid classes. They are prepared by reaction with an excess of methanol in the presence of an acid or base acting as a catalyst. FAME are preferred because of their high volatility and good GC separations [Ulbert 1999]. The expected products from the O-acyl and N-acyl chains are fatty acid methyl esters, while alk-1-enyl ethers yield dimethylacetals (DMA) [Cruz-Hernandez et al., 2006; Mossoba and Kramer, 2009]. Because of the high volatility of short chain FAME (4:0-8:0), which are present in milk fat and ruminant fats, derivatives other than methyl esters have been evaluated (e.g. isopropyl esters) [Wolff et al., 1995].

The chemical nature and the composition of a lipid sample can be very complex. The lipid may include free fatty acids (FFA), neutral lipids, phospholipids, glycolipids and sphingolipids [Kramer and Zhou, 2001]. The catalyst should quantitatively convert the acyl moieties contained in all these compounds to FAME, and not react with any functional group present on the FA alkyl chains.

Methylation using base catalysts such as sodium methoxide is preferred for the determination of ruminant-derived fats that contain CLA because of the mild conditions of reaction, causing negligible degradation of labile FA [Kramer et al 1997]. However, base catalysts are selective, and do not convert all FA into FAME. Free fatty acids (FFA), N-acyl lipids and alk-1-enyl ethers are not trans-esterified by basic catalysis [Cruz-Hernandez et al. 2006; Mossoba and Kramer 2009].

Acid catalyzed preparation of FAME is applicable to the derivatization of all common lipid classes except ethers. The most commonly used acid reagents for the preparation of FAME are methanolic solutions of H_2SO_4, BF_3, or HCl [Christie, 2003; Mossoba and Kramer, 2009]. The application of acid catalyzed methylation requires stronger conditions of reaction compared to basic catalysis, and might result in the isomerization of CLAs [Kramer et al. 1997].

In the simplest cases, the GC separation of FAME allows their identification based on their elution pattern or relative retention times (RRT). In some instances, e.g. unknown components, the use of a mass selective detector is essential. In these cases, it is preferable to prepare derivatives other than methyl esters such as pyrrolidides, dimethyloxazoline (DMOX) or picolinyl derivatives. These derivatives provide MS characteristic fragmentation spectra that allow unambiguous localization of functional groups such as double bonds and methyl branches. [Christie, 1989].

EQUIVALENT CHAIN LENGTH OF FAME

The retention of FA derivatives on polar GC-columns is generally determined by their boiling points, and the number and position of double bonds on the aliphatic chain. In isothermal GC separations, the logarithm of the retention time of the saturated FAME and the number of carbons in their carbon chain show a linear relationship [Mjos 2006b; James 1956]. Retention times of FAME are often analyzed using equivalent chain length values (ECL). ECL values for the different fatty acids are principally dependent on the stationary phase selection, and are therefore used to compare retention data where the other chromatographic parameters (temperature program, column length, column flow) might vary.

Generally, for FAME with the same chain length, the retention times increase when the double bond(s) move from the carbonyl group toward the methyl end of the molecule. This observation was confirmed for mono-unsaturated FAME [Gunstone et al. 1967; Christie 1988] and poly-unsaturated FAME [Christie 1968, Christie 1988], with some deviations when double bonds are located close to the carbonyl group, i.e. positions Δ2 to Δ4. The n-2 and Δ3/2 positions deviate from the regular pattern by having significantly higher ECL values than isomers with double bonds in nearby positions. Investigations regarding the ECL values of dienes have mainly been carried out using their methylene-interrupted isomers [Christie, 1989].

1.7.5 GC-MASS SPECTROMETRY OF FATTY ACIDS

The coupling of gas chromatography with mass spectrometry (MS) using highly polar capillary columns has become an important analytical tool for identification and detection of FA. In order to be detected by a mass spectrometer, the targeted molecule (M) in the gas phase must be ionized. The ionization is carried out either by bombarding the molecule with high-energy electrons (EI) (e.g. 70 eV), or by chemical ionization (CI). The CI process can be divided in two reaction steps. In the first step, a stable cluster of reagent ions is produced from the reagent gas (e.g. acetonitrile, butane or methane) by electron bombardment. In the second step, the M eluting from the GC column reacts with the ions contained in the reagent gas cluster [Hübschmann, 2008].

If the chain length and number of unsaturations of FAME are the only required information, an EI-MS spectra containing an abundant $[M+H]^+$ ion is generally sufficient to achieve identification. Since the EI fragmentation of unsaturated FAME does not provide any diagnostic fragment, the location of the double bond is challenging. In addition, it is known that double bonds of FAME migrate during electron impact ionization, presumably driven by charges localized on the double bonds [Lawrence and Brenna, 2006]. Use of FA derivatives such as DMOX (4,4-dimethyloxazoline) or picolinyl esters is an effective approach for localization of the double bonds. However, variations in the chromatographic separation of these derivatives compared to that of FAME make the translation of those identifications a challenging process. Thus, utilizing a technique that determines the position of the double bond using FAME is highly preferred.

Brenna et al. have developed a GC/MS technique capable of identifying the double bond position in FAME by covalent adduct chemical ionization (CACI). This technique relies on the self reaction of acetonitrile, used as the CI reagent gas, to form the (1-methyleneimino)-1-ethylenium (MIE; $CH_2=C=N^+=CH_2$) ion with m/z 54. The MIE ion reacts with the double bonds of FAME to form heterocyclic covalent adducts $[M + 54]^+$. The $[M+54]^+$ ion is then fragmented in MS/MS mode and provides the diagnostic ions that indicate the position of the double bonds (Fig. 7).

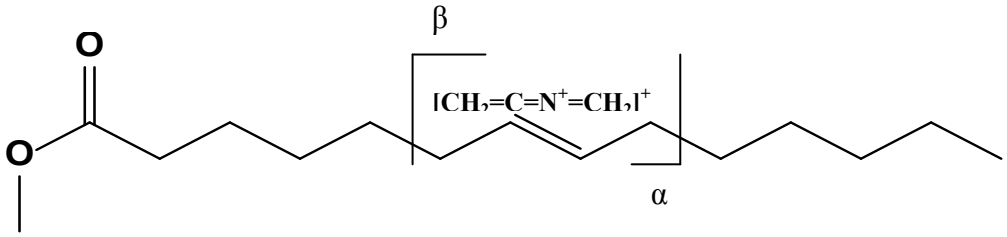


FIGURE 7 CACI-MS/MS diagnostic fragmentation of FAME for the localization of the double bonds

1.7.6 INFRARED SPECTROSCOPY

Fourier-Transform Infrared (FT-IR) spectroscopy is one of the most rapid techniques for the analysis of lipids. An FT-IR spectrometer [Reedy and Mossoba, 1999] consists of a source of continuous infrared radiation that emits light from an element that withstands prolonged heating and exposure to air, an interferometer, and a detector. The interferometer allows the collection of IR spectra in the mid-infrared region (4000–600 cm^{-1}).

When a test sample such as an oil containing TFA is placed between the beam splitter and the detector, it absorbs selected infrared wavelengths. Changes in the IR radiation intensity reaching the detector as a function of time yield an interferogram, the raw infrared spectrum. The interferogram is successively converted by the mathematical Fourier transformation into the FT-IR spectrum of absorbance *vs.* wavelength. *Trans* double bonds in oils and fats can be quantified by FT-IR by measuring the absorption at approximately 966 cm^{-1} [e.g. AOCS Official Method Cd 14-95]. However, samples consisting of free fatty acids must first be esterified particularly when low levels of *trans* of TFA are investigated (less than 15%) [Firestone and Sheppard, 1993]. The absorption band near 935 cm^{-1}, due to the O-H out-of plane deformation in the –C(O)OH moieties, can interfere with the determination of the TFA band at 966 cm^{-1}. Table 2 shows the diagnostic IR absorption bands of unsaturated FAME.

Frequency (cm^{-1})	**Group**	**Mode of vibration**
3025	=C-H (*trans*)	Stretching (very weak)
3010	=C-H (*cis*)	Stretching
2955	-C-H (CH_3)	Stretching, asym
2926	-C-H (CH_2)	Stretching, asym
2855	-C-H (CH_2)	Stretching, sym
1746	-C=O	Stretching
1654/1658	-C=C- (*cis*)	Stretching (very weak)
1465	-C-H (CH_2, CH_3)	Bending, scissoring
1418	=C-H (cis)	Bending, rocking
1377	-C-H (CH_3)	Bending, sym
1238	-C-O, -CH_2-	Stretching, bending
1163	-C-O, -CH_2-	Stretching, bending
1033	-C-O	Stretching
968	-HC=CH- (*trans*)	Bending out of plane
914	-HC=CH- (*cis*)	Bending out of plane (very weak)
723	-(CH2)n-, -HC=CH- (*cis*)	Bending, rocking

Table 2. Diagnostic IR absorption bands for FAME [Mjos and Petersen, 2001].

1.8 LIQUID CHROMATOGRAPHY IN FATTY ACID SEPARATION

1.8.1 SILVER ION CHROMATOGRAPHY

Silver ion–HPLC (Ag^+-HPLC) has been widely applied to the separation and quantitation of FAME. The FAME are separated based on their number, geometric configuration, and position of double bonds. This technique does not provide relevant selectivity based on the chain length of FAME. Ag^+-HPLC is based on the formation of complexes between the d orbitals of the silver ions and the π electrons of the FAME double bonds.

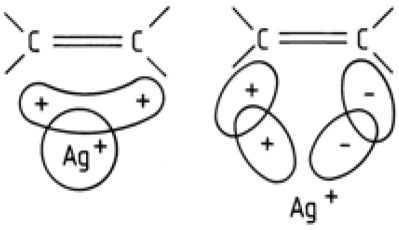


FIGURE 8 Model of interaction between silver ions and FAME double bonds.

The strength and the stability of these quasi-complexes are determined by the following rules:

- The stability decreases with increasing chain-length
- The stability decreases with an increasing number of substituents at the double bond in the order $R.CH=CH_2$ > $R_2C=CH_2$ > *cis* R.CH=CH.R > *trans* R.CH=CH.R > $R_2.C=CH.R$ > $R_2C=CR_2$
- Conjugated polyenes form less stable complexes than methylene-interrupted equivalents with the same number of double bonds. The stability also increases with increasing distance between the double bonds.

Earlier Ag^+-HPLC methods relied on self-prepared Ag^+ columns, prepared by loading silver nitrate on strong cation exchange HPLC columns. The current most common application of Ag^+–HPLC is the fractionation of saturated, *trans*-monounsaturated FA (MUFA), *cis*-MUFA, and polyunsaturated FAME prior to GC analysis to aid in the identification of

complex FAME mixtures. *Trans*- and *cis*-MUFA are well separated into two distinctive groups by using a single analytical size Ag^+–HPLC column and 0.1–0.2% acetonitrile in hexane as the elution solvent. The separation of the positional isomers of MUFA and CLAs is generally achieved using multiple columns (usually three) in series [Sehat et al., 1999]. For TFA quantitation, Christie et al. (1989b) suggest collecting saturated and *trans*-monounsaturated FAME together, and then using the content of saturated FAME as the reference for GC quantitation of TFA. An alternative approach, based on the same principle, is the fractionation of FAME by Ag^+-SPE using strong cation exchange cartridges loaded with silver ions (Discovery_ Ag-Ion SPE; Sigma-Aldrich, St. Louis, MO). FAME can be separated based on the double-bond number/geometric configuration by applying the simple procedure developed by the SPE tube manufacturer (Kramer et al., 2008).

1.8.2 REVERSED-PHASE HPLC

Reversed-phase HPLC is an alternate separation technique to Ag^+-HPLC chromatography for the separation of FAME. It was successfully used in the separation of conjugated FA from biological matrices [Banni et al., 2001]. Juaneda and Destaillats [2002 & 2007] described a procedure for separating *trans* monounsaturated FAME as a single peak prior to GC quantitation. Tsuzuki and Uchida [2009], focusing on the separation of MUFA, evaluated the separation offered by different chromatographic columns, mobile phase compositions, and elution temperatures. Delmonte and co-workers [2008], using one or three Vyndac 201TP54 (250 x 4.6 mm, 5µm particle size; Grace Davison, Deerfield, IL) HPLC columns in series, maintained at 15 °C, and 5% hexane in MeCN as the elution solvent at 1 mL/min, separated positional isomers of *cis*-17:1 FAME with double bond positions 6 to 14.

1.9 MULTI DIMESIONAL GAS CHROMATOGRAPHY

1.9.1 MULTI DIMENSIONAL GAS CHROMATOGRAPHY

Multidimensional gas chromatography (MDGC) requires two columns, ideally of different selectivity, to enable multidimensional separations. MDGC has been widely reported in many areas of industrial and environmental analysis [Lewis, 2002]. The direct coupling of two GC columns is a multichromatography and is basically a combination of two stationary phases in series in order to enhance the selectivity in the separation. For analyzing complex samples, MDGC can provide considerable improvements in the separation of individual compounds. The focus of MDGC has been in two major areas: (I) increasing peak capacity of the system, and (II) increasing the speed of the separation system [Luke et al., 1968].

The general approach to improving resolution is to modify the column physical parameters such as increasing column length, decreasing the column internal diameter, or a combination of both. However, in many cases, changing these parameters may only offer minor improvements in resolving the target compounds. It is well-known that doubling the length of a column results in only a $\sqrt{2N}$ (N for theoretical plates) increase in the number of theoretical plates on the column.

Two-dimensional GC (2D-GC) has a requirement that target analytes be subjected to two or more mutually independent separation steps and that the components remain separated until completion of the overall steps. Technically, the effluent from first column/first dimension (1D) is isolated, passed to the second column/second dimension (2D) and analyzed. Thus, the simplest MDGC setup is to use a valve pressure switching system that is able to pass zones of effluent from 1D to 2D.

In 1968, Deans introduced a basic principle of pressure switching which has been used to date for “heart-cutting” [Deans, 1968]. FIGURE 9 shows the basic layout of the Deans switching system (Agilent Technologies). The sample is injected onto the primary column. The effluent from this column goes to the primary detector via the restrictor (deactivated tubing). When the solenoid valve is activated, gas pressure from the pressure control module (PCM) switches to the other side of the pneumatic coupler, transferring the effluent to the secondary column.

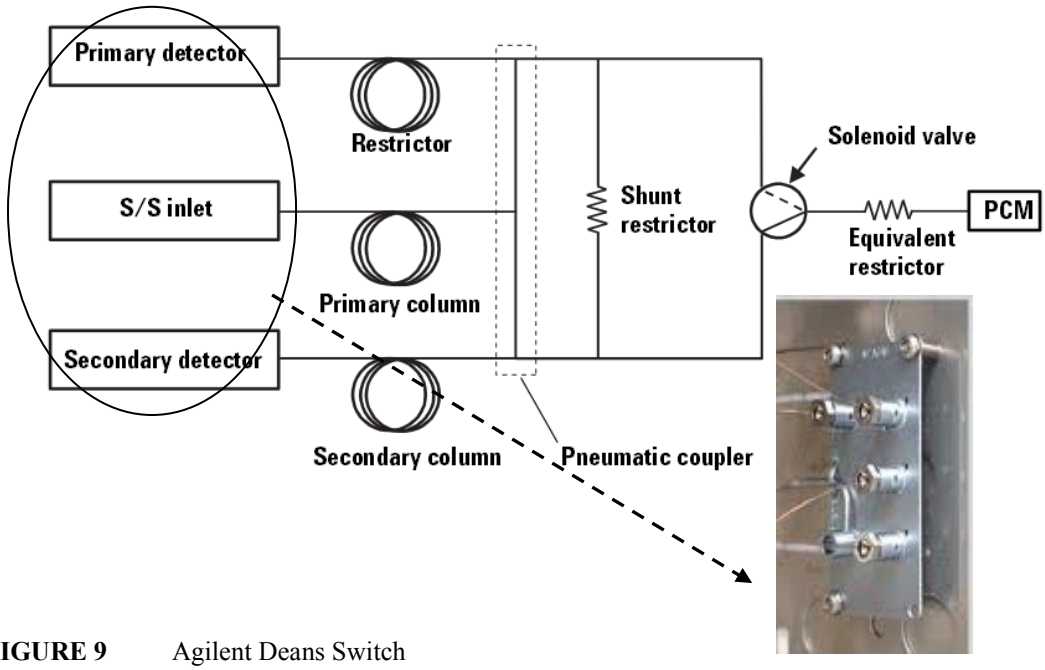


FIGURE 9 Agilent Deans Switch

1.9.2 COMPREHENSIVE GC X GC

In the analysis of complex samples such as milk fat and marine oils, the primary goal is to increase the peak capacity of the system by one or two orders of magnitude. The peak capacity (n_c) is the maximum number of components that can be placed, side by side, into the available separation space at a given resolution. Ideally, in a comprehensive system, a 2D column should be available for each transfer of the effluent from the 1D. Effluent from the 1D can be focused at the end of the column or in an interface and rapidly "injected" into the 2D in the form of very narrow pulses. This is normally done by a valve (switching valve) or with a modulator. The theoretical n_c of a coupled system, such as a "Deans switch", is the sum of the n_c of each individual column [Bertsch, 1999]. In a comprehensive system, it is the product of the n_c of the 1D and 2D column.

The most common modulators are the "Thermal-Sweeper-Modulator" (TSM) of Phillips [FIGURE 10] and the "cryogenic-modulator" of Marriott [FIGURE 11]. In our studies, we have used the "Loop-Modulator" system of Zoex Corporation [FIGURE 12]. The Zoex system is a modification of the TSM in which the "Loop-Modulator" consists of a length of column looped through the paths of intersecting hot and cold jets. The cooling (trapping) and

heating (releasing), modulation cycle, of the analytes happens every few seconds on the loop. Two cold spots are used for producing very sharp "injections" onto the 2D column

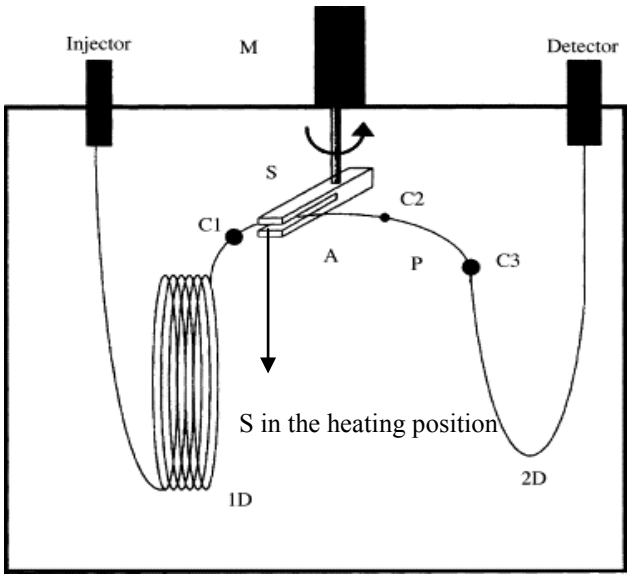


FIGURE 10 Thermal-Sweeper-Modulator of Phillips. C1, C2 and C3 are column connections; A: accumulator consisting of an uncoated capillary column as well P (called a pigtail); S: slotted heater. The heater moves over the accumulator and releases the accumulated effluent from the 1D

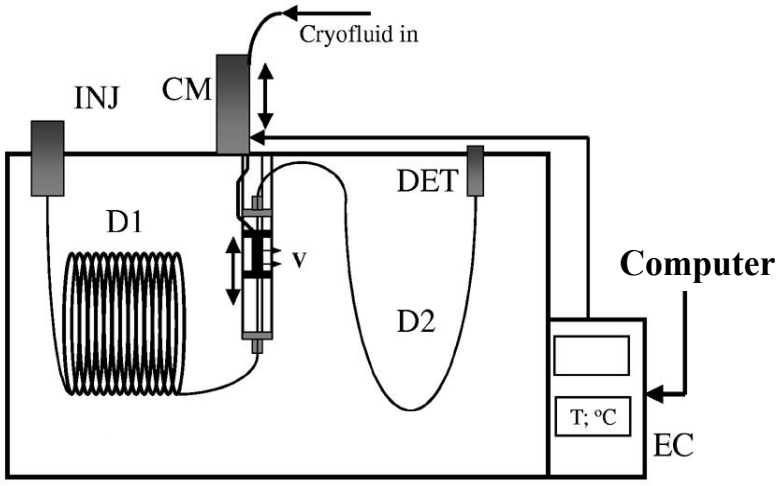


FIGURE 11 GC X GC diagram including the longitudinal cryogenic modulator of Marriott. INJ: injector; DET: detector; D1 and D2: column 1 and column 2; V: exit vents from the cryotrap; EC: electronic controller. The cryogenic cold trap focuses the effluent from 1D and moves along the column which is than exposed to the heat of the oven and thus it becomes released and "injected" onto the 2D.

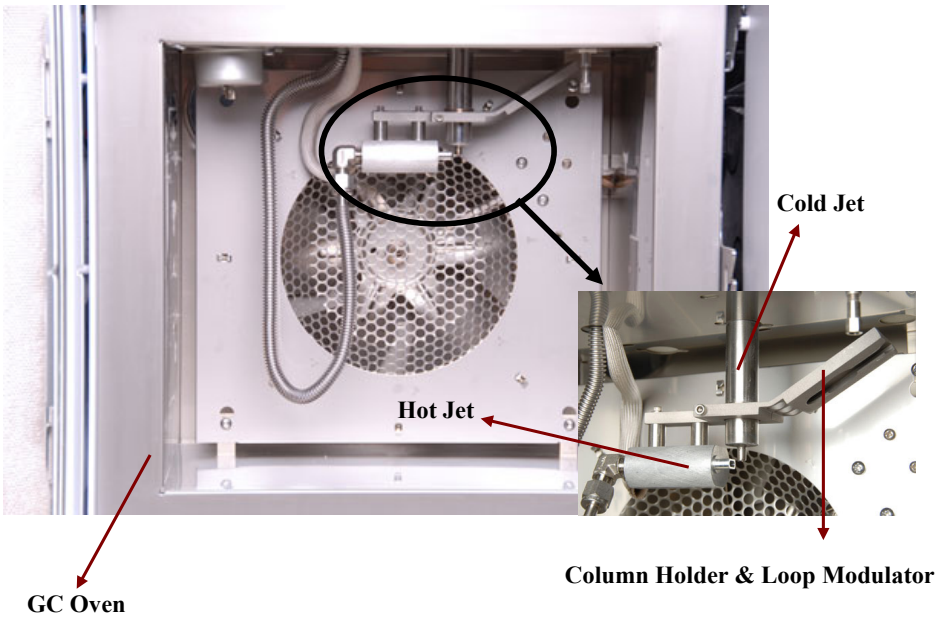


FIGURE 12 Zoex Thermal Modulator

CHAPTER 2

Preparation, chromatographic separation and relative retention times of *cis/trans* heptadecaenoic (17:1) fatty acids.

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Preparation, chromatographic separation and relative retention times of *cis*/*trans* heptadecaenoic (17:1) fatty acids

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ABSTRACT

In recent years, several countries have implemented new regulations regarding limitations or labeling of the *trans* fatty acid (*t*FA) content in foods. In order to comply with the new requirements, gas chromatographic methods for fatty acid (FA) analysis have been refined toward the quantitation of a larger number of FAs . Increased attention is also being paid to those present in lower quantities. This article describes a simple procedure for obtaining, pure or in mixtures, geometric and positional isomers of a commercially available monounsaturated FA . *cis* 10–17:1 Fatty acid methyl ester (FAME) was isomerized into its positional/geometrical isomers by repeated hydrobromination/dehydrobromination of its double bond. Reaction products were fractionated into *cis* and *trans* geometric isomers by silver ion HPLC. Pure *cis*-17:1 FAME positional isomers were obtained by reversed-phase HPLC fractionation and identified by gas chromatography – covalent adduct chemical ionization MS/MS using acetonitrile as the reacting gas. The isomerization with *p*-toluenesulfinic acid of the purified FAME yielded the corresponding *trans* isomers; these products were analyzed by GC with flame ionization detection using a Supelco 2560 capillary column in order to determine their elution order and retention times (t_R). A novel procedure was developed to determine t_T for 17:1 FAME positional/geometrical isomers relative to that of the commercially available *cis* 10–17:1 FAME.

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1. Introduction

In recent years, several countries have implemented new regulations regarding limitations or labeling of *trans* fatty acid (*t*FA) content in foods [1–4]. Current analytical methods for quantitation of *t*FA in foods are primarily based on gas chromatography (GC) [5–7]. Methodologies based on infrared spectroscopy (IR), by transmission or total attenuated reflection (ATR), are also available and have been refined in recent years [8–13]. However, the higher limits of quantitation for *t*FA by IR based methods compared with those possible with GC, and the inability of IR methods to provide information regarding the content of other fatty acids (FA), make these methods less desirable for *t*FA quantitation in foods [5,12,13]. *trans* monounsaturated fatty acids (MUFAs), the primary source of *t*FA in food, are of limited availability as reference materials. 18:1 MUFAs occur in large amounts in foods, and the GC separation/quantitation of their geometric/positional isomers has been widely studied [5,6,14]. With the exception of limited data regarding the separation of 16:1 fatty acid methyl esters (FAMEs) positional/geometrical isomers [14,15], identification of the GC elution profile of MUFAs, other than 18:1 FAMEs, is limited to that of the available reference materials.

The current study focuses on the preparation of 17:1 FAMEs, pure or in mixture, that can be used as reference for identification purpose. 17:1 FAs are considered minor components in foods. However, Ollivier et al. reported that 88 of the 564 samples of French virgin olive oil analyzed in their survey had a 17:1 FAs content higher than 0.3% [16]. Alves et al. identified as *cis* 9–17:1 FA the major 17:1 FA present in ruminant fats, observing that *cis* 8- and *cis* 10–17:1 FA are also present as minor positional isomers [17]. Both studies are based on the identification of the 17:1 FAMEs by refined GC–MS methodologies.

The procedure described here for the isomerization of *cis* 10–17:1 FAME can be applied to the isomerization of other MUFAs with the double bond not located in proximity to the carboxylic group, or in the terminal position. The process is based on the hydrobromination of the MUFA double bond yielding monobrominated FAs , followed by dehydrobromination yielding back the double bond in the same or adjacent position. Both reactions have been extensively studied in an earlier period of FA research (1930–1960), and the relevant literature was reviewed by Sonntag in 1961 [18]. Observations and conclusions reported by Sonntag

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should be evaluated in the light of analytical and separation techniques available at that time.

Silver ion HPLC (Ag^+-HPLC) has been widely applied to the separation of FAs based on double bond number, geometric configuration, and position [19–22]. In the case of medium chain length MUFAs, Ag^+-HPLC was observed to provide remarkable separation based on double bond geometric configuration, partial separation based on double bond position, and limited separation based on the FA chain length [22].

In recent years, reversed-phase HPLC has found only limited application in the separation of FAs [23,24]. Destaillats et al. used reversed-phase HPLC to collect the MUFA fraction from food samples as "a single peak" prior to GC analysis [23,24]. The reversed-phase separation applied in the current study is based on the unique selectivity of the Vydac 201TP54 column (Grace Vydac) eluting at sub ambient temperature. The Vydac 201TP54 column has been used for the separation of several compounds based on its selectivity toward analytes' double bonds, including polycyclic aromatic hydrocarbons, vitamin A, vitamin E and β-carotene [25–28]. In the current study, Ag^+-HPLC was used to separate MUFAs into *cis*/*trans* isomers and reversed-phase HPLC was used to separate positional *cis*-17:1 FAME isomers.

The identification of the FA double bond position by GC–EI-MS requires the derivatization of the fatty acids into suitable derivatives such as picolinyl esters or 2-alkenyl-4,4-dimethyl oxazoline derivatives (DMOX)[29,30]. In the current study MUFA FAME chain length and double bond position identifications are achieved without derivatization, through the methodology developed by Brenna and co-workers [31–34]. MUFAs as FAMEs are analyzed with a GC coupled to an ion trap by acetonitrile covalent adduct chemical ionization MS/MS (GC–CACI–MS/MS). Acetonitrile, used as a chemical ionization reagent, produces by self reaction the 1-methyleneimino-1-ethenylium ion (*m/z* 54) that reacts with double bond of monounsaturated FAMEs yielding covalent adduct ions of mass $(M + 54)^+$. Collisional dissociation of these ions causes cleavage adjacent to either allylic sites or the double bond, permitting its unambiguous localization.

p-Toluenesulfinic acid (PTSA) provides *cis*/*trans* isomerization of double bonds without modifying the double bond position on the aliphatic chain [35]. The conditions for the GC separation of FAME isomers were chosen in accordance with Ce-1h05 AOCS Official Method of Analysis [36].

The separation of FAMEs provided by the cyanopropyl capillary columns used for FAME analysis was observed to be affected by slight changes in column polarity [37] caused by column age or different production lots. As an example, the elution order of 21:0 FAME and conjugated linoleic acid positional isomers was shown to vary when analyzing the same test sample with CP-Sil 88 columns (Varian, Walnut Creek, CA) of different age [37]. *cis* 10–17:1 FAME was chosen to calculate relative retention times (RRT) values based on the fact that slight changes in capillary column polarity provide limited effect on the relative retention of FAME with the same chain length and unsaturation.

2. Materials and methods

FAME reference materials, including *cis* 10-heptadecenoic FAME, were purchased from Nu-Chek-Prep. (Elysian, MN, USA). HBr solution in glacial acetic acid (Purum grade, >33%, Fluka), anhydrous *tert*-butanol (> 99.5%, Sigma), potassium *tert*-butoxide (reagent grade, 95%, Sigma), 1,4-dioxane, and PTSA sodium salt (>95%, Aldrich) were purchased from Sigma Aldrich (St. Louis, MO, USA). UV grade acetonitrile, light petroleum (bp 30–60 °C, PE), isooctane and hexane were purchased from J. T. Baker (Phillipsburg, NJ, USA). Anhydrous diethyl ether (DE), suitable for FA analysis, and verified to be peroxide free, was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Pre-packed tubes containing 3 g of sodium sulphate were purchased from Alltech (currently Grace Davison, Deerfield, IL, USA) or containing 1 g from Varian. BF_3 14% in methanol was purchased from Supelco (Bellefonte, PA, USA).

2.1. Synthetic process

2.1.1. Addition of HBr

100–500 mg of monounsaturated FAME were placed in a 20 ml screw cap test tube and purged with argon. About 10 ml of 33% HBr in glacial acetic acid were placed in a separate screw cap test tube, and both test tubes were placed in a Nestlab 40 RTE chiller (Thermo Electron, Newington, NH, USA) at −15 °C. Two millilitre of chilled HBr in acetic acid were pipetted into the FAME test tube, while minimizing the amount of entering air. The chiller was covered with aluminum foil to prevent light exposure and the test tube was kept 3 h at −15 °C. The chiller temperature was then raised to 5 °C, and maintained constant for 48 h. Ten millilitre of 50:50 (v/v) DE/PE were added to the tube, swirled, and the content was transferred to a 125 ml separatory funnel. The test tube was rinsed two times with 10 ml of 50:50 PE/DE. Fifty millilitre of 50:50 saturated $NaHCO_3$/distilled water were slowly added to the separatory funnel and the pH was verified to be about neutral after the addition was completed. The neutralization reaction produces a large amount of carbon dioxide, and the separatory funnel must be continuously vented during mixing. The organic phase was gently washed with 50 ml of distilled water, filtered through an anhydrous sodium sulfate tube, and the solvent was removed under a gentle stream of argon. The addition of 2–3 drops of 6 M HCl was observed to reduce the formation of emulsions.

2.1.2. Elimination of HBr

1 M potassium *tert*-butoxide solution in anhydrous *tert*-butanol was prepared by dissolving 11.2 g of potassium *tert*-butoxide in 100 ml of anhydrous *tert*-butanol. Five millilitre of potassium *tert*-butoxide solution were added to the 20 ml crew cap test tube containing the reaction products of the HBr addition. The test tube was purged with argon, placed for 24 h in a silicon oil bath maintained at 100 °C, and covered with aluminum foil. After cooling the test tube to room temperature, 10 ml of 50:50 DE/PE were added and the content transferred to a125 ml separatory funnel. The test tube was rinsed two more times with 10 ml of DE/PE. Five millilitre of 6 M HCl were added, followed by 50 ml of 50:50 saturated $NaHCO_3$/distilled water. The aqueous phase pH was verified to be about neutral, and if still basic, corrected by adding 6 M HCl. The $NaHCO_3$ solution must be added slowly and mixing of the separatory funnel must be performed with continuous venting. The organic phase was washed with 50 ml of distilled water (with the addition of two drops of 6 M HCl), and dried through an anhydrous sodium sulfate tube. The solvent was removed under a gentle stream of argon.

2.1.3. Methylation

Final products of reaction were methylated according to previously published principles [38]. About 20–100 mg of the previous reaction products were placed in a 20 ml screw cap test tube, followed by 2–3 ml of 14% BF_3 in methanol. The tube was purged with argon and placed for 15 min in a silicon oil bath at 100 °C. After chilling to room temperature, 2–4 ml of isooctane were added and the tube was swirled. The test tube was then filled with NaCl saturated solution, mixed, and the organic phase was filtered through an anhydrous sodium sulfate tube. The extraction of FAME was repeated with 2–4 more ml of isooctane.

2.1.4. PTSA isomerization

The isomerization was carried out according to previously published principles [35]. PTSA was produced by acidification of its sodium salt. Thirty millilitre of deionized water, 2 ml of 37% HCl, 30 ml of diethyl ether and 1 g of PTSA sodium salt were added in sequence to a 125 ml separatory funnel. The contents were mixed until the PTSA sodium salt was fully dissolved and the organic phase appeared clear. The diethyl ether phase was washed with 30 ml of deionized water plus 1 ml of 37% HCl, and dried under vacuum. Fifty milligram of PTSA (free acid) were dissolved in 50 ml of 1,4-dioxane (1 mg/ml). One milligram or less of a pure 17:1 FAME positional isomer, purified by HPLC, was dried at the bottom of a 2 ml screw cap autosampler vial and 250 μl of PTSA solution was added. The vial was purged with argon, tightly capped, and held 15 min in a GC oven at 100 °C. At room temperature, 300 μl of 1N NaOH were added, followed by 1 ml of isooctane. After mixing, the lower phase was removed and the organic layer was washed twice with 500 μl of deionized water. The organic phase containing the FAMEs was transferred to a clean 2 ml autosampler vial, dried under a stream of argon, and dissolved in 1 ml of isooctane. Amber silanized vials with a 10 mm opening were used as reaction vessels and for storage.

2.2. Separation and analysis.

2.2.1. Fractionation by Ag^+-HPLC

Fractionation was performed with a Waters 2695 separation module (Milford, MA, USA) equipped with a Waters 2996 photodiode array detection (DAD) system and a Waters Fraction Collector II. Three Ag^+- HPLC columns (ChromSpher 5 Lipids, 250 × 4.6 mm, 5 μm particle size, Varian) were used in series. The mobile phase was 0.15% MeCN in isooctane at 1.0 ml/min and the injection volume was 100 μl. The fractionation was monitored by UV at 200 nm.

2.2.2. Fractionation by reversed-phase HPLC

Fractionation was performed with a Waters 2695 separations module equipped with a Waters 2996 DAD system and Waters Fraction Collector II. One (or three in series) Vydac 201TP54 column(s) (250 × 4.6 mm, 5 μm particle size, Grace Davison, Deerfield, IL, USA) was/were maintained at −15 °C in a Nestlab RTE 40 chiller. The mobile phase was 5% hexane in MeCN at 1.0 ml/min, and the injection volume was 10–100 μl. Injection volumes greater than 10–15 μl required the sample to be dissolved in the mobile phase. The fractionation was monitored by UV at 200 nm.

2.2.3. Analysis by GC–CACI–MS/MS

Fatty acid identification was performed using a Varian Saturn 2200 ion trap coupled to a Varian 3800 gas chromatograph equipped with a Varian 1177 split/splitless injector and a CP Sil 88 (100 m × 0.25 mm I.D., 0.2 μm thickness, Varian) capillary column. Helium was used as carrier at 1.0 ml/min. The GC oven was maintained at 180 °C, and the injector at 250 °C. The split ratio was 1:20 or 1:100, and the injection volume was 1 μl. The ion trap was operated under automatic reaction control (ARC) according to the conditions described by Van Pelt and Brenna [31]. MS/MS experiments were carried out in multiple reaction monitoring (MRM) mode with the target set to 5000, and the resonant excitation amplitude was set to 0.4, 0.7 or 1.0 V. Other MS/MS parameters were mass isolation window 3 *m/z* units, and excitation storage level 85 or 148.2 *m/z* units. Axial modulation amplitude was set to 4.0 V, trap temperature to 160 °C, manifold temperature to 40 °C, transfer line temperature to 200 °C.

2.2.4. Analysis by GC–FID

Samples were analyzed with an Agilent 6890N gas chromatograph equipped with flame ionization detection (FID) and a Supelco 2560 capillary column (100 m × 0.25 mm I.D. , 0.2 μm thickness). The oven was maintained at 180 °C; the detector and the split/splitless injection port were maintained at 250 °C. Hydrogen as carrier was eluted at 1.0 ml/min with a split ratio of 1:100. Injection volume was 1 μl.

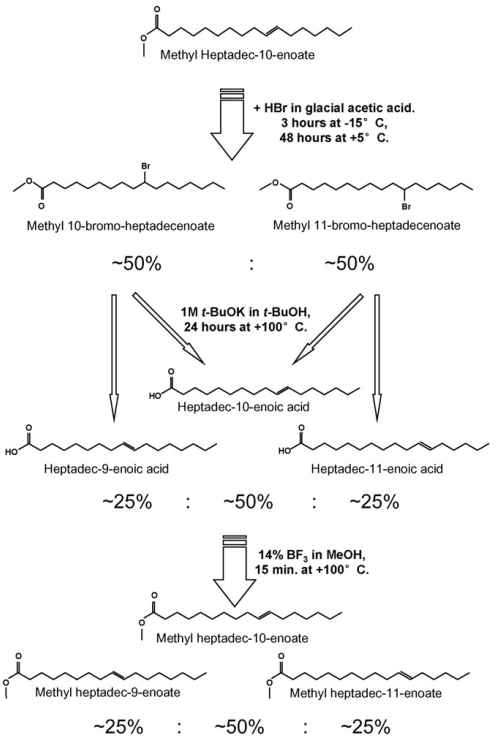


Fig. 1. Flow chart of the synthetic process. Steps 1 and 2 are repeated until the desired isomerization is achieved. Products of the reaction are re-converted into FAME by BF_3 in methanol before GC analysis or fractionation.

3. Results and discussion

Fig. 1 shows the sequence of reactions. The addition of HBr to a MUFA with the double bond in position *n* yields two saturated FA positional isomers brominated in position *n* and *n* + 1. The subsequent reaction of elimination, performed by the strong non-nucleophilic base potassium *tert*-butoxide, yields back three *trans* and three *cis* FAs with a double bond in position *n* − 1, *n* or *n* + 1 in the ratio 1:2:1. The abundance ratio of the FA produced can be affected by steric or electronic factors if the reacting double bond is located in proximity to both ends of the MUFA. The addition/elimination of HBr can be reiterated until the targeted isomerization is reached. After obtaining the desired MUFA isomerization, the products of reaction are re-converted to methyl esters before being used as reference materials for GC analysis or fractionation by HPLC [38].

Fig. 2 (upper panel) shows the products of reaction after one repetition of the reaction process described in Fig. 1. The Ag^+-HPLC fractionation distinguishes between the *cis* and *trans* 17:1 FAME geometrical isomers. *trans* isomers, thermodynamically favored,

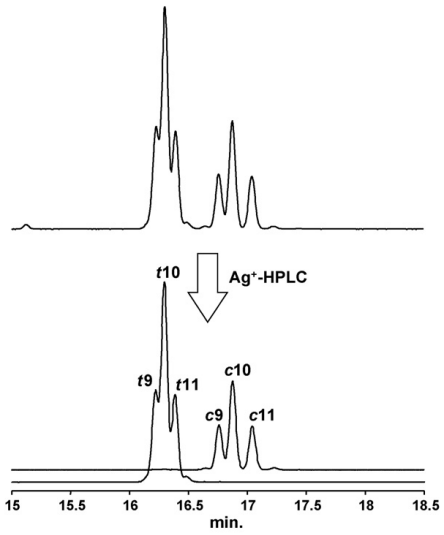


Fig. 2. GC separations of products from the reaction of *cis* 10–17:1 FAME according to the procedure described in Fig. 1. FAME were analyzed before (upper panel) and after (lower panel) Ag^+-HPLC fractionation in *cis* and *trans* isomers. Supelco 2560 capillary column (100 m × 0.25 mm I.D. , 0.2 μm thickness) maintained at 180 °C, hydrogen carrier gas at 1.0 ml/min, detection by FID.

are produced in about twice the amount as the *cis* isomers. *cis* and *trans* positional isomers, identified by GC–MS/MS (as later described), are produced in the ratio 1:2:1 according to theoretical expectations. No other side reaction products were observed in the GC separation. The addition/elimination was then repeated six times, and the final products were methylated with BF_3 in methanol. While the addition of HBr in acetic acid to the 17:1 FAME double bond was observed to proceed without the presence of solvent, 1 ml of toluene was added to the FA when reacting 19:1 or longer chain FAMEs. The solvent is necessary because products of the first addition/elimination cycle are solid at +5 °C, decreasing the reaction rate of the following hydrobromination reaction. The elimination of HBr with potassium *tert*-butoxide , during the first repetition of the synthetic process, was observed to be quantitative within 90 min. The reaction time of 24 h ensured the completeness of the HBr elimination independently from the FA chainlength or bromine position.

The addition and elimination reactions were carried out using anhydrous solvents, in the dark, and in argon purged test tubes in order to avoid radical reactions. The reaction products were observed not to be altered by the addition of 100 μl of water to the reagents when carrying out the bromination and dehydrobromination of *cis* 10–17:1 FAMEs.

FAMEs were observed to be hydrolyzed to free fatty acids (FFA) during the reaction of elimination, or the following FA extraction. FFAs showed undistinguishable reactivity to FAME when repeating the hydrobromination and dehydrobromination reactions. Products of reaction were reconverted to methyl esters only after the desired isomerization was achieved.

Fig. 3 shows the Ag^+-HPLC separation of *cis* 10–17:1 FAME after six addition/elimination cycles, followed by methylation. The *cis*- and *trans*-17:1 FAMEs are clearly separated into two groups, and no attempts were made to fractionate single positional isomers.

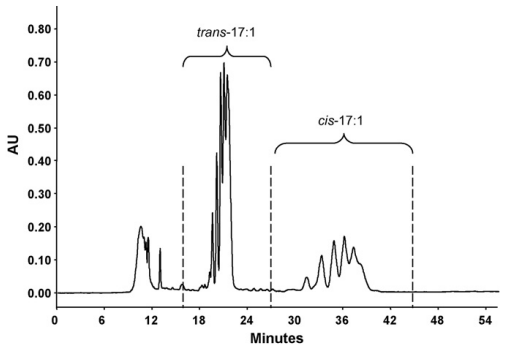


Fig. 3. Ag^+-HPLC *cis*/*trans* fractionation of mixtures of *cis* and *trans* positional isomers. Addition/elimination of HBr to *cis* 10–17:1 was repeated six times before methylation and fractionation. Three ChromSpher 5 Lipids columns in series (250 × 4.6 mm, 5 μm particle size, Varian), 0.15% MeCN in isooctane mobile phase at 1.0 ml/min, UV detection at 200 nm.

The identification of the *cis* and *trans* fractions was based on the literature [19–22] and on the analysis of available reference materials. The *cis*-17:1 FAME fraction was preferred for reversed-phase HPLC fractionation of positional isomers because it provides a more suitable chromatographic separation. Fig. 4 shows the separation of the *cis*-17:1 FAME fraction by reversed-phase HPLC using one Vydac 201TP54 column maintained at −15 °C, and 5% hexane in MeCN as the mobile phase. Retention times and separation among positional isomers were observed to increase by lowering the elution temperature. The optimal temperature was chosen as a compromise among peak tailing, separation of the critical pair *cis* 6–17:1 and *cis* 11–17:1 FAME, and reasonable separation time. Peak tailing was observed to affect more dramatically the separation of *trans*-17:1 than *cis*-17:1 FAME positional isomers by lowering the elution temperature. According to the chilling system used, fine adjustment of the elution temperature might be required for the separation of the pair *cis* 6–17:1 and *cis* 11–17:1 FAME. *cis* 14–17:1 FAME, not shown in the separation, elutes as a broad peak at 120 min.

Fig. 4 (upper panel) shows the separation of FAME from *cis* 6–17:1 to *cis* 11–17:1 FAME using 3 Vydac 201TP54 columns in series under the same experimental conditions. This separation was

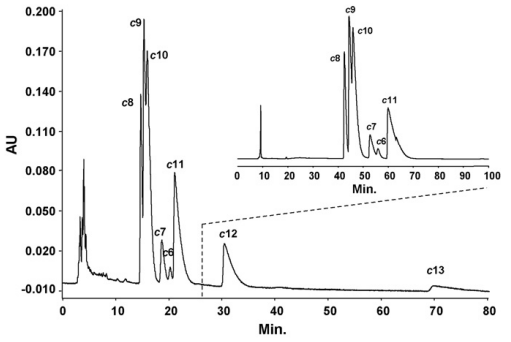
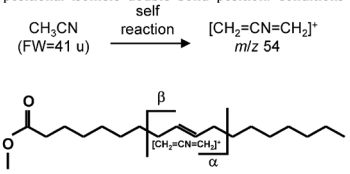


Fig. 4. Reversed-phase HPLC separation of the *cis*-17:1 FAME fraction obtained by Ag^+-HPLC (Fig. 3). Lower panel: One Vydac 201TP54 column at −15 °C, 5% hexane in MeCN mobile phase, UV detection at 200 nm. Upper panel: separation obtained using three columns in series under the same conditions.

preferred for the collection of pure isomers. It allowed the near baseline separation of *cis* 8–17:1 FAME, and a more suitable separation for fractionation purpose between *cis* 9–17:1 and *cis* 10–17:1. As an alternative approach, the *cis*-17:1 FAME mixture was fractionated at +5 °C using a single column by collecting the positional isomers from *cis* 6- to *cis* 11–17:1 in a single fraction, then in sequence, the *cis* 12-, *cis* 13- and *cis* 14–17:1 FAME in pure form. The *cis* 6 to *cis* 11–17:1 FAME positional isomers were then fractionated by applying the separation shown in Fig. 4 (upper panel).

Identification of positional isomers shown in Figs. 2 and 4 was obtained by acetonitrile covalent addition chemical ionization–MS/MS, using a gas chromatograph coupled with an ion trap. Table 1 shows the fragmentation pattern that led to the identification of the double bond position, and the expected diagnostic MS/MS ions per each double bond location. This methodology was developed by Brenna and co-workers [31–34] and applied without modifications. Fig. 5 shows the MS/MS spectra collected in MRM mode of the purified *cis*-17:1 positional isomers. The identification of the purified positional isomers from *cis* 6- to *cis* 11–17:1 FAME was obtained by setting the MS/MS excitation storage level to 148.2 *m*/*z* units, while 85 *m*/*z* units was used for positional isomers from *cis* 12- to *cis* 14–17:1 FAME. The double

Table 1
Acetonitrile CACI–MS/MS diagnostic ions for the identification of the 17:1 FAME positional isomers double bond position. Conditions as described in the text.



FAME	α (*m*/*z*)	β (*m*/*z*)
4–17:1	182	264
5–17:1	196	250
6–17:1	210	236
7–17:1	224	222
8–17:1	238	208
9–17:1	252	194
10–17:1	266	180
11–17:1	280	166
12–17:1	294	152
13–17:1	308	138
14–17:1	322	124

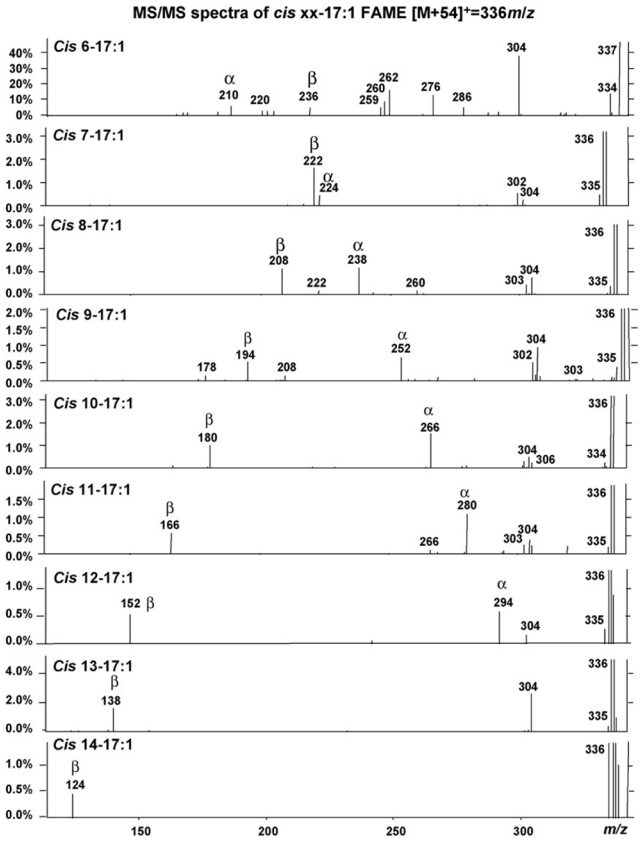


Fig. 5. Acetonitrile CACI–MS/MS spectra of the purified *cis*-17:1 FAME positional isomers. Conditions as described in the text.

bond position of each FA was clearly identified based on the α and β fragmentation ions. *cis* 13- and *cis* 14–17:1 FAME were identified based only on the presence in the MS/MS spectrum of the weak β ion. In the case of these two fatty acids, the α ion was not observed, probably because it would require the cleavage of the terminal ethyl or methyl group.

Pure *cis*-17:1 FAME positional isomers were isomerized by PTSA, to obtain mixtures of both geometrical isomers. *trans* isomers were identified in GC separations by comparing the analysis of isomerized and non-isomerized *cis* isomer test samples. As expected, the more thermodynamically stable *trans* isomers were produced in larger amounts than the *cis* counterparts, and, during GC, eluted before their *cis* geometric isomer. Fig. 6 shows the stacked GC separations of the purified and isomerized 17:1 FAME positional isomers under the experimental conditions described in the AOCS Ce-1h05 Official Method of Analysis [36]. Among *cis* isomers, *cis* 7–17:1 FAME presented the lowest retention time, slightly lower than the one of *cis* 6–17:1 FAME. Similar to the separation of *trans* 18:1 FAME under the same conditions, *trans* 6–7–8–17:1 FAME would co-elute if contained in the same mixture. By contrast, *trans* 13–17:1 FAME would co-elute with *trans* 12–17:1 rather than with *trans* 14–17:1. The retention times of these FAMEs are reported in Table 2. *cis* 10–17:1 was chosen as a reference for relative retention time calculations because of its commercial availability and its chemical similarity to the other FAs identified in this study. Retention times relative to *cis* 10–17:1 can be used to calculate the retention time of these non-commercially available isomers when using Supelco 2560 columns of different age or production lot, thus allowing identification of these FAs in food samples. *cis* 10–17:1 FAME is contained in several commercially available FAME mixes, such as the Nu Chek-Prep GLC 463. Data reported in Table 2 were obtained after dissolving the FAME in isooctane.

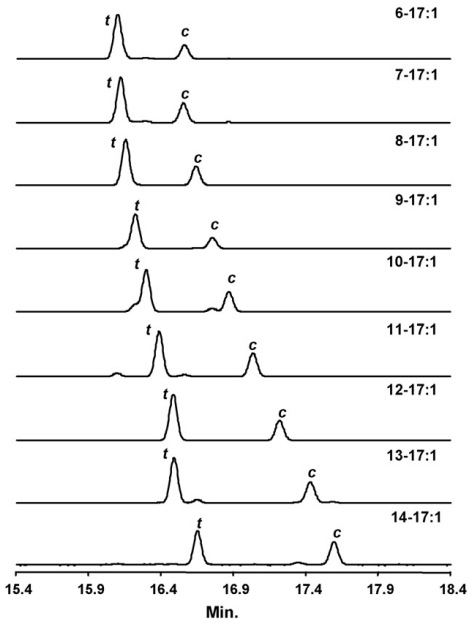


Fig. 6. GC separations of purified fractions of 17:1 FAME positional isomers, and isomerized with PTSA. Supelco 2560 capillary column (100 m × 0.25 mm I.D., 0.2 μm thickness) maintained at 180 °C, hydrogen carrier gas at 1.0 ml/min, detection by FID.

Table 2
Table of the *cis* and *trans*-17:1 FAME relative retention times, analyzed by GC under the conditions specified in Fig. 6. Retention times are calculated relative to 21:0 FAME and to *cis* 10–17:1 FAME as references. FAME solutions were dissolved in isooctane.

FAME	t_R *trans* (min)	t_R *cis* (min)	RRT *cis* 10–17:1		RRT 21:0	
			trans	*cis*	*trans*	*cis*
6–17:1	16.10	16.56	0.91827	0.96793	0.37743	0.39784
7–17:1	16.12	16.55	0.92057	0.96698	0.37840	0.39748
8–17:1	16.15	16.64	0.92392	0.97603	0.37979	0.40121
9–17:1	16.21	16.75	0.93071	0.98780	0.38256	0.40603
10–17:1	16.29	16.86	0.93877	1.00000	0.38586	0.41103
11–17:1	16.38	17.03	0.94860	1.01796	0.38991	0.41842
12–17:1	16.48	17.21	0.95916	1.03788	0.39424	0.42659
13–17:1	16.48	17.42	0.95976	1.06060	0.39450	0.43594
14–17:1	16.65	17.59	0.97723	1.07807	0.40169	0.44314

4. Conclusions

The isomerization procedure described here allows the preparation of MUFA mixtures that can be used as reference material for GC analysis, or fractionated into pure isomers. Isomerized MUFAs can first be fractionated into *cis* and *trans* geometric isomers by Ag^+-HPLC, and then each fraction can be separated into positional isomers by reversed-phase HPLC at sub ambient temperature. A combination of Ag^+-HPLC and reversed-phase HPLC can also be used. MUFAs, as FAMEs, were identified by acetonitrile covalent adduct chemical ionization–MS/MS using a gas chromatograph coupled with an ion trap. *cis* 10–17:1 FAME was isomerized by repeating six times the hydrobromination/dehydrobromination of its double bond. The HPLC fractionation of the reaction products yielded all the *cis*-17:1 FAME isomers from double bond position 6 to 14. Purified FAME were characterized by GC–MS/MS, isomerized with PTSA to yield both the cis/*trans* geometrical isomers and analyzed by GC–FID. Retention times were calculated relative to *cis* 10–17:1 FAME, a commercially available FAME contained in several GC FAME standard mixtures. Retention time values reported in Table 2 can be used to rebuild the elution pattern of the 17:1 FAME positional isomers using a Supelco 2560 column, based on the experimentally determined relative retention time of *cis* 10–17:1 FAME.

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References

[1] Department of Health and Human Services, US Food and Drug Administration (FDA), food labeling: *trans* fatty acids in nutrition labeling; nutrient content claims, and health claims; final rule. Fed. Reg. 68 (2003) 41, 434–41, 506.
[2] Health Canada, Regulations amending the food and drug regulations (nutritional labeling, nutrient content claims and health claims), Canada Gazette 137 (Pt II) 154–409.
[3] W.M.N. Ratnayake, C. Zehaluk, in: C.C. Akoh, O.M. Lai (Eds.), Healthful Lipids, AOCS Press, Champaign, IL, 2005, p. 1.
[4] J. Schrimpf-Moss, V. Wilkening, in: D.R. Kodali, G.R. List (Eds.), *trans* Fats Alternatives, AOCS Press, Champaign, IL, 2005, p. 26.
[5] M.M. Mossoba, J.K.G. Kramer, P. Delmonte, M.P. Yurawecz, J.I. Rader, in: D.R. Kodali, G.R. List (Eds.), *trans* Fats Alternatives, AOCS Press, Champaign, IL, 2005, p. 47.
[6] C. Cruz-Hernandez, Z. Deng, J. Zhou, A.R. Hill, M.P. Yurawecz, P. Delmonte, M.M. Mossoba, M.E. Dugan, J.K. Kramer, J. AOAC Int. 87 (2004) 545.
[7] P. Delmonte, J.I. Rader, Anal. Bioanal. Chem. 389 (2007) 77.

[8] D. Firestone (Ed.), American Oil Chemists' Society Official Methods and Recommended practices, 5th ed., AOCS Press, Champaign, IL, 1999, Official method Cd 14d-99.
[9] M. Adam, M.M. Mossoba, T. Lee, J. Am. Oil Chem. Soc. 77 (2000) 457.
[10] M.M. Mossoba, M. Adam, T. Lee, J. AOAC Int. 84 (2001) 1144.
[11] M. Milosevic, V. Milosevic, J.K.G. Kramer, H. Azizian, M.M. Mossoba, Lipid Technol. 16 (2004) 252.
[12] M.M. Mossoba, J.K.G. Kramer, V. Milosevic, M. Milosevic, H. Azizian, J. Am. Oil Chem. Soc. 84 (2007) 339.
[13] M.M. Mossoba, V. Milosevic, M. Milosevic, J.K.G. Kramer, H. Azizian, Anal. Bioanal. Chem. 389 (2007) 87.
[14] J.K.G. Kramer, M. Hernandez, C. Cruz-Hernandez, J. Kraft, M.E.R. Dugan, Lipids 43 (2008) 259.
[15] D. Precht, J. Molkentin, Eur. J. Lipid Sci. Technol. 102 (2000) 102.
[16] D. Ollivier, J. Artaud, C. Pinatel, J.P. Durbec, M. Guérère, J. Agric. Food Chem. 51 (2003) 5723.
[17] S.P. Alves, C. Marcelino, P.V. Portugal, R.J. Bessa, J. Dairy Sci. 89 (2006) 170.
[18] N.O.V. Sonntag, in: K.S. Markley (Ed.), Fatty Acids, Their Chemistry, Properties, Production, and Uses, Part II, Interscience, New York, 1961, p. 1073.
[19] R. Wood, T. Lee, J. Chromatogr. A 254 (1983) 237.
[20] R. Adlof, J. Chromatogr. A 659 (1994) 95.
[21] R. Adlof, L.C. Copes, E.A. Emken, J. Am. Oil Chem. Soc. 72 (1995) 571.
[22] 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.
[23] P. Juaneda, J. Chromatogr. A 954 (2002) 285.
[24] F. Destaillats, P.A. Golay, F. Joffre, M. de Wispelaere, B. Hug, F. Giuffrida, L. Fauconnot, F. Dionisi, J. Chromatogr. A 1145 (2007) 222.
[25] C.H. Risner, J. Chromatogr. Sci. 26 (1988) 113.
[26] C. Cortés, M.J. Esteve, A. Frígola, F. Torregrosa, J. Agric. Food Chem. 52 (2004) 2203.
[27] E.A. Koutsos, A.J. Clifford, C.C. Calvert, K.C. Klasing, J. Nutr. 133 (2003) 1132.
[28] W.A. MacCrehan, E. Schönberger, Clin. Chem. 33 (1987) 1585.
[29] W.W. Christie, Lipids 33 (1998) 343.
[30] J.T. Hamilton, W.W. Christie, Chem. Phys. Lipids 105 (2000) 93.
[31] C.K. Van Pelt, J.T. Brenna, Anal. Chem. 71 (1999 1981).
[32] P. Lawrence, J.T. Brenna, Anal. Chem. 78 (2006) 1312.
[33] A.L. Michaud, M.P. Yurawecz, P. Delmonte, B.A. Corl, D.E. Bauman, J.T. Brenna, Anal. Chem. 75 (2003) 4925.
[34] A.L. Michaud, P. Lawrence, R. Adlof, J.T. Brenna, Rapid Commun. Mass Spectrom. 19 (2005) 363.
[35] J.M. Snyder, C.R. Scholfield, J. Am. Oil Chem. Soc. 59 (1982) 469.
[36] D. Firestone (Ed.), American Oil Chemists' Society Official Methods and Recommended practices, 5th ed., AOCS Press, Champaign, IL, 1999, Official method Ce 1h-05.
[37] C. Cruz-Hernandez, J.K.G. Kramer, J. Kraft, V. Santercole, M. Or-Rashid, P. Deng, M.E.R. Dugan, P. Delmonte, M.P. Yurawecz, in: M.Z. Yurawecz, J.K.G. Kramer, O. Gudmundsen, M.W. Pariza, S. Banni (Eds.), Advances in Conjugated Linoleic Acid Research, vol. 3, AOCS Press, Champaign, IL, 2006, p. 45.
[38] W.R. Morrison, L.M. Smith, J. Lipid Res. 5 (1964) 600.

CHAPTER 3

Review of Methods for Preparation and Gas Chromatographic Separation of *trans* and *cis* Reference Fatty Acids.

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SPECIAL GUEST EDITOR SECTION

Review of Methods for Preparation and Gas Chromatographic Separation of *trans* and *cis* Reference Fatty Acids

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In recent years, several countries have implemented new regulations regarding the limitation or labeling of the *trans* fatty acid (TFA) content of foods and dietary supplements. GC methods for fatty acid (FA) analysis have been updated by improving the separation of TFAs from other FAs, especially *trans*- and *cis*-18:1, and by focusing more attention on the FAs contained in fats and oils in lower amounts. FA analysis is affected by the limited availability of reference materials. Identifications are frequently made simply by comparison with separations reported in the literature. This report describes the preparation of mixtures containing fatty acid methyl esters (FAMEs) that are not available as reference materials. These mixtures can be used for FAME identifications. The prepared mixtures are analyzed under the experimental conditions of the American Oil Chemists' Society (AOCS) Official Method Ce 1h-05 and AOCS Recommended Practice Ce 1j-07.

In recent years, several countries have implemented new regulations regarding the limitation or labeling of the *trans* fatty acid (TFA) content of foods and dietary supplements (1–3). The TFA content of a food or dietary supplement is calculated by summing the content of fatty acids (FAs) with at least one nonconjugated double bond in the *trans* configuration (1, 2). Methodologies for TFA and FA measurement have been updated to meet new regulatory requirements. Current official methods of analysis based on IR spectroscopy [American Oil Chemists' Society (AOCS) Cd 14d-99, AOAC **2000.10**], which measures the IR absorption band at 966 cm^{-1} by attenuated total reflection FTIR (ATR-FTIR), can quantify TFA contents of fats and oils at the level of 5% (w/w) of total fat or higher (4–6). A new and promising approach, measuring the second negative derivative of the 966 cm^{-1} IR absorption band, was shown to lower the TFA ATR-FTIR LOQ to 1%, w/w (7–10). GC with flame ionization detection (GC-FID), the most frequently applied technique for quantifying FAs in fats and oils, provides lower LOQ values and the simultaneous measurement of other FAs reported on the nutrition label. The total TFA content of the sample is calculated by summing the content of single TFAs. The most frequently used and versatile official method for FA analysis, AOAC Official Method **996.06**, is applicable to the determination of total, saturated, and unsaturated fat in food. This method was developed and validated before TFA regulations were established (11). It includes a table containing the retention time of 58 fatty acid methyl esters (FAMEs) relative to that of the 11:0 FAME internal standard, and states that when peaks of unknown identity are observed during the chromatographic run, the analyst should attempt to identify such peaks by MS, FTIR, etc. (11). Major limitations to the application of AOAC **996.06** are the incomplete separation of *trans*- and *cis*-18:1 FAME, the absence from the FAME retention time table of TFAs contained in many fats and oils, and the absence of validation data for TFA quantitation.

In 2005, AOCS approved Official Method Ce 1h-05 for the determination of *cis*-, *trans*-, saturated, monounsaturated, and polyunsaturated fatty acids (PUFAs) in vegetable or nonruminant animal oils and fats by capillary GLC (12). Ce 1h-05, based on the separation offered by the CP-Sil 88 (100 m × 0.25 mm id, 0.2 mm thickness; Varian Inc., Walnut Creek, CA) and Supelco 2560 (100 m × 0.25 mm id, 0.2 μm thickness; Supelco Inc., Bellefonte, PA) capillary columns operated at an isothermal elution temperature of 180°C, provides improved separation of *trans*- and *cis*-18:1, and includes validation data for the quantitation of TFAs. AOCS Ce 1h-05 includes labeled chromatograms of common fats and oils, which are to be used for FAME identification.

Guest edited as a special report on "*Trans* Fats: Update on Health Effects, Methodology, and Levels in Processed Foods" by G. Sarwar Gilani and W.M. Nimal Ratnayake.

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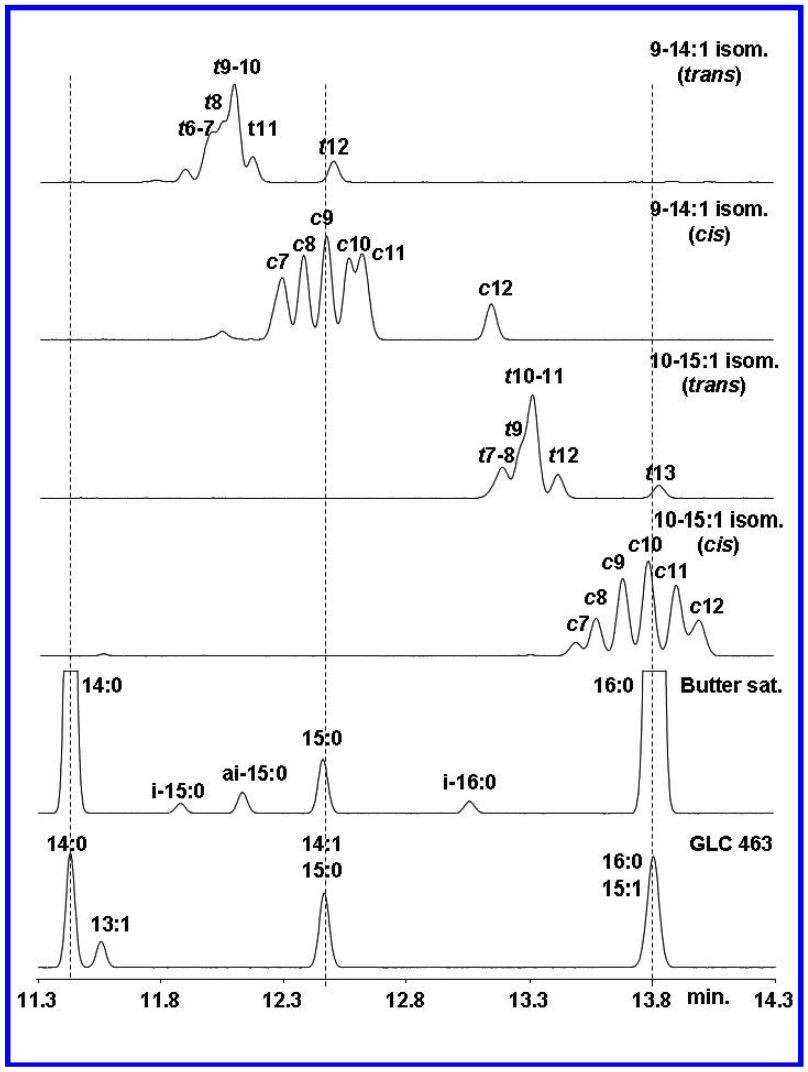

Figure 1A. GC separation of mixtures. From the top: *trans* and *cis* fractions of *cis* 9-14:1 and *cis* 10-15:1 FAMEs brominated/debrominated five times, saturated FAMEs fractionated from milk fat (as FAMEs), Reference Mixture GLC 463. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 26 cm/s linear velocity, 180°C isothermal elution.

Designed for the analysis of pure fats and oils, Ce 1h-05 must be coupled with a FAME preparation procedure such as AOCS Official Method Ce 2-66 or ISO 5509 (13, 14), and, if needed, with a fat extraction procedure. Ce 1h-05 is not suitable for the analysis of samples that contain short-chain FAMEs, such as dairy fats, or that contain long-chain PUFAs. However, the method provides superior separation of 18:1 and 18:2 FAME positional/geometrical isomers.

In 2007, in order to complement Ce 1h-05, AOCS introduced Recommended Practice Ce 1j-07 for the determination of *cis*-, *trans*-, saturated, monounsaturated, and PUFAs in dairy and ruminant fats by capillary GLC (15). Based on the separation offered by the same CP-Sil 88 and Supelco 2560 capillary columns, and a temperature program from 45 to 215°C (16), AOCS Ce 1j-07 provides separation of short-chain FAME in exchange for a slightly greater overlap

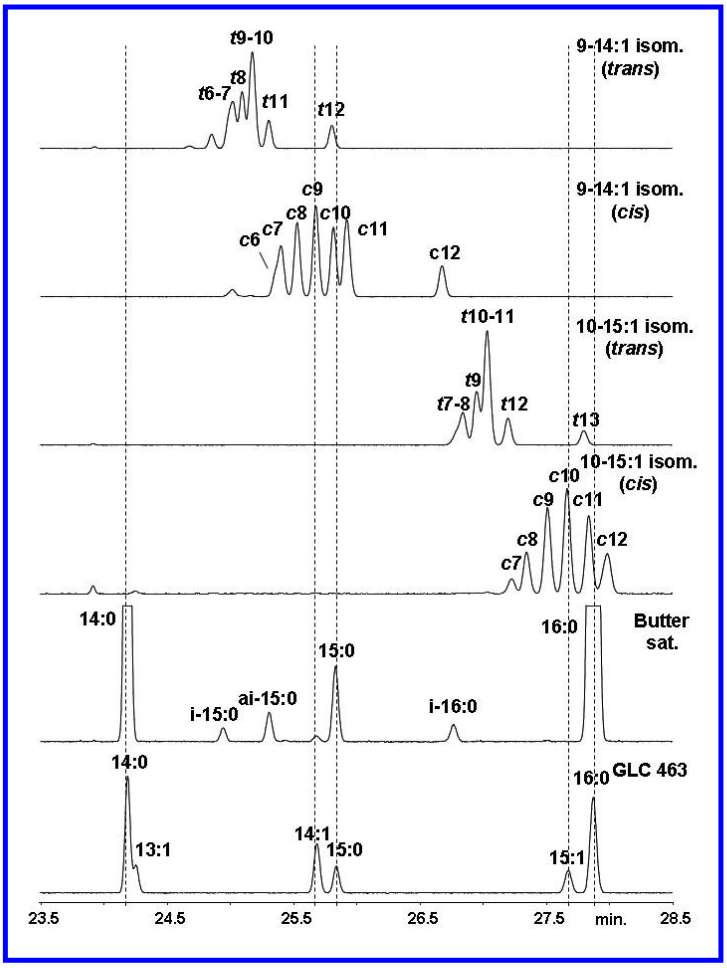

Figure 1B. GC separation of mixtures. From the top: *trans* and *cis* fractions of *cis* 9-14:1 and *cis* 10-15:1 FAMEs brominated/debrominated five times, saturated FAMEs fractionated from milk fat (as FAMEs), Reference Mixture GLC 463. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 23 cm/s linear velocity, oven temperature program: initial temperature 45°C (hold 4 min); ramp 12°C/min to 175°C (hold 27 min); ramp 4°C/min to 215°C (hold 20 min).

in the separation of *cis*- and *trans*-18:1 FAME. AOCS Ce 1j-07 contains reference chromatograms for FAME identification, but does not yet include validation data. AOCS Ce 1j-07 must also be coupled with a FAME preparation procedure, such as AOCS Official Method Ce 2-66 or ISO 5509 (13, 14), and, if needed, with fat extraction.

The identification of FAME in both methods relies on the comparison with the FAME separation of selected oils and fats, and of available reference materials. AOCS Ce 1j-07 also includes a table of FAME retention times, but these are not related to that of an internal standard. Unknown samples might contain FAs different from those contained in the oils and fats selected for the reference chromatography and not available as reference materials, or FAs other than those indicated in the reference chromatography might be present at the same retention time. Accurate identification in these situations is not possible.

This report describes the preparation of mixtures containing FAMEs not available as reference materials, in order to develop a more complete FAME elution pattern. FAME mixtures are produced by a combination of chemical reactions and/or chromatographic fractionations. FAME

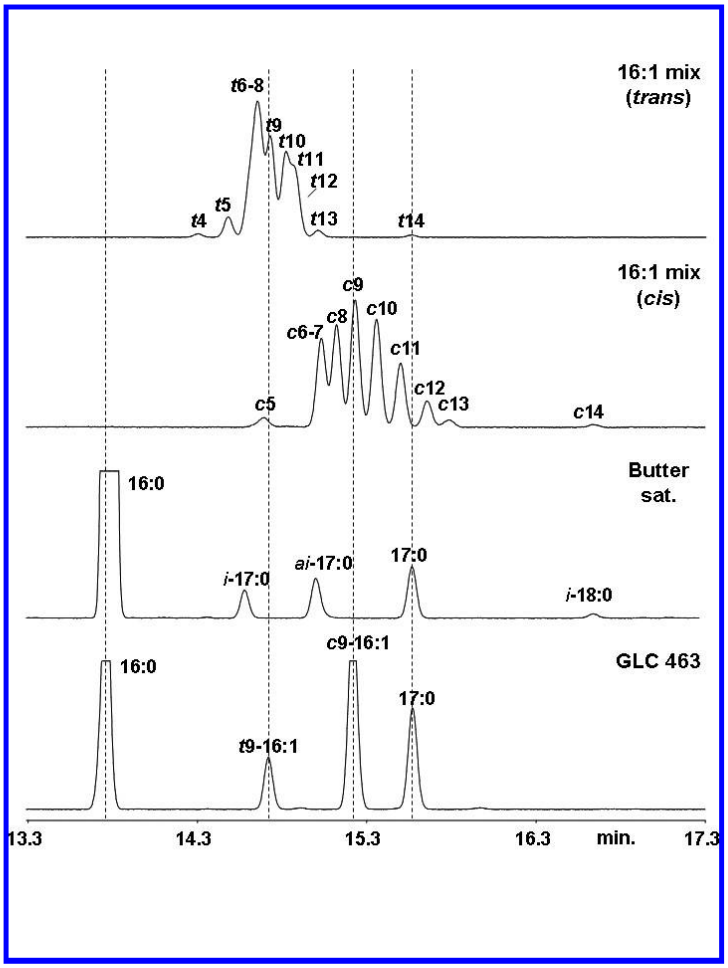

Figure 2A. GC separation of mixtures. From the top: *trans* and *cis* fractions of *cis* 9-16:1 FAME brominated/debrominated six times, saturated FAMEs fractionated from milk fat (as FAMEs), and Reference Mixture GLC 463. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 26 cm/s linear velocity, 180°C isothermal elution.

identification is achieved by a combination of comparison with available literature and GC/MS/MS analysis. The FAME mixtures are then analyzed under the chromatographic conditions of AOCS Ce 1j-07 and AOCS Ce 1h-05 in order to develop the short- and medium-chain FAME elution pattern under the conditions of both methods.

Small differences in the GC column polarity, generally caused by different production lots or age, or slight differences in the oven temperature handling, can alter the elution order of FAs with different unsaturation (17, 18). The chromatographic separations included here were all obtained using the same Supelco 2560 capillary column, the same Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA), ultrapure H_2, and are compared with the separation of GLC 463 Reference Mixture (Nu Check Prep, Elysian, MN).

Techniques for Preparative FA Separation

Silver Ion (Ag^+)–TLC

Ag^+ TLC is the simplest approach to FAME fractionation based on FA double-bond number and geometric configuration. A detailed procedure for Ag^+ TLC fractionation of milk FAME was described by

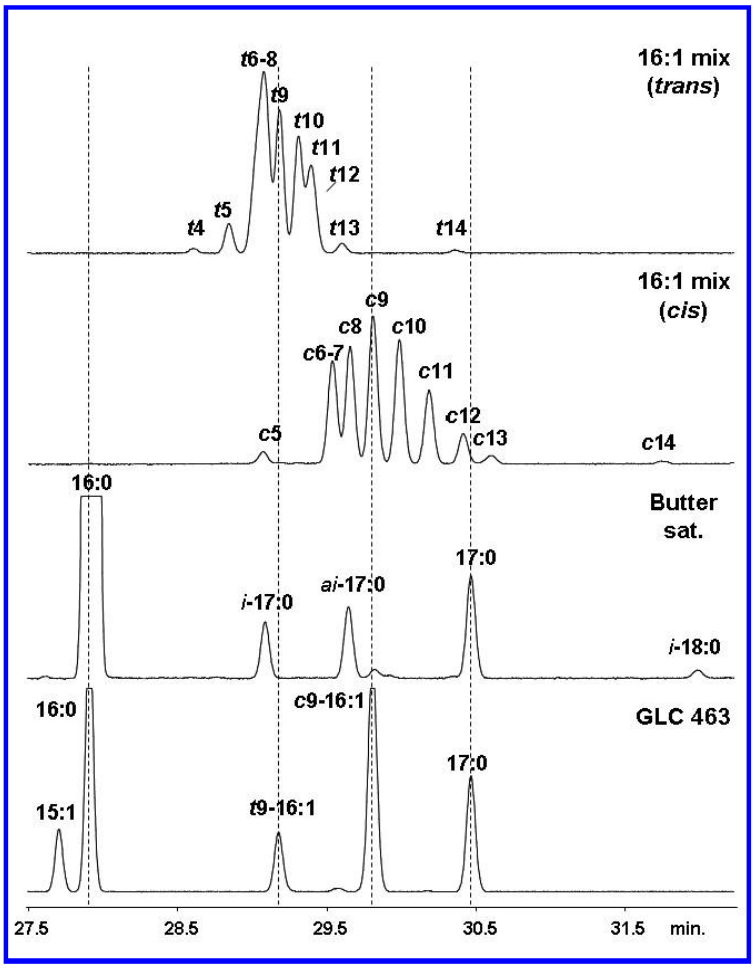

Figure 2B. GC separation of mixtures. From the top: *trans* and *cis* fractions of *cis* 9-16:1 FAME brominated/debrominated six times, saturated FAMEs fractionated from milk fat (as FAMEs), Reference Mixture GLC 463. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 23 cm/s linear velocity, oven temperature program: initial temperature 45°C (hold 4 min); ramp 12°C/min to 175°C (hold 27 min); ramp 4°C/min to 215°C (hold 20 min).

Cruz-Hernandez et al. (16). Silica Gel G plates are prewashed with methanol–chloroform (50 + 50, v/v), activated by heating for 1 h at 110°C, soaked in 5% $AgNO_3$ in acetonitrile (w/v), and reactivated for 1 h at 110°C. The TLC plates are developed using hexane–diethyl ether (90 + 10, v/v) eluent, and the FAMEs are separated in bands containing, in progressive order of retention, saturated FAs, *trans*-monounsaturated FAs (MUFAs), *cis*-MUFAs with conjugated linoleic acid (CLA), and PUFAs. Fournier et al. (19) described the fractionation of geometrically isomerized EPA and DHA by Ag^+-TLC. Geometrical isomers of EPA and DHA are separated in bands based on the number of double bonds in the *trans* configuration. The application and principles of TLC separation of lipid classes and FAMEs are reviewed in detail by Christie (20).

Ag^+–HPLC

Ag^+–HPLC has been widely applied to the separation and quantitation of FAs. The FAs are separated based on the number of double bonds, their configuration, and to some extent, on their chain length. Earlier methods relied on columns self-loaded with silver ions (21) and transformation

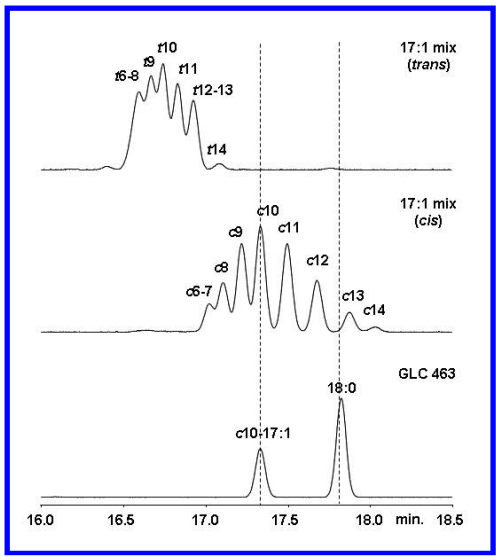

Figure 3A. GC separation of mixtures. From the top: *trans* and *cis* fractions of *cis* 10-17:1 FAME brominated/debrominated six times, Reference Mixture GLC 463. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 26 cm/s linear velocity, 180°C isothermal elution.

of FAs into aromatic esters (21–26). Adlof described the separation of FAs as FAMEs with UV monitoring at 206 nm using acetonitrile in hexane as the elution solvent (27, 28). Eulitz et al. (29) described a procedure for separation and quantitation of CLA positional isomers using three Chromspher 5 Lipids columns (Varian Inc.) in series, 0.1% acetonitrile (MeCN) in hexane as mobile phase, and UV detection at 233 nm. This procedure was then optimized by Delmonte et al. (30–32). The current most common application of Ag^+–HPLC is the fractionation of saturated, *trans*-monounsaturated, *cis*-monounsaturated, and polyunsaturated FAMEs prior to GC quantitation (16, 33, 34). *Trans*- and *cis*-MUFAs are well separated into two distinctive groups by using a single analytical size Ag^+–HPLC column and 0.1–0.2% acetonitrile in hexane elution solvent (16, 35). The separation of FA positional isomers of MUFAs and CLA is generally achieved using multiple columns (usually three) in series. For TFA quantitation, Christie (20) suggests collecting saturated and *trans*-monounsaturated FAMEs together, and then using saturated FAMEs as the relative reference for GC analysis. An alternative approach, based on the same principle, is the fractionation of FAME by SPE using strong cation exchange cartridges loaded with silver ions (Discovery® Ag-Ion SPE; Sigma-Aldrich, St. Louis, MO). FAME can be separated based on the double-bond number/geometric configuration applying the procedure developed by the SPE tube manufacturer (36).

Reversed-Phase HPLC

Reversed-phase HPLC is a less popular approach to FA separation than argentation chromatography. However, some recent applications have revived interest in this technique for FA separation. Destaillats and Juaneda (34, 37) described a procedure for separating *trans* monounsaturated FAME as a single peak prior to GC quantitation. Tsuzuki and Uchida (38), focusing on the separation of MUFAs, evaluated the separation offered by different chromatographic columns, mobile phase compositions, and elution temperatures. Delmonte et al. (39), using one or three Vydac 201TP54 (250 × 4.6 mm, 5 μm particle size; Grace Davison, Deerfield, IL) HPLC columns in series, maintained at –15°C, and 5% hexane in MeCN as the elution solvent at 1 mL/min, separated from a synthetic mixture the *cis*-17:1 FAMEs with double-bond positions 6 to 14.

High-Speed Counter Current Chromatography (CCC)

Liquid–liquid chromatography, in the form of counter current distribution (CCD), has been extensively applied to FA fractionation (40–44). As an example, Scholfield et al. (42) separated the FAMEs from alkali isomerized linolenic acid by argentation CCD using 0.2 N $AgNO_3$ in 90% methanol and hexane as the repartitioning solvents. The primary advantages of liquid–liquid chromatography compared to HPLC are the

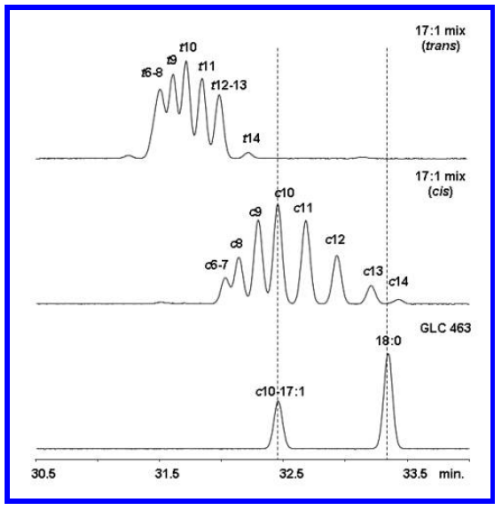

Figure 3B. GC separation of mixtures. From the top: *trans* and *cis* fractions of *cis* 10-17:1 FAME brominated/debrominated six times, Reference Mixture GLC 463. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 23 cm/s linear velocity, oven temperature program: initial temperature 45°C (hold 4 min); ramp 12°C/min to 175°C (hold 27 min); ramp 4°C/min to 215°C (hold 20 min).

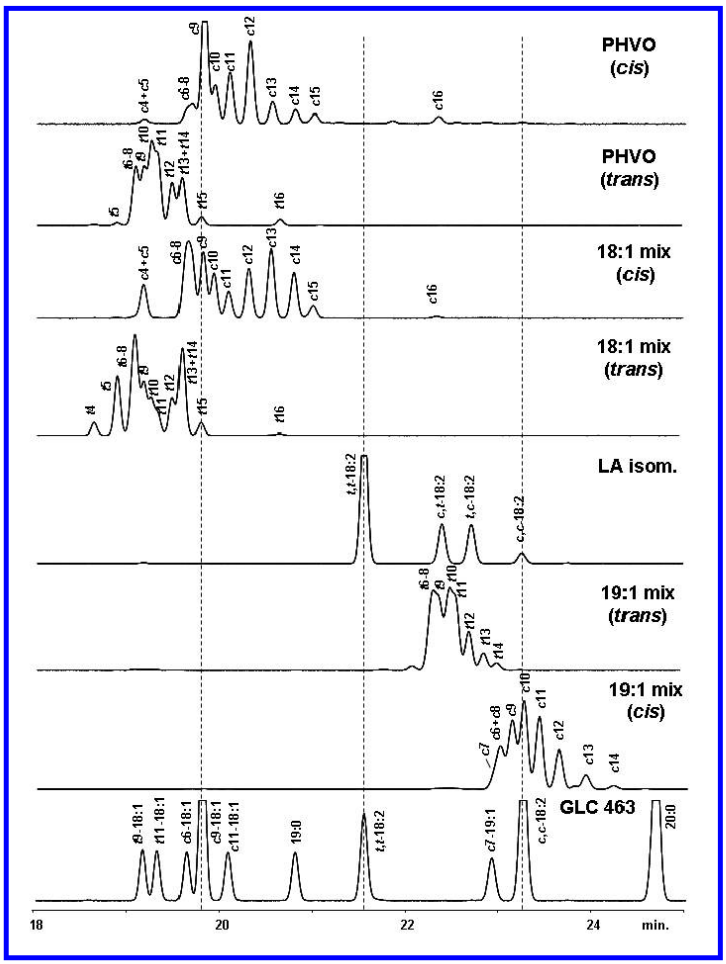

Figure 4A. GC separation of mixtures. From the top: *trans* and *cis* fractions of partially hydrogenated vegetable oil (PHVO) and of a mixture containing *cis* 6-, *cis* 9-, *cis* 13-18:1 FAME brominated/debrominated two times, linoleic acid FAME isomerized by PTSA, *trans* and *cis* fractions of *cis* 10-19:1 FAME brominated/debrominated six times, Reference Mixture GLC 463. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 26 cm/s linear velocity, 180°C isothermal elution.

higher load capacities, which allow the separation of grams of compounds in a single run, the lower amounts of solvent needed per gram of purified compound, and the recovery of all noneluted compounds by subsequent extraction of the liquid stationary phase. Large CCD apparatuses have now been replaced by benchtop CCC systems (45 48), and recently, by high-speed CCC (HSCCC; 49). Bousquet and Le Goffic (48) separated the FAs 16:3, 18:4, 20:5, and 22:6 from the *Skeletonema costatum* algae by reversed-phase CCC, injecting 2.4 g FAs for each separation. Cao and Ito (49) separated the main FAs contained in grape seeds by HSCCC. A comprehensive guide to CCC method development, including a collection of separation conditions already reported in the literature, was recently published by Ito (50).

Urea Fractionation

In 1940, while working on the analysis of milk, Bengen (51, 52) accidentally discovered that urea, in water, ethanol and methanol, forms well-defined crystalline inclusion compounds with aliphatic compounds having a straight chain of at least six carbons, but not with branched chain and cyclic compounds. Since that time, urea

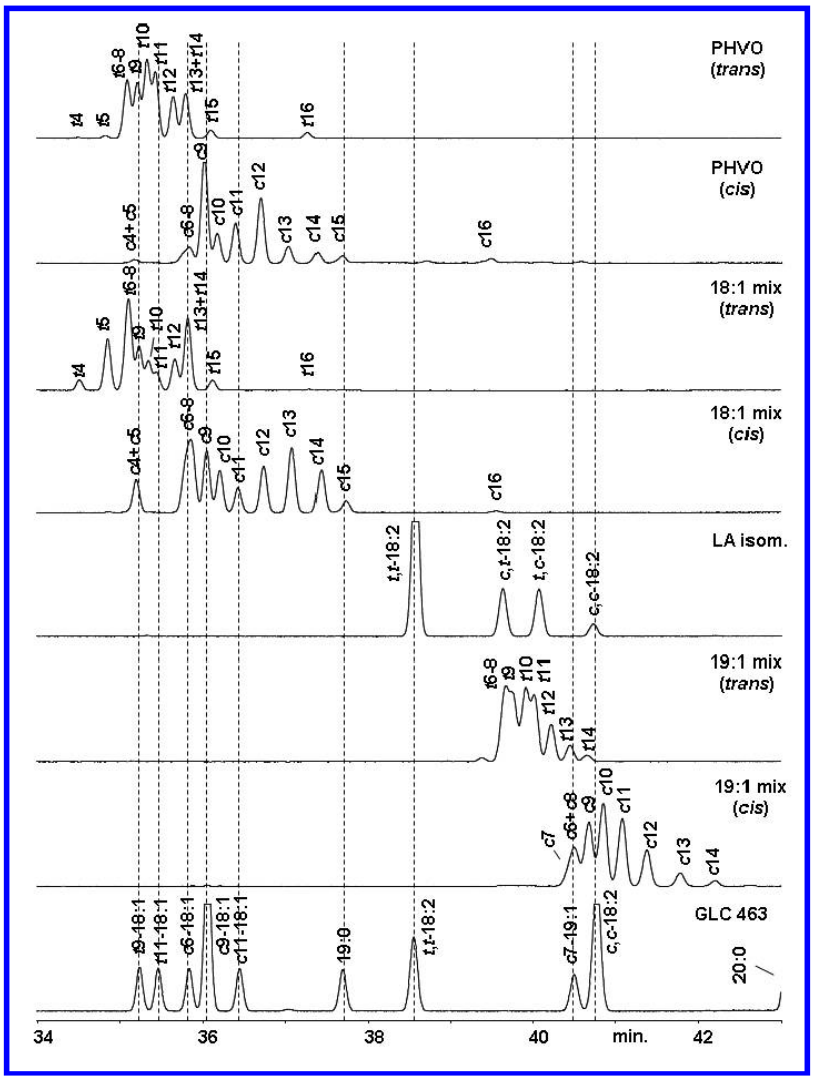

Figure 4B. GC separation of mixtures. From the top: *trans* and *cis* fractions of PHVO and of a mixture containing *cis* 6-, *cis* 9-, *cis* 13-18:1 FAME brominated/debrominated two times, linoleic acid FAME isomerized by PTSA, *trans* and *cis* fractions of *cis* 10-19:1 FAME brominated/debrominated six times, Reference Mixture GLC 463. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 23 cm/s linear velocity, oven temperature program: initial temperature 45°C (hold 4 min); ramp 12°C/min to 175°C (hold 27 min); ramp 4°C/min to 215°C (hold 20 min).

fractionation has been widely applied to the fractionation of FAs. FAs are separated by urea fractionation based on the fact that some of them form adducts preferentially. The stability of urea complexes increases with increasing FA chain length. At constant chain length, the complex stability decreases by increasing the number of unsaturations, and FAs with *trans* double bonds are preferred over FAs with *cis* double bonds (53, 54). A simple procedure for urea fractionation of FAMEs was described by Christie (20). Twenty grams of urea are added to 10 g FAMEs dissolved in 100 mL methanol. The mixture is warmed until the urea dissolves, then slowly cooled to room temperature with occasional mixing. About 4 h later,

the crystals are filtered and washed with methanol saturated with urea. The liquid phase is acidified with 60 mL 1% HCl, and FAMEs are extracted with 50 mL hexane. The precipitated complexes are broken by the addition of water, and FAMEs are recovered with hexane.

Chemical Reactions for Preparing Mixtures of Unavailable FAMEs

P-Toluene Sulfinic Acid Isomerization

P-toluene sulfinic acid (PTSA) can react with isolated double bonds causing their geometric isomerization but not migration. The product of such a reaction is a mixture of FA geometric isomers. *Trans* double bonds are formed in higher relative amounts than the *cis* counterpart if the reaction proceeds to equilibrium. PTSA isomerization can be carried out according to the procedure originally described by Snyder and Scholfield (55). PTSA is generally produced by acidification of its commercially available sodium salt. A 30 mL volume of deionized water, 2 mL 37% HCl, 30 mL diethyl ether, and 1 g PTSA sodium salt are added in sequence to a 125 mL separatory funnel. The contents are mixed until the PTSA sodium salt is fully dissolved and the organic phase appears clear. The diethyl ether phase containing the acid is washed with 30 mL deionized water acidified with 1 mL 37% HCl, and dried under vacuum. A small-scale modification of the original isomerization procedure was described by Delmonte et al. (39). A 1 mg or smaller amount of an unsaturated FAME is dried at the bottom of a 2 mL screw-cap autosampler vial, and 250 µL of 1 mg/mL PTSA solution in 1,4-dioxane is added. The vial, purged with argon and tightly capped, is held 15 min in a GC oven at 100°C. The contents are neutralized at room temperature by adding 300 µL 1 N NaOH and extracted with 1 mL isooctane. The organic layer is washed twice with 500 µL deionized water, dried with a stream of argon (or nitrogen) in a clean 2 mL autosampler vial, and dissolved in 1 mL isooctane. Amber sylanized vials are preferred as reaction vessels.

Partial Hydrogenation with Hydrazine

Hydrogenation (or reduction) of the FA double bonds is a procedure commonly applied by industry and research laboratories to reduce the number of FA double bonds. FAs are hydrogenated by heating them in the presence of a stoichiometric amount of hydrogen gas and traces of a catalyst such as platinum, nickel palladium, or copper. Scholfield (56) partially hydrogenated linoleic acid by heating 300 mg FA and an equimolar amount of hydrogen at 140°C in the presence of 0.5% nickel catalyst, or at 50°C in the presence of 0.09% platinum. Hydrazine is a preferred reagent for small-scale partial or full hydrogenation of FAs, based on the fact that it does not cause double-bond geometric/positional isomerization and it removes the hazard associated with use of hydrogen. This reaction can be carried out according to principles described by Christie (57). An example of its application is the partial hydrogenation of γ-linolenic acid (γ-LA) or α-LA to produce methylene-interrupted dienes that are intermediates in the preparation of CLA isomers (30–32). A half gram of γ-LA or α-LA free fatty acid (FFA) is stirred for 2.5 h at 45°C in an open-to-air container with 100 mL 10% hydrazine hydrate in methanol solution. The reaction is stopped by the addition of 100 mL H_2O, followed by 40 mL 6 N HCl. Partially hydrogenated FFAs are extracted three times with 50 mL diethyl ether/petroleum ether [(DE/PE) (50 + 50, v/v)]. The combined extracts are dried over anhydrous Na_2SO_4, and the solvent is removed in a stream of argon.

Conjugation with Alkali

Conjugation with alkali is a reaction widely applied to the preparation of FAs with conjugated double bonds from methylene-interrupted precursors. The availability of the 50:50 (w/w) *c*9,*t*11-18:2 and *t*10,*c*12-18:2 CLA isomeric mixture produced by alkali conjugation of linoleic acid has been essential for CLA research. When reacting FAs with two methylene-interrupted double bonds, one of the double bonds remains in its position and retains its configuration, while the other migrates to the conjugated position. If the migrating double bond is in the *cis* configuration, it will quantitatively convert to *trans*; if it is in the *trans* configuration, it will achieve about 50:50 *cis*/*trans* geometric configuration (29, 58). The reaction of FAs with three or more methylene-interrupted double bonds produces partially and fully conjugated polyenes in variable ratios according to the selected reaction time, temperature, and alkali concentration (59). The conjugation of highly unsaturated FAs, especially at high temperature, is in competition with the reaction of self-cyclization and polymerization. A practical procedure was described by Delmonte et al. (30–32) for producing CLA mixtures from methylene-interrupted dienes. A 75 mL amount of 6.6% KOH in ethylene glycol is heated at 150–160°C in a 100 mL three-neck round-bottom flask. A condenser is mounted on the main neck; a thermometer and the argon purge line are mounted on the other two. Ethylene glycol must be dry. Traces of water can be eliminated by heating the solvent for 1 h under nitrogen purge before adding KOH. A half gram of methylene-interrupted diene FFAs (e.g., linoleic acid) is added to the flask, and the solution is stirred for 2 h at 150–160°C. Once it reaches room temperature, the solution is moved to a separatory funnel and neutralized with 6 N HCl until acidic. The FFAs are extracted three times with DE/PE (50 + 50). Extracts are combined, filtered through anhydrous sodium sulfate, and dried in a stream of argon (or N_2). Increasing alkali concentration or temperature might result in nonselective double-bond migration and geometric isomerization.

Addition/Elimination of Hydrobromic Acid (HBr)

Addition followed by elimination of HBr to a monounsaturated FAME produces a mixture of its positional isomers with the double bond in position n–1, n, and n+1 in the ratio 1:2:1 (39). Starting from a pure FA, the combination of reactions can be repeated until the desired level of double-bond position isomerization is reached. The formation

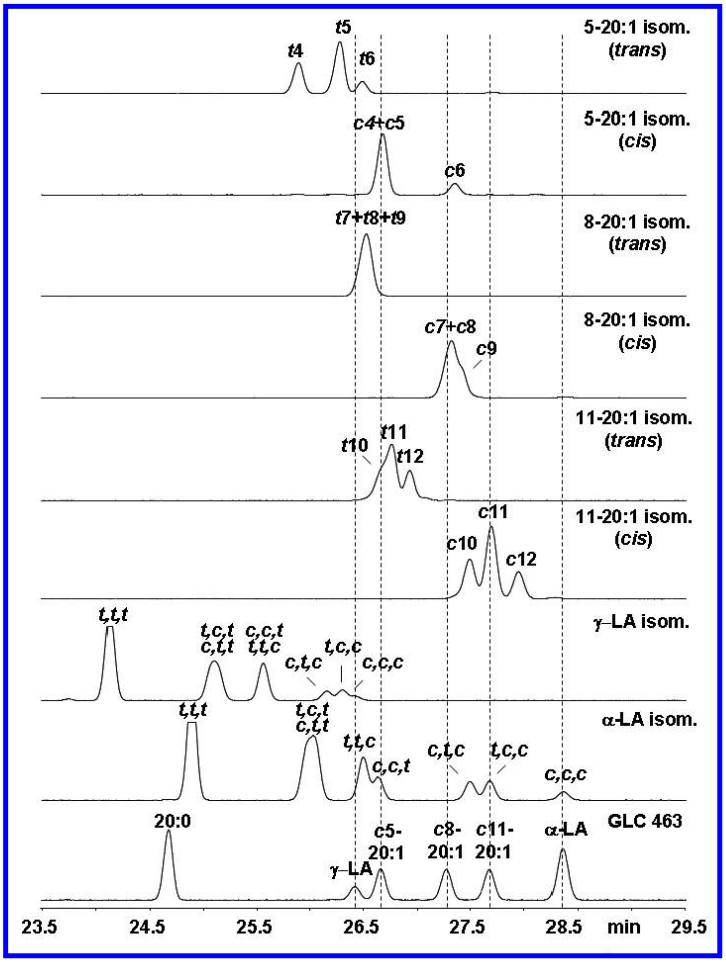

Figure 5A. GC separation of mixtures. From the top: *trans* and *cis* fractions of *cis* 5-, *cis* 8-, and *cis* 11-20:1 FAME brominated/debrominated one time, γ-linoleic acid and α-linoleic acid FAME isomerized by PTSA, Reference Mixture GLC 463. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 26 cm/s linear velocity, 180°C isothermal elution.

of *trans* double bonds is slightly preferred over the *cis* counterparts. Electronic and hindrance factors might affect selectivity when the double bond is located near either extremity of the FA. Both reactions, addition and elimination of HBr to unsaturated FAs, were widely applied during an earlier period of FA research (1930–1960) and the principles were reviewed in detail by Sonntag in 1961 (60). Delmonte et al. (39) described a simple procedure for the isomerization of *cis* 10-17:1 FAME (Nu Chek Prep) that can be applied to the isomerization of every MUFA with the double bond not in the terminal position, or close to the carboxyl group. About 100–500 mg MUFA and 1 mL toluene are placed in a 20 mL screw-cap test tube purged with argon. The tube is placed in a chiller maintained at 15°C, and 2 mL chilled 33% HBr in glacial acetic acid are added. The tube, protected from light exposure, is then maintained for 3 h at 15°C, followed by 48 h at 5°C. The time of reaction might be reduced for shorter-chain FAME, but 48 h are recommended for completeness of the reaction. The products of the reaction are transferred to a 125 mL separatory funnel, along with 20 mL DE/PE (50 + 50, v/v), and 50 mL saturated $NaHCO_3$ distilled water (50 + 50) is slowly added. The neutralization reaction produces a large amount of carbon dioxide, and the separatory funnel must be continuously vented during mixing. The

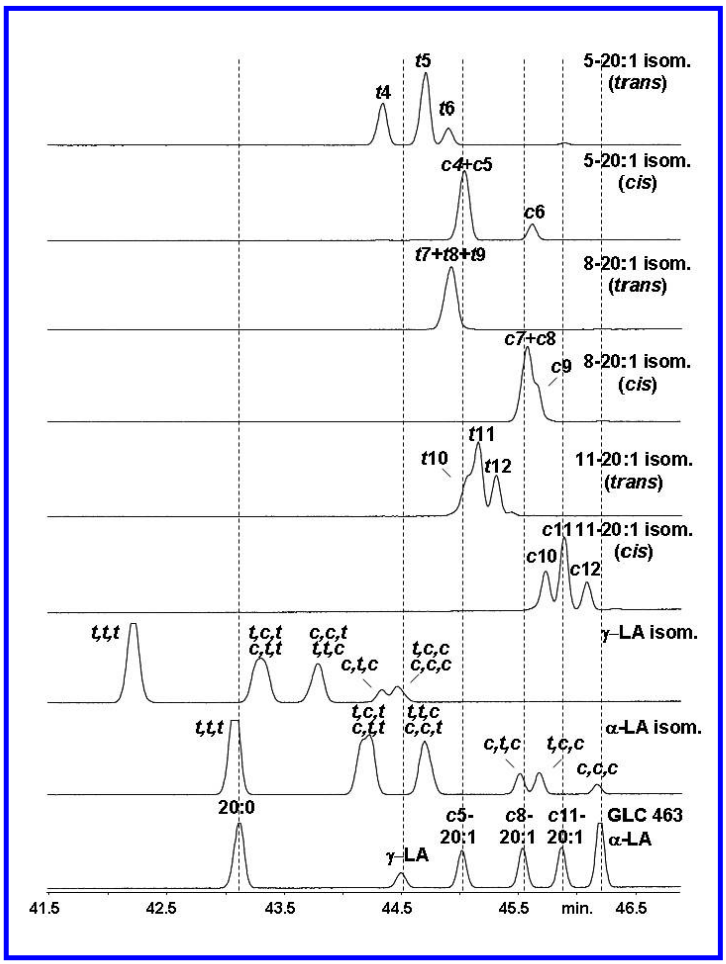

Figure 5B. GC separation of mixtures. From the top: *trans* and *cis* fractions of *cis* 5-, *cis* 8-, and *cis* 11-20:1 FAME brominated/debrominated one time, γ-linoleic acid and α-linoleic acid FAME isomerized by PTSA, Reference Mixture GLC 463. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 23 cm/s linear velocity, oven temperature program: initial temperature 45°C (hold 4 min); ramp 12°C/min to 175°C (hold 27 min); ramp 4°C/min to 215°C (hold 20 min).

aqueous phase is re-extracted with 10 mL DE/PE (50 + 50, v/v), and the combined extracts are washed with 50 mL distilled water acidified with 2 3 drops of 6 N HCl. The organic phase is filtered through anhydrous Na_2SO_4 into a screw-cap test tube, and the solvent is removed under a gentle stream of argon. A 5 mL amount of 1 M potassium *t*-butoxide in *t*-butanol is added to the brominated product. The test tube is purged with argon and placed for 24 h at 100°C in a silicon oil bath protected from light exposure. The products of reaction are transferred at room temperature to a 125 mL separatory funnel along with 20 mL DE/PE (50 + 50, v/v). A 5 mL volume of 6 M HCl is added, followed by 50 mL saturated $NaHCO_3$ distilled water (50 + 50). If the pH of the aqueous phase is still basic after mixing, more 6 M HCl must be added until the pH is slightly acidic. The aqueous layer is re-extracted with 10 mL DE/PE (50 + 50, v/v), and the combined extracts are washed with 50 mL distilled water acidified with 2 3 drops of 6 M HCl. The organic phase is filtered through anhydrous Na_2SO_4 into a screw-cap test tube, and the solvent is removed under a gentle stream of argon. The dry FAs can be reacted again, or, because of the partial hydrolysis to FFAs occurring during the process, can be

re-converted to FAME using boron trifluoride (BF_3) in methanol before chromatographic analysis.

Iodine Isomerization

Isomerization of conjugated double bonds by reversible addition of iodine is a reaction used to produce mixtures of all geometric isomers of a given conjugated FA. Eulitz et al. (29) described the application of this reaction to the isomerization of CLA. When carried to equilibrium, the isomerization of a conjugated diene produces a mixture of its geometric isomers in the approximate ratio of 88% *t,t*; 5.5% *t,c*; 5.5% *c,t*; and 1% *c,c*. About 5–20 mg of conjugated diene FAMEs are dissolved in 2 mL petroleum ether in a glass screw-cap test tube. A few drops of 0.06 mg/mL iodine solution in petroleum ether are added until a light pink color appears. The tube should be maintained for 30 min in the presence of indirect light; the reaction is stopped by adding 5 mL aqueous 0.01 N $Na_2S_2O_3$ and shaking for 10 s. The organic layer is repeatedly washed with 5 mL 0.01 N $Na_2S_2O_3$ to remove the remaining iodine until no more light pink color develops, and is then washed with 5 mL distilled water and filtered through anhydrous Na_2SO_4.

Identification of FAME by Combination of GC-Covalent Adduct Chemical Ionization (CACI)-MS/MS and Ag^+-HPLC

The identification of the MUFAs contained in the mixtures was achieved by GC coupled with CACI-MS/MS, using acetonitrile as the chemical reagent gas. This technique, developed by the team of Brenna (61–64), allows the identification of the double-bond positions of unsaturated FAMEs by using an ion trap mass spectrometer, without the need for preparing other derivatives. MeCN, used as a chemical ionization reagent, produces the 1-methyleneimino-1-ethenylium ion (*m/z* 54; MIE) by self-reaction. The MIE ion reacts with double bonds of unsaturated FAMEs, yielding the covalent adduct ions of mass $(M + 54)^+$. Collisional dissociation of the $(M + 54)^+$ adduct causes cleavage at both allylic sites or the double bond, providing two fragments that unambiguously identify double-bond positions. Delmonte et al. applied this technique to the identification of 17:1 FAMEs with double-bond positions from 6 to 14. Other authors, applying the same principle, showed that this technique was capable of identifying FAs with two or more double bonds (62, 64). In the case of CLA, this technique can identify the geometric configuration of the two conjugated double bonds based on the relative abundance of the two allylic fragmentation ions (62, 63). However, with the exception of CLA FAMEs, this technique cannot identify the double-bond geometric configuration. As a consequence, FAMEs must be fractionated by Ag^+-HPLC, Ag^+-TLC, or Ag^+-SPE based on the number and the geometric configuration of double bonds prior to CACI-MS/MS analysis.

Preparation of Selected FAME Reference Mixtures

GC Separation Conditions

All the chromatograms shown in series A in Figures 1–5 were acquired under the experimental conditions specified by AOCS Official Method Ce 1h-05 using an Agilent 6890N gas chromatograph equipped with a Supelco 2560 capillary column and a flame ionization detector. Hydrogen carrier gas was used at 1 mL/min with the linear velocity of 26 cm/s. The split ratio was set to 1:100 and the typical injection volume was 1 μL. The injection port was maintained at 250°C, the detector at 250°C, and the oven at 180°C (isothermal elution).

All the chromatograms shown in series B in Figures 1–5 were acquired using the temperature program (16) specified under the experimental conditions of AOCS Recommended Practice Ce 1j-07 using the same gas chromatograph and the same capillary column. Hydrogen carrier gas was used at 1 mL/min with a linear velocity of 23 cm/s. The split ratio was set to 1:100, and the typical injection volume was 1 μL. The injection port was maintained at 235°C and the detector at 325°C. The oven temperature program was as follows: initial temperature 45°C, hold 4 min; ramp 12°C/min to 175°C, hold 27 min; ramp 4°C/min to 215°C, hold 20 min (16).

Straight-Chain Saturated and Branched-Chain FAME

Straight-chain saturated FAMEs up to 26:0 are commercially available, relatively inexpensive, and are the main component of most commercial FAME reference mixtures. Branched-chain FAMEs are only partially available as reference materials and are quite expensive (Matreya LLC, Pleasant Gap, PA). The simplest approach to the preparation of a mix containing branched-chain FAMEs is the separation by Ag^+-HPLC or Ag^+-TLC of the saturated FAME fraction from milk fat FAMEs. The separation by Ag^+-HPLC can be achieved using a single analytical size silver ion-loaded column and 0.1–0.2% MeCN in hexane eluent at 1.0 mL/min, and collecting the fraction from the dead volume (included) to the *trans*-monoenes (excluded). Because saturated FAMEs will not show a UV signal, the collection window must be set based on the *trans*-monoenes elution monitored at 200 nm. The mix will contain all the straight-chain FAME up to 18:0 FAME, the *iso*- (*I*-) and *anteiso*- (*ai*-) isomers of odd chain length FAMEs up to 17:0 FAME, and the *iso*- isomers of even chain length FAME up to 18:0 FAME, with *iso*-18:0 FAME contained in trace amounts. Recently, an Ag^+-SPE procedure was reported to isolate saturated and branch-chain FAME from milk fat (65). Alternatively, branched FAs can be separated from lanolin FAs (as FAMEs), but this preparation is complicated by the presence of hydroxyl-FAs and by the more complex GC elution profile of the saturated FAME fraction. Under the conditions of AOCS Ce 1h-05 and AOCS Ce 1j-07, for a given FAME chain length, the *iso*- isomer elutes first, followed by the *anteiso*- isomer, and then the straight-chain isomer.

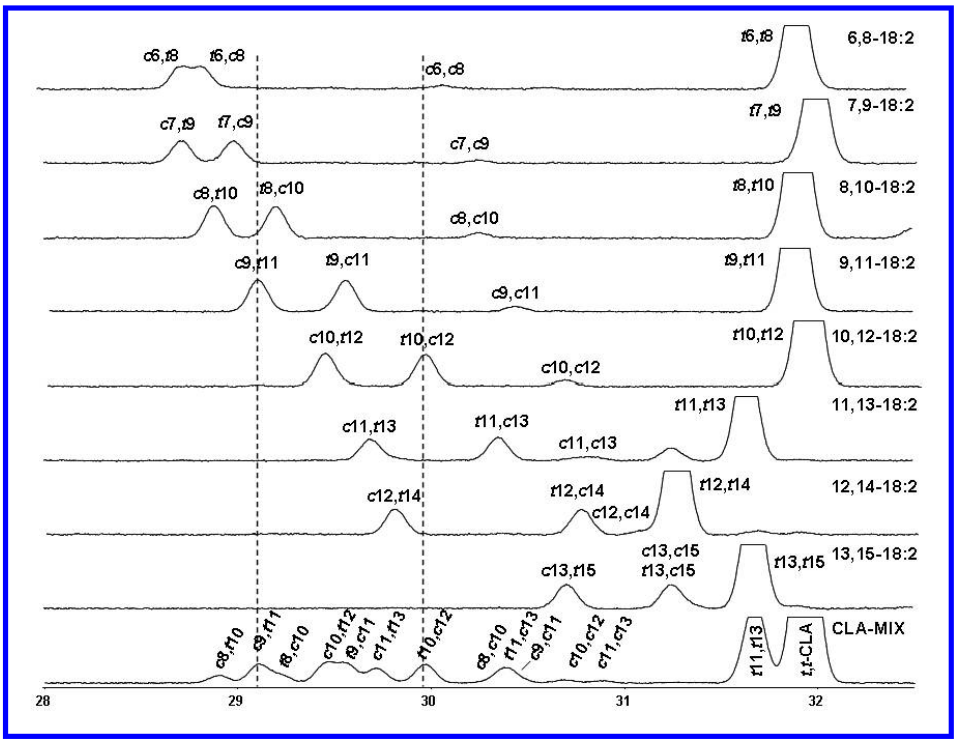

Figure 6A. GC separation of mixtures. From the top: geometric isomers of CLA positional isomers from double-bond position 6,8- to 13,15-18:2 prepared as described in ref. 30, and four isomers CLA mix (Nu Check Prep) isomerized with iodine. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 26 cm/s linear velocity, 180°C isothermal elution.

14:1 and 15:1 FAMEs

The commercial availability of 14:1 and 15:1 FAMEs is limited to that of *cis/trans* 9-14:1 FAMEs, *cis/trans* 10-15:1 FAMEs and 14-15:1 FAME (Nu Chek Prep). 14:1 FAMEs are found in milk and dairy products (66), while the odd chain 15:1 FAMEs are less common. Positional isomer mixtures of 14:1 and 15:1 FAMEs can be produced by repeated bromination/debromination of the commercially available isomers. Four or five repetitions are recommended. The final products of reaction are reconverted to FAME using BF_3 in methanol and separated into *cis* and *trans* isomers by Ag^+-HPLC or Ag^+-TLC. Figures 1A and 1B show the separation of the mixtures prepared under the experimental conditions of AOCS Ce 1h-05 and AOCS Ce 1j-07 compared with the separation of saturated FAMEs isolated from butter. Separation of the *trans* and *cis* 14:1 and 15:1 isomers is improved using a GC temperature program (16) compared to the isothermal setting specified by AOCS Ce 1h-05. The accurate quantitation of 14:1 and 15:1 FAMEs according to these methods would require preventive argentation fractionation to remove saturated FAMEs. Identifications of the double-bond positions of the FAMEs contained in these mixtures are achieved by GC-CACI-MS/MS. For identification purposes, a clear separation of the *cis* or *trans* positional isomers can be achieved by isothermal elution at 110°C for the 14:1 FAMEs, and at 120°C for the 15:1 FAMEs. These results are consistent with previous reports that showed a marked improvement of the *trans* monounsaturated fatty acid isomers separation at reduced isothermal temperature settings of 120°C (16, 67, 68).

16:1 FAMEs

In several research manuscripts, 16:1 FAME positional/geometric isomers and saturated 17:0 FAME isomers are only partially quantified, or are quantified based on analyst assumptions. The commercial availability of the 16:1 FAMEs is limited to that of *cis/trans* 9-16:1 FAMEs present in the GLC 463 Reference Mixture. A mixture containing all the positional isomers of 16:1 FAME can be produced by repeated bromination/debromination of the *cis* or *trans* 9-16:1 FAME. It is suggested that the isomerization be repeated 6–8 times. The products of reaction are separated into *cis* and *trans* isomers by Ag^+-HPLC or Ag^+-TLC, and the positional isomers contained

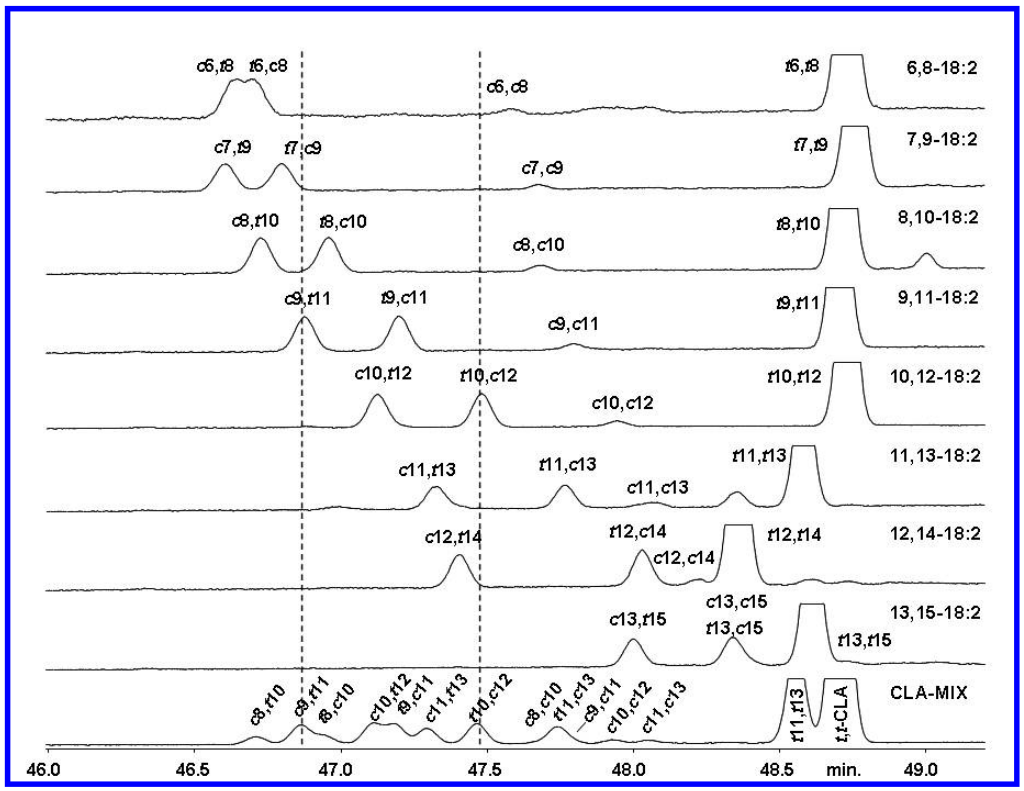

Figure 6B. GC separation of mixtures. From the top: geometric isomers of CLA positional isomers from double-bond position 6,8- to 13,15-18:2 prepared as described in ref. 30, and four isomers CLA mix (Nu Check Prep) isomerized with iodine. Conditions: Supelco 2560 capillary column, hydrogen carrier gas at 1 mL/min and 23 cm/s linear velocity, oven temperature program: initial temperature 45°C (hold 4 min); ramp 12°C/min to 175°C (hold 27 min); ramp 4°C/min to 215°C (hold 20 min).

in each mixture are identified by GC-CACI-MS/MS. *Trans* 3-16:1 FA, a common constituent of plant chloroplasts and unlikely to be contained in this mixture, can be separated from the lipid extract of spinach leaves (69). Natural products are often a good source of unique FAs.

Figures 2A and 2B show the separation of the mixtures obtained from the Ag^+–HPLC fractionation of *cis* 9-16:1 FAME isomerized six times, compared with saturated FAMEs from butter and GLC 463 reference mixture. The overlap between the *cis*- and *trans*-16:1 FAMEs is minimal when they are analyzed under the conditions of both methods, but careful quantitation requires removal of 17:0 and its branch-chain FAME isomers with argentation chromatography. Precht and Molkentin (67) noted that the overlap of the 16:1 and branch-chain 17:0 FAME in milk fat could easily lead to misidentification of these isomers, and they recommended a prior separation using Ag^+-TLC for authentic identification of the FAME. It was recently shown that a comparison at two temperature settings could also be used to resolve this isomer mixture (65).

17:1 FAMEs

The commercial availability of 17:1 FAMEs is limited to that of the *cis/trans* 10-17:1 FAMEs. Pure 17:1 FAME positional isomers from double-bond position 6 to 14 were prepared by Delmonte et al. (39). A mixture of 17:1 FAME geometric/positional isomers was produced initially by repeating the bromination/debromination process six times. The products were remethylated and separated into *cis* and *trans* isomers by Ag^+-HPLC. The *cis* fraction was separated into pure positional isomers by reversed-phase HPLC using one or three (in series) Vydac 201TP54 HPLC columns maintained at –15°C and 5% hexane in MeCN as the mobile phase. Figures 3A and 3B show the separation of *cis*-17:1 and

trans-17:1 FAME obtained by repeating the isomerization six times, compared to the GLC 463 reference mixture. The temperature program (Figure 3B) provided a better separation of *cis*- and *trans*-17:1 FAME than did the 180°C isothermal separation (Figure 3A).

18:1, 19:1, and 18:2 FAMEs

Pure *cis*- and *trans*-18:1 FAMEs are commercially available with the double bond in positions 6, 9, and 11–13 (Sigma-Aldrich). *Cis* and *trans* 7-18:1 FAMEs are not consistently available, and 15-18:1 FAMEs seem to be no longer available. *Cis*/*trans*-19:1 FAMEs are available with the double bond in positions 7 and 10 (Nu Chek Prep). A mix of the four geometric isomers of linoleic acid was recently introduced by Sigma (No. L8404). The simplest approach to the preparation of a mix containing the *cis*-18:1 FAMEs with double-bond positions from 4 to 16 and a mix containing all the *trans*-18:1 FAMEs with the same double bond positions is to fractionate a partially hydrogenated vegetable oil (PHVO) containing approximately 35–50% of TFAs, or milk fat, by Ag^+-HPLC or Ag^+-TLC. An alternative approach, if such PHVO is not available, is the repeated bromination/debromination of a mixture containing 6-, 9-, and 13-18:1 FAMEs in equal amounts. The isomerization should be repeated twice (*cis*-18:1 and *trans*-18:1 mixes shown in Figures 4A and 4B), but a third repetition increases the relative amount of 16-18:1 FAME and might produce 3-18:1.

A mixture containing most of 19:1 FAME positional isomers can be prepared by repeating the bromination/debromination of *cis* or *trans* 10-19:1 FAME 6–8 times. The isomerization of 7-19:1 FAME can be used to complement the previous mixture with isomers having the double bond from position 4 to 7. The *cis*-19:1 and *trans*-19:1 mixtures whose separation is shown in Figures 4A and 4B were prepared by reacting six times *cis* 10-19:1 FAME, and separating the products by Ag^+-HPLC. The mixture containing the four geometric isomers of linoleic acid can be purchased or prepared by isomerization of linoleic acid with PTSA according to the procedure described above. A mixture containing positional and geometric isomers of linoleic acid, along with CLA (about a 50% reaction yield), can be prepared by bromination/debromination of linoleic acid. The identification of the products of preparation is particularly challenging, and a second repetition of the process leads to the loss of the targeted FAs because of the competition of aggressive side reactions. Finally, the direct analysis of FAME prepared from milk fat might be considered in order to identify some 18:2 FAME such as *cis* 9, *trans* 13-18:2 FAME that are generally present in these samples (70). Figures 4A and 4B show the separation of the mixtures prepared following the procedures described in this section, compared to the one of GLC 463 reference mixture. If *cis*-19:1 FAMEs, *trans*-19:1 FAMEs, and linoleic acid isomers are all present in the same sample, neither of the two GC methods would be capable of providing unambiguous quantitation of these FAMEs.

20:1 and 18:3 FAMEs

20:1 FAMEs are commercially available as *cis* 5-, *cis* 8-, *cis* 11-, and *trans* 11-20:1 FAME (Nu Chek Prep). α-Linolenic acid is available as the all *cis* isomer and recently, as a mix of geometric isomers (No. L6031; Sigma-Aldrich). γ-Linolenic acid is only available as the all *cis* isomer. Mixtures containing all *cis* and *trans* 20:1 FAMEs with double-bond positions from 4 to 12 can be prepared by brominating/debrominating 5-20:1, 8-20:1, and 11-20:1 FAMEs individually one time only. If isomers with double bonds in positions higher than 12 are desired, the isomerization of 11-20:1 FAME can be repeated as needed. The three commercially available positional isomers of 20:1 FAME can be combined and reacted together to obtain a single mixture. The mixture containing all the geometric isomers of α-linolenic acid can be purchased or prepared by isomerization of α-linolenic acid with PTSA. The mixture containing all the geometric isomers of γ-linolenic acid, currently not commercially available, should be prepared by isomerization with PTSA. Figures 5A and 5B show the GC separation of the mixtures prepared following the procedures described in this section along with the separation of GLC 463. Because slight changes in GC column polarity can affect the relative elution of FAME with different double-bond numbers, the comparison with GLC 463 is needed when reproducing separations obtained with different columns.

Conjugated Linoleic Acid FAME

The availability of CLA is limited to that of the geometric isomers of 9,11-18:2 and 10,12-18:2 FAMEs, to *cis* 11, *trans* 13-18:2 FAME (Matreya LLC) and to a reference mixture containing *trans* 8, *cis* 10-, *cis* 9, *trans* 11-, *trans* 10, *cis* 12-, *cis* 11, *trans* 13-18:2 FAMEs plus traces of their *cis*, *cis*, and *trans*, *trans* isomers (Nu Check Prep). A practical reaction for producing all the geometric isomers of a given CLA isomer is isomerization with iodine, applied as described earlier. The iodine isomerization of the four isomer CLA mix produces a mixture of all the geometric isomers of the four positional isomers (from 8, 10 to 11, 13–18:2). Delmonte et al. first described the preparation and characterization of *trans* 7, *cis* 9-18:2 FAME (32), and then the preparation of all CLA geometric and positional isomers with double-bond positions from 6,8 to 13,15 (30). Figures 6A and 6B show the separation of the CLA solutions prepared following the procedure described in the original manuscript under the GC elution conditions of AOCS Ce 1h-05 and AOCS Ce 1j-07, and compared with the four-isomer mix isomerized with iodine. Following the procedure described by Delmonte et al., a mixture containing the four isomers *cis* 6, *trans* 8-, *trans* 7, *cis* 9-, *cis* 9, *trans* 11-, and *trans* 10, *cis* 12-18:2 FAMEs can be prepared by partial hydrogenation with hydrazine of γ-linolenic acid, followed by conjugation with hot alkali. Similarly, the reduction/conjugation of α-linolenic acid yields the four isomers *cis* 9, *trans* 11-, *trans* 10, *cis* 12-, *cis* 12, *trans* 14-, *trans* 13, *cis* 15-18:2 FAMEs. These mixtures will also contain other FAs that do not interfere with the GC separation

of CLA FAMEs. The separation of 21:0 FAME is not shown in Figures 5A and 5B. The exact co-elution of 21:0 FAME with the CLA separation pattern varies from column to column and often depends on column age. Most often, 21:0 FAME co-elute in the window from *trans* 9, *cis* 11- to *trans* 10, *cis* 12-18:2 FAME (16, 18).

Conclusions

The set of chemical reactions and separation techniques described in this review allows the preparation of most FAs expected to be found in fats and oils not containing PUFAs. The chromatographic separations, acquired under the experimental conditions described by AOCS Official Method Ce 1h-05 and AOCS Recommended Practice Ce 1J-07, can be used as reference chromatography for FAME identification. While neither of the two methods can completely separate all the FAMEs contained in the reference mixtures prepared as described in this review, samples containing short-chain FAME should be preferably analyzed by a temperature program method (16), and samples containing medium-chain FAME should be preferably analyzed under isothermal conditions.

References

(1) U.S. Food and Drug Administration (2003) *Fed. Reg.* **68**, 41434 41506

(2) Schrimpf-Moss, J., & Wilkening, V. (2005) D.R. Kodali & G.R. List (Eds), *Trans Fats Alternatives*, AOCS Press, Champaign, IL, pp 26 33

(3) Health Canada (2003) *Canada Gazette* **137**(Pt II), 154 409

(4) Mossoba, M.M., & Kramer, J.K.G. (2009) *Official Methods for the Determination of Trans Fat*, AOCS Press, Urbana, IL

(5) *Official Methods of Analysis* (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2000.10**

(6) *Official Methods and Recommended Practices*, AOCS Press, Champaign, IL, Official Method Cd 14d-99

(7) Milosevic, M., Milosevic, V., Kramer, J.K.G., Azizian, H., & Mossoba, M.M. (2004) *Lipid Technol.* **16**, 229 231

(8) Mossoba, M.M., Kramer, J.K.G., Milosevic, V., Milosevic, M., & Azizian, H. (2007) *J. Am. Oil Chem. Soc.* **84**, 339 342

(9) Mossoba, M.M., Milosevic, V., Milosevic, M., & Kramer, J.K.G., & Azizian, H. (2007) *Anal. Bioanal. Chem.* **389**, 87 92

(10) Mossoba, M.M., Seiler, A., Kramer, J.K.G., Milosevic, V., Milosevic, M., Azizian, H., & Steinhart, H., *J. Am. Oil Chem. Soc.*, in press; doi 10.1007/s11746-009-1444-x

(11) *Official Methods of Analysis* (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **996.06**

(12) *Official Methods and Recommended Practices*, AOCS Press, Champaign, IL, Official Method Ce 1h-05

(13) *Official Methods and Recommended Practices*, AOCS Press, Champaign, IL, Official Method Ce 2-66

(14) International Organization for Standardization (2004) *Animal and Vegetable Fats and Oils-Preparation of Methyl Esters of Fatty Acids*, ISO Method 5509, Geneva, Switzerland

(15) *Official Methods and Recommended Practices, 2006 2007 Methods Additions and Revisions*, AOCS Press, Champaign, IL, Recommended Practice Ce 1j-07

(16) Cruz-Hernandez, C., Deng, Z., Zhou, J., Hill, A.R., Yurawecz, M.P., Delmonte, P., Mossoba, M.M., Dugan, M.E., & Kramer, J.K.G. (2004) *J. AOAC Int.* **87**, 545 562

(17) Delmonte, P., & Rader, J.I. (2007) *Anal. Bioanal. Chem.* **389**, 77 85

(18) Cruz-Hernandez, C., Kramer, J.K.G., Kraft, J., Santercole, V., Or-Rashid, M., Deng, Z., Dugan, M.E.R., Delmonte, P., & Yurawecz, M.P. (2006) M.P. Yurawecz, J.K.G. Kramer, O. Gudmundsen, M.W. Pariza, & S. Banni (Eds), *Advances in Conjugated Linoleic Acid Research,* Vol. 3, AOCS Press, Champaign, IL

(19) Fournier, V., Juanéda, P., Destaillats, F., Dionisi, F., Lambelet, P., Sébédio, J.L., & Berdeaux, O. (2006) *J. Chromatogr. A* **1129**, 21 28

(20) Christie, W.W. (2003) *Lipid Analysis*, 3rd Ed., The Oily Press, Bridgewater, UK

(21) Christie, W.W. (1987) *J. High Res. Chromatogr.* **10**, 148 150

(22) Nikolova-Damyanova, B., Herslof, B.G., & Christie, W.W. (1992) *J. Chromatogr. A* **609**, 133 140

(23) Christie, W.W., & Breckenridge, G.H.M. (1989) *J. Chromatogr. A* **469**, 261 269

(24) Momchilova, S., Nikolova-Damyanova, B., & Christie, W.W. (1998) *J. Chromatogr. A* **793**, 275 282

(25) Wood, R., & Lee, T. (1983) *J. Chromatogr. A* **254**, 237 246

(26) Nikolova-Damyanova, B., & Momchilova, S. (2002) *J. Liq. Chromatogr. RT* **25**, 1947 1965

(27) Adlof, R. (1994) *J. Chromatogr. A* **659**, 95 99

(28) Adlof, R., Copes, L.C., & Emken, E.A. (1995) *J. Am. Oil Chem. Soc.* **72**, 571 574

(29) Eulitz, K., Yurawecz, M.P., Sehat, N., Fritsche, J., Roach, J.A., Mossoba, M.M., Kramer, J.K.G., Adlof, R.O., & Ku, Y. (1999) *Lipids* **34**, 873 877

(30) Delmonte, P., Kataok, A., Corl, B.A., Bauman, D.E., & Yurawecz, M.P. (2005) *Lipids* **40**, 509 514

(31) Delmonte, P., Yurawecz, M.P., Mossoba, M.M., Cruz-Hernandez, C., & Kramer, J.K.G. (2004) *J. AOAC Int.* **87**, 563 568

(32) Delmonte, P., Roach, J.A., Mossoba, M.M., Morehouse, K.M., Lehmann, L., & Yurawecz, M.P. (2003) *Lipids* **38**, 579 583

(33) *Official Methods and Recommended Practices* (1999) 5th Ed., AOCS Press, Champaign, IL, Recommended Practice Ce 1g-96

(34) Destaillats, F., Golay, P.A., Joffre, F., De Wispelaere, M., Hug, B., Giuffrida, F., Fauconnot, L., & Dionisi F. (2007) *J. Chromatogr. A* **1145**, 222 228

(35) Delmonte, P., Kramer, J.K.G., & Yurawecz, M.P. (2006) 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

(36) *Technical Report T406062* (2006) Sigma-Aldrich Chemical Co., St. Louis, MO

(37) Juaneda, P. (2002) *J. Chromatogr. A* **954**, 285 289

(38) Tsuzuki, W., & Uchida, K. (2009) *Lipids* **44**, 373 379

(39) Delmonte, P., Hu, Q., Fardinkia, A.R., & Rader, J.I. (2008) *J. Chromatogr. A* **1214**, 30 36

(40) Scholfield, C.R., Butterfield, R.O., & Dutton, H.J. (1966) *Lipids* **1**, 163 165

(41) Scholfield, C.R., Jones, E.P., Butterfield, R.O., & Dutton, H.J. (1963) *Anal. Chem.* **35**, 1588 1591

(42) Scholfield, C.R., Butterfield, R.O., Peters, E., Glass, C.A., & Dutton, H.J. (1967) *J. Am. Oil Chem. Soc.* **44**, 50 54

(43) Dutton, H.J., Holman, R.T., Lundberg, W.O., & Malkinn, T. (1954) *Progress in the Chemistry of Fats and Other Lipids*, Pergamon Press, Oxford, UK

(44) Allen, C.F., Good, P., Davis, H.F., Chisum, P., & Fowler, S.D. (1966) *J. Am. Oil Chem. Soc.* **43**, 223 231

(45) Foucault, A.P. (1991) *Anal. Chem.* **63**, 569A 579A

(46) Murayama, W., Kosuge, Y., Nakaya, N., Nunogaki, Y., Nunogaki, K., Cazes, J., & Nunogaki, H. (1988) *J. Liq. Chromatogr.* **11**, 283 300

(47) Alvarez, J.G., Cazes, J., Touchstone, J.C., & Grob, R.L. (1990) *J. Liq. Chromatogr.* **13**, 3603 3614

(48) Bousquet, O., & Le Goffic, F. (1995) *J. Chromatogr. A* **704**, 211 216

(49) Cao, X., & Ito, Y. (2003) *J. Chromatogr. A* **1021**, 117 124

(50) Ito, Y. (2005) *J. Chromatogr. A* **1065**, 145 168

(51) Bengen, M.F. (March 18, 1940) German Patent Application, OZ 12438

(52) Bengen, M.F. (1951) *Angew. Chem.* **63**, 207 208

(53) Swern, D. (1952) *J. Am. Oil Chem. Soc.* **29**, 614 615

(54) Swern, D. (1953) *J. Am. Oil Chem. Soc.* **30**, 5 8

(55) Snyder, J.M., & Scholfield, C.R. (1982) *J. Am. Oil Chem. Soc.* **59**, 469 470

(56) Scholfield, C.R. (1972) *J. Am. Oil Chem. Soc.* **49**, 583 585

(57) Christie, W.W. (1989) *Gas Chromatography and Lipids: A Practical Guide*, The Oily Press, Scotland, UK

(58) Nichols, P.L., Jr, Herb, S.F., & Riemenschneider, R.W. (1951) *J. Am. Oil Chem. Soc.* **73**, 247 252

(59) Mounts, T.L., Dutton, H.J., & Glover, D. (1971) *Lipids* **5**, 997 1005

(60) Sonntag, N.O.V. (1961) K.S. Markley (Ed.), *Fatty Acids, Their Chemistry, Properties, Production, and Uses*, Part II, Interscience Publishers, Inc., New York, NY, pp 1073 1185

(61) Lawrence, P., & Brenna, J.T. (2006) *Anal. Chem.* **78**, 1312 1317

(62) Michaud, A.L., Yurawecz, M.P., Delmonte, P., Corl, B.A., Bauman, D.E., & Brenna, J.T. (2003) *Anal. Chem.* **75**, 4925 4930

(63) Michaud, A.L., Lawrence, P., Adlof, R., & Brenna, J.T. (2005) *Rapid Commun. Mass Spectrom.* **19**, 363 368

(64) Van Pelt, C.K., & Brenna, J.T. (1999) *Anal. Chem.* **71**, 1981 1989

(65) Kramer, J.K.G., Hernandez, M., Cruz-Hernandez, C., Kraft, J., & Dugan, M.E.R. (2008) *Lipids* **43**, 259 273

(66) Alves, S.P., & Bessa, R.J. (2009) *J. Chromatogr. A* **1216**, 5130 5139

(67) Precht, P., & Molkentin, J. (2000) *Eur. J. Lipid Sci. Technol.* **102**, 102 113

(68) Precht, D., & Molkentin, J. (2000) *Nahrung* **44**, 222 228

(69) Destaillats, F., Wolff, R.L., Precht, D., & Molkentin, J. (2000) *Lipids* **35**, 1027 1032

(70) Ratnayake, W.M., Plouffe, L.J., Pasquier, E., & Gagnon, C. (2002) *J. AOAC Int.* **85**, 1112 1118

CHAPTER 4

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

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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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CP-Sil-88
SLB-IL111

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

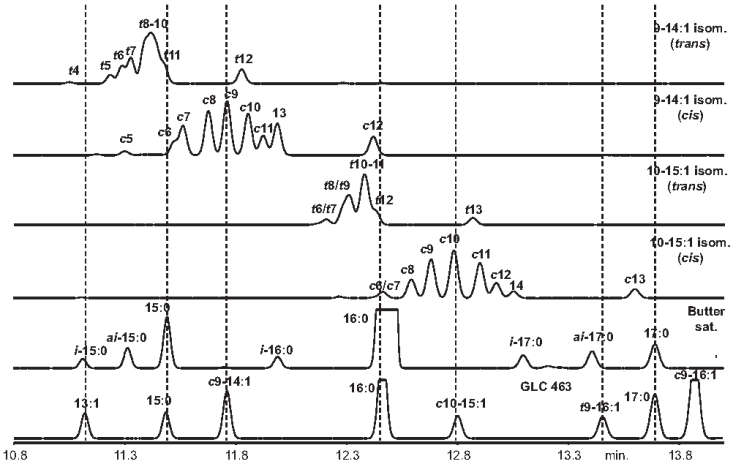

Fig. 1. Partial GC chromatogram of the 14:1 and 15:1 region. From top: *trans* and *cis* fractions obtained from *cis* 9-14:1 and *cis* 10-15:1 FAMEs after five successive brominations and debrominations, saturated FAMEs fractionated from milk fat (as FAMEs), and reference FAME mixture GLC 463. Conditions: 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-/*c*9-/*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.

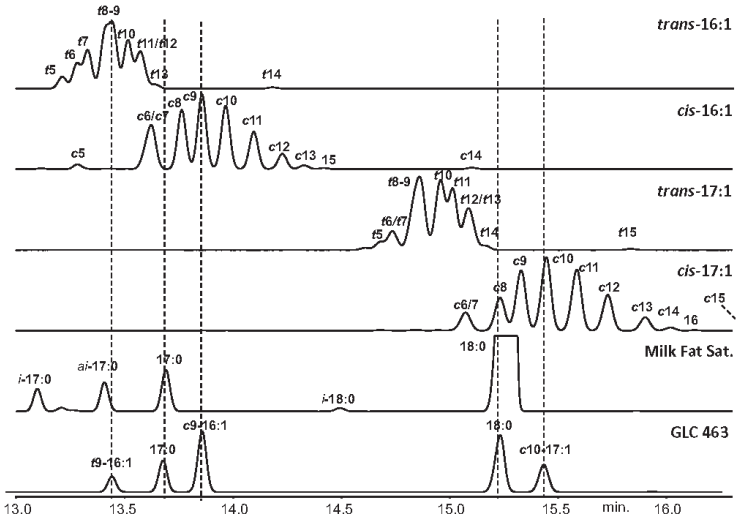

Fig. 2. Partial GC chromatogram of the 16:1 and 17:1 region. From top: *trans* and *cis* fractions obtained from *cis* 9-16:1 and *cis* 10-17:1 FAMEs after five successive brominations and debrominations, saturated FAMEs fractionated from milk fat (as FAMEs), and reference FAME mixture GLC 463. Conditions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

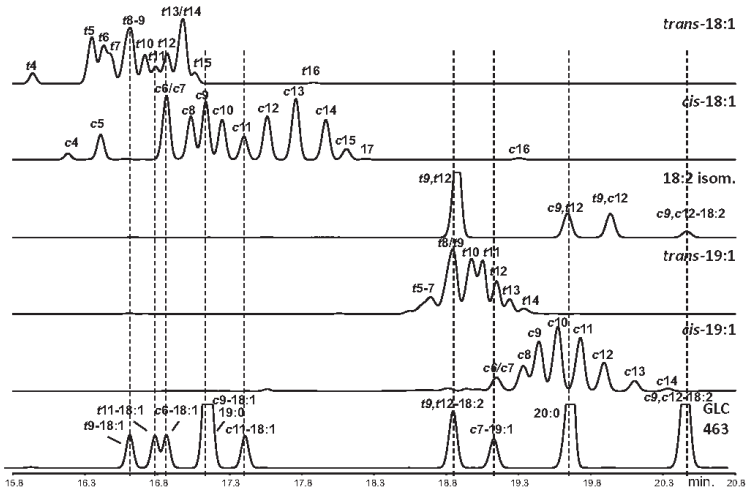

Fig. 3. Partial GC chromatogram of the 18:1, 19:1 and 18:2 region. From top: *trans* and *cis* fractions of 18:1 obtained from a mixture of *cis* 6-, *cis* 9- and *cis* 13-18:1 FAMEs after two successive brominations and debrominations, linoleic acid FAME isomerized by PTSA, *trans* and *cis* fractions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

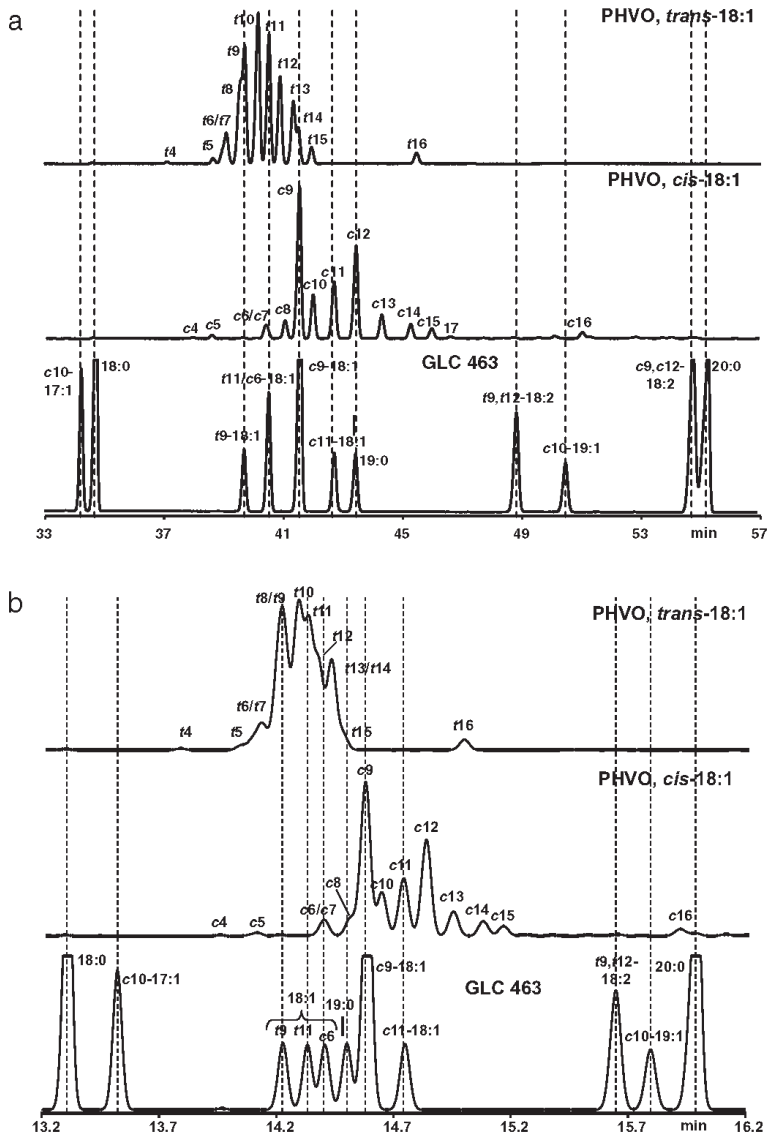

Fig. 4. (A) Partial GC chromatogram of the 18:0 to 20:0 region. From top: *trans* and *cis* fractions of partially hydrogenated vegetable oil (PHVO) and reference FAME mixture GLC 463. Conditions: Supelco 100 m SLB-IL111 capillary column (100 m × 0.25 mm, 0.2 μm thickness), hydrogen carrier gas at 1 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

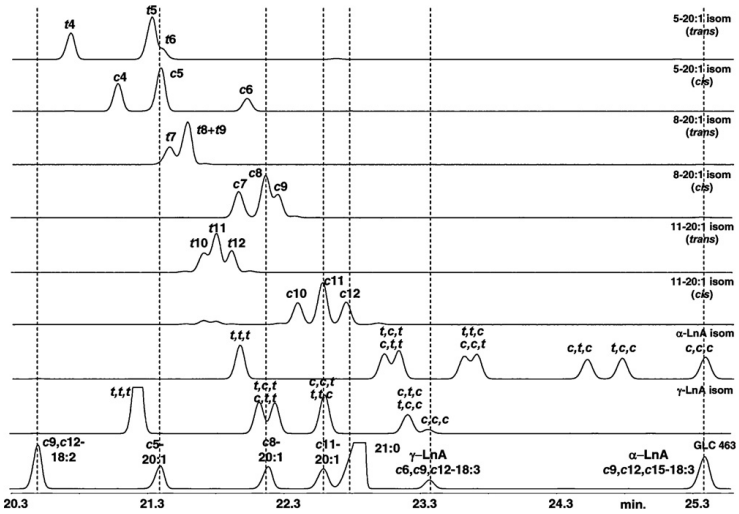

Fig. 5. Partial GC chromatogram of the 20:1 and 18:3 region. From top: *trans* and *cis* fractions of *cis* 5-, *cis* 8- and *cis* 11-20:1 FAMEs after one successive bromination and debromination, α-LnA and γ-LnA isomerized by PTSA, and reference FAME mixture GLC 463. Conditions as in Fig. 1.

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

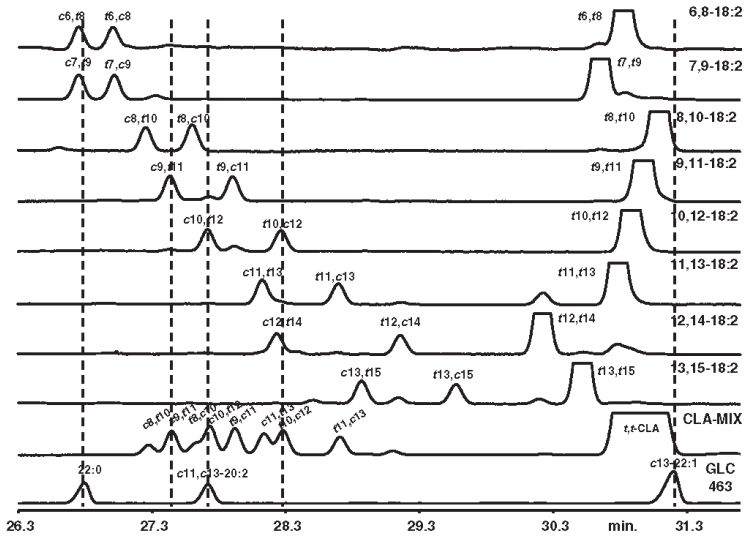

Fig. 6. Partial GC chromatogram of the CLA region. From top: iodine isomerized CLA isomers with double bond position 6,8- to 13,15-18:2, iodine isomerized CLA mixture UC-59-M and reference FAME mixture GLC 463. Conditions as in Fig. 1.

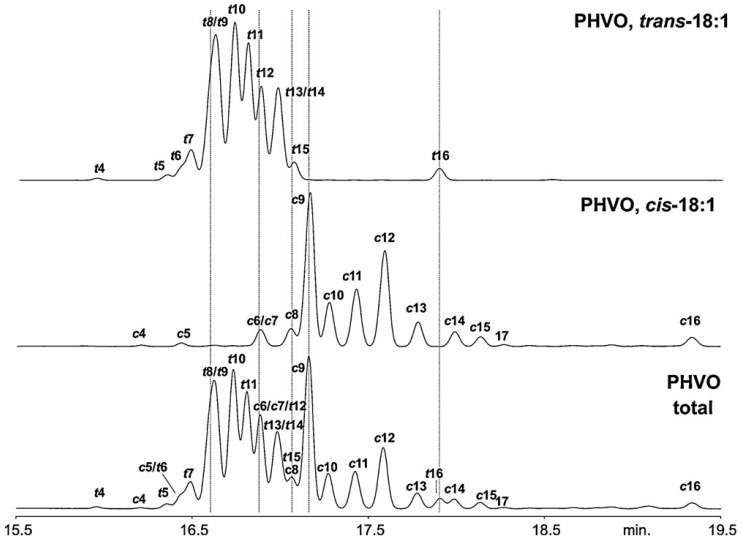

Fig. 7. Partial GC chromatogram of the 18:1 region. From top: *trans* and *cis* fractions of partially hydrogenated vegetable oil (PHVO), and total PHVO. Conditions as in Fig. 1.

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

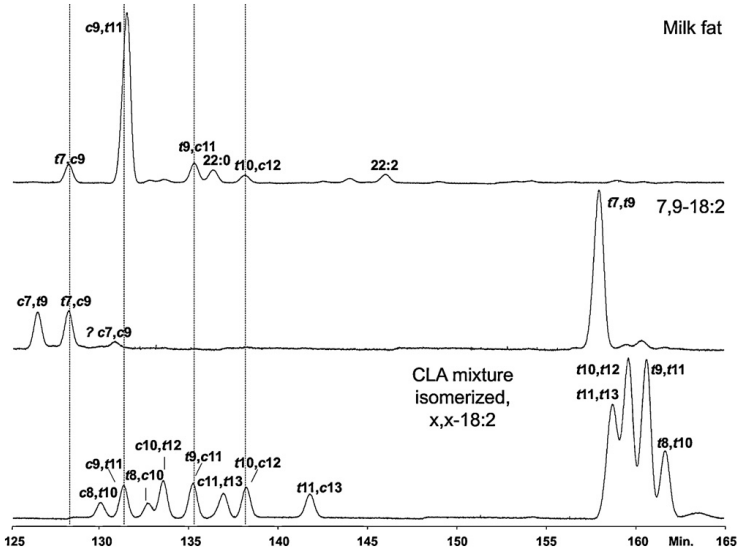

Fig. 8. Partial GC chromatogram of the CLA region. From top: milk fat sample, 7,9-18:2 isomerized with iodine and iodine isomerized CLA mixture UC-59-M. Conditions: SLB-IL111 capillary column (100 m × 0.25 mm, 0.2 μm thickness), hydrogen carrier gas at 1 mL/min, 130 °C isothermal elution.

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.

References

[1] M.M. Mossoba, J.K.G. Kramer, Official Methods for the Determination of Trans Fat, 2nd ed., AOCS Press, Urbana, IL, 2009.
[2] D. Precht, J. Molkentin, Int. Dairy J. 6 (1996) 791.
[3] J.K.G. Kramer, C. Cruz-Hernandez, J. Zhou, Eur. J. Lipid Sci. Technol. 103 (2001) 600.
[4] W.M.N. Ratnayake, J. AOAC Int. 87 (2004) 523.

[5] 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.
[6] Department of Health and Human Services, FDA 21 CFR Part 101 [Docket No. 94P-0036]. Food labeling: *trans* fatty acids in nutrition labeling; nutrient content claims, and health claims; final rule, Federal Register 68, No. 133, July 11, 2003, p. 41434.
[7] Regulations Amending the Food and Drug Regulations (Nutrition Labelling, Nutrient Content Claims and Health Claims). Department of Health, Canada Gazette, Part 11. January 1, 2003. http://canadagazette.gc.ca/partII/2003/20030101/html/sor11-e.html.
[8] K. Duhem, in: F. Destaillats, J.-L. Sébédio, F. Dionisi, J.-M. Chardigny (Eds.), Trans Fatty Acids in Human Nutrition, 2nd ed., The Oily Press, Bridgewater, England, 2009, pp. 381–394.
[9] D. Firestone (Ed.), American Oil Chemists' Society Official Methods and Recommended Practices, 6th ed., AOCS Press, Urbana, IL, 2009, official method Ce 1h-05.
[10] W.M.N. Ratnayake, S.L. Hansen, M.P. Kennedy, J. Am. Oil Chem. Soc. 83 (2006) 475.
[11] 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, M.W. Pariza, S. Banni (Eds.), Advances in Conjugated Linoleic Acid Research, vol. 3, AOCS Press, Champaign, IL, 2006, pp. 45–93.
[12] P. Delmonte, A.R. Fardin Kia, Q. Hu, J.I. Rader, J. AOAC Int. 92 (2009) 1310.
[13] J.K.G. Kramer, M. Hernandez, C. Cruz-Hernandez, J. Kraft, M.E.R. Dugan, Lipids 43 (2008) 259.
[14] G. Castello, S. Vezzani, G. D'Amato, J. Chromatogr. A 779 (1997) 275.
[15] M.M. Mossoba, J. Moss, J.K.G. Kramer, J. AOAC Int. 92 (2009) 1284.
[16] D. Precht, J. Molkentin, Milchwissenschaft 58 (2003) 30.
[17] S.P. Alves, R.J.B. Bessa, J. Chromatogr. A 1216 (2009) 5130.
[18] D. Precht, J. Molkentin, Eur. J. Lipid Sci. Technol. 102 (2000) 102.
[19] S.P. Alves, R.J.B. Bessa, Eur. J. Lipid Sci. Technol. 109 (2007) 879.
[20] C. Ragonese, P.Q. Tranchida, P. Dugo, G. Dugo, L.M. Sidisky, M.V. Robillard, L. Mondello, Anal. Chem. 81 (2009) 5561.
[21] 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.
[22] Supelco Ionic Liquid GC Columns, Introduction to the Technology, 2010. sigma-aldrich.com/il-gc (accessed 09.08.10).
[23] J.L. Anderson, J. Ding, T. Welton, D.W. Armstrong, J. Am. Chem. Soc. 124 (2002) 14247.
[24] P. Delmonte, Q. Hu, A.R. Fardin Kia, J.I. Rader, J. Chromatogr. A 1214 (2008) 30.
[25] P. Delmonte, A. Kataoka, B.A. Corl, D.E. Bauman, M.P. Yurawecz, Lipids 40 (2005) 509.
[26] K.J. Shingfield, C.K. Reynolds, B. Lupoli, V. Toivonen, M.P. Yurawecz, P. Delmonte, J.M. Griinari, A.S. Grandison, D.E. Beever, Anim. Sci. 80 (2005) 225.
[27] K. Huang, X. Han, X. Zhang, D.W. Armstrong, Anal. Bioanal. Chem. 389 (2007) 2265.
[28] S.A. Mjøs, J. Chromatogr. A 1015 (2003) 151.
[29] 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.
[30] L.S. Piperova, B.B. Teter, I. Bruckental, J. Sampugna, S.E. Mills, M.P. Yurawecz, J. Fritsche, K. Ku, R.A. Erdman, J. Nutr. 130 (2000) 2568.
[31] D. Precht, J. Molkentin, F. Destaillats, R.L. Wolff, Lipids 36 (2001) 827.
[32] F.D. Gunstone, I.A. Ismail, M. Lie Ken Jie, Chem. Phys. Lipids 1 (1967) 376.
[33] J. Molkentin, D. Precht, Chromatographia 41 (1995) 267.
[34] J.K.G. Kramer, C.B. Blackadar, J. Zhou, Lipids 37 (2002) 823.
[35] F. Destaillats, P.A. Golay, F. Joffre, M. de Wispelaere, B. Hug, F. Giuffrida, L. Fauconnot, F. Dionisi, J. Chromatogr. A 1145 (2007) 222.
[36] K.J. Shingfield, S. Ahvenjärvi, V. Toivonen, A. Ärölä, K.V.V. Nurmela, P. Huhtanen, J.M. Griinari, Anim. Sci. 77 (2003) 165.
[37] R.L. Wolff, D. Precht, J. Am. Oil Chem. Soc. 75 (1998) 421.
[38] M.M. Mossoba, R.E. McDonald, J.A.G. Roach, D.D. Fingerhut, M.P. Yurawecz, N. Sehat, J. Am. Oil Chem. Soc. 74 (1997) 125.
[39] D. Precht, J. Molkentin, Nahrung 43 (1999) 233.
[40] R.L. Wolff, J. Am. Oil Chem. Soc. 71 (1994) 907.
[41] D. Precht, J. Molkentin, Nahrung 44 (2000) 222.

CHAPTER 5

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

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

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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.

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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

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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

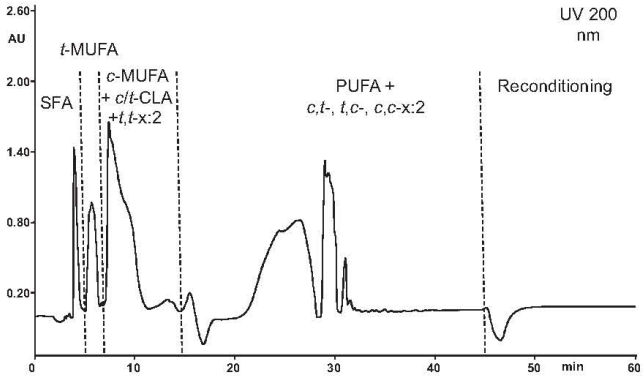


Fig. 1. Milk fat FAME Ag^+-HPLC separation used to collect the fractions subsequently analyzed by gas chromatography. One semipreparative Chromshper 5 lipid column (10 mm × 250 mm, 5 μm particle size, Varian Inc.), flow rate 5 ml/min., 10:90 saturated MeCN in isooctane solution/isooctane until about 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].

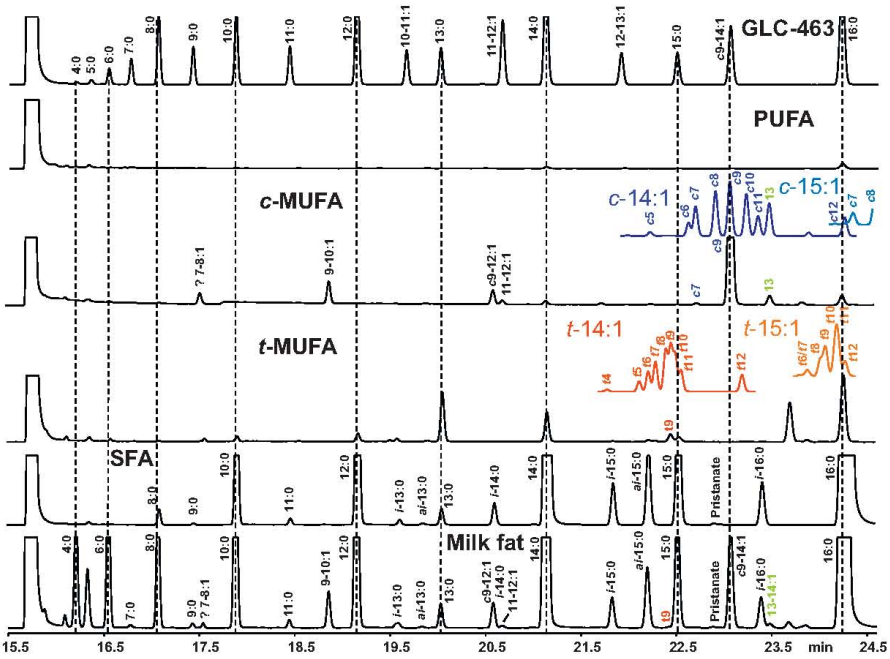
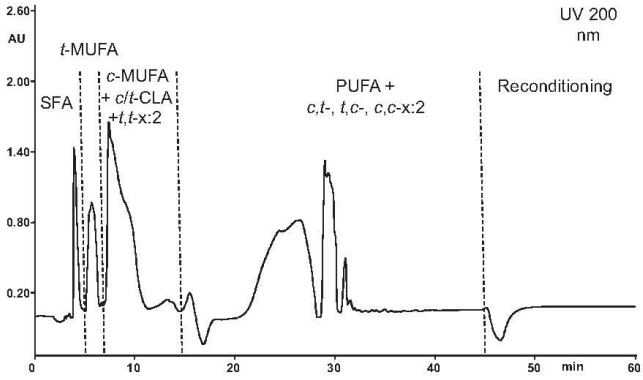


Fig. 2. Partial GC chromatogram of the 4:0 to 16:0 elution profile region. From the bottom: non fractionated milk fat FAMEs, milk fat fractions containing SFAs, *t*-MUFAs, *c*-MUFAs, PUFAs, and reference FAME mixture GLC 463, conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 mm, 0.2 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.



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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].

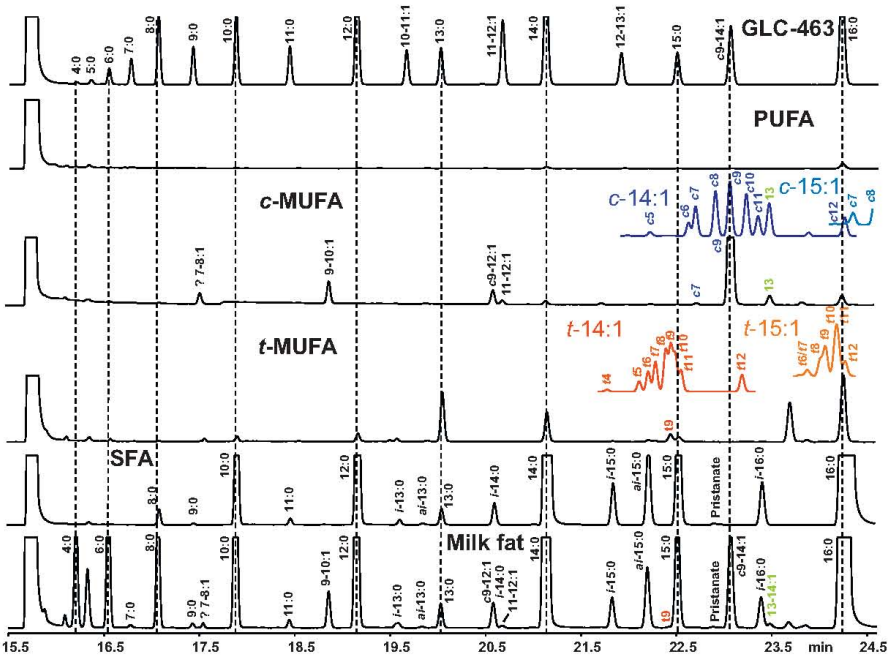

Fig. 2. Partial GC chromatogram of the 4:0 to 16:0 elution profile region. From the bottom: non fractionated milk fat FAMEs, milk fat fractions containing SFAs, *t*-MUFAs, *c*-MUFAs, PUFAs, and reference FAME mixture GLC 463, conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 mm, 0.2 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.

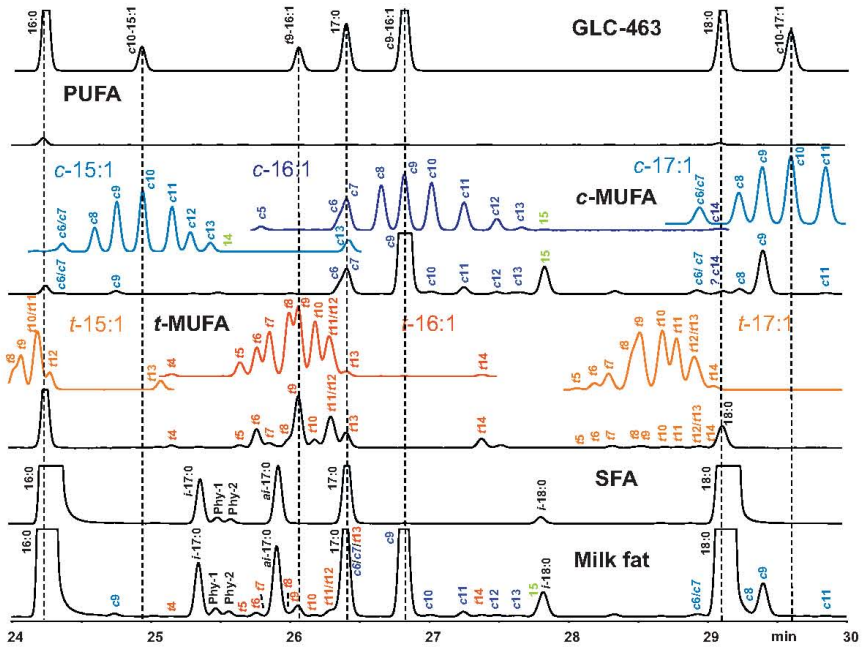

Fig. 3. Partial GC chromatogram of the 16:0 to 18:0 elution profile region. From the bottom: non fractionated milk fat FAMEs, milk fat fractions containing SFAs, *t*-MUFAs, *c*-MUFAs, PUFAs, and reference FAME mixture GLC 463, conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 mm, 0.2 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

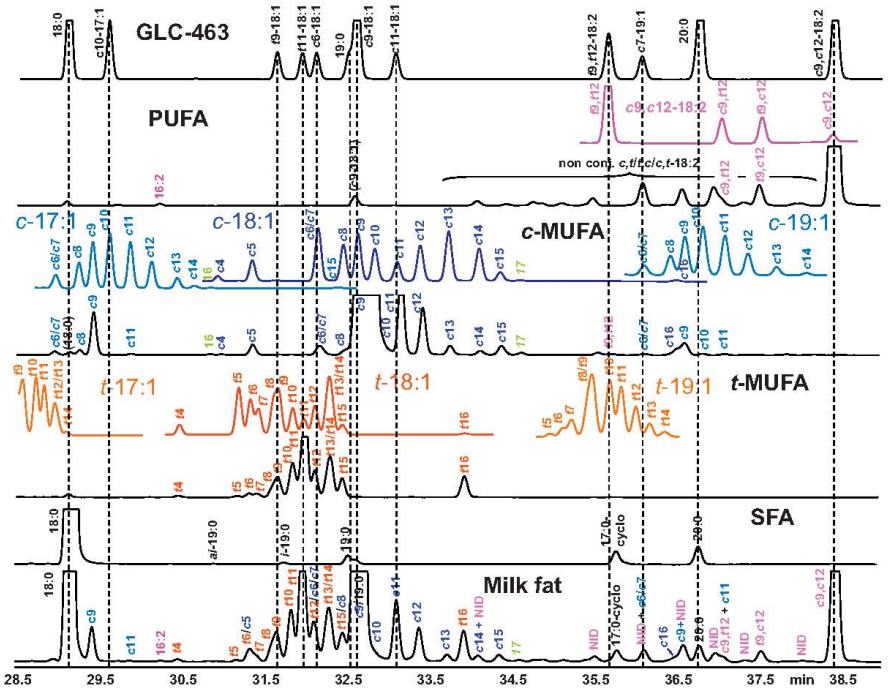

Fig. 4. Partial GC chromatogram of the 18:0 to *c*9,*c*12-18:2 elution profile region. From the bottom: non fractionated milk fat FAMEs, milk fat fractions containing SFAs, *t*-MUFAs, *c*-MUFAs, PUFAs, and reference FAME mixture GLC 463, conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 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

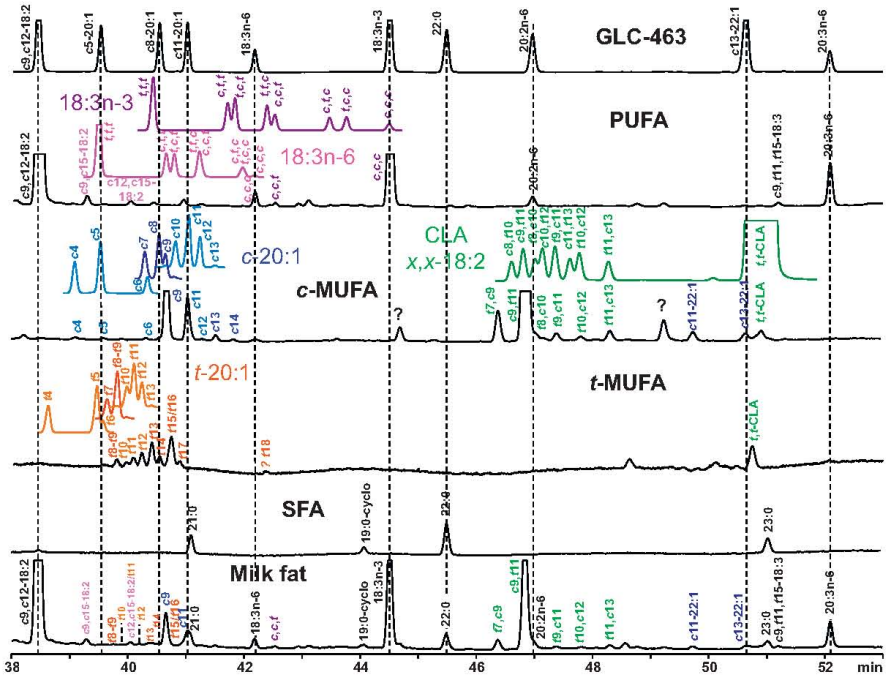

Fig. 5. Partial GC chromatogram of the *c*9,*c*12-18:2 to 20:3 elution profile region. From the bottom: non fractionated milk fat FAMEs, milk fat fractions containing SFAs, *t*-MUFAs, *c*-MUFAs, PUFAs, and reference FAME mixture GLC 463, conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 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

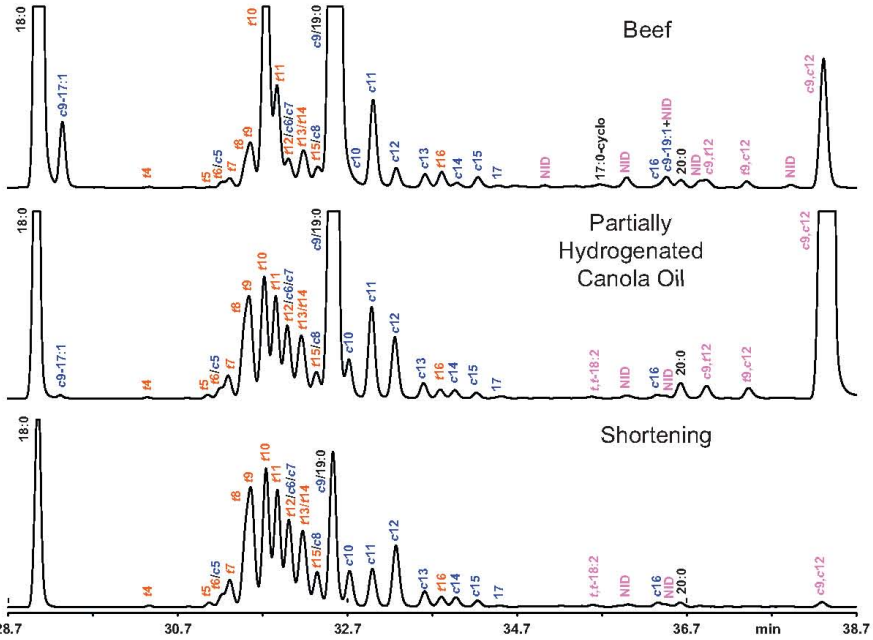

Fig. 7. Partial GC chromatogram of the 18:0 to *c*9,*c*12-18:2 elution profile region. From top: FAMEs prepared from a beef fat, a hydrogenated canola oil, and a shortening sample. Conditions: Supelco 200 m SLB-IL111 capillary column (200 m × 0.25 mm, 0.2 μm thickness), temperature/flow gradients as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

CHAPTER 6

Separation of Fatty Acid Methyl Esters by GC-Online Hydrogenation × GC.

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Separation of Fatty Acid Methyl Esters by GC-Online Hydrogenation × GC

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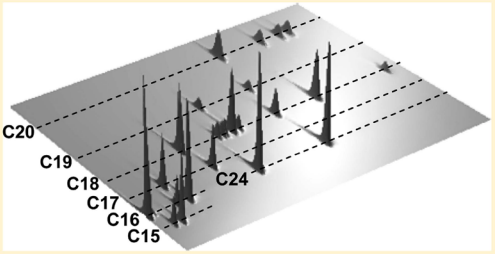
ABSTRACT: The separation of fatty acid methyl esters (FAME) provided by a 200 m × 0.25 mm SLB-IL111 capillary column is enhanced by adding a second dimension of separation (^{2}D) in a GC × GC design. Rather than employing two GC columns of different polarities or using different elution temperatures, the separation in the two-dimensional space is achieved by altering the chemical structure of selected analytes between the two dimensions of separation. A capillary tube coated with palladium is added between the first dimension of separation (^{1}D) column and the cryogenic modulator, providing the reduction of unsaturated FAMEs to their fully saturated forms. The ^{2}D separation is achieved using a 2.5 m × 0.10 mm SLB-IL111 capillary column and separates FAMEs based solely on their carbon skeleton. The two-dimensional separation can be easily interpreted based on the principle that all the saturated FAMEs lie on a straight diagonal line bisecting the separation plane, while the FAMEs with the same carbon skeleton but differing in the number, geometric configuration or position of double bonds lie on lines parallel to the ^{1}D time axis. This technique allows the separation of trans fatty acids (FAs) and polyunsaturated FAs (PUFAs) in a single experiment and eliminates the overlap between PUFAs with different chain lengths. To our knowledge, this the first example of GC × GC in which a chemical change is instituted between the two dimensions to alter the relative retentions of components and identify unsaturated FAMEs.



Oils and fats extracted from products of marine origin, such as fish and algae, contain a large number of saturated, monounsaturated, and polyunsaturated fatty acids.[1,2] Gas chromatography using mid- and high-polarity capillary columns was shown to provide only a partial separation of the FAMEs prepared from these oils and fats.[1,2] Santercole et al.[1] recently compared the separations of menhaden oil FAMEs using a mid polarity GC column (polyethylene glycol phase, PEG) and a high polarity column (100% cyanopropyl siloxane phase, CPS). The CPS column used by the authors provided a more detailed separation of positional and geometric isomers of unsaturated fatty acids compared to the PEG counterpart but none of the sets of chromatographic conditions evaluated could separate all of the FAMEs contained in the samples investigated.[1]

About a decade ago, Armstrong et al. showed that room temperature ionic liquids (RTIL) possess the volatility, viscosity, solubility and polarity proprieties required for use as stationary phases for capillary GC.[3,4] Used as stationary phases in GC, they showed dual nature retention selectivity, separating polar molecules as a polar stationary phase and nonpolar molecules as a nonpolar stationary phase.[3−5] The development of dicationic,[6−8] trigonal tricationic,[9] and cross-linked[10] RTILs led to the novel IL capillary columns currently used for the separation of FAMEs.[11−14] Capillary columns coated with RTILs are increasingly used for GC × GC separations due to their unique selectivity and thermal stability.[15−17,19−27] The use of a column coated with an RTIL (trihexyl(tetradecyl)-phosphonium bis(trifluoromethane)sulfonamide) for GC × GC separations was first tested by Seeley et al., using standard mixtures of organic compounds with a wide range of functional groups.[15] Used for ^{1}D or ^{2}D, RTIL columns have been used successfully for the separation of phosphorus−oxygen (P−O) containing compounds,[16] FAMEs,[17,19] PCBs,[20,21] perfume allergens,[22] pesticides in water,[23] and petroleum fractions containing nitrogen and sulfur compunds.[24,25] A GC × GC × GC application for the separation of phosphorus−oxygen (P−O) containing compounds in diesel samples has also been reported.[26] The use of RTIL columns for GC separations has recently been reviewed in greater detail by Ragonese et al.[27]

Delmonte et al. tested the novel SLB-IL111 ionic liquid column and showed that the higher polarity of its stationary phase provides a higher selectivity for FAMEs based on the number, geometric configuration, and position of double bonds than other phases used for FAME analysis.[12] On the basis of these findings, the authors developed a procedure capable of separating most FAMEs prepared from milk fat using a 200 m × 0.25 mm SLB-IL111 capillary column.[13] Among other improvements, this methodology allowed the separation of almost all positional/geometric isomers of 18:1 and 18:2 milk fat FAMEs. However, certain FAMEs differing in chain length

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and number of double bonds still coeluted. All of the *trans*-18:1 FAMEs up to *trans*-15−18:1 eluted before *cis*-9−18:1. The biologically active *trans*-vaccenic acid (*t*11−18:1) was clearly separated from other *trans*-18:1, and in the conjugated linoleic acid region (CLA), *t*7,*c*9- was separated from *c*9,*t*11−18:2.[13]

Comprehensive two-dimensional gas chromatography can provide a more detailed separation of FAMEs by combining the selectivity of two different gas chromatographic separations.[28] The orthogonality between the two separations is commonly achieved by employing GC columns of different polarities, different elution temperatures for ^{1}D and ^{2}D, or a combination of both. Several combinations of experimental conditions have been developed targeting FAMEs prepared from different lipid sources and biodiesel blends.[17−19,29−37] Nonpolar capillary columns separate FAMEs based primarily on their chain lengths and are often preferred for the ^{1}D separation. Highly polar capillary columns provide selectivity based on the number, geometric configuration and position of double bonds and are preferred for the ^{2}D separation. Gu et al.[17] tested several combinations of capillary columns for the GC × GC separation of FAMEs prepared from samples of marine origin. The authors obtained a group-type GC × GC separation of FAMEs with different carbon numbers and number of unsaturations by using an apolar polydimethyl siloxane capillary column for ^{1}D combined with a polar ionic liquid column for ^{2}D. Tranchida et al.[18] separated FAMEs prepared from menhaden oil using an SLB-5ms for ^{1}D combined with an SP2560 for ^{2}D. These platforms could separate a large number of PUFAs and divide them into groups based on their carbon number but provided limited separation based on the geometric configuration and position of double bonds.[17,18]

Online hydrogenation of FAMEs using a capillary fused silica tube coated with Pt or Pd before or after GC separation was used in the early stages of lipid research.[38−40] Schomburg et al.[38] prepared a 300 mm × 0.35 mm I.D. capillary reactor by dynamic or static coating, loading Pt or Pd on the capillary tube as an acetyl−acetonate salt dissolved in $MeCl_2$. The salt was successively dried on the inner surface of the tube, and decomposed into metallic Pt or Pd by heating at 200 °C in a mild flow of carrier gas. The efficiency of the Pt capillary reactor was tested by analyzing several types of unsaturated compounds, including unsaturated FAMEs. The authors recommended using Pd over Pt for the reduction of double bonds in FAMEs because the stronger reactivity of the latter might cause the reduction of the ester group. Le Quere et al.[40] characterized unsaturated cyclic fatty acid monomers using a post column 60 cm × 0.32 mm capillary tube coated with Pd prior to the MS detector.

This study describes the development of a novel approach to the GC × GC separation of FAMEs in which the analytes undergo chemical reduction between ^{1}D and ^{2}D, and ^{2}D separates the products of the reaction. Unsaturated FAMEs (U-FAME) are reduced to their fully saturated form by passing them through a capillary tube coated with Pd (reactor) in the presence of H_2 as the carrier gas. The GC × GC separation is achieved by using two capillary columns coated with the same highly polar stationary phase, and maintained at the same temperature. To our knowledge, this is the first GC × GC application in which the two-dimensional separation is achieved by modifying the chemical structure of the analytes instead of by applying different chromatographic conditions.

MATERIALS AND METHODS

Fatty acid methyl ester reference mixture GLC 463 and other FAMEs reference materials used for identification purposes were purchased from Nu-chek Prep, Inc. (Elysian, MN). Menhaden oil and Pd acetyl acetonate were purchased from Sigma Aldrich (St. Louis, MO). The FAMEs prepared from human colon adenocarcinoma cells HT-29 were kindly provided by Christian Degen of University of Jena (Germany). The origin and preparation of this sample are described in a previous study.[41]

Capillary Reactor Preparation. Fifty milligrams of Pd acetyl acetonate was weighed in a 10 mL volumetric flask and dissolved with $MeCl_2$. The solution was loaded into a 4 m x 0.15 mm deactivated fused silica capillary tube using a 1 mL glass syringe, allowing several drops to exit from the end side. The capillary tube was dried for 2 days under vacuum with an end plugged with silicone vacuum grease and then maintained for 2 h at 200 °C in the GC oven under a mild flow of hydrogen. If the crystallization and thermal decomposition of the acetyl acetonate salt produce a homogeneous layer of Pd, the expected thickness is 5 nm. A piece of uncoated silica capillary tube was placed between the injector port and the Pd reactor to avoid contamination. The capillary tube coated with metallic Pd was cut in 50 cm portions to be used as capillary reactors.

GC × GC Conditions. Separations were achieved with an Agilent 7890A gas chromatograph (Agilent Tech., Wilmington, DE) combined with a Zoex ZX2 dual stage cryogenic modulator (Houston, TX). The injection port of the gas chromatograph was connected to a 1034 kPa electronic pressure control module and hydrogen was used as the carrier gas. The fluidic path of the system is shown in Figure 1. A

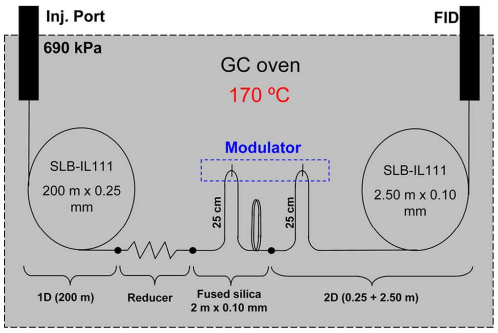

Figure 1. Scheme of GC × GC apparatus.

Supelco SLB-IL111 capillary column (200 m x 0.25 mm I.D., 0.2 μm coating, Bellefonte, PA) was used for ^{1}D separation and the same capillary column but with different dimensions (2.75 m x 0.10 mm I.D., 0.08 μm coating) was used for the ^{2}D separation. The cryogenic modulator first loop consisted of a 2 m x 0.10 mm deactivated capillary tube and the modulation (first stage) was set at 25 cm from the beginning of the tube. The second stage of modulation was achieved using part of the ^{2}D column and the modulation spot was set at 25 cm from the column front end leaving 2.50 m of ^{2}D column for separation. The 50 cm x 0.15 mm Pd capillary reactor was placed between the ^{1}D column and the modulator. The timing of the

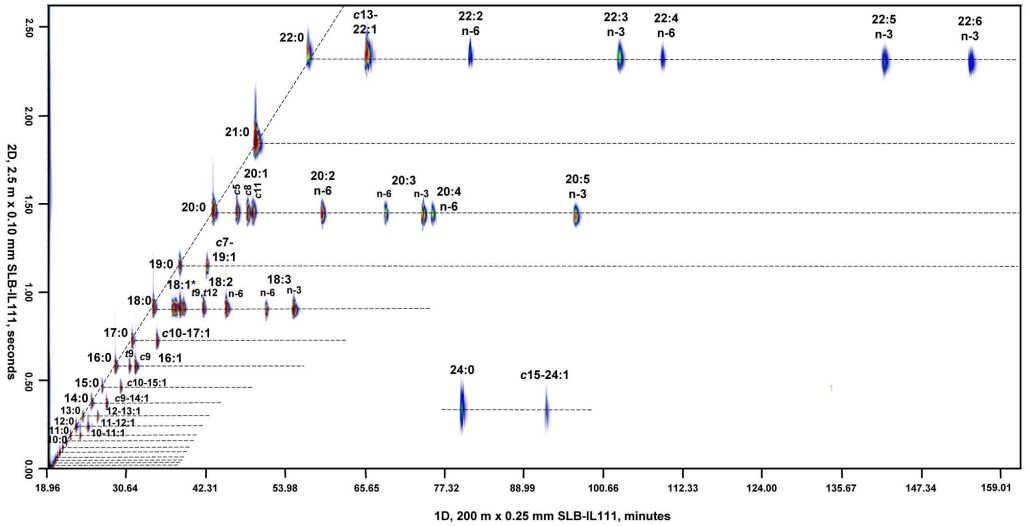

Figure 2. GC × GC separation of the FAME contained in reference mixture GLC 463 (Nu-chek Prep., Inc.). Instrumental configuration as shown in Figure 1. *In order of elution, *t*9-, *t*11-, *c*6-, *c*9-, and *c*11−18:1.

modulation was set to 3.5 s, the temperature of the hot jet was set at 350 °C with a pulse of 350 ms. The column oven temperature was set to 170 °C, and the injector port at 300 °C. Chromatography was acquired in constant pressure mode at 690 kPa and the split flow was set to 125.69 mL/min. The FID was maintained at 250 °C and fed with 30 mL/min of nitrogen make up gas, 400 mL/min air, and 25 mL/min of hydrogen. All capillary connections were made with Ultimate unions (Agilent Technologies).

GC × GC Conditions, Temperature Program. The separation was achieved using the same instrumental configuration used for isothermal separation, with the exception of the 100 m × 0.25 mm SLB-IL111 used for ^{1}D. The elution temperature was set to 130 °C per 23 min, then increased 1.3 °C/min to 220 °C. The inlet pressure was maintained at 483 kPa per 23 min, and then raised at 1.514 kPa/min until 588 kPa. The modulation time was set to 2 s, and the chromatograms were adjusted by shifting the ^{2}D phase −1.8 s.

GC-MS Identification of FAMEs. Identities of FAMEs were confirmed or achieved by monodimensional GC-MS using a Micromass GCT TOF mass spectrometer (Manchester, U.K.) coupled with an Agilent 6890N GC. The separation was achieved using a 100 m × 0.25 mm SLB-IL111 capillary column maintained at 170 °C and H_2 carrier gas at the pressure of 80 kPa. Experiments were carried out in CI^+ mode using isobutane as the ionization gas. The source was maintained at 150 °C, the electron energy was 70 eV, the emission current 250 μA, and the CI gas flow was set to 10. The molecular ion of FAMEs identified the chain length and the number of unsaturations.

■ RESULTS AND DISCUSSION

The current preferred approach for quantitation of FAMEs is monodimensional GC using the longest and most polar capillary columns available. Methods for the quantitation of trans fatty acids and PUFAs require the separation of FAMEs based on the number and geometric configuration of their double bonds. Rather than enhancing these separations by adding a second dimension of separation, most GC × GC methods use a low polarity column for ^{1}D, thus separating FAMEs based primarily on their chain length, combined with a short polar ^{2}D column providing limited separation based on the number, position and geometry of double bonds.

In this study, we selected for ^{1}D separation the same chromatographic conditions we prefer for monodimensional GC analyses: a highly polar 200 m × 0.25 mm SLB-IL111 column maintained at 170 °C. After they were separated by the ^{1}D column, the U-FAMEs are reduced to their carbon skeleton by the capillary reactor, and the products of reaction are separated using a 2.5 m × 0.10 mm SLB-IL111 maintained at the same temperature as ^{1}D. This GC × GC system requires a very simple hardware configuration: the two capillary columns are maintained in the same oven, and there is no need for supplying extra carrier gas at the midpoint before the ^{2}D column, as in the case of using larger diameter columns for ^{2}D separation (i.e., flow modulation).

Fatty acid methyl esters prepared from samples of marine origin are characterized by a high sample dimensionality, primarily derived from the presence of double bonds on their alkyl chains.[42] The number, position (individual or cumulative using $n - x$ nomenclature) and geometric configuration of double bonds generate a number of sample dimensions higher than the number of separation dimensions that can be combined in a single chromatographic system. The hydrogenation of double bonds reduces the dimensionality of the sample solely to chain length, or to the chain length and the position of the methyl group substituent if *iso*- and *anteiso*-FAMEs are also considered. While the ^{1}D column provides a complex peak distribution by retaining FAMEs based on all sample dimensions, the ^{2}D provides an ordinate separation based only on one sample dimension (or two, including *iso*-/*anteiso*-FAMEs). The result is a GC × GC separation in which FAMEs are eluting from ^{1}D in a "disordered" manner, and are

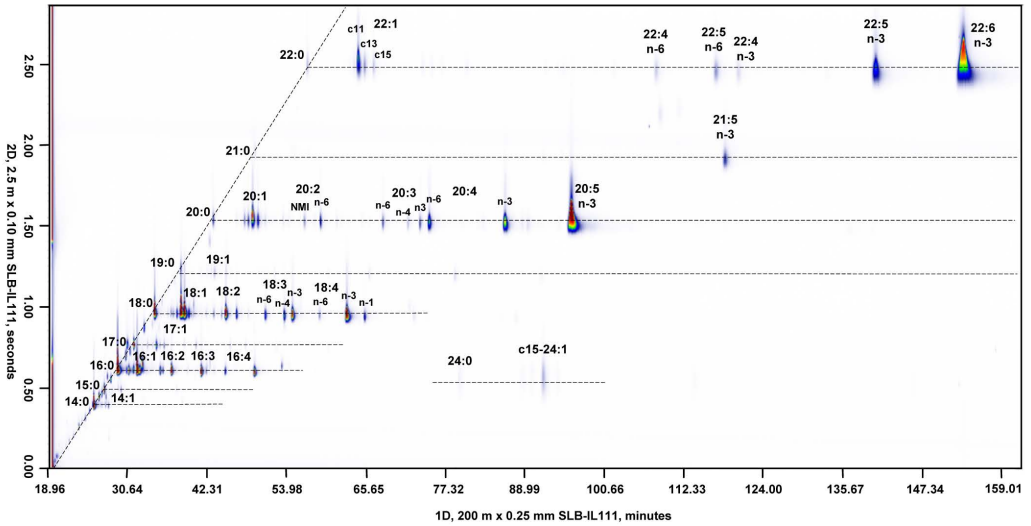

Figure 3. GC × GC separation of the FAME prepared from a menhaden fish oil sample. Instrument configuration as shown in Figure 1. NMI, nonmethylene interrupted.

then "ordered" by ^{2}D into lines based on their chain length dimension. If the desired separation is limited to straight chain C8−C26 FAMEs, a theoretical ^{2}D peak capacity of 19 can be sufficient to separate all the products of hydrogenation. The low peak capacity required for performing the ^{2}D separation allows the rapid modulation time of 3.5 s.

The highly polar column used for ^{2}D was selected based on its lower retention for long chain saturated FAMEs relative to short/medium chain saturated FAMEs, in comparison with lower polarity columns. It also allowed the use of a single GC oven, thus simplifying the hardware configuration. The disadvantage of this selection is the lower solubility of the FAMEs in the highly polar liquid phase, resulting in a lower loading capacity. Capillary reactors were prepared by loading either 0.5 or 1.0% (w/v) Pd acetyl acetonate in $MeCl_2$ onto the capillary tube. Capillary reactors coated using the 1.0% solution as short as 5 cm were observed to provide quantitative reduction of U-FAMEs. The 50 cm version prepared using the 0.5% solution proved to be less dependent on the uniformity of the Pd coating layer and was preferred for this study. Combinations of excessive Pd load and/or length of the capillary reactor, such as use of a capillary of at least 1 cm prepared using a 5% Pd acetyl acetonate solution, were observed to generate ^{1}D peak tailing and the degradation of FAMEs. This was recognized by the appearance of peaks in proximity to the ^{2}D unretained band.

Figure 2 shows the separation of the FAMEs contained in reference mixture GLC 463. The two-dimensional separation can be easily interpreted using two basic rules: 1) all the saturated FAMEs lie on a straight diagonal line bisecting the separation plane, as expected from the lack of orthogonality between the ^{1}D and ^{2}D chromatographic conditions, and 2) the FAMEs with the same carbon skeleton but differing in the number/geometric configuration/position of double bonds lie on lines parallel to the ^{1}D time axis, based on the fact that the reduction converted them into the same compound. The result is a two-dimensional separation in which the ^{1}D separation shows limited loss of resolution compared to its monodimensional equivalent, as shown by the fact that the 18:1 and 20:1 FAMEs contained in this reference mixture are still separated from each other. The most critical coelutions affecting the same separation by monodimensional GC are instead resolved by ^{2}D: the n:1 FAMEs are separated from the saturated $(n + 1)$:0 (eg, 18:1 vs 19:0), the 18:2 FAMEs are separated from the 20:0 and 19:1, and the 18:3 FAMEs are separated from the 20:1 and 21:0.

The pressure of 690 kPa applied to the injection port provided an average linear velocity of 17 cm/s for ^{1}D and 230 cm/s for ^{2}D, based on the retention times of hexane, an unretained compound. Two dimension chromatograms were adjusted by shifting the ^{2}D phase by the estimated holdup time, 1.1 s. The 0 s time of the chromatogram corresponds to 1.1 s of separation, while 3.5 s correspond of 1.1 s of the subsequent modulation cycle. The 3.5 s modulation time allowed the separation of FAMEs with up to 23 carbons in a single modulation cycle, while FAMEs with 24 or more carbons if present elute during the subsequent modulation cycle. If the sample does not contain FAMEs with more than 21 carbons, as in the case of several vegetable oils, the ^{2}D modulation time can be reduced to 2 s. The combination of the long ^{1}D column (200 m) and the 3.5 s modulation time allowed a modulation ratio (MR) of at least 3 for FAMEs with at least 10 carbons. The isothermal and constant flow elution conditions applied in this study caused broad late eluting peaks, and the last eluted FAME (22:6) showed an MR of 15 or more according to its load on the column.

Figure 3 shows the separation of FAMEs prepared from menhaden oil. Identification of FAMEs was achieved by combining information obtained from the separations of available reference materials, fractionation using silver ion chromatography,[13] GC-MS analysis, and available literature.[1,2,12,13] The FAMEs prepared from menhaden oil were

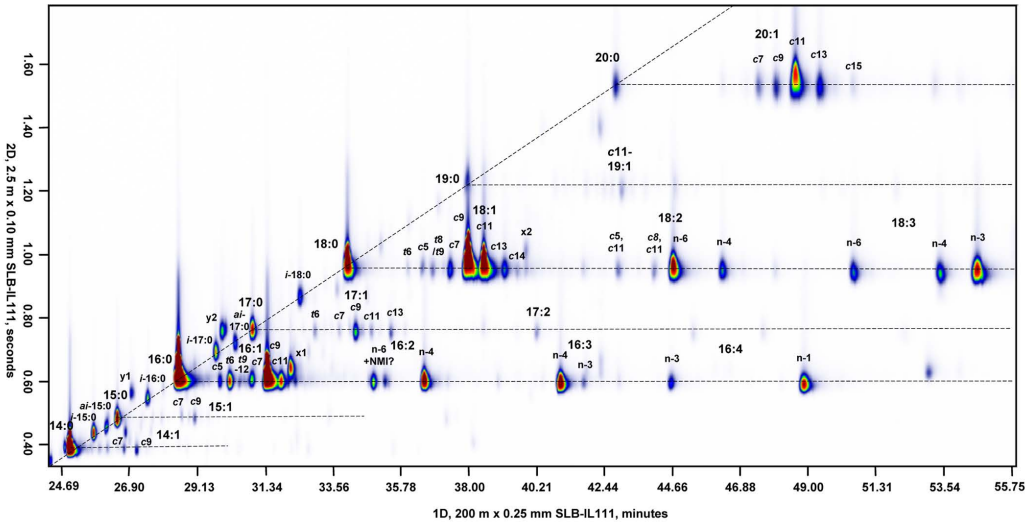

Figure 4. Partial GC × GC separation from 14:0 to 18:3 $n-3$ of the FAME prepared from a menhaden oil sample. Instrument configuration as shown in Figure 1. y1, pristanic acid methyl ester; y2, phytanic acid methyl ester; x1, FAME with 17 carbons and one unsaturation; x2, FAME with 19 carbons and one unsaturation; NMI, nonmethylene interrupted.

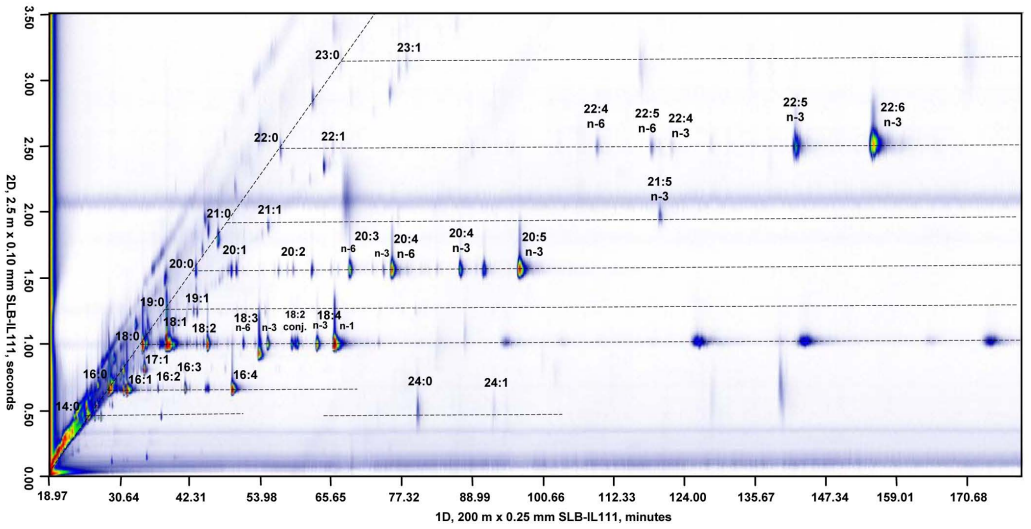

Figure 5. GC × GC separation of the FAME prepared from human colon adenocarcinoma cells HT-29 incubated with conjugated fatty acids. Instrument configuration as shown in Figure 1.

fractionated by silver ion chromatography into saturated FAMEs, *trans*-monounsaturated FAMEs (MUFA), *cis*-MUFAs, and PUFAs as previously described.[13] The FAMEs in the saturated FAMEs fraction were identified by comparison with available reference material and separations reported in the literature.[1,2,12,13] The FAMEs isolated in the *cis*- and *trans*-MUFAs fractions were identified using previously prepared and characterized reference mixtures.[12,13,43,44] The identification of the FAMEs in the PUFA fraction was achieved by integrating identifications reported in the literature[1,2,13] using different separation columns with GC-MS data obtained in this study. The molecular ion of PUFA indicated the n:x structure of the FAME, and, for each n:x series, the location of double bonds was obtained by comparison with identifications reported in the literature.[1,2] The separation of PUFA up to 22:6 requires 165 min, and FAMEs with different chain lengths are separated by

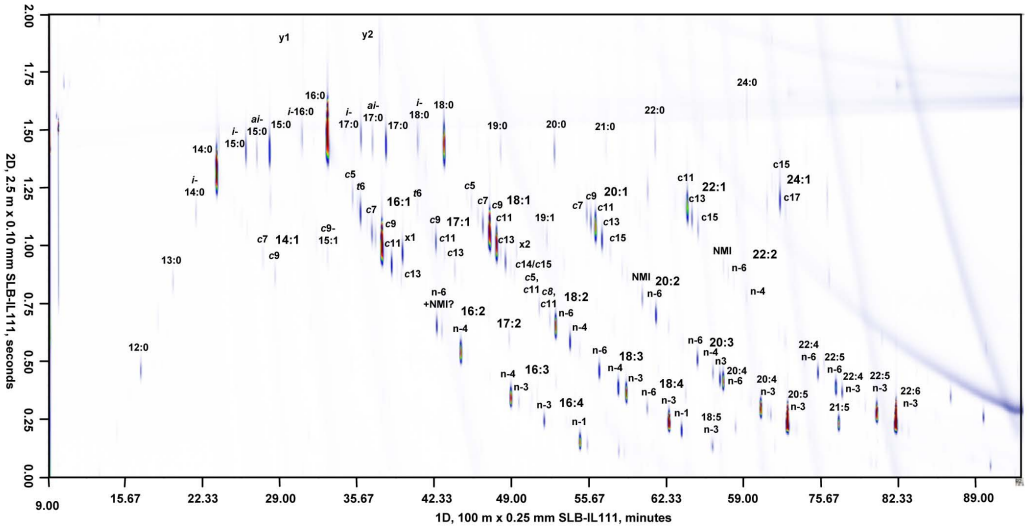

Figure 6. GC × GC separation of the FAME prepared from a menhaden fish oil sample. Instrument configuration as shown in Figure 1, with modifications: ^{1}D column 100 m × 0.25 mm SLB-IL111, and elution temperature program. y1, pristanic acid methyl ester; y2, phytanic acid methyl ester; x1, FAME with 17 carbons and one unsaturation; x2, FAME with 19 carbons and one unsaturation; NMI, nonmethylene interrupted.

^{2}D on different lines. The separation on the two-dimensional space shows that several C16, C18, and C20 U-FAMEs require a second dimension of separation in order to be resolved. Valuable information provided by this separation technique is the identification of the FAME carbon skeleton, including the presence of branches. Branched chain *n*:*x* FAMEs (i.e., iso and anteiso isomers) are eluted by ^{2}D on lines located between the C(n − 1) and C*n* FAME. If branched chain FAMEs with unsaturations are present, the ^{2}D retention time indicates the iso or anteiso structure and the ^{1}D retention time provides information regarding the number and geometric configuration of double bonds. Obtaining the same information utilizing other analytical techniques including mass spectrometry might be challenging, especially if the targeted FAME is present in low concentration or it coelutes with other FAMEs.

Figure 4 shows the separation of the FAMEs prepared from menhaden oil from 14:0 to 18:3. The two-dimensional separation of the 16:1 and 17:0 FAME isomers provides a dramatic improvement over the most refined separation obtained by monodimensional GC: the 16:1 isomers are separated by ^{1}D along the C16 line, while the 17:0 branch chain isomers are eluted on the saturated FAME diagonal line. The x1 compound analyzed by mass spectrometry showed a skeleton constituted of 17 carbons and one unsaturation or a ring. The ^{2}D retention time indicates that this FAME is reduced by Pd because it elutes below the diagonal line bisecting the plane, and it is not an *n*-/*iso*-/*anteiso*-17:1 FAME because its ^{2}D retention time is different. The 17:1 and 16:2 FAMEs are separated on different lines and, as in the case of several other coelutions, they could be resolved only by taking advantage of the ^{2}D separation. Of particular interest was the identification of the 16:4 FAME coeluting with *c*9,*c*12−18:2 in ^{1}D, of the 16:4 FAME coeluting with *c*11−20:1 and 21:0, and the 16:2 FAME coeluting with *c*5−18:1 in the middle of the 18:1 cluster. The separation described here not only provides a unique separation capability but it also allows a simple identification of the separated FAMEs.

The application presented in this study is expected to be suitable for the separation of FAMEs prepared from complex samples of various origins. Figure 5 shows the separation of FAMEs prepared from human colon adenocarcinoma cells HT-29 incubated with conjugated fatty acids. More details regarding the composition and the biological significance of this sample can be found in the original study.[41] In this separation, the diagonal line of saturated FAMEs bisecting the plane is accompanied by at least two similar other lines, probably not belonging to FAMEs. No attempt was made to identify these compounds. The identification of the FAMEs contained in this sample is challenging, but the model of interpretation described in this study simplifies the identification of most FAMEs based on the ^{2}D lines on which they are eluted. A single dimension of separation can provide a very limited resolution of the FAMEs prepared from this sample, while the chromatographic system described here can separate these FAMEs from each others and from other interfering compounds. As an example, while the C16 PUFAs are separated in the two dimension plane from other compounds contained in this sample, their separation and identification by monodimensional GC is a very challenging task.

The GC × GC separation of marine oil FAMEs can be further optimized by applying an elution temperature program. Figure 6 shows the separation of FAMEs prepared from menhaden oil using a 100 m × 0.25 mm SLB-IL111 for ^{1}D and a temperature program from 130 to 220 °C. The chromatogram is adjusted by shifting the phase 1.8 s. The saturated FAMEs with 14 or more carbons are eluted in an almost straight line parallel to the time axis, and for each chain length, the unsaturated FAMEs are eluted on crescents. The temperature elution program provided an optimized utilization of the separation space, shortened the separation to 90 min,

and allowed the reduction of the modulation time to 2 s. The GC × GC separation shown in Figure 6 is characterized by a reversed pattern compared with the separations provided by the system constituted by a nonpolar column for ^{1}D and a polar column for ^{2}D:[17,18] for each chain length, the saturated FAME elutes first at the top of the crescent and unsaturated FAMEs follow in order of increasing number of double bonds. The result is still a group-type GC × GC separation based on the FAME chain length and number of unsaturations, but the groups are arranged in reversed ^{2}D elution order.

■ CONCLUSION

The GC × GC chromatographic system described here provides an enhancement of our preferred method for trans fatty acid separation by adding a second dimension of separation capable of resolving most of the FAMEs not separated by ^{1}D. The FAMEs can be easily identified based on a simple interpretation model: saturated FAMEs lie on a diagonal line bisecting the separation plane and FAMEs with the same carbon skeleton lie on lines parallel to the ^{1}D time axis. The chromatographic conditions described have been shown to allow the separation of the FAMEs prepared from the most complex samples, including fish oil and biological extracts. The proposed approach, reducing unsaturated FAMEs to their saturated form between the two dimensions of separation, provided an enhanced separation compared to GC × GC systems based solely on the orthogonality of the two separations. Reducing U-FAMEs prior to ^{2}D separation also allowed faster modulation times, because the reaction products (saturated FAME) show significantly lower retention times when they are separated on highly polar columns. We invite the users of GC × GC to consider whether or not the described chromatographic system can be considered orthogonal, despite the fact that there is no orthogonality between the separation columns selected for ^{1}D and ^{2}D.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Santercole, V.; Delmonte, P.; Kramer, J. K. *Lipids* **2012**, *47*, 329–344.
(2) *AOCS Official Method Ce 1i-07*, AOCS Press: Urbana, IL, 2007.
(3) Armstrong, D. W.; He, L.; Liu, Y. S. *Anal. Chem.* **1999**, *71*, 3873–3876.
(4) Anderson, J. L.; Armstrong, D. W. *Anal. Chem.* **2003**, *75*, 4851–4858.
(5) Anderson, J. L.; Ding, J.; Welton, T.; Armstrong, D. W. *J. Am. Chem. Soc.* **2002**, *124*, 14247–14254.
(6) Anderson, J. L.; Ding, R.; Ellern, A.; Armstrong, D. W. *J. Am. Chem. Soc.* **2005**, *127*, 593–604.
(7) Huang, K.; Han, X.; Zhang, X.; Armstrong, D. W. *Anal. Bioanal. Chem.* **2007**, *389*, 2265–2275.
(8) Breitbach, Z. S.; Armstrong, D. W. *Anal. Bioanal. Chem.* **2008**, *390*, 1605–1617.
(9) Payagala, T.; Zhang, Y.; Wanigasekara, E.; Huang, K.; Breitbach, Z. S.; Sharma, P. S.; Sidisky, L. M.; Armstrong, D. W. *Anal. Chem.* **2009**, *81*, 160–173.
(10) Anderson, J. L.; Armstrong, D. W. *Anal. Chem.* **2005**, *77*, 6453–6462.
(11) Ragonese, C.; Tranchida, P. Q.; Dugo, P.; Dugo, G.; Sidisky, L. M.; Robillard, M. V.; Mondello, L. *Anal. Chem.* **2009**, *81*, 5561–5568.
(12) Delmonte, P.; Fardin Kia, A. R.; Kramer, J. K.; Mossoba, M. M.; Sidisky, L.; Rader, J. I. *J. Chromatogr. A* **2011**, *1218*, 545–554.
(13) Delmonte, P.; Fardin-Kia, A. R.; Kramer, J. K.; Mossoba, M. M.; Sidisky, L.; Tyburczy, C.; Rader, J. I. *J. Chromatogr. A* **2012**, *1233*, 137–146.
(14) Destaillats, F.; Guitard, M.; Cruz-Hernandez, C. *J. Chromatogr. A* **2011**, *1218*, 9384–9389.
(15) Seeley, J. V.; Seeley, S. K.; Libby, E. K.; Breitbach, Z. S.; Armstrong, D. W. *Anal. Bioanal. Chem.* **2008**, *390*, 323–332.
(16) Reid, V. R.; Crank, J. A.; Armstrong, D. W.; Synovec, R. E. *J. Sep. Sci.* **2008**, *31*, 3429–3436.
(17) Gu, Q.; David, F.; Lynen, F.; Vanormelingen, P.; Vyverman, W.; Rumpel, K.; Xu, G.; Sandra, P. *J. Chromatogr. A* **2011**, *1218*, 3056–3063.
(18) Tranchida, P. Q.; Franchina, F. A.; Dugo, P.; Mondello, L. *J. Chromatogr. A* **2012**, *1255*, 171–176.
(19) Villegas, C.; Zhao, Y.; Curtis, J. M. *J. Chromatogr. A* **2010**, *1217*, 775–784.
(20) Zapadlo, M.; Krupcík, J.; Májek, P.; Armstrong, D. W.; Sandra, P. *J. Chromatogr. A* **2010**, *1217*, 5859–5867.
(21) Zapadlo, M.; Krupčík, J.; Kovalczuk, T.; Májek, P.; Spánik, I.; Armstrong, D. W.; Sandra, P. *J. Chromatogr. A* **2011**, *1218*, 746–751.
(22) Purcaro, G.; Tranchida, P. Q.; Ragonese, C.; Conte, L.; Dugo, P.; Dugo, G.; Mondello, L. *Anal. Chem.* **2010**, *82*, 8583–8590.
(23) Purcaro, G.; Tranchida, P. Q.; Conte, L.; Obiedzińska, A.; Dugo, P.; Dugo, G.; Mondello, L. *J. Sep. Sci.* **2011**, *34*, 2411–2417.
(24) Dutriez, T.; Borras, J.; Courtiade, M.; Thiébaut, D.; Dulot, H.; Bertoncini, F.; Hennion, M. C. *J. Chromatogr. A* **2011**, *1218*, 3190–3199.
(25) Mahé, L.; Dutriez, T.; Courtiade, M.; Thiébaut, D.; Dulot., H.; Bertoncini, F. *J. Chromatogr. A* **2011**, *1218*, 534–544.
(26) Siegler, W. C.; Crank, J. A.; Armstrong, D. W.; Synovec, R. E. *J. Chromatogr. A* **2010**, *1217*, 3144–3149.
(27) Ragonese, C.; Sciarrone, D.; Tranchida, P. Q.; Dugo, P.; Mondello, L. *J. Chromatogr. A* **2012**, *1255*, 130–144.
(28) Adahchour, M.; Beens, J.; Vreuls, R. J. J.; Brinkman, U. A. T. *Trends Anal. Chem.* **2006**, *25*, 438–454.
(29) Mondello, L.; Casilli, A.; Tranchida, P. Q.; Dugo, P.; Dugo, G. *J. Chromatogr. A* **2003**, *1019*, 187–196.
(30) Adahchour, M.; Jover, E.; Beens, J.; Vreuls, R. J. J.; Brinkman, U. A. T. *J. Chromatogr. A* **2005**, *1086*, 128–134.
(31) de Geus, H. J.; Aidos, I.; de Boer, J.; Luten, J. B.; Brinkman, U. A. T. *J. Chromatogr. A* **2001**, *910*, 95–103.
(32) Western, R. J.; Lau, S. S. G.; Mariott, P. J.; Nichols, P. D. *Lipids* **2002**, *37*, 715–724.
(33) Harynuk, J.; Vlaeminck, B.; Zaher, P.; Marriott, P. J. *Anal. Bioanal. Chem.* **2006**, *386*, 602–613.
(34) Seeley, J. V.; Seeley, S. K.; Libby, E. K.; McCurry, J. D. *J. Chromatogr. Sci.* **2007**, *45*, 650–656.
(35) Tiyapongpattana, W.; Wilairat, P.; Marriott, P. J. *J. Sep. Sci.* **2008**, *31*, 2640–2649.
(36) Adam, F.; Bertoncini, F.; Coupard, V.; Charon, N.; Thiebaut, D.; Espinat, D.; Hennion, M. C. *J. Chromatogr. A* **2008**, *1186*, 236–244.

(37) De Koning, S.; Janssen, H. G.; Brinkman, U. A. T. *LC-GC Eur.* **2006**, *18*, 590−600.
(38) Schomburg, G.; Hubinger, E.; Husmann, H.; Weeke, F. *Chromatographia* **1982**, *16*, 228−232.
(39) Henneberg, D.; Henrichs, U.; Schomburg, G. *J. Chromatogr.* **1975**, *112*, 343−352.
(40) LeQuere, J. L.; Semon, E.; Lanher, B.; Sebedio, J. L. *Lipids* **1989**, *24*, 347−350.
(41) Degen, C.; Ecker, J.; Piegholdt, S.; Liebisch, G.; Schmitz, G.; Jahreis, G. *Biochim. Biophys. Acta* **2011**, *1811*, 1070−1080.
(42) Giddings, J. C. *J. Chromatogr. A* **1995**, *703*, 3−15.
(43) Delmonte, P.; Kia, A. R.; Hu, Q.; Rader, J. I. *J. AOAC Int.* **2009**, *92*, 1310−1326.
(44) Delmonte, P.; Hu, Q.; Kia, A. R.; Rader, J. I. *J. Chromatogr. A* **2008**, *1214*, 30−36.

CHAPTER 7

Separation of menhaden oil fatty acid methyl esters using highly polar gas liquid chromatography coupled with identification using time of flight mass spectrometry.

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Separation of the fatty acids in menhaden oil as methyl esters with a highly polar ionic liquid gas chromatographic column, and identification by time of flight mass spectrometry.

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Running title: **Separation of marine oil FA using ionic liquid GC columns.**

Key words: ionic liquid, SLB-IL111, GC, gas chromatography, menhaden oil, marine oil, PUFA, GC-TOF/MS.

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ABBREVIATIONS:

AOCS	American Oil Chemists' Society
CI	chemical ionization
CLA	conjugated linoleic acid
CPS	cyanopropylsiloxane
DHA	docosahexaenoic acid
DMOX	4,4-dimethyloxazoline
ECL	equivalent chain length
EI	electron impact ionization
EPA	eicosapentaenoic acid
FA	fatty acid
FCL	fractional chain length
FD	furane dimethyl
FAME	fatty acid methyl ester
FID	flame ionization detector
FM	furane mono-methyl
GC	gas chromatography
HPLC	high performance liquid chromatography
MI	methylene interrupted
MS	mass spectrometry
MUFA	monounsaturated fatty acid
NMI	non-methylene interrupted
PEG	polyethylene glycol
PUFA	polyunsaturated fatty acid
RT	retention time
SFA	saturated fatty acid
TMTD	tetramethyltridecanoate
TOF	time of flight

ABSTRACT

The fatty acids contained in marine oils or products are traditionally analyzed by gas chromatography using capillary columns coated with polyethylene glycol phases. Recent reports indicate that 100% cyanopropyl siloxane phases should also be used when the analyzed samples contain *trans* fatty acids. We investigated the separation of the fatty acid methyl esters prepared from menhaden oil using the more polar SLB-IL111 (200m x 0.25mm) ionic liquid capillary column and the chromatographic conditions previously optimized for the separation of the complex mixture of fatty acid methyl esters prepared from milk fat. Identifications of fatty acids were achieved by applying Ag^+-HPLC fractionation and GC-TOF/MS analysis in CI^+ mode with isobutane as the ionization reagent. Calculation of equivalent chain lengths confirmed the assignment of double bond positions. This methodology allowed the identification of 125 fatty acids in menhaden oil, including isoprenoid and furanoid fatty acids, and the novel 7-methyl-6-hexadecenoic and 7-methyl-6-octadecenoic fatty acids. The chromatographic conditions applied in this study showed the potential of separating in a single 90 min analysis, among others, the short chain and *trans* fatty acids contained in dairy products, and the polyunsaturated fatty acids contained in marine products.

INTRODUCTION

The oils and fats extracted from marine products, including fish and algae, are known to contain over 100 different fatty acids (FA) with a wide range of chain lengths and unsaturations [1-3]. Their determination is most commonly achieved by gas chromatography (GC) after derivatization to their corresponding FA methyl esters (FAME). For ease of identification and to avoid overlap, Ackman [4] proposed the use of polyethylene glycol (PEG) phases with medium polarity that minimized the overlap of unsaturated FAME with different chain lengths. These GC columns have subsequently been recommended in the American Oil Chemists' Society (AOCS) Official Method Ce 1i-07 [2] for the analysis of saturated FA, *cis*-monounsaturated FA (MUFA), and *cis*-polyunsaturated FA (PUFA) in marine and other oils. The selectivity for FAME of these capillary columns is based primarily on the FAME chain length, secondarily on the number of double bonds, and also on the double bond positions [4,5,6]. Capillary columns coated with PEG show little selectivity based on

the geometric configuration of FAME unsaturations and are not suitable for the quantification of *trans* FA [3]. *Trans* FA in fish and fish products may occur as a result of heating or processing [7-9] or when the product is combined with other ingredients that may contain *trans* FA [10]. Dietary supplements of fish oil may also contain *trans* FA [11,12]. Current efforts to enrich ruminant products with docosahexaenoic acid (DHA) will require the separation of many natural *trans* FA [13] and *trans* FA metabolites formed from DHA and other PUFA in the rumen [14,15].

The selectivity toward the number, geometric configuration and position of double bonds of FAME can be increased by selecting capillary columns coated with phases of higher polarity. Use of capillary columns coated with 100% cyanopropyl siloxane (CPS) is the current preferred approach for the separation of positional and geometric isomers of unsaturated FAME, specifically for *trans* FA [3,16-20]. However, it is evident that isothermal elutions [17,19,21], single temperature programs [18,19], combinations of temperature programs [18] or use of a combination of results achieved using different GC columns [16,21] are unable to resolve all the FA present in marine [3,21] or ruminant lipids [16,18].

The recent availability of capillary columns coated with ionic liquids of higher polarity than 100% CPS phases provided new selectivities for the analysis of FAME. Delmonte et al. [22] demonstrated that the coating of the SLB-IL111 capillary column is characterized by a significantly higher selectivity toward the number, geometric configuration and position of the FAME double bonds as shown using synthetic mixtures of FAME positional/geometric isomers. The authors used the same capillary column to resolve the FAME prepared from milk fat and further increased the separation potential by doubling its length from 100 m to 200 m, still retaining the 0.25 mm internal diameter. This methodology provided an improved resolution of all the *trans*-18:1 FAME including the separation between *t*10- and *t*11-18:1, the elution of all *trans*-18:1 FAME up to *t*15-18:1 prior to oleic acid, an improved separation of the *cis*-18:1 isomers, including of *c*6- and *c*7- from *c*8-18:1, the separation of a higher number of *c/t*-18:2 FAME isomers, the resolution of the conjugated linoleic acid (CLA) isomers *t*7,*c*9- and *c*9,*t*11-18:2, and the identification of the n-1 MUFA series [23]. The improved resolution among the *cis*-18:1 positional isomers using this ionic liquid column also allowed the identification of the Δ6-MUFA series in human hair and nail samples [24].

In our previous publication [3] using a 100 m x 25 mm 100% CPS capillary GC column operated with two different temperature programs, we were able to resolve most, but not all of the FAME prepared from menhaden oil. In that study [3], we could not confirm the identity of the FAME for which reference materials were not available.

In the current study, we apply the methodology successfully developed using a 200 m x 25 mm SLB-IL111 capillary column for the analysis of milk fat [23] to the analysis of a sample of marine origin, specifically menhaden oil. We evaluate whether these chromatographic conditions allow for the simultaneous separation of short-chain FA, *trans* FA present in partially hydrogenated oils and ruminant fats, CLAs, long-chain PUFA, and geometric/positional isomers of PUFA. In addition, we establish the structures of most of the FAME by combining information provided by Ag^{+}-HPLC fractionation followed by GC analysis, high resolution GC-TOF/MS, and calculation of equivalent chain lengths.

MATERIALS AND METHODS

The GLC 463 reference mixture and other FAME reference materials used in this study were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). Methyl phytanate and methyl pristanate were purchased from Larodan (Malmo, Sweden). Menhaden oil was purchased from Sigma Aldrich (St. Louis, MO). The CLA mixture UC-59M purchased from Nu-Chek Prep, Inc. was isomerized using iodine [19,25], and individual CLA isomers were prepared as previously described [26,27]. α-linolenic (18:3n-3) and γ-linolenic (18:3n-6) acids were purchased from Nu-Chek Prep, Inc. and isomerized using *p*-toluene sulfinic acid as previously described [16,19,28]. The synthetic mixtures containing the positional isomers of *cis* and *trans* monounsaturated FAME were prepared by hydrobromination/dehydrobromination of reference materials purchased from Nu-Chek Prep, Inc. [19,28]. 4,4-dimethyloxazoline (DMOX) derivatives were prepared as described previously [27] to identify unsaturated branched-chain FAME.

GC-FID analysis of FAME. Gas chromatographic separations were achieved by applying previously described conditions [23] using an Agilent 7890N gas chromatograph (Agilent Tech., Wilmington, DE, USA) equipped with a flame ionization detector. The separation column was prepared by coupling two SLB-IL111 columns (100 m x 0.25 mm, 0.2 μm thickness, Supelco Inc., Bellefonte, PA, USA) with an Agilent Ultimate Union (Agilent Tech., Wilmington, DE). The oven was maintained at 170 °C for 50 min, then the temperature was raised 6 °C/min to 185 °C and maintained constant 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. During the separation the column head pressure increased from 51 to 74 psi. The separation time was 87.5 min, with 5 min post injection re-equilibration time. 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 mode) 2 min after injection. The injection volume was 1 μL.

Ag^+-HPLC fractionation. Fatty acid methyl esters prepared from menhaden oil were fractionated as previously described [23] using an Alliance 2695 separation module (Waters, Milford, USA) equipped with a Waters 2996 PDA detector, a Waters Fraction Collector II, and a semi-preparative Chromspher 5 Lipids column (10 x 250 mm, 5 μm particle size, Varian Inc. Walnut Creek, CA). The HPLC system and the fraction collector were operated as previously described with no modifications, including the same collection timing [23].

GC-MS identification of FAME. Identities of FAME were confirmed using a Micromass GCT TOF mass spectrometer (Manchester, UK) coupled with an Agilent 6890N gas chromatograph. The separation was achieved using an SLB-IL111 capillary column (100 m x 0.25 mm, 0.20 mm film thickness) maintained at 170 °C with H_2 as the carrier gas at the pressure of 80 kPa. The mass spectrometer was operated in the CI^+ or EI mode. When CI^+ mode was selected, isobutane was used as the ionization gas. The source was maintained at 150 °C, the electron energy was 70 eV, the emission current was 250 μA, and the CI gas flow was set to 10%. When the mass spectrometer was operated in EI mode, the ionization energy was also set to 70 eV.

RESULTS

The Ag^+-HPLC separation of FAME prepared from menhaden oil provided an elution profile similar to the profile reported previously for milk fat FAME [23]. The application of the same experimental conditions and collection parameters provided 4 fractions containing the FAME of saturated FA (SFA, fraction I); *trans*-MUFA and *t,t*-CLAs (fraction II); *cis*-MUFA plus *c,t*-/*t,c*-CLAs and non-conjugated *t,t* dienoic FA such as *t*9,*t*12-18:2 (fraction III); the non-conjugated *c,t* / *t,c* / *c,c* dienoic FA and the FA with more than 2 double bonds (fraction IV).

Each of the Figures 1 through 5 shows, from the bottom, the GC separation of the FAME prepared from menhaden oil, the 4 Ag^+-HPLC fractions in order of collection (SFA, *trans*-MUFA, *cis*-MUFA, PUFA), and reference mixture GLC 463. The inserts show the separation of reference mixtures that facilitate identifications of selected FAME or demonstrates their absence. With the exception of the FAME reported in Figure 1, which were easily identified using reference materials and elution principles, identification of the remaining FAME in Figures 2-5 was achieved or verified using a high resolution TOF/MS operated in CI^+ mode. Isobutane, used as the CI reagent gas, provided mild ionization and the molecular ion $[M+H]^+$ as the most abundant ion for all FAME including PUFA, which indicated the chain length and the number of unsaturations of FAME. The presence of the $[M-MeOH+H]^+$ ion was used to verify that the investigated compound was a FAME, which was further confirmed by comparison with the elution of authentic FAME standards, or previously reported FAME separations [2,3].

Figures 6 to 9 show the partial GC-TOF/MS regions corresponding to Figures 2 to 5. There were slight differences in the separation patterns shown in Figures 6-9 compared to those in Figures 2-5. These differences are attributed to the use of a 100 m rather than a 200 m SLB-IL111 column for the GC/MS analysis, and the former was operated at 170 °C with no thermal or flow gradient. Each separation segment shows, at the bottom, the total ion current (TIC) chromatogram, followed by single ion traces of the molecular ion $[M+H]^+$ of the FAME eluting in the selected region.

Figure 1 shows the separation of the short- and mid-chain FAME up to 16:0 present in menhaden oil. Most of the FAME contained in this section were saturated and were identified by comparison with the FAME contained in GLC 463. The branched-chain *iso*- (*i*) and *anteiso*- (*ai*) FAME eluted at the equivalent chain lengths (ECL) X.47 and X.72, respectively, consistent with previous observations [3] and their retention times matched the values previously observed for milk fat FAME [22,23]. In this segment are eluted two isoprenoid FAME, methyl 4,8,12-trimethyl tridecanoate (4,8,12-TMTD) and methyl 2,6,10,14-tetramethyl pentadecanoate (methyl pristanate). Both isoprenoid FAME eluted in the SFA HPLC fraction. These FAME were previously reported in menhaden oil [29], and in this study were identified by GC-TOF/MS in CI^+ mode based on their molecular ions m/z 271.33 and 313.40. The identification of methyl pristanate was also confirmed by comparison with the elution of its reference material. The only unsaturated FAME observed in this separation segment (Figure 1, menhaden oil) were *c*7- and *c*9-14:1, which were present in trace amounts and identified by comparison with the *cis*-14:1 synthetic mixture.

Figure 2 shows the separation of FAME from 16:0 to 18:0 acquired by GC-FID, while Figure 6 shows the same separation segment acquired by GC-TOF/MS. Inserts show the separation of *cis*- and *trans*- 15:1, 16:1 and 17:1 FAME positional isomers and are included in Figure 2 to facilitate identifications. All of the most abundant FAME eluting in this area are separated, with the exception of the coeluting of *c*7-16:1 and 17:0. Methyl 7-methyl-6-hexadecenoate (7-Me-6-16:1 FAME) eluted among the *cis*-16:1 FAME and showed a molecular ion of m/z 283.34 corresponding to a FAME with 17 carbons and one unsaturation (Figure 6). The GC-TOF/MS spectrum of 7-Me-6-16:1 FAME acquired in EI mode is reported in Figure 10 (upper panel). This spectrum was identical to the one originally reported by Sano [30] with m/z 282, 138, 155 and 115 as the diagnostic ions. The elution of this FAME in the *trans* FA Ag^+-HPLC fraction (II) would suggest that its double bond is in the *trans* configuration. However further investigation is needed to establish its geometry. The EI spectrum of 7-Me-6-16:1 as DMOX derivative showed the same fragmentation pattern reported by Christie [31], with m/z 126, 113, 55, 208 and 166 (in order of relative abundance) as the predominant fragmentation ions, and m/z 321 as the molecular ion. As in the case of the spectrum of its FAME equivalent, the interpretation of the fragmentation pattern is rather complex.

The isoprenoid FA phytanic acid reported in menhaden oil by Ratnayake et al. [29] was identified in Figure 6 based on its molecular ion m/z 327.41. *Trans* 6-16:1 is the main *trans* fatty acid present in this fish oil sample and was well separated from other FAME, while *t*6-, *t*10-, and *t*12/*t*13-17:1 were found in trace amounts. The *n*-, *i*- and *ai*- 17:0 and the *i*-18:0 were identified based on their elution in the Ag^+-HPLC SFA fraction (Figure 2), and by GC-TOF/MS based on their molecular weight (Figure 6). *Trans*- and *cis*-MUFA were identified based on the comparison with the separation of synthetic mixtures (inserts), their elution in the *trans*- or *cis*- MUFA Ag^+-HPLC fraction, and their corresponding molecular weights.

Figure 3 shows the separation of FAME from 18:0 to linoleic acid (*c*9,*c*12-18:2). Inserts showing the separation of *cis*- and *trans*- 17:1, 18:1 and 19:1 FAME positional isomers and the geometric isomers of 9,12-18:2 are included as references. Figure 7 shows the same FAME separation segment investigated by GC-TOF/MS. This partial GC region includes the simultaneous elution of several SFA, MUFA and PUFA. The 16:2, 16:3 and 18:2 positional isomers eluted in the PUFA HPLC fraction (IV) and were identified by GC-MS based on their molecular ions. The X:2 FAME (i.e. 16:2, 18:2, 20:2) provided $[M]^+$ as the most abundant ion instead of $[M+H]^+$. The 16:2 FAME, in particular, were identified based on their $[M-MeOH+H]^+$ ion instead of $[M]^+$ to avoid the interference with the isotopes of the $[M-MeOH+H]^+$ ions of 18:1 FAME. The identities of all FAME were consistent with the results obtained from the GC-online hydrogenation × GC separation of the same menhaden oil sample [32]. However, the locations of the double bonds could not be determined in either study and were therefore assigned based on comparison with previously published separations [2,3]. Several *cis*-MUFA were found eluting in the *cis*-MUFA Ag^+-HPLC fraction, of which *c*9- and *c*11-18:1 were the most abundant. The *trans*-MUFA fraction contained only trace amounts of *t*8-11-18:1 FAME. Methyl 7-methyl-6-octadecenoate (7-Me-6-18:1) to our knowledge has not been reported in menhaden oil, and it showed a molecular ion of m/z 310 corresponding to a FAME with 19 carbons and one unsaturation. This FAME eluted in the *trans*-MUFA Ag^+-HPLC fraction (II) as its homologous 7-Me-6-16:1 FAME. Figure 10 (lower panel) shows the GC-TOF/MS spectrum of 7-Me-6-18:1 FAME acquired in EI mode. This FAME was identified by Caballeira et al. [33] in the phospholipids fraction of *Holothuria mexicana* using a synthetic reference material, and in this study was characterized by the occurrence of the same diagnostic ions m/z 310, 195, 155, 138 and 115 (Figure 10, lower panel).

The SFA fraction also contained 18:0, 20:0 and trace amounts of *n*-, *i*-, and *ai*- isomers of 19:0.

Figure 4 shows the separation of FAME from *c*9,*c*12-18:2 to 20:3n-6. The separations of *cis*- and *trans*- 20:1 positional isomers, the isomerized CLA reference mixture (UC-59M), 18:3n-3 and 18:3n-6 are inserted. Figure 8 shows the same FAME separation segment investigated by GC-TOF/MS. This segment includes predominantly PUFA which are contained in the PUFA Ag^{+}-HPLC fraction IV. Identification of the 16:4, 18:2, 18:3, 20:2, 18:4 and 20:3 positional isomers is shown in Figure 8. All the PUFA contained in this interval were separated except for the n:4 series: 16:4n-3 showed limited separation from 18:2n-6, 16:4n-1 co-eluted with *c*12-20:1 and was not completely resolved from *c*11-20:1, and 18:4n-1 was only partially resolved between *c*13-22:1 and 23:0 (Fig. 4). The investigated menhaden oil sample did not contain CLA and *trans* isomers of 18:3n-3 or 18:3n-6. The *cis*-MUFA fraction contained predominantly *c*11-20:1 and *c*11-22:1 and several other minor *cis* positional isomers. These MUFA were identified by comparison with the elution of the MUFA isolated from a partially hydrogenated fish oil sample by silver ion solid phase extraction (Kramer, unpublished data). The *trans*-MUFA content was limited to traces of *t*11-/*t*13-20:1 and the SFA fraction contained the 21:0, 22:0 and 23:0 FAME. The slight difference in the relative elution of 22:0 and *c*7,*c*13-20:2 between Figures 4 and 8 was due to differences in the chromatographic conditions, i.e., isothermal/constant flow rate elution versus temperature and flow rate program. The *trans*-MUFA fraction shows the elution of the first member of the furan FA family, FD 9,3.

Furan FA are reported in several fish species and are characterized by a furan ring in the middle of the alkyl chain [34]. Naturally occurring furan FA are characterized by the presence of one (FM series) or two methyl substituents (FD series) on the furan ring. In this manuscript, the furan FA are designated as FM *a*,*b* or FD *a*,*b*, in which the letter *a* indicates the length of the chain towards the carboxyl group, while *b* indicates the length of the terminal chain which is either 3 or 5 carbons. Furan FAME eluted in the *trans*-MUFA fraction (II) and were identified by GC-TOF/MS in EI mode. For common furan FAME, the base peak in the MS spectra is caused by α cleavage to the furan ring towards the carboxyl end of the molecule [34]. The mass difference between the molecular ion and the base peak indicates the length of the carboxyl group side chain [34].

The presence of the ions m/z 123 and m/z 109 (lower intensity) indicates an FD structure, while the presence of the ions m/z 109 and m/z 95 (lower intensity) indicates an FM structure.

Figure 5 shows the separation of FAME from 20:3n-6 to 22:6n-3 and Figure 9 shows the separation of the FAME present in the same partial GC chromatogram investigated by GC-TOF/MS. This segment also contained predominantly PUFA with the exception of the 24:0 and 26:0 FAME contained in the SFA fraction and of *c*13-/*c*15-/*c*17-24:1 which were present in the *cis*-MUFA fraction along with a *cis*-23:1 FAME. The identifications of the PUFA contained in this segment are shown in Figure 8 and are based on the $[M+H]^+$ ion of these FAME. Most of these PUFA are also components of the GLC 463 reference mixture (top chromatogram). Six furan FAME were identified in the *trans*-MUFA Ag^+-HPLC fraction II. Identification of these compounds was achieved by GC-TOF/MS in EI mode applying the principles described in the section for Figure 4. Under the experimental conditions selected for this study (injecting 1 µl of 20 mg/ml FAME solution with 1:100 split ratio), the reported furan FAME were observed in quantities slightly above the limit of detection of the instrument, and occasionally co-eluted with other FAME. The elution order of 22:5n-6, 21:5n-3 and 22:4n-3 was established by GC-TOF/MS and was not affected by changes in the temperature program (Figure 9). All the FAME identified in this segment were well separated from each other and 22:6n-3, the last FAME considered in this separation, eluted at 81.08 min.

DISCUSSION

The main aim of the study was to evaluate whether a 200 m x 0.25 mm SLB-IL111 capillary column, operated under the previously optimized conditions, could be used to improve the separations of the FAME prepared from menhaden oil that are provided by the 100% CPS columns. Despite its marginal economic significance, menhaden oil has served as a valuable reference material in many studies and in the establishment of the AOCS current official method for marine lipids analysis [2] because it contains most of the FA occurring in common marine fats and oils. It is also commercially available in pure form. A previous attempt to analyze menhaden oil using a 100 m x 0.25 mm CPS capillary column (SP-2560) and a temperature program with a plateau at 175 °C provided the resolution of 70 peaks, the coelution of 13 peaks, and 9 small peaks that could not be identified [3].

Of the 13 co-eluting FAME, 7 were resolved by conducting an additional separation with the same GC column but using a temperature program that had a plateau at 150 °C [3]. Two sets of FAME clusters (*t*6/*t*7/*t*8-18:1 and *c*6/*c*7/*c*8-18:1) could not be separated unless the elution temperature was further reduced to 120 °C [13,16,18]. However, five FAME could not be resolved as individual peaks using either temperature program and they included 18:2n-6, 16:4n-1, 18:3n-3, *t*9-19:1 and 23:0 [3]. A similar conclusion was reached after combining the results of marine oil analyses using a BPX-70 column (bonded cyanopropyl polysilphenylene siloxane polymer phase equivalent to 70% CPS) and an SP-2560 column [21].

In the present study, as shown in Figures 1 to 5, we were able to identify 125 FAME prepared from menhaden oil. The applied methodology, using a 200 m x 0.25 mm SLB-IL111 capillary column operated with a temperature and flow rate program, could separate these FAME in 107 peaks. About 10 coelutions are caused by FAME present in amounts near their limit of detection, and only 8 were caused by FAME present in amounts near or above their limit of quantification. The 8 co-elutions were: 17:0 with *c*7-16:1 plus *t*13-16:1 (Figure. 2); 19:0 with *c*9-18:1 and 18:2n-6 with 16:4n-3 (Figure 3); 16:4n-1 with 21:0 and 20:2n-6 with a 18:4 (RT 46.88 min, Figure 4); *c*13-24:1 with FD 11,3; *c*17-24:1 with 20:5n-4; and 26:0 with FD 11,5 (Figure 5). Based on the results of the Ag^{+}-HPLC fractionation, only minor amounts of *t*13-16:1, *c*11-17:1, a 18:4 (RT 46.88 min) and 20:5n-4 were observed, which reduces to 5 the quantitatively significant co-elutions. More coeluting and unidentified FAME might be present in menhaden oil in quantities below the limit of detection of the methodology applied in this study. These FAME can be resolved and identified by applying extensive complementary fractionation and concentration procedures [35], followed by multiple GC analyses. We previously reported the difficulty in separating SFA from major *cis*-MUFA using highly polar ionic liquid columns [22,23], and this was also the case here with the co-elution of 17:0 with *c*7-16:1 and 19:0 with *c*9-18:1. Specific pairs of FAME can be better resolved by fine tuning the elution temperature program, but that would likely lead to the co-elution of other FAME as previously shown [22,23]. Despite a few limitations observed in this investigation using a 200 m x 0.25 mm SLB-IL111 capillary column, this separation of the menhaden oil FAME was a marked improvement over that obtained using a 100 m x 0.25 mm SP-2560. More FAME were resolved in a single analysis than occurred with use of an SP-2560 operated using two different temperature programs and then combining the results from the two analyses [3].

In addition, the latter method was more time consuming, involved two separate analyses, and required combining the results of two chromatograms [3]. Furthermore, the methodology developed using the 200 m ionic liquid column provided the means of resolving more PUFA, branched-chain FA, furanoid FA, and particularly geometric FA isomers, which could not be separated using 30 m x 0.25 mm PEG columns as recommended in the AOCS Official Method for marine lipid analysis [2].

The identification of all the FAME prepared from menhaden oil continues to be challenging. In our previous publication [3], we based the identification of the FAME on authentic FAME standards, principles of Ag^+-HPLC fractionation, and published reports [1,2,4,5,7]. However, published separations had mainly been conducted using GC columns other than the 100 m x 0.25 mm highly polar CPS phases. In this study, we established the FAME structures by utilizing the available FAME reference materials and standard mixtures, Ag^+-HPLC fractionation followed by GC analysis, and by conducting complementary GC-TOF/MS analysis. The high resolution TOF/MS provided reliable and reproducible identifications even for PUFA present at low concentrations. Chemical ionization using CH_4 as the ionization reagent gas was shown to provide a less marked fragmentation of unsaturated FAME compared to EI [36]. and the MS spectra might be used to obtain structural information based on the fragmentation pattern. Still, the fragmentation caused by CH_4 as CI reagent did not allow the observation of the molecular ion of PUFA present in small amounts [36]. Chemical ionization using isobutane as the reagent gas provided soft ionization that produced an abundant molecular ion $[M+H]^+$ including for highly unsaturated PUFA contained in small amounts. As shown in Figures 6 to 9, extracting the expected $[M+H]^+$ ion from the TIC chromatogram was sufficient to identify the chain-length and the number of double bonds of most FAME. The presence of the $[M-MeOH+H]^+$ ion was used to verify that the investigated peaks were FAME. We further compared our results to those obtained applying GC-online hydrogenation × GC. In this novel application, the FAME are separated using a 200 m x 0.25 mm SLB-IL111 capillary column, then are fully hydrogenated by passage through a capillary tube coated with Pd, and re-separated in their reduced forms [32]. The elution of the FAME prepared from menhaden oil on the two dimensional separation plane confirmed the identifications of both their chain lengths and number of double bonds.

While the current GC-TOF/MS method provided definitive information regarding the chain length and number of double bonds of the FAME prepared from menhaden oil, it did not establish the positions of double bond. Preparation and analysis of the DMOX derivatives or other suitable derivatives (picolinyl esters, pyrrolidide) or adducts (4-methyl-1,2,4-triazoline-3-5-dione) could provide this information [37-39]. However, investigation of minor and partially overlapping peaks would still present a challenge because their signals fall below the sensitivity of the instrumentation or because mass spectra provide limited information. The ECL calculations based on the elution times on GC columns have been used and are commonly recommended to serve as a guide in the assignment of the double bond positions of highly unsaturated methylene-interrupted (MI) PUFA [37,39]. It is well known that the ECL of MI PUFA having the same number of double bonds, in the same geometric configuration, and in the same position relative to the terminal carbon of the FAME have similar fractional chain length (FCL) values when analyzed using the same GC column under isothermal/isobaric conditions [40,41]. Since most of the PUFA in fish oils have MI structures, the ECL and FCL calculations are helpful in the identification of homologous PUFA. Table 1 presents the ECL and FCL values calculated based on the retention times of the menhaden oil PUFA separated using the described chromatographic conditions, and applying the formulas reported by Bannon et al [41]. The PUFA with similar structure reported in Figures 1 to 5 were characterized by similar FCL values (Table 1). Menhaden oil contains several MI PUFA with chain lengths ranging from 16 to 22 carbon atoms that can be identified using the n-6, n-4, n-3 and n-1 nomenclature. The Δ- nomenclature of PUFA is also provided in Table 1. It should be noted that ECL and FCL calculations strictly apply when using isothermal/isometric GC conditions. This observation explains why the calculated ECL/FCL values are more consistent for FAME eluting during the isothermal portion of the separation, i.e., the first 50 min. After the beginning of the temperature gradient at 50 min the retention times of the FAME are shortened, causing the deviation of the corresponding ECL/FCL values from their expected counterparts calculated under constant temperature and flow conditions. Increasing the flow rate of the carrier gas from 1.6 to 3.0 ml/min after the elution of the 18:1 and 18:2 FAME (35 min) provided a minor effect on the ECL and FCL values (Table 1). Increasing the elution temperature from 170 to 185°C after the elution of 18:1, 18:2 and 18:3 FAME (50 min.) allowed the separation of these FAME at their optimal separation temperature, and then reduced the time of elution of the highly retained PUFA. Menhaden oil also contained several non-methylene interrupted (NMI) FAME listed in Table 1.

Two of the FAME had the same terminal structure and a similar FCL, with the double bonds separated by 6 methylene (CH_2) units (*c*5,*c*11-18:2 and *c*7,*c*13-20:2). There were two additional NMI FAME with unknown structures (16:2NMI, 17:2) which FCL values did not match those of the NMI FAME stated above. Additional studies are needed to establish the structures of these NMI FAME.

In this study, we were able to confirm the presence of *t*6-16:1 in menhaden oil. This FAME was clearly resolved on the 200 m SLB-IL111 column from the other *trans*-16:1 FAME contained in the *trans*-16:1 synthetic mixture (Fig. 2). The occurrence of *t*6-16:1 FAME was previously reported by Ackman in a number of marine species [1]. We could only tentatively identify this FAME in menhaden oil using a 100% CPS column [3], since that phase did not allow for the separation of *t*6-, *t*7- and *t*8-16:1 [19], while the use of an SLB-IL111 allowed the separation of *t*6- from other *trans*-16:1 FAME (Figure 2). It is of interest to note that small amounts of *t*6-17:1 (Figure 2) and *t*8-18:1 (Figure 3) were also observed, suggesting that *t*6-18:1 may be chain elongated in fish.

The occurrence of furan FA in fish lipids was first reported by Glass et al. [42] and further studies indicated that unsaturations can be present in their alkyl chains. Ishii et al. [34] reported the presence at least 30 unusual furan FA in the hepatopancreas of crayfish. Furan FA were isolated (along with some branched-chain FA) by applying hydrogenation followed by urea fractionation. This procedure provided the comprehensive content in furan FA with a given carbon skeleton. The current study reports only the elution pattern of fully saturated furan FAME that are present at concentrations above the limit of detection of this methodology. The low concentration of the furan FA in menhaden oil and their coelution (as MEs) with other FAME indicates the need of a prior isolation/concentration of these compounds before their quantification by GC-FID, or the application of a more selective/sensitive detection. The chromatographic conditions used for Ag^+-HPLC fractionation eluted furan FAME in the *trans*-MUFA fraction (II). A more precise evaluation of the furan FAME elution by Ag^+-HPLC indicated that these FAME elute after the SFA and slightly ahead of the *trans*-MUFA.

Isoprenoid FA are derived from the metabolism of phytol [43] and were identified in menhaden oil by Ratnayake et al. [29]. Methyl 4,8,12-TMTD, methyl pristanate and methyl phytanate are present in fish oils as two diastereoisomers [43,44] and they were eluted as

partially separated doublets in both the unfractionated sample and the SFA Ag^+-HPLC fraction (I). These isoprenoid FA, which unlikely are *de novo* synthesized in fish [43], were all separated from other FAME occurring in menhaden oil and might be used as dietary markers.

We also wished to settle the question of whether menhaden oil contains naturally occurring trace amounts of CLA isomers. This occurrence was suspected since in a previous study, using a SP-2560 column for FAME separation, small amounts of *c*9,*t*11-18:2 and *t*9,*t*11-18:2 were observed [3]. In this study no peaks were observed in the *cis*-MUFA fraction (III) where the *c*,*t*-/*t*,*c*-CLA are expected to elute (Figure 4), and no peaks were observed in the *trans*-MUFA fraction (II) where *t*,*t*-CLA are expected to elute based on Ag^+-HPLC elution principles [13]. These obbervations were confirmed by comparison with the separation of the isomerized CLA mixture inserted in Figure 4, and the separation of previously synthetized CLA isomers from 6,8- to 13,15-18:2 FAME [26,27]. Ackman previously reported the absence of CLA in fish oil unless there was a contamination [45] and we can now support this conclusion. However, it should be noted that partially or fully conjugated FA are also formed from PUFA during heating of vegetable oils [46] and fish oils [8,9], and therefore, partially or fully conjugated FA (including CLAs) might occur in refined menhaden oil. If mixtures of fish and ruminant products containing CLAs were to be investigated using the current GC conditions, rumenic acid (*c*9,*t*11-18:2) would co-elute with a18:4 FAME (RT 46.88 min), and *t*,*t*-CLA positional isomers would coelute with 18:4n-1, 23:0, or *c*13- and *c*15-22:1 (Figure 4).

While the occurrence of 7-Me-6-16:1 FA in marine fish oil has already been reported [30,47], the one of methyl 7-Me-6-18:1 has not. In our previous work, separating the same matrix by GC - online reduction × GC [32], we observed the presence of their two corresponding FAME but could not provide their identification. The GC - online reduction × GC results showed two peaks eluting slightly above the C16 and C18 lines, which would be consistent with the presence of a double bond (reduced by the Pd catalyst) and a methyl substituent in the central part of the alkyl chain. The interpretation of their mass spectra as ME or DMOX derivatives is rather complex [31,47] and likely requires the evaluation of more than one rearrangement. However, the mass spectra of both compounds show unique fragmentation patterns that could allow for their identification by comparison with spectra of the same compounds published elsewhere [30,31,33,47,48,49]. Even though methyl 7-Me-7-16:1 was reported in marine fish

oils [29,48,49], we observed the occurrence of only methyl 7-Me-6-16:1 FAME as shown by the single peak contained in the *trans*-MUFA fraction (II) in Figure 2.

We also considered whether the use of a 100 m x 0.25 mm SLB-IL111 column would be suitable for routine analysis of fish oil FAME rather than its 200 m x 0.25 mm version. The results indicate that most of the PUFA (Table 1) can be well separated from each other using either column versions with the exception of few overlaps as 16:4n-3 with 18:2n-6, and 18:4 (RT46.88 min) with 20:2n-6 FAME. However, the real difference between the separations provided by these two columns differing in lengths is the lower resolution for the 100 m column of the positional isomers of unsaturated FAME, such as the separation of *t*10- from *t*11-18:1 [23], and the more extensive overlap of the PUFA with the SFA and MUFA [22,23]. The lower resolution provided by the 100 m column would be of particular concern for the analysis of marine oils mixed with other fat products, such as ruminant fats, milk fats, vegetable oils, or partially hydrogenated fats. By contrast, the methodology developed using the 200 m SLB-IL111 column was shown to successfully separate (as FAME) the complex mixtures of SFA, MUFA, di-unsaturated FA, CLAs and short-chain FA constituting plant lipids and ruminant fats [22,23].

CONCLUSIONS

The set of chromatographic conditions described in this study provided the separation of over 100 FAME prepared from menhaden oil, including the positional and geometric isomers of MUFA, n-6, n-4, n-3 and n-1 MI PUFA with chain lengths from C16 to C22, several NMI PUFA, saturated and branched-chain FAME. Silver ion chromatography provided a very useful simplification of the complex elution pattern of the menhaden oil FAME. High resolution GC-TOF/MS, operated in CI^+ mode using isobutane as the ionization gas, provided unambiguous identifications of the molecular ions of all FAME, including most of the co-eluting FAME. The identifications of methylene interrupted PUFA were verified by evaluating their ECL and FCL values. In a previous publication, we demonstrated that the chromatographic conditions applied in this study provide superior separations of FAME prepared from partially hydrogenated fats [22] and dairy products [23] compared to other available methodologies. In this study, we demonstrated that the same methodology can also be applied to the separation of FAME prepared from fats and oils of marine origin.

REFERENCES

1. Ackman RG (1989) Fatty acids. In: Ackman RG, (ed) Marine Biogenic Lipids, Fats, and Oils, Vol. 1, CRC Press, Boca Raton, Fl, pp 103-137.

2. AOCS Official Method Ce 1i-07 (2007). Determination of saturated, *cis*-monounsaturated and *cis*-polyunsaturated fatty acids in marine and other oils containing long-chain polyunsaturated fatty acids (PUFA) by capillary GLC, AOCS, Urbana, IL.

3. Santercole V, Delmonte P, Kramer JKG (2012) Comparison of separations of fatty acids from fish products using a 30-m Supelcowax-10 and a 100-m SP-2560 column. Lipids 47:329-344.

4. Ackman RG (1986) WCOT (capillary) gas-liquid chromatography. In: Hamilton RJ, Rossell JB, (eds) Analysis of oils and fats, Elsevier Applied Science Publishers, New York, pp 137-206.

5. Ackman RG (1967) The chain-length overlap problem in gas-liquid chromatography with polyester liquid phases. Lipids 2:502-505.

6. Kramer JKG, Fouchard RC, Jenkins KJ (1985) Differences in chromatographic properties of fused silica capillary columns, coated, crosslinked, bonded, or crosslinked and bonded with polyethylene glycols (CARBOWAX 20M) using complex fatty acid methyl ester mixtures. J Chromatogr Sci 23:54-56.

7. Sébédio J-L, Ratanayake WWN, Ackman RG, Prevost J (1993) Stability of polyunsaturated omega-3 fatty acids during deep fat frying of Atlantic mackerel (*Scomber scombrus* L.). Food Res Int 26:163-172.

8. Fournier V, Juaneda P, Destaillats F, Dionisi F, Lambelet P, Sebedio J-L, Berdeaux O (2006). Analysis of eicosapentaenoic and docosahexaenoic acid geometrical isomers formed during fish oil deodorization. J Chromatogr A 1129:21-28.

9. Fournier V, Destaillats F, Juanéda P, Dionisi F, Lambelet P, Sébédio J-L, Berdeaux O (2006). Thermal degradation of long-chain polyunsaturated fatty acids during deodorization of fish oil. Eur J Lipid Sci Technol 108:33-42.

10. Kolanowski W, Laufenberg G (2006) Enrichment of food products with polyunsaturated fatty acids by fish oil addition. Eur Food Res Technol 222:472- 477.

11. Azizian H, Kramer JKG, Ehler S, Curtis JM (2010) Rapid quantitation of fish oil fatty acids and their ethyl esters by FT-NIR models. Eur J Lipid Sci Technol 112:452–462.

12. Sciotto C, Mjøs SA (2012) *Trans* isomers of EPA and DHA in omega-3 products on the European market. Lipids 47:659-667.

13. Cruz-Hernandez C, Deng Z, Zhou J, Hill AR, Yurawecz MP, Delmonte P, Mossoba MM, Dugan MER, Kramer JKG (2004) Methods for analysis of conjugated linoleic acid and trans-18:1 isomers in dairy fats by using a combination of gas chromatography, silver-ion thin-layer chromatography/gas chromatography, and silver-ion liquid chromatography. J AOAC Int 87:545-562.

14. Kairenius P, Toivonen V, Shingfield KJ (2011) Identification and ruminal outflow of long-chain fatty acid biohydrogenation intermediates in cows fed diets containing fish oil. Lipids 46:587-606.

15. Aldai N, Hervás G, Belenguer Á, Frutos P, Mantecóm AR, Kramer JKG (2012) Evaluating the *in vitro* metabolism of docosahexaenoic acid in sheep rumen fluid. Lipids 47:821-825.

16. Kramer JKG, Blackadar CB, Zhou J (2002). Evaluation of two GC columns (60-m SUPELCOWAX 10 and 100-m CP Sil 88) for analysis of milkfat with emphasis on CLA, 18:1, 18:2 and 18:3 isomers, and short- and long-chain FA. Lipids 37:823B835.

17. Ratnayake WMN, Hansen SL, Kennedy MP (2006) Evaluation of the CP-Sil 88 and SP-2560 GC columns used in the recently approved 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 method. J Am Oil Chem Soc 83:475-488

18. Kramer JKG, Hernandez M, Cruz-Hernandez C, Kraft J, Dugan MER (2008) Combining results of two GC separations partly achieves determination of all cis and trans 16:1, 18:1, 18:2 and 18:3 except CLA isomers of milk fat as demonstrated using Ag ion SPE fractionation. Lipids 43:259–273

19. Delmonte P, Fardin Kia A-R, Hu Q, Rader JI (2009) Review of methods for preparation and gas chromatographic separation of *trans* and *cis* reference fatty acids. J. AOAC Internat. 92:1310-1326.

20. Mossoba MM, Moss J, Kramer JKG (2009) *Trans* fat labeling and levels in US foods: assessment of gas chromatographic and infrared spectroscopic techniques for regulatory compliance. J AOAC Int 92:1284–1300.

21. Mjøs SA, Haugsgjerd BO (2011) *Trans* fatty acid analyses in samples of marine origin: The risk of false positives. J Agr Food Chem 59:3520-3531.

22. Delmonte P, Fardin Kia A-R, Kramer JKG, Mossoba MM, Sidisky L, Rader JI (2011) Separation characteristics of fatty acid methyl esters using SLB-1L111, a new ionic liquid coated capillary gas chromatographic column. J Chromatogr 1218:545-554.

23. Delmonte P, Fardin Kia A-R, Kramer JKG, Mossoba MM, Sidisky L, Tyburczy C, Rader JI (2012) Evaluation of highly polar ionic liquid gas chromatographic column for the determination of the fatty acids in milk fat. J Chromatogr A 1233:137-146.

24. Destaillats F, Guitard M, Cruz-Hernandez C (2011) Identification of Δ6-monounsaturated fatty acids in human hair and nail samples by gas-chromatography mass-spectrometry using ionic-liquid based capillary column. J Chromatogr A 1218:9384-9389.

25. Eulitz K, Yurawecz MP, Sehat N, Fritsche J, Roach JAG, Mossoba MM, Kramer JKG, Adlof RO, Ku Y (1999) Preparation, separation, and confirmation of the eight geometrical *cis/trans* conjugated linoleic acid isomers 8,10- through 11,13-18:2. Lipids 34:873-877.

26. Delmonte P, Roach JAG, Mossoba MM, Morehouse KM, Lehmann L, Yurawecz MP (2003) Synthesis and isolation of trans-7,cis-9 octadecadienoic acid and other CLA isomers by base conjugation of partially hydrogenated gamma-linolenic acid. Lipids 38:579-583.

27. Delmonte P, Roach JAG, Mossoba MM, Losi G, Yurawecz MP (2004) Synthesis, isolation, and GC analysis of all the 6,8-to 13,15-cis/trans conjugated linoleic acid isomers. Lipids 39:185-191.

28. Delmonte P, Qing H, Fardin Kia A-R, Rader JI (2008) Preparation, chromatographic separation and relative retention times of *cis*/*trans* heptadecaenoic (17:1) fatty acids. J Chromatogr A 1214:30-36.

29. Ratnayake MWN, Olsson B, Ackman RG (1989) Novel Branched-Chain Fatty Acids in Certain Fish Oils. Lipids 24, 630-637.

30. Sano Y (1967) Studies on the Antarctic whale oils by gas-liquid chromatography using a hydrogen flame ionization detector-VIII. Identification of 7-methyl-6-hexadecenoic acid in whale blubber oil. Yukagaku 16: 605-610

31. The AOCS Lipid Library [http://lipidlibrary.aocs.org/ms/ms12/index.htm; accessed on 05/20/2013]

32. Delmonte P, Fardin Kia A-R, Rader JI (2013) Separation of fatty acid methyl esters by GC- online hydrogenation × GC. Anal Chem DOI: 10.1021/ac302707z.

33. Caballeira NM, Maldonado ME (1988) 7-Methyl-8-hexadecenoic acid: A novel fatty acid from the marine sponge *Desmapsama anchorata*. Lipids 23:690-693

34. Ishii K, Okajima H, Koyamatsu T, Okada Y, Watanabe H (1988) The composition of furan fatty acids in the crayfish. Lipids 23:694-700.

35. Schröder M, Vetter W (2013) Detection of 430 fatty acid methyl esters from a transesterified butter sample. J Am Oil Chem Soc 90: 771-790.

36. Dayhuff LE, Wells MJ (2005) Identification of fatty acids in fishes collected from the

Ohio River using gas chromatography-mass spectrometry in chemical ionization and electron impact modes. J Chromatogr A 1098:144-149.

37. Spitzer V (1997) Structure analysis of fatty acids by gas chromatography – low resolution electron impact mass spectrometry of their 4,4 –dimethyloxazoline derivatives – a review. Prog Lipid Res 35:387-408.

38. Spitzer V (1999) Gas Chromatography/(Electron Impact) Mass Spectrometry Analysis of Conjugated Linoleic Acid (CLA) Using Different Derivatization Techniques. In: Yurawecz MP, Mossoba MM, Kramer JKG, Pariza MW, Nelson GJ (eds) Advances in Conjugated Linoleic Acid Research, Volume 1. AOCS Press, Champaign, IL, pp 110-125.

39. Yu QT, Liu BN, Zhang JY, Huang ZH (1989) Location of double bonds in fatty acids of fish oil and rat testis lipids. Gas chromatography-mass spectrometry of the oxazoline derivatives. Lipids 24:79-83.

40. Christie WW (2003) Lipid analysis, isolation, separation, identification and structural analysis of lipids (3rd Ed), The Oily Press, Bridgwater, England.

41. Bannon CD, Craske JD, Norman LM (1988) Effect of overload of capillary gas-chromatographic columns on the equivalent chain length of C18 unsaturated fatty acid methyl esters. J Chromatogr 447:43-52.

42. Glass RL, Krick TP, Sand DM, Rahn CH,, Schlenk H (1975) Furnaoid fatty acids from fish lipids. Lipids 10:695-702

43. Ackman RG, Hooper SN (1967) Examination of isoprenoid fatty acids as distinguishing chatactheristics of specific marine oils with particular reference to whale oils Comp. Biochem. Physiol. 24, 549-565.

44. Ackman RG, Hansen RP (1967). The occurrence of diastereomers of phytanic and pristanic acids and their determination by gas-liquid chromatography. Lipids 2:357-362.

45. Ackman RG (1999) Conjugated linoleic acid (CLA) in lipids of fish tissue. In: Yurawecz MP, Mossoba MM, Kramer JKG, Pariza MW, Nelson GJ (eds) Advances in conjugated linoleic acid research, vol 1. AOCS Press, Champaign, pp 283-295.

46. Juanéda P, de la Péririère SB, Sébédio J-L, Grégoire S (2003) Influence of heat and refining on formation of CLA isomers in sunflower oil. J Am Oil Chem Soc 80:937-940.

47. The AOCS Lipid Library [http://lipidlibrary.aocs.org/ms/ms03b/index.htm; accessed on 05/20/2013]

48. Ackman RG, Safe L, Hooper SN, Paradis M (1972) 7-Methyl-7-hexadecenoic acid: Isolation from lipids of the ocean sunfish *Mola mola* (Linnaeus) 1758. Lipids 8:21-24

Pascal JC, Ackman RG (1975) Occurrence of 7-methyl-7-hexadecenoic acid, the corresponding alcohol, 7-methyl-6-hexadenoic acid, and 5-methyl-4-hexadecenoic acid in sperm whale oils. Lipids 10:478-482

LEGENDS TO FIGURES

Fig. 1. Partial GC chromatogram of the 4:0 to 16:0 elution segment. From the bottom: FAME prepared from menhaden oil before fractionation, Ag^+-HPLC fractions containing SFA (I), *trans*-MUFA (II), *cis*-MUFA (III), PUFA (IV), and reference mixture GLC 463. Conditions: Supelco SLB-IL111 capillary column (200 m x 0.25 mm, 0.2 μm thickness), temperature/flow gradients as described in the experimental section. Colored inserts show the synthetic *cis* (blue) and *trans* (red) 14:1 and 15:1 positional isomer mixtures. Abbreviations: *i*- = iso; *ai*- = anteiso. 4,8,12-TMTD = 4,8,12-trimethyl-tridecanoate ME.

Fig. 2. Partial GC chromatogram of the 16:0 to 18:0 elution segment. From the bottom: FAME prepared from menhaden oil before fractionation, Ag^+-HPLC fractions containing SFA (I), *trans*-MUFA (II), *cis*-MUFA (III), PUFA (IV), and reference mixture GLC 463. Conditions: SLB-IL111 capillary column (200 m x 0.25 mm, 0.2 μm thickness), temperature/flow gradients as described in the experimental section. 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, NMI= non methylene interrupted. Colored inserts show the synthetic *cis* (blue) and *trans* (red) 15:1, 16:1 and 17:1 positional isomers mixtures. 7-Me-6-16:1 = methyl 7-methyl 6-hexadecenoate

Fig. 3. Partial GC chromatogram of the 18:0 to *c*9,*c*12-18:2 elution segment. From the bottom: FAME prepared from menhaden oil before fractionation, Ag^+-HPLC fractions containing SFA (I), *trans*-MUFA (II), *cis*-MUFA (III), PUFA (IV), and reference mixture GLC 463. Conditions: SLB-IL111 capillary column (200 m x 0.25 mm, 0.2 μm thickness), temperature/flow gradients as described in the experimental section. Colored inserts show 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). 7-Me-6-18:1 = methyl 7-methyl-6-octadecenoate.

Fig. 4. Partial GC chromatogram of the *c*9,*c*12-18:2 to 20:3 elution segment. From the bottom: FAME prepared from menhaden oil before fractionation, Ag^+-HPLC fractions containing SFA (I), *trans*-MUFA (II), *cis*-MUFA (III), PUFA (IV), and reference mixture GLC 463. Conditions: SLB-IL111 capillary column (200 m x 0.25 mm, 0.2 μm thickness), temperature/flow gradients as described in the experimental section. CLA = conjugated linoleic acid. Colored inserts show 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).

Fig. 5. Partial GC chromatogram of the 20:3 to 22:6 elution segment. From the bottom: FAME prepared from menhaden oil before fractionation, Ag^+-HPLC fractions containing SFA (I), *trans*-MUFA (II), *cis*-MUFA (III), PUFA (IV), and reference mixture GLC 463. Conditions: SLB-IL111 capillary column (200 m x 0.25 mm, 0.2 μm thickness), temperature/flow gradients as described in the experimental section. FM *a*,*b* = furan FA with a one methyl substituent on the furanoid ring, *a* indicates the length of the alkyl chain containing the carboxyl group, *b* the length of the terminal alkyl chain; FD *a*,*b*: furan FA with two methyl substituents on the furanoid ring.

Fig. 6. Partial GC-MS chromatogram of the 16:0 to 18:0 elution segment. From the bottom: TIC chromatogram, selected ion m/z 271.330 $[M+H]^+$ ion of 16:0, m/z 285.350 $[M+H]^+$ ion of 17:0, m/z 269.317 $[M+H]^+$ ion of 16:1, m/z 327.406 $[M+H]^+$ ion of phytanate, m/z 283.336 $[M+H]^+$ ion of 17:1, m/z 299.370 $[M+H]^+$ ion of 18:0. Conditions: SLB-IL111 capillary column (100 m x 0.25 mm, 0.2 μm thickness),

constant elution temperature 170°C, constant elution pressure 80 kPa, TOF mass spectrometer operated in CI^+ mode with isobutane as the CI reagent gas.

Fig. 7. Partial GC-MS chromatogram of the 18:0 to *c*9,*c*12-18:2 elution segment. From the bottom: TIC chromatogram, selected ion m/z 299.372 $[M+H]^+$ ion of 18:0, m/z 283.339 $[M+H]^+$ ion of 17:1, m/z 235.261 $[M\text{-}MeOH+H]^+$ ion of 16:2, m/z 297.354 $[M+H]^+$ ion of 18:1, m/z 313.391 $[M+H]^+$ ion of 19:0, m/z 265.284 $[M+H]^+$ ion of 16:3, m/z 294.330 $[M]^+$ ion of 18:2. Conditions as described in Figure 6.

Fig. 8. Partial GC-MS chromatogram of the *c*9,*c*12-18:2 to 20:3 elution segment. From the bottom: TIC chromatogram, selected ion m/z 294.334 $[M]^+$ ion of 18:2, m/z 325.393 $[M+H]^+$ ion of 20:1, m/z 263.271 $[M+H]^+$ ion of 16:4, m/z 341.428 $[M+H]^+$ ion of 21:0, m/z 293.320 $[M+H]^+$ ion of 18:3, m/z 322.373 $[M]^+$ ion of 20:2, m/z 291.306 $[M+H]^+$ ion of 18:4, m/z 353.434 $[M+H]^+$ ion of 22:1, m/z 321.369 $[M+H]^+$ ion of 20:3. Conditions as in described Figure 6.

Fig. 9. Partial GC-MS chromatogram of the 20:3 to 22:6 elution segment. From the bottom: TIC chromatogram, selected ion m/z 321.369 $[M+H]^+$ ion of 20:3, m/z 319.346 $[M+H]^+$ ion of 20:4, m/z 381.461 $[M+H]^+$ ion of 24:1, m/z 317.332 $[M+H]^+$ ion of 20:5, m/z 331.319 $[M+H]^+$ ion of 21:5, m/z 347.407 $[M+H]^+$ ion of 22:4, m/z 345.358 $[M+H]^+$ ion of 22:5, m/z 343.343 $[M+H]^+$ ion of 22:6. Conditions as described in Figure 6.

Fig. 10. GC-EI-TOF/MS spectra of methyl 7-methyl-6-hexadecenoate (upper), and methyl 7-methyl-6-octadecenoate (lower).

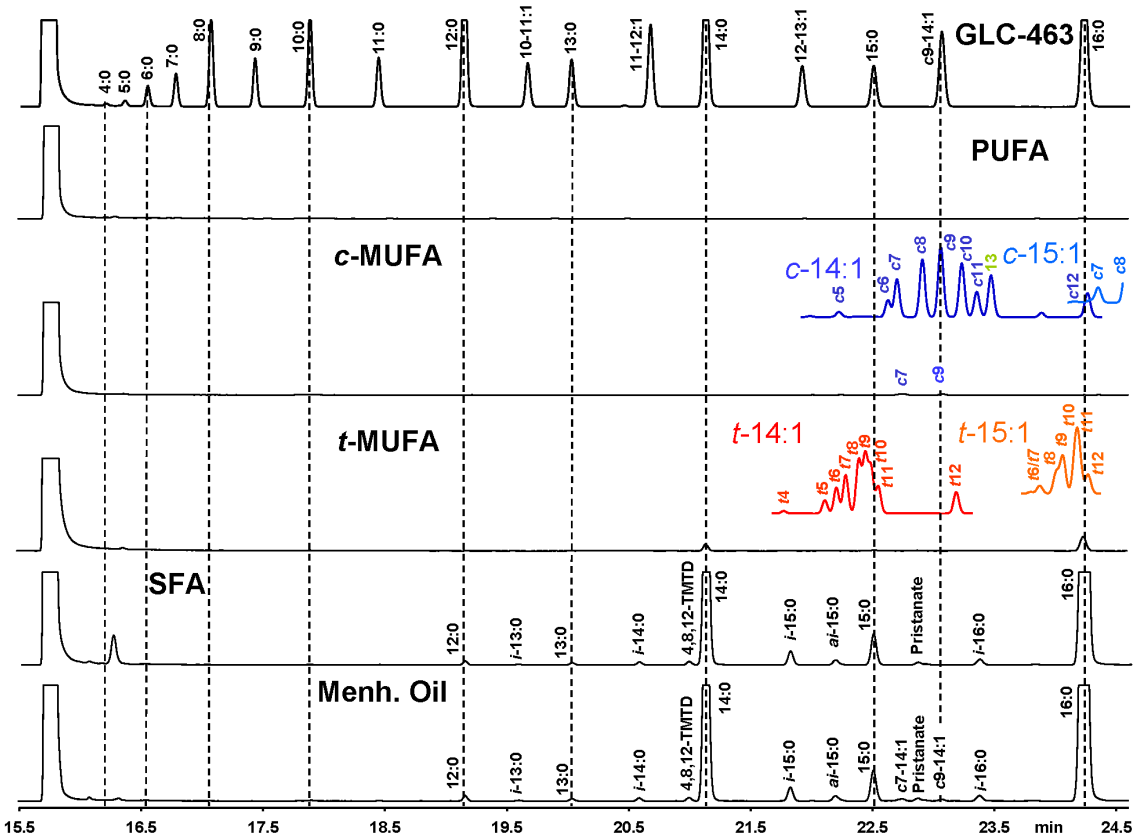
Table 1

Table 1. Equivalent chain length (ECL) and fractional chain length (FCL) calculation of the PUFAs in menhaden oil

Fatty Acid Methyl Esters					Fractional Chain Length (FCL)						
n-nomenclature	Δ-nomenclature	RT (min)	Log(RT)	ECL	FA:2	FA:3	FA:4	FA:5	FA:6	Unkn.	6(CH2)
16:2n-7	*c*6,*c*9-16:2	30.221	1.480	18.327	2.327						
18:2n-7	*c*8,*c*11-18:2	37.908	1.579	20.301	2.301						
18:2n-6	*c*9,*c*12-18:2	38.378	1.584	20.418	2.418						
20:2n-6	*c*11,*c*14-20:2	46.880	1.671	22.321	2.321						
22:2n-6	*c*13,*c*16-22:2	56.553	1.752	24.188	2.188						
18:3n-6	*c*6,*c*9,*c*12-18:3	42.110	1.624	21.293		3.293					
20:3n-6	*c*8*c*11*c*14-20:3	52.025	1.716	23.356		3.356					
18:4n-6	*c*3*c*6*c*9*c*12-18:4	46.880	1.671	22.321			4.321				
20:4n-6	*c*5*c*8*c*11*c*14-20:4	54.681	1.738	23.851			3.851				
22:4n-6	*c*7*c*10*c*13*c*16-22:4	66.386	1.822	25.797			3.797				
22:5n-6	*c*4*c*7*c*10*c*13*c*16-22:5	69.398	1.841	26.242				4.242			
16:2n-4	*c*9,*c*12-16:2	31.353	1.496	18.643	2.643						
18:2n-4	*c*11,*c*14-18:2	39.519	1.597	20.694	2.694						
20:2n-4	*c*13,*c*16-20:2	48.283	1.684	22.614	2.614						
16:3n-4	*c*6,*c*9,*c*12-16:3	35.256	1.547	19.651		3.651					
18:3n-4	*c*8,*c*11,*c*14-18:3	43.739	1.641	21.651		3.651					
20:3n-4	*c*10,*c*13,*c*16-20:3	53.493	1.728	23.633		3.633					
20:5n-4	*c*4,*c*7,*c*10,*c*13,*c*16-20:5	60.744	1.784	24.905				4.905			
16:3n-3	*c*7,*c*10,*c*13-16:3	35.942	1.556	19.817		3.817					
18:3n-3	*c*9,*c*12,*c*15-18:3	44.434	1.648	21.799		3.799					
20:3n-3	*c*11,*c*14,*c*17-20:3	54.166	1.734	23.757		3.757					
16:4n-3	*c*4,*c*7,*c*10,*c*13-16:4	38.262	1.583	20.389			4.389				
18:4	*all cis*-18:4	49.152	1.692	22.792						4.792	
20:4n-3	*c*8,*c*11,*c*14,*c*17-20:4	58.778	1.769	24.575			4.575				
22:4n-3	*c*10,*c*13,*c*16,*c*19-22:4	70.387	1.847	26.384			4.384				
20:5n-3	*c*5,*c*8,*c*11,*c*14,*c*17-20:5	62.240	1.794	25.149				5.149			
22:5n-3	*c*7,*c*10,*c*13,*c*16,*c*19-22:5	76.992	1.886	27.284				5.284			
21:5n-3	*c*6,*c*9,*c*12,*c*15,*c*18-21:5	69.923	1.845	26.318				5.318			
22:6n-3	*c*4,*c*7,*c*10,*c*13,*c*16,*c*19-22:6	81.082	1.909	27.803					5.803		
16:4n-1	*c*6,*c*9,*c*12,*c*15-16:4	41.104	1.614	21.065			5.065				
18:4n-1	*c*8,*c*11,*c*14,*c*17-18:4	50.714	1.705	23.103			5.103				
Non-Methylene Interrupted											
16:2NMI	??	29.888	1.475	18.231						2.231	
17:2	??	34.610	1.539	19.492						2.492	
18:2n-7,13	*c*5,*c*11-18:2	36.953	1.568	20.061							2.061
20:2n-7,13	*c*7,*c*13-20:2	45.450	1.658	22.013							2.013

RT, retention time in min; ECL, equivalent chain length; FCL fractional chain length; FA:2, chain length has 2 double bonds, etc; 6(CH2), 6 methylene groups between double bonds; NMI, non-methylene interrupted fatty acids.

Fig. 1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t-14:1
t-15:1
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4,8,12-TMTD
i-15:0
ai-15:0
Pristanate
i-16:0
c7-14:1
15.5
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18.5
19.5
20.5
21.5
22.5
23.5
min
24.5

Page 31 of 39 **Fig. 2**

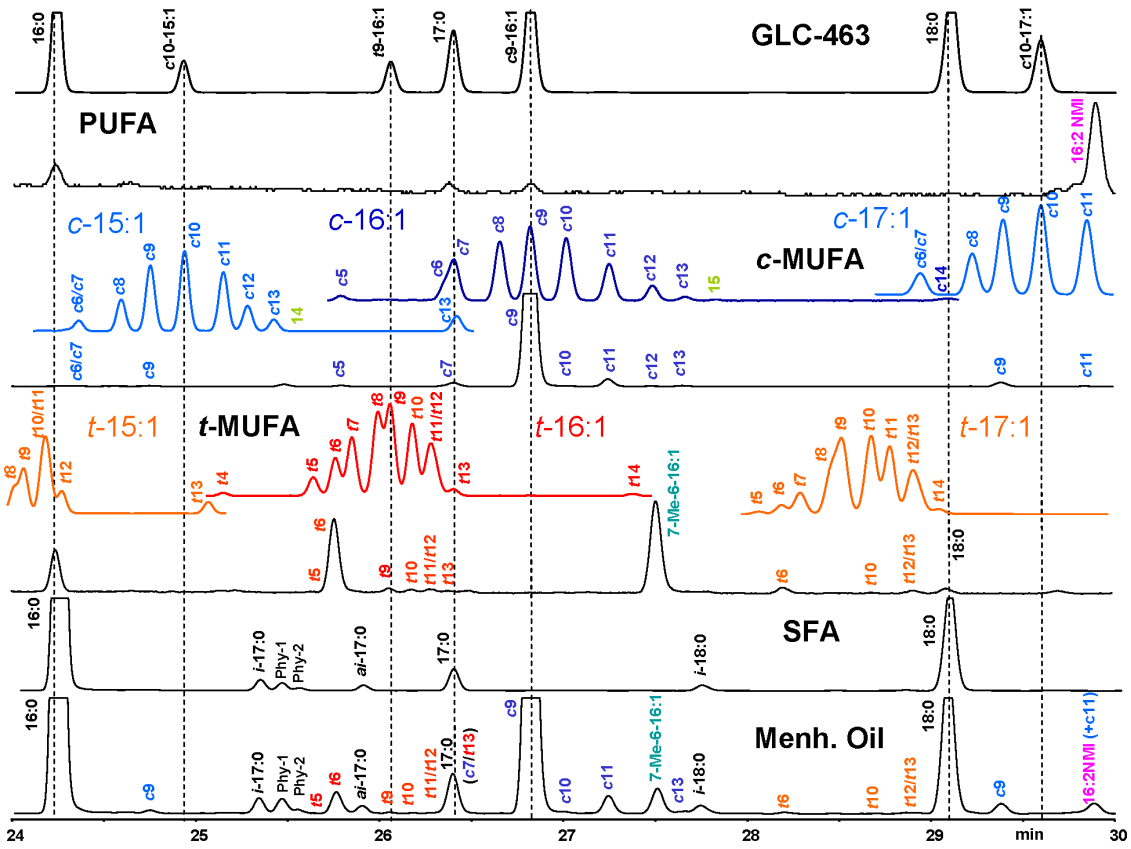
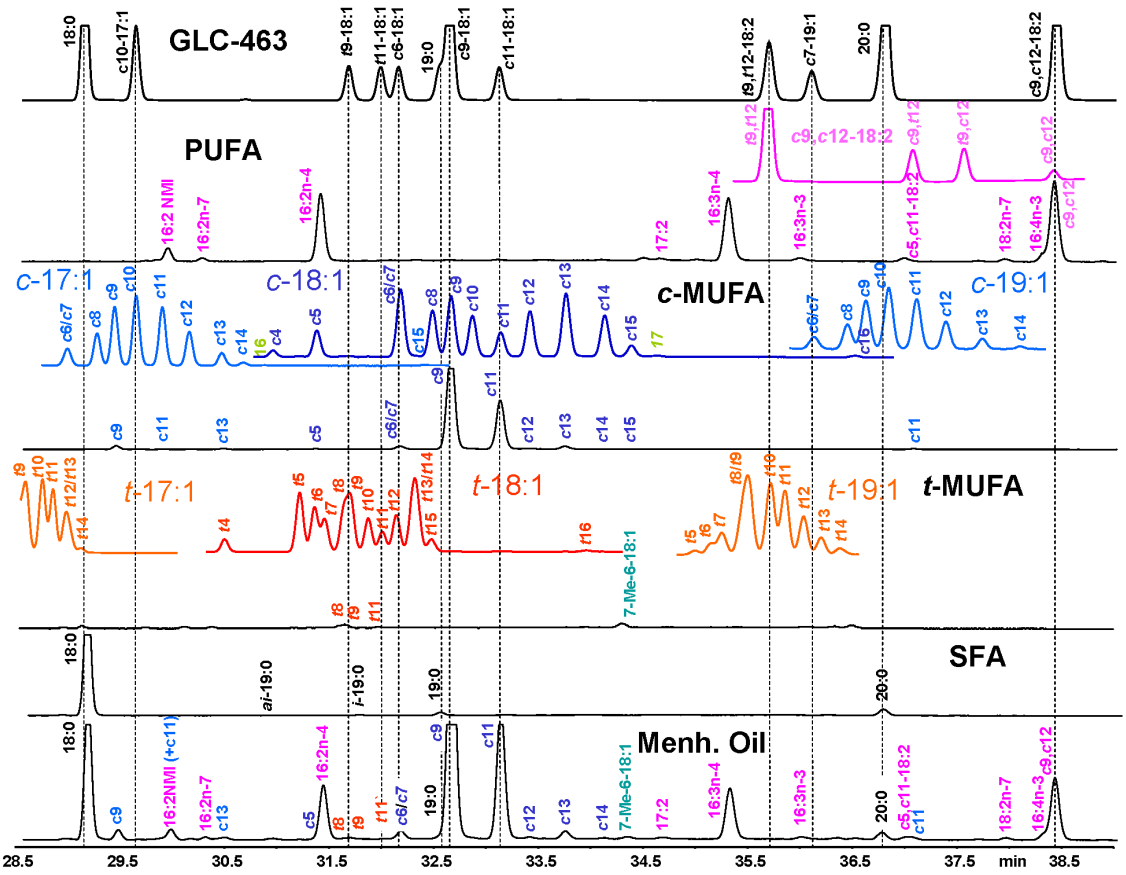


Fig. 3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SFA
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c-19:1
t-17:1
t-18:1
t-19:1
18:0
c10-17:1
t9-18:1
t11-18:1
c6-18:1
19:0
c9-18:1
c11-18:1
t9,t12-18:2
c7-19:1
20:0
c9,c12-18:2
16:2 NMI
16:2n-7
16:2n-4
17:2
16:3n-4
16:3n-3
c5,c11-18:2
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16:4n-3
7-Me-6-18:1
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min
38.5

Page 33 of 39 **Fig. 4**

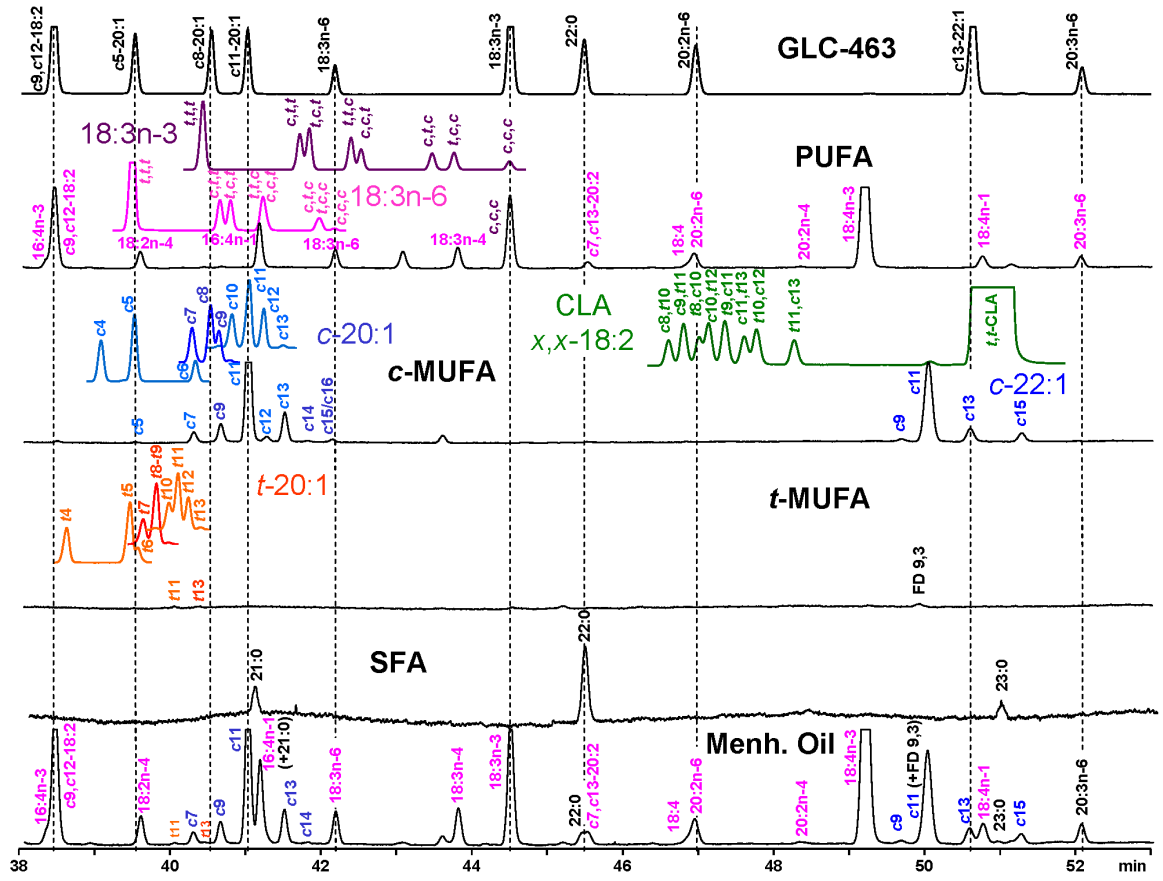
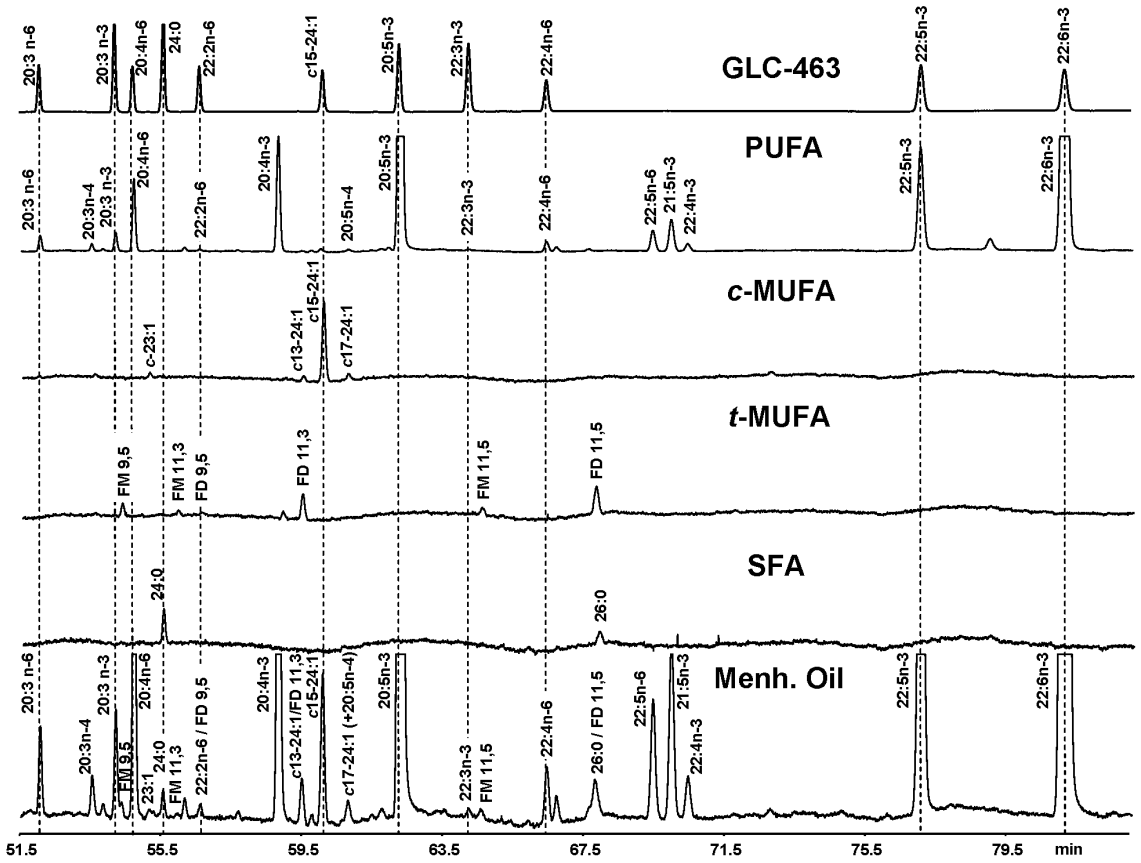


Fig. 5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c-23:1
c13-24:1
c17-24:1
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FD 9,5
FD 11,3
FM 11,5
FD 11,5
26:0
23:1
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c17-24:1 (+20:5n-4)
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63.5
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71.5
75.5
79.5
min

Page 35 of 39

Figure 6

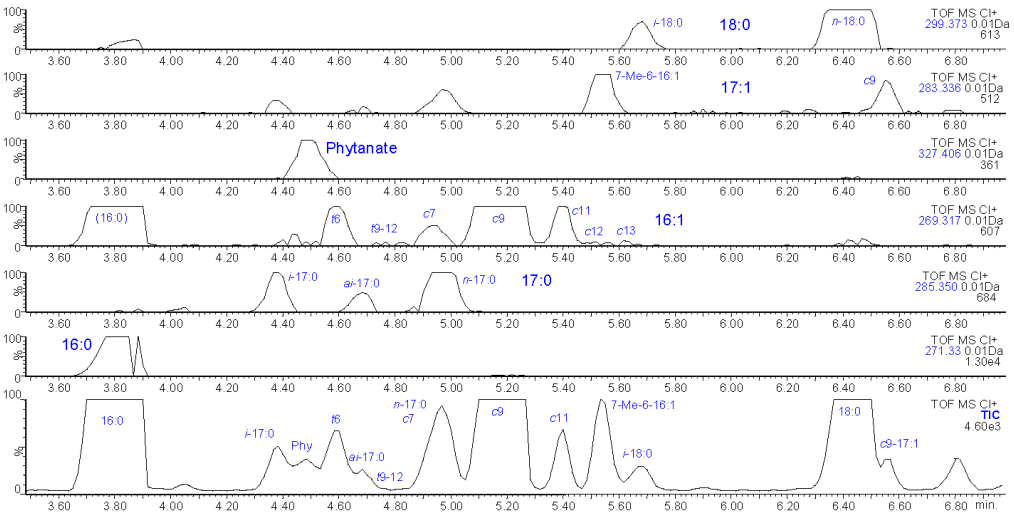
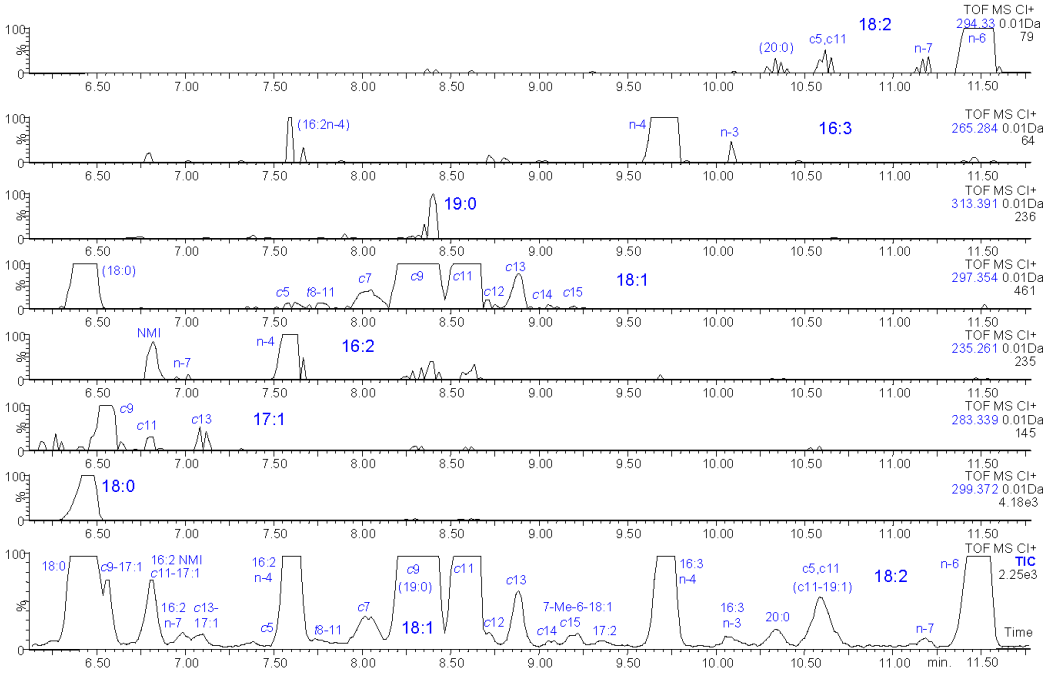


Figure 7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(16:2n-4)
n-4
n-3
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c7
c9
c11
c12
c13
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c15
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n-4
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145
c9
c11
c13
17:1
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TIC
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c13
c12
18:1
7-Me-6-18:1
c14
c15
17:2
16:3
n-4
16:3
n-3
20:0
c5,c11
(c11-19:1)
18:2
n-7
n-6
Time
min.

Page 37 of 39

Figure 8

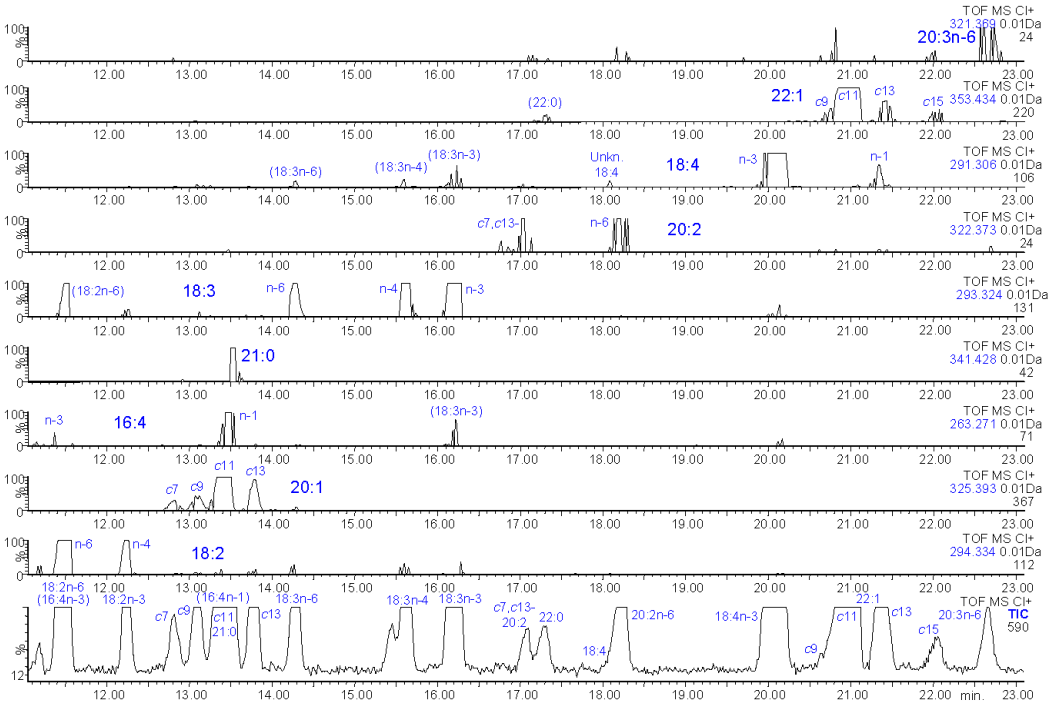
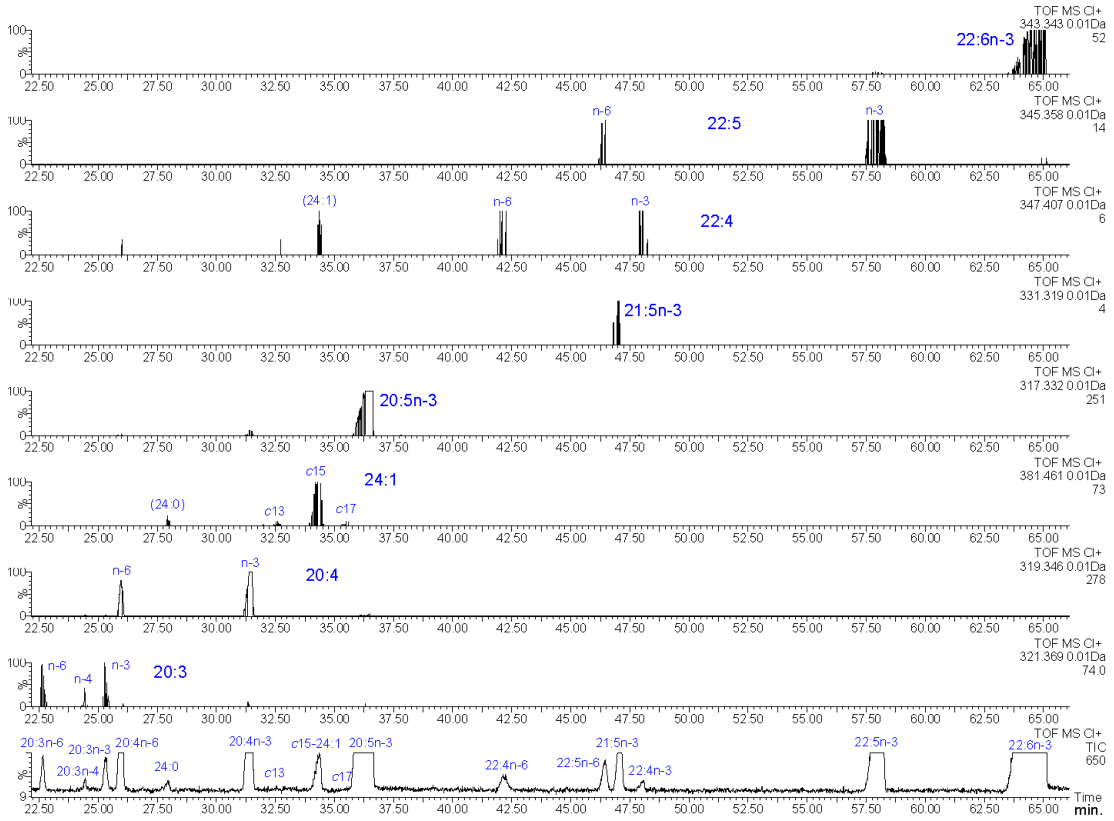


Figure 9


TOF MS CI+
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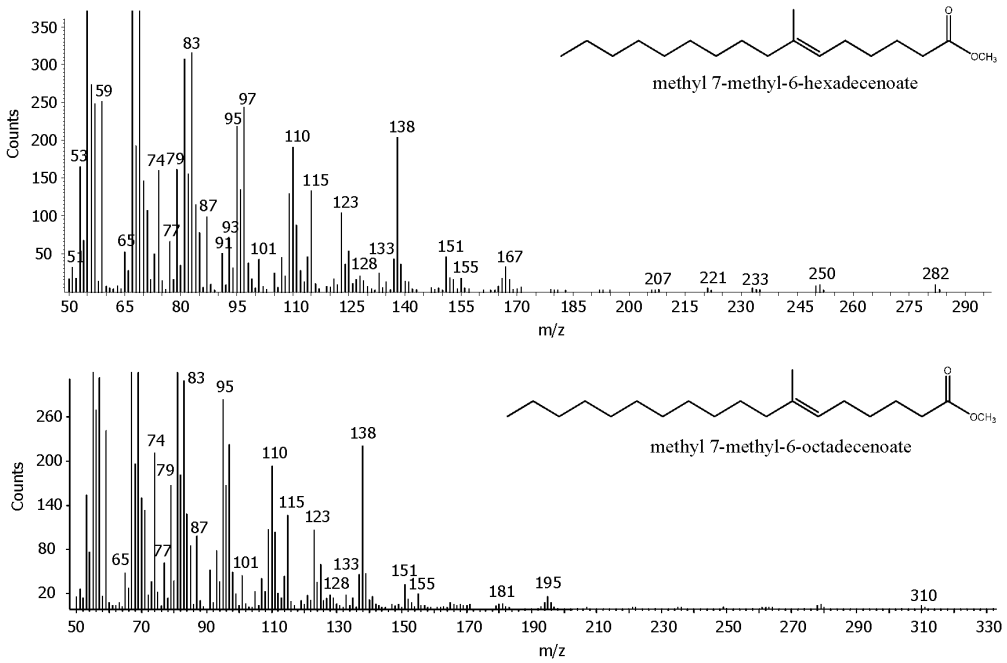
52
22:6n-3
TOF MS CI+
345.358 0.01Da
14
n-6
22:5
n-3
TOF MS CI+
347.407 0.01Da
6
(24:1)
n-6
n-3
22:4
TOF MS CI+
331.319 0.01Da
4
21:5n-3
TOF MS CI+
317.332 0.01Da
251
20:5n-3
TOF MS CI+
381.461 0.01Da
73
c15
24:1
(24:0)
c13
c17
TOF MS CI+
319.346 0.01Da
278
n-6
n-3
20:4
TOF MS CI+
321.369 0.01Da
74.0
n-6
n-4
n-3
20:3
TOF MS CI+
TIC
650
20:3n-6
20:3n-3
20:4n-6
20:3n-4
24:0
20:4n-3
c15-24:1
20:5n-3
c13
c17
22:4n-6
22:5n-6
21:5n-3
22:4n-3
22:5n-3
22:6n-3
Time
min.

Figure 10


methyl 7-methyl-6-hexadecenoate
Counts
m/z
53
59
65
74
79
83
87
91
93
95
97
101
110
115
123
128
133
138
151
155
167
207
221
233
250
282
methyl 7-methyl-6-octadecenoate
Counts
m/z
65
74
77
79
83
87
95
101
110
115
123
128
133
138
151
155
181
195
310

CHAPTER 8

Characterization of Pine Nuts in the U.S. Market, Including Those Associated with "Pine Mouth", by GC-FID.

Ali Reza Fardin-Kia; Sarah M. Handy; Jeanne I. Rader

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Characterization of Pine Nuts in the U.S. Market, Including Those Associated with "Pine Mouth", by GC-FID

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ABSTRACT: Taste disturbances following consumption of pine nuts, referred to as "pine mouth", have been reported by consumers in the United States and Europe. Nuts of *Pinus armandii* have been associated with pine mouth, and a diagnostic index (DI) measuring the content of Δ5-unsaturated fatty acids relative to that of their fatty acid precursors has been proposed for identifying nuts from this species. A 100 m SLB-IL 111 GC column was used to improve fatty acid separations, and 45 pine nut samples were analyzed, including pine mouth-associated samples. This study examined the use of a DI for the identification of mixtures of pine nut species and showed the limitation of morphological characteristics for species identification. DI values for many commercial samples did not match those of known reference species, indicating that the majority of pine nuts collected in the U.S. market, including those associated with pine mouth, are mixtures of nuts from different *Pinus* species.

KEYWORDS: *pine nuts, "pine mouth", diagnostic index, DI, SLB-IL 111 column, Δ5-unsaturated polymethylene-interrupted fatty acids*

INTRODUCTION

Nuts from certain *Pinus* species are highly nutritious foods because of their relatively high fat (ca. 40−60%) and protein (ca. 30−40%) contents.[1] Pine nuts have been a staple in the diets of several Native American tribes and for peoples across the Mediterranean region and Asia for hundreds or thousands of years.[2,3] Pine nuts are considered to be an abundant natural resource, and world production was estimated to be about 20000 tons/year.[4] Various species of pine nuts are eaten raw or roasted and are used for products such as pastry, sauces, and chocolates.

Nuts from *Pinus koraiensis*, *Pinus pinea* L., and *Pinus gerardiana* have been a part of global commerce for many years,[3] and *Pinus sibirica* has recently entered the international marketplace.[2] Nuts from the edible species *Pinus edulis*, *Pinus lambertiana*, and *Pinus monophylla* are primarily consumed in the United States. Until recently, nuts from *Pinus armandii*, primarily produced and consumed in Asia, and *Pinus cembra*, primarily consumed in Europe, were considered to be locally important but not major contributors to international trade compared with *P. koraiensis*, *P. pinea* L., and *P. gerardiana*.[2]

Some consumers have reported taste disturbances, commonly referred to as "pine mouth", following consumption of pine nuts. The first case of "pine mouth" was reported in Belgium in 2000.[5] Since then, several publications have reported consumer complaints of a bitter taste in the mouth, which can persist from 8 days to 2 weeks after the consumption of pine nuts.[6,7] The French Food Safety Agency[8] described a growing number of complaints of pine mouth syndrome among French consumers but was unable to identify a cause for the taste disturbances. In the largest overview to date, Flesch et al.[9] provided a descriptive report of more than 3000 cases of nut-related dysgeusia that were reported in France between May 2008 and January 2010. No species information was provided, and an etiological agent was not identified. Between July 2008 and November 2010, the U.S. Food and Drug Administration (FDA) received 197 consumer complaints of taste disturbances related to the consumption of pine nuts, and 15 samples associated with these complaints were collected and analyzed.[10] To date, no chemical compound in the collected samples has been identified as a potentially causative agent for the taste disturbances.

The French Food Safety Agency reported the presence of nuts from *P. armandii* in some of the products associated with consumer complaints.[8] *P. armandii* is not among the 29 species of pine nuts traditionally used for human consumption.[3] The identification of the species of pine nuts in commercial samples became an area of active investigation on the basis of the report by the French Food Safety Agency,[8] and the hypothesis was that consumption of pine nuts from *P. armandii*, a species not previously consumed in some markets, might be linked to the appearance of taste disturbances.[7]

Wolff et al.[11] demonstrated that the fatty acid (FA) composition of gymnosperms provided useful chemometric data for taxonomy and phylogeny of this group. Whereas the most abundant fatty acids (FAs) in conifer nuts are oleic acid (9-18:1) and linoleic acid (9,12-18:2), the Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA) are characteristic of *Pinus* species.[12,13] The primary Δ5-UPIFA in conifer nuts are taxoleic acid (5,9-18:2), pinolenic acid (5,9,12-18:2), and sciadonic acid (5,11,14-20:3).[14−16] All of the unsaturated FAs reported in pine nuts have double bonds in the *cis* configuration. Destaillats et al.[7] proposed the use of a diagnostic index (DI) calculated as the ratio between the Δ5-UPIFA and their metabolic FA precursors as a tool for identifying the botanical origin of pine nuts in commercial samples.

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In this study, we report the FA composition of a series of reference pine nuts and market samples, including pine mouth-associated samples. We evaluated the use of the DI for identification of pine nut species and compared the results of species identification obtained with the DI method with the results from a DNA-based method recently developed in our laboratory by Handy et al.[17]

MATERIALS AND METHODS

Collection of Samples. Shelled and unshelled pine nuts were purchased from a wide range of sources to obtain a variety of reference pine nuts and samples representative of the U.S. market. We obtained a total of 15 reference samples, 13 market samples, and 17 samples that were associated with consumer complaints of pine mouth.

Reference samples contained nuts from a single pine species, and this identity was indicated by the vendor. In cases in which we obtained reference samples of the same species from multiple sources, the samples were identified by letters A–C. The International Nut and Dried Fruit Foundation, Reus, Spain, generously provided samples of pine nuts from *P. armandii* (A), *P. koraiensis* (A), and *P. sibirica* (A). Lawyer Nursery, Inc., Plains, MT, provided seeds from *P. gerardiana* (A); *P. armandii* (B); *P. monophylla*, *P. pumila* (A); *P. sibirica* (B); *P. cembra*, *P. koraiensis* (B); and *P. lambertiana*, *P. edulis*, and *P. wallichiana*. Schumacher Co., Inc., Sandwich, MA, provided seeds from *P. armandii* (C), *P. griffithii*, *P. koraiensis* (C), *P. pumila* (B), *P. tabuliformis*, and *P. yunnanensis*. Wild-crafted pine nuts from *P. edulis*, *P. lambertiana*, and *P. monophylla* were obtained from wildcrops.com.

Korean pine tree seeds were obtained from TreeSeedsforSale.com, Burlington, VT. Seeds from *P. cembroides* (Texas USA) and *P. monophylla* (Nevada USA) were obtained from Kew Gardens by M. Eason, Ladybird Johnson Wildflower Center, University of Texas, Austin, TX, USA. Pine nuts in the shell of *P. armandii*, *P. sibirica*, *P. koraiensis*, and *P. gerardiana* (B) from the Oregon State University (Corvallis, OR, USA) botanical collection were generously provided by Dr. A. Liston. Authenticated seeds from *P. kesiya* (synonym *P. insularis*) were obtained from Botanical Liaisons, Boulder, CO, USA, and authenticated shelled pine nuts of *P. pinea* L. were collected in Sardinia, Italy.

Organic cedar nuts labeled as originating in Russia were obtained from www.FloresFarm.com. Shelled pine nuts labeled as originating in China were purchased at a supermarket in Falls Church, VA, USA. Pine nuts from Pakistan were obtained from Kohenoor International, Hyderabad Sindh, Pakistan. Siberian pine nut oil was obtained from siberiantigernaturals.com and from Igourmet.com. Mediterranean pine nuts were obtained from NutsOnline.com. Other raw pine nuts without species identification were obtained from WholeSalePineNuts.com, NutsOnline.com, and Igourmet.com. Complaint samples from the U.S. market were provided by FDA inspectors. Two samples of pine nuts withdrawn from the Danish market because of consumer complaints were obtained from the Danish Veterinary and Food Administration, Region East, Ringsted, Denmark.

Chemicals. Fatty acid methyl ester (FAME) reference materials (GLC-463, linoleic acid and linolenic acid) were purchased from Nu-Chek Prep (Elysian, MN, USA). Δ5 reference FAMEs were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA) and Larodan Fine Chemicals (Malmo, Sweden). Methanolic hydrochloric acid (HCl/MeOH, 3 N) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Isooctane, methanol (MeOH), and hexane for gas chromatography were purchased from J. T. Baker (Phillisburg, NJ, USA).

Sample Preparation and Methylation. If needed, pine nuts were shelled before grinding. Shelled pine nuts (about 5 g) were ground in a mortar previously washed with MeOH and isooctane. A modification of the method of Destaillats et al.[7] was used to prepare FAMEs as follows:

About 200–250 mg of ground pine nuts was placed in a 20 mL screw cap test tube, and 3 mL of 3 N HCl/MeOH and 2 mL of MeOH were added. The tube was purged with argon and placed in a silicon oil bath (VWR International LLC, Radnor, PA, USA) set at 80 °C for 75 min. After the tube had cooled to room temperature, 3 mL of hexane was added, and the test tube was swirled. The test tube was then filled with a saturated solution of aqueous sodium chloride (NaCl), and the contents were mixed, and then 1 mL of the organic phase was transferred into a new test tube and washed two times with 5 mL of deionized water. The organic phase was filtered through an SPE tube containing anhydrous sodium sulfate and transferred into a 2 mL amber sialinized glass vial. The solvent was removed under a stream of argon at room temperature, and the FAMEs were reconstituted with 1.5 mL of isooctane.

To evaluate the FA composition and botanical identification of known mixtures of pine nuts, we prepared two-component mixtures that contained 50% by weight of each of the reference pine nut species *P. koraiensis* (A), *P. armandii* (A), and *P. sibirica* (A). Mixtures were identified as follows: M1, *P. koraiensis* and *P. armandii*; M2, *P. koraiensis* and *P. sibirica*; and M3, *P. armandii* and *P. sibirica*. FAMEs from the mixed samples were handled as described above.

Gas Chromatographic Analysis with Flame Ionization Detection (GC-FID). GC analyses were performed with a 6890N GC equipped with an FID (Agilent Technologies, Wilmington, DE, USA) and an SLB-IL 111 capillary column (100 m × 0.25 mm, 0.2 μm thickness, Supelco, Bellefonte, PA, USA). Hydrogen was used as carrier gas at 1 mL/min constant flow with the linear velocity of 26 cm/s. The oven temperature was maintained at 168 °C (isothermal elution), and the injection port and FID temperatures were 250 °C. The split ratio was set to 1:100, and a typical injection volume was 1 μL. The injection port liner was a "split only SSF" from Agilent Technologies. FID additional gases were H_2 at 30 mL/min, air at 400 mL/min, and makeup gas (N_2) at 30 mL/min. The separation time was 30 min.

Separations under the chromatographic conditions described by Destaillats et al.[7] were achieved with the same GC equipped with a fused-silica BPX-70 capillary column (10 m × 0.1 mm i.d., 0.2 μm film thickness; SGE, Melbourne, Australia). Hydrogen was used as carrier gas with a constant flow of 0.6 mL/min. Oven temperature programming was 50 °C isothermal for 1 min, increased to 200 °C at 20 °C/min and then to 250 °C at 50 °C/min. The split ratio was 1800:1, and a typical injection volume was 0.5 μL. The FID was maintained at 250 °C and the injector at 250 °C. Additional FID gases were the same as those listed above.

Each sample was prepared in triplicate, and each replicate was analyzed three times by GC. Means and standard deviations were calculated for each set of injections. FA composition is expressed as weight percent (%, w/w) of total FAs. The identification of FAMEs was made by comparing the FAME retention times with those of commercially available standards (Δ5 FAMEs and GLC-463) and the available literature.

Calculation of the Diagnostic Index (DI). DI values for botanical species identification according to Destaillats et al.[7] were calculated as follows:

$$[(5,\ 9\text{-}18{:}\ 2 + 5,\ 9,\ 12\text{-}18{:}\ 3 + 5,\ 11,\ 14\text{-}20{:}\ 3) / (9\text{-}18{:}\ 1 \text{ and } 11\text{-}18{:}\ 1 + 9,\ 12\text{-}18{:}\ 2 + 11,\ 14\text{-}20{:}\ 2)] \times 10 \quad (1)$$

The 9-18:1 and 11-18:1 FAs were baseline resolved under the chromatographic conditions selected in this study, and DI values were calculated as follows:

$$[(5,\ 9\text{-}18{:}\ 2 + 5,\ 9,\ 12\text{-}18{:}\ 3 + 5,\ 11,\ 14\text{-}20{:}\ 3) / (9\text{-}18{:}\ 1 + 9,\ 12\text{-}18{:}\ 2 + 11,\ 14\text{-}20{:}\ 2)] \times 10 \quad (2)$$

Genetic Differentiation of Pine Nuts. A single seed from each of the reference samples and 3–13 seeds from market samples 21 and 24–26 and from all pine mouth-associated samples were analyzed according to the method of Handy et al.[17]

RESULTS

Chromatographic Separations. The chromatographic separations presented here were obtained using the 100 m SLB-IL 111 capillary column. We also used a 10 m BPX-70 GC column to compare our results with those obtained by Destaillats et al.[7]

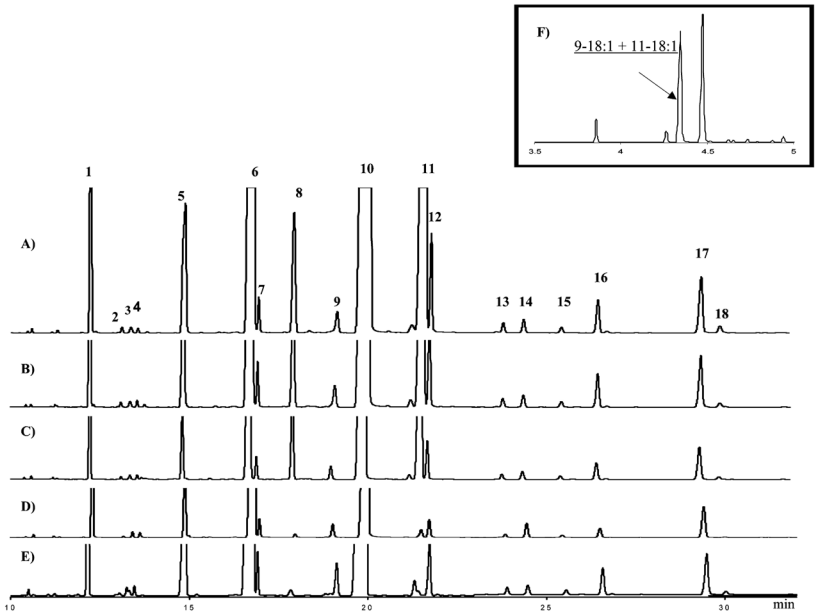

Figure 1. GC-FID chromatogram of pine nut species using the SLB-IL 111 column (Supelco, 100 m × 0.25 mm, 0.2 μm thickness): (A) *P. koraiensis*; (B) *P. armandii*; (C) *P. sibirica*; (D) *P. gerardiana*; (E) *P. pinea* L. (F) GC-FID chromatogram of *P. pinea* L. using a BPX-70 column (10 m × 0.1 mm, 0.2 μm film thickness). Peaks: (1) 16:0; (2) anteiso (ai)-17:0 + 7-16:1; (3) 17:0; (4) 9-16:1; (5) 18:0, (6) 9-18:1; (7) 11-18:1; (8) 5,9-18:2; (9) 20:0; (10) 9,12-18:2; (11) 5,9,12-18:3; (12) 11-20:1; (13) 5,11-20:2; (14) 9,12,15-18:3; (15) 22:0; (16) 11,14-20:2; (17) 5,11,14-20:3; (18) 5,9,12,15-18:4.

Typical GC profiles of FAMEs prepared from *P. koraiensis*, *P. armandii*, *P. sibirica*, *P. gerardiana*, and *P. pinea* L. are shown in Figure 1A–E. The most abundant FAs in these species were 16:0, 18:0, 9-18:1, and 9,12-18:2. All of the unsaturated FAs identified in the analyzed pine nuts showed double bonds only in the *cis* configuration. In addition to these FA, *P. koraiensis*, *P. armandii*, and *P. sibirica* also showed two peaks identified as taxoleic acid (5,9-18:2, peak 8) and pinolenic acid (5,9,12-18:3, peak 11), which were almost completely absent in *P. pinea* L. and *P. gerardiana*. The branched chain FA anteiso-17:0 (peak 2) coeluted with 7-16:1, whereas the two 18:1 FAs, 9-18:1 and 11-18:1 (peaks 6 and 7), were clearly separated. The profile of a sample of *P. pinea* L. (Figure 1F) analyzed using the 10 m BPX-70 column showed that 9-18:1 and 11-18:1 coeluted as a single peak under the fast conditions described by Destaillats et al.,[7] whereas they were resolved into two distinct peaks using the SLB-IL 111 column and the conditions described for this study.

Fatty Acid Composition of Reference Pine Nut Samples. The FA composition and DI value calculated according to eq 2 for reference pine nut species are reported in Table 1. We obtained samples from multiple sources for *P. gerardiana* (1A, 1B), *P. koraiensis* (6A, 6B, 6C), *P. armandii* (7A, 7B, 7C), *P. pumila* (9A, 9B), and *P. sibirica* (10A, 10B). Oleic acid (9-18:1) was found in *P. gerardiana* and *P. pinea* L. at concentrations between 37 and 40% of total FA and at concentrations between 21 and 30% of total FA in *P. koraiensis*, *P. armandii*, and *P. sibirica*.

A total of five Δ5-UPIFA (5,9-18:2; 5,9,12-18:3; 5,11-20:2; 5,11,14-20:3, and 5,9,12,15-18:4) were identified. The major Δ5-UPIFA in *P. koraiensis*, *P. armandii*, and *P. sibirica* were 5,9-18:2 (taxoleic acid, 2–4% of total FA) and 5,9,12-18:3 (pinolenic acid, 13–20% of total FA). Among the remaining Δ5-UIPFA, 5,11,14-20:3 was found at about 1% of total FA in these three species, whereas 5,11-20:2 and 5,9,12,15-18:4 were each found at <0.5% of total FA. In contrast, *P. gerardiana* and *P. pinea* L. contained pinolenic acid at about 0.3% of total FA.

The DI values ranged from 0.05 for *P. gerardiana* to 4.33 for *P. yunnanensis*. The strikingly low DI values for reference samples 1–4 are driven by the relatively high levels of FA 9-18:1 (e.g., 37–46% of total FA) and lower levels of FAs 5,9-18:2, 5,9,12-18:3, and 5,11,14-20:3 compared with corresponding levels in the other samples analyzed.

Pine Nuts Collected in the U.S. Market, Including Those Associated with Pine Mouth. Table 2 lists the DI values, countries of origin if known, and sources of pine nuts and pine nut oils collected from the U.S. market. The DI values of these samples ranged from 0.10 to 3.27 (Table 2). In most cases, particularly among the samples with the higher DI values, the values did not match with any of the reference pine nut samples. This observation indicated that the samples collected in the U.S. market were mixtures of pine nut species.

In addition to the determination of their DI values, the 17 pine mouth-associated samples were subjected to DNA "finger-printing" for speciation (Table 3). The DI values of the pine mouth-associated samples ranged from 2.41 and 3.37, and most did not match the DIs of the known reference pine nut samples. Genetic analysis showed that 12 of the 17 samples were mixtures of several pine nut species (Table 3). Genetic analysis of four of the market samples (21 and 24–26) also showed them to be mixtures.

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Table 1. Fatty Acid Composition and Diagnostic Index (DI) of Reference Pine Nuts[a]

sample	species	16:0	SD	ai-17:0[b]	SD	17:0	SD	9-16:1	SD	18:0	SD
1A	*P. gerardiana*	6.14	0.04	0.07	0.01	0.09	0.01	0.09	0.01	2.32	0.08
1B	*P. gerardiana*	5.63	0.06	0.06	0.01	0.10	0.01	0.10	0.01	2.20	0.09
2	*P. edulis*	6.95	0.03	0.02	0.01	0.08	0.02	0.13	0.00	2.36	0.00
3	*P. monophylla*	7.05	0.00	0.03	0.01	0.09	0.01	0.06	0.02	3.10	0.00
4	*P. pinea* L.	6.20	0.02	0.03	0.00	0.15	0.00	0.10	0.01	3.64	0.11
5	*P. lambertiana*	5.60	0.03	0.06	0.01	0.10	0.02	0.10	0.03	1.60	0.05
6A	*P. koraiensis*	4.71	0.01	0.05	0.00	0.07	0.00	0.06	0.00	2.13	0.03
6B	*P. koraiensis*	5.09	0.00	0.04	0.01	0.07	0.10	0.08	0.01	2.35	0.09
6C	*P. koraiensis*	5.30	0.03	0.04	0.00	0.07	0.00	0.08	0.02	2.05	0.00
7A	*P. armandii*	4.70	0.03	0.04	0.01	0.09	0.01	0.07	0.01	1.88	0.02
7B	*P. armandii*	4.48	0.01	0.04	0.00	0.10	0.00	0.11	0.02	2.19	0.02
7C	*P. armandii*	4.97	0.02	0.06	0.01	0.10	0.04	0.11	0.03	2.43	0.01
8	*P. cembra*	4.66	0.09	0.07	0.00	0.08	0.03	0.07	0.02	2.54	0.03
9A	*P. pumila*	4.07	0.04	0.07	0.02	0.08	0.02	0.07	0.01	2.20	0.09
9B	*P. pumila*	4.00	0.02	0.06	0.01	0.06	0.02	0.08	0.01	2.16	0.01
10A	*P. sibirica*	4.39	0.05	0.07	0.00	0.08	0.01	0.04	0.02	2.49	0.02
10B	*P. sibirica*	4.29	0.01	0.07	0.04	0.07	0.01	0.07	0.00	2.93	0.00
11	*P. griffithii*	4.90	0.01	0.13	0.02	0.08	0.01	0.12	0.01	2.75	0.12
12	*P. wallichiana*	4.67	0.03	0.14	0.07	0.07	0.03	0.12	0.03	2.72	0.02
13	*P. tabuliformis*	5.22	0.02	0.09	0.05	0.09	0.02	0.20	0.03	2.15	0.05
14	*P. kesiya*	5.58	0.01	0.12	0.09	0.16	0.08	0.20	0.01	1.71	0.02
15	*P. yunnanesis*	4.13	0.01	0.13	0.00	0.12	0.01	0.19	0.00	1.72	0.03
sample	species	9-18:1	SD	11-18:1	SD	5,9-18:2	SD	20:0	SD	9,12-18:2	SD
1A	*P. gerardiana*	36.83	0.09	0.86	0.01	0.19	0.04	0.46	0.04	50.87	0.07
1B	*P. gerardiana*	39.52	0.05	0.52	0.00	0.13	0.06	0.40	0.06	49.8	0.08
2	*P. edulis*	44.40	0.02	0.80	0.01	0.15	0.02	0.50	0.05	42.66	0.02
3	*P. monophylla*	46.40	0.03	0.60	0.09	0.51	0.02	0.68	0.04	39.06	0.11
4	*P. pinea* L.	39.47	0.01	0.57	0.02	0.10	0.00	0.60	0.02	44.78	0.03
5	*P. lambertiana*	22.20	0.03	0.71	0.11	2.69	0.08	0.22	0.02	52.50	0.05
6A	*P. koraiensis*	24.79	0.05	0.49	0.06	2.19	0.06	0.38	0.05	46.71	0.09
6B	*P. koraiensis*	29.69	0.03	0.78	0.08	2.26	0.05	0.38	0.04	43.21	0.06
6C	*P. koraiensis*	25.48	0.10	0.71	0.01	2.34	0.05	0.29	0.02	47.03	0.03
7A	*P. armandii*	22.02	0.19	0.49	0.09	3.11	0.02	0.39	0.03	46.92	0.03
7B	*P. armandii*	25.93	0.03	0.62	0.08	3.87	0.03	0.42	0.06	43.95	0.01
7C	*P. armandii*	22.62	0.02	0.78	0.00	3.66	0.06	0.40	0.01	45.56	0.06
8	*P. cembra*	22.62	0.11	0.46	0.01	1.47	0.09	0.31	0.02	45.34	0.05
9A	*P. pumila*	21.99	0.02	0.51	0.01	2.84	0.11	0.27	0.00	45.97	0.04
9B	*P. pumila*	23.18	0.01	0.63	0.02	3.18	0.01	0.24	0.01	44.60	0.03
10A	*P. sibirica*	24.16	0.04	0.37	0.05	1.96	0.02	0.34	0.00	43.78	0.02
10B	*P. sibirica*	21.38	0.09	0.47	0.03	1.92	0.11	0.34	0.06	44.69	0.08
11	*P. griffithii*	16.97	0.12	0.73	0.07	2.34	0.04	0.38	0.01	47.35	0.01
12	*P. wallichiana*	16.55	0.06	0.77	0.01	2.36	0.07	0.39	0.01	46.83	0.11
13	*P. tabuliformis*	20.39	0.05	1.01	0.00	4.12	0.05	0.26	0.02	41.99	0.03
14	*P. kesiya*	19.24	0.01	0.83	0.04	3.08	0.02	0.30	0.03	41.95	0.11
15	*P. yunnanesis*	17.46	0.04	0.92	0.02	2.23	0.02	0.26	0.04	44.27	0.08
sample	species	5,9,12-18:3	SD	20:1	SD	5,11-20:2	SD	9,12,15-18:3	SD	22:0	SD
1A	*P. gerardiana*	0.30	0.04	0.66	0.02	0.05	0.01	0.06	0.03	0.40	0.02
1B	*P. gerardiana*	0.24	0.03	0.63	0.01	0.7	0.02	0.24	0.01	0.13	0.00
2	*P. edulis*	0.38	0.02	0.64	0.02	0.06	0.01	0.19	0.00	0.12	0.00
3	*P. monophylla*	0.80	0.09	0.52	0.08	0.05	0.01	0.27	0.01	0.18	0.02
4	*P. pinea* L.	0.33	0.02	0.86	0.02	0.16	0.02	0.60	0.03	0.12	0.01
5	*P. lambertiana*	10.77	0.01	0.82	0.01	0.03	0.03	0.19	0.01	0.27	0.03
6A	*P. koraiensis*	15.00	0.01	1.25	0.00	0.13	0.02	0.19	0.00	0.10	0.05
6B	*P. koraiensis*	12.38	0.02	1.44	0.01	0.02	0.05	0.14	0.00	0.17	0.04
6C	*P. koraiensis*	13.02	0.02	1.25	0.02	0.16	0.01	0.16	0.02	0.09	0.02
7A	*P. armandii*	16.60	0.00	1.01	0.02	0.16	0.02	0.27	0.03	0.12	0.10
7B	*P. armandii*	14.50	0.01	1.09	0.01	0.21	0.08	0.25	0.03	0.14	0.09
7C	*P. armandii*	15.52	0.01	1.05	0.01	0.03	0.00	0.19	0.00	0.25	0.01
8	*P. cembra*	18.45	0.02	1.31	0.09	0.05	0.02	0.13	0.05	0.33	0.01
9A	*P. pumila*	18.75	0.04	1.13	0.00	0.02	0.00	0.19	0.01	0.21	0.02

Table 1. continued

sample	species	5,9,12-18:3	SD	20:1	SD	5,11-20:2	SD	9,12,15-18:3	SD	22:0	SD
9B	*P. pumila*	18.71	0.01	1.16	0.01	0.04	0.00	0.18	0.01	0.18	0.01
10A	*P. sibirica*	18.95	0.05	1.14	0.07	0.14	0.01	0.21	0.04	0.08	0.02
10B	*P. sibirica*	19.85	0.03	1.28	0.04	0.04	0.04	0.14	0.05	0.27	0.00
11	*P. griffithii*	20.37	0.01	0.83	0.02	0.01	0.03	0.15	010.C	0.35	0.03
12	*P. wallichiana*	21.19	0.02	0.91	0.00	0.02	0.00	0.17	0.01	0.36	0.01
13	*P. tabuliformis*	16.89	0.01	0.92	0.01	0.03	0.01	0.41	0.04	0.42	0.04
14	*P. kesiya*	17.87	0.03	0.82	0.02	0.48	0.02	0.35	0.00	0.24	0.03
15	*P. yunnanesis*	19.66	0.02	0.83	0.01	0.02	0.01	0.44	0.02	0.50	0.00

sample	species	11,14-20:2	SD	5,11,14-20:3	SD	5,9,12,15-18:4	SD	DI	SD
1A	*P. gerardiana*	0.38	0.01	0.19	0.06			0.08	0.02
1B	*P. gerardiana*	0.18	0.01	0.11	0.01			0.05	0.01
2	*P. edulis*	0.23	0.02	0.29	0.01	0.02	0.00	0.09	0.03
3	*P. monophylla*	0.19	0.02	0.30	0.06	0.30	0.00	0.19	0.02
4	*P. pinea* L.	0.57	0.01	1.85	0.00	0.03	0.13	0.27	0.02
5	*P. lambertiana*	0.84	0.03	1.27	0.01	0.05	0.01	1.95	0.02
6A	*P. koraiensis*	0.59	0.01	1.05	0.04	0.07	0.01	2.53	0.17
6B	*P. koraiensis*	0.76	0.01	1.07	0.00	0.07	0.13	2.13	0.03
6C	*P. koraiensis*	0.69	0.02	1.15	0.01	0.07	0.07	2.26	0.04
7A	*P. armandii*	0.60	0.00	1.36	0.02	0.12	0.02	3.03	0.10
7B	*P. armandii*	0.67	0.02	1.35	0.04	0.08	0.00	2.80	0.09
7C	*P. armandii*	0.76	0.01	1.38	0.03	0.09	0.06	2.98	0.03
8	*P. cembra*	0.80	0.00	1.13	0.01	0.16	0.06	3.06	0.02
9A	*P. pumila*	0.61	0.03	0.89	0.02	0.10	0.04	3.28	0.02
9B	*P. pumila*	0.62	0.01	0.81	0.01	0.09	0.01	3.32	0.01
10A	*P. sibirica*	0.54	0.04	1.09	0.04	0.14	0.01	3.21	0.15
10B	*P. sibirica*	0.86	0.02	1.18	0.10	0.13	0.00	3.43	0.11
11	*P. griffithii*	0.90	0.08	1.44	0.02	0.15	0.01	3.70	0.02
12	*P. wallichiana*	0.93	0.04	1.56	0.01	0.18	0.04	3.90	0.01
13	*P. tabuliformis*	1.12	0.01	4.27	0.03	0.40	0.06	3.98	0.02
14	*P. kesiya*	1.12	0.02	5.44	0.01	0.44	0.01	4.24	0.04
15	*P. yunnanesis*	1.21	0.03	5.35	0.02	0.50	0.03	4.33	0.02

[a]Fatty acid composition is expressed as wt % of total fatty acids. Pine nuts of the same species from multiple sources are indicated by letters. Values are the means ± SD of results of triplicate injections of each of three fatty acid methyl ester preparations per sample. The names of all reference pine nuts are exactly as provided by the supplier. *P. griffithii* is a synonym for *P. wallichiana* and, therefore, these two are not different species. DI = [(5,9-18:2 + 5,9,12-18:3 + 5,11,14-20:3)/(9-18:1 + 9,12-18:2 + 11,14-20:2)] × 10. [b]ai-17:0 and 7-16:1.

Table 2. Sources of Pine Nuts Collected from the U.S. Market[a]

sample	pine nuts	country of origin	distributor	DI	SD
16	pinon nuts (Indian nuts)	?	online store	0.10	0.03
17	pine nuts	Pakistan (vendor)	wholesaler	0.12	0.07
18	pine nuts	?	online store	0.21	0.02
19	Mediterranean pine nuts (pignolias)	?	online store	0.25	0.03
20	pine nuts (pignolias)	?	online store	2.41	0.02
21	pine nuts (pignolias)	?	online store	2.44	0.02
22	pine nuts	?	online store	2.55	0.03
23	pine nuts	China (product label)	retailer	2.60	0.01
24	organic pine nuts	?	online store	2.67	0.02
25	organic pine nuts	?	online store	2.78	0.03
26	pine nuts	?	online store	3.04	0.01
27	pine nut oil	Siberia (product label)	online store	3.15	0.01
28	cedar nuts	Russia (product label)	online store	3.27	0.04

[a]Pine nuts were collected from retail and wholesale distributors and from online stores. Country of origin information, if available, was provided by vendors or listed in product labels. Values are the mean ± SD of results of triplicate injections of each of three fatty acid methyl ester preparations per sample. A question mark (?) indicates that the country of origin was not known. DI = [(5,9-18:2 + 5,9,12-18:3 + 5,11,14-20:3)/(9-18:1 + 9,12-18:2 + 11,14-20:2)] × 10.

The FA composition of selected commercial samples collected from the U.S. market is reported in Table 4. Samples M1, M2, and M3 are mixtures of 50% by weight (w/w) of *P. koraiensis* and *P. armandii* (M1), *P. koraiensis* and *P. sibirica* (M2), and *P. armandii* and *P. sibirica* (M3). Samples 16−19 are pine nuts with DI values of <0.5, and samples 21 and 24−26 are pine nuts with higher DI values (>2.44). Samples 35 and 42 are pine nut samples with high DI values that were associated with

Table 3. Genetic Assessment and Diagnostic Index of Pine Nuts from the U.S. Market[a]

sample	DI	SD	genetic assessment
Pine Nut Samples from the U.S. Market Associated with Pine Mouth			
29	2.41	0.02	mix of *P. koraiensis* and *P. armandii*
30	2.43	0.01	mix of *P. koraiensis* and *P. armandii*
31	2.50	0.02	mix of *P. koraiensis* and *P. armandii*
32	2.55	0.02	mix of *P. koraiensis* and *P. armandii*
33	2.62	0.06	mix of *P. armandii* and *P. gerardiana*
34	2.67	0.01	mix of *P. koraiensis* and *P. armandii*
35	2.79	0.02	mix of *P. koraiensis* and *P. armandii*
36	2.91	0.03	*P. armandii*
37	2.96	0.01	*P. armandii*
38	3.00	0.04	mix of *P. cembra/sibirica* and *P. armandii*
39	3.00	0.03	*P. armandii*
40	3.00	0.01	*P. armandii*
41	3.09	0.05	mix of *P. cembra/sibirica* and *P. armandii*
42	3.10	0.03	mix of *P. koraiensis*, *P. cembra/sibirica*, and *P. armandii*
43	3.18	0.02	mix of *P. cembra/sibirica*, *P. pumila*, and *P. armandii*
44	3.16	0.02	*P. armandii*
45	3.37	0.03	mix of *P. cembra/sibirica* and *P. armandii*
Pine Nut Samples from the U.S. Market			
21	2.44	0.02	mix of *P. koraiensis* and *P. armandii*
24	2.67	0.02	mix of *P. koraiensis* and *P. armandii*
25	2.78	0.03	mix of *P. koraiensis* and *P. armandii*
26	3.04	0.01	mix of *P. cembra/siberica* and *P. armandii*

[a]Species assignments were made as described under Materials and Methods. Samples 21, 24–26, and 29–43 were obtained from the U.S. market, and samples 44 and 45 were provided by the Danish Veterinary and Food Administration, Region East, Ringsted, Denmark. Values are the mean ± SD of results of triplicate injections of each of two or three fatty acid methyl ester preparations per sample. DI = [(5,9-18:2 + 5,9,12-18:3 + 5,11,14-20:3)/(9-18:1 + 9,12-18:2 + 11,14-20:2)] × 10.

pine mouth. The DI of the mixture of *P. koraiensis* + *P. sibirica* was found to be 2.90 ± 0.03, which is close to that of the reference *P. armandii* (3.03 ± 0.10). For the three laboratory-prepared mixtures M1, M2, and M3, the DI value was lower than that of the reference component species with the higher DI value. Genetic analysis of samples 21, 24–26, 35, and 42 confirmed that they were mixtures of several species. The FA compositions of all of these mixtures were indeterminant (i.e., they did not match a FA profile and DI value of a reference species and thus did not allow a specific species assignment to be made).

■ DISCUSSION

Chromatographic Separation. For decades, FA analysis by GC has been used as a relatively rapid and simple fingerprinting method to determine the origin of oils and fats. Recently, such analysis has been applied to the taxonomy of conifers. The FA composition of conifer seeds differs according to genus, subgenus, section, and subsection and thus can be used as a taxonomic marker.[11,18−21]

Delmonte et al.[22] recently provided a detailed description of the separation characteristics of the SLB-IL 111 column for FAMEs. The ionic liquid SLB-IL 111 is a fused-silica capillary column capable of providing an enhanced separation of unsaturated FAMEs compared to the highly polar cyanopropyl siloxane columns currently used for FAME analysis (CP-Sil 88, SP 2560).[22] The isothermal elution temperature of 168 °C was selected because it provided the most balanced compromise for the separation of mono- and polyunsaturated FAs in fats and oils, including those found in pine nuts.[20] All characteristic Δ5-UPIFAs (peaks 8, 11, 13, 17, and 18) were separated without coelutions.

Destaillats et al.[7] proposed separating the FAMEs prepared from pine nuts by fast GC using a 10 m BPX-70 column and calculating DI values using eq 1. The separation under the conditions Destaillats et al.[7] recommended was rapid and did not fully resolve 9-18:1 and 11-18:1. The minor content of 11-18:1 was included in the calculation of the DI[7] regardless of the fact that it is not a precursor of any of the Δ5-UPIFAs quantified. The SLB-IL 111 column operated under the conditions described in this study separated the 9- and 11-18:1 FAs and made possible the calculation of a more accurate DI relating the Δ5-UPIFA and their metabolic precursors. Previous papers in which Δ5-UPIFAs were analyzed also used columns and conditions that resolved 9-18:1 and 11-18:1 FAs. Separation of these two isomers has been reported using a 50 m CP-Sil 88 column,[23] a 50 m BPX-70 column,[14] and a 30 m PEG column.[11] However, Nasri et al.[24] did not report the separation of 9-18:1 and 11-18:1 FAs on a CP WAX 52 CB (50 m) column.

Wolff et al.[14] identified 9-18:1 as the metabolic precursor of taxoleic acid (5,9-18:2) and, thus, only this isomer should be included in the calculation of the DI value. The exclusion of the 11-18:1 isomer from the equation proposed by Destaillats et al.[7] generally resulted in slightly higher DI values because the denominator was slightly reduced. Whereas the separation of the two isomers 9-18:1 and 11-18:1 might not appear to be important for calculation of the DI value, it has considerable significance when studies are conducted on the biosynthesis of Δ5-UPIFAs. Thus, the use of GC conditions capable of separating these two FAs is mandatory for such studies.

On the basis of our previous work utilizing the 100 m ionic liquid SLB-IL 111 GC column, we considered that the column might provide an enhanced separation of FAs of possible significance for pine nut speciation, which might have coeluted on columns used in previous studies. Despite the improved separations achieved with the SLB-IL 111 column, our results did not reveal a more unique FA profile for individual pine nut species that might assist in determining their botanical origin. In the case of *P. armandii*,[7,25] we did not find any new or characteristic differences in the FA profile.

Pine Nut Samples Collected from the U.S. Market, Including Those Associated with Pine Mouth. We demonstrated that more accurate DI values can be obtained by use of the SBL-IL 111 column. Using the SLB-IL 111 column, we analyzed a total of 45 pine nut samples and calculated the DI according to eq 2 (Tables 2–4). The DI values for many of the market samples and pine mouth-associated samples were indeterminate. These findings suggested that many of the pine nut samples were mixtures of several species.

The deliberate mixing of known pine nuts (samples M1, M2, and M3) results in different FA distributions and, hence, different DI values, which themselves can lead to an inaccurate botanical identification. In analyzing our pine nut mixes, we obtained DI values close to, or different from, that of *P. armandii* (Table 4). The 50% by weight (w/w) mixing of two species not associated with pine mouth (i.e., *P. koraiensis* and *P. sibirica*) provided an FA profile and DI value similar to those of *P. armandii*.

Journal of Agricultural and Food Chemistry Article

Table 4. Fatty Acid Composition and Diagnostic Index (DI) of Mixed Pine Nut Samples[a]

sample	16:0	SD	ai-17:0	SD	17:0	SD	9-16:1	SD	18:0	SD	9-18:1	SD	11-18:1	SD	5,9-18:2	SD	20:0	SD
M1	4.67	0.02	0.05	0.02	0.07	0.01	0.05	0.01	2.35	0.00	24.94	0.02	0.43	0.01	2.09	0.01	0.40	0.01
M2	4.47	0.02	0.04	0.05	0.07	0.01	0.04	0.00	2.54	0.01	24.44	0.02	0.39	0.00	2.12	0.00	0.42	0.02
M3	4.76	0.01	0.05	0.01	0.08	0.02	0.05	0.03	2.42	0.01	22.90	0.01	0.40	0.02	2.25	0.00	0.40	0.01
16	7.15	0.01	0.02	0.04	0.05	0.13	0.18	0.01	2.35	0.04	44.83	0.00	0.63	0.03	0.17	0.02	0.46	0.03
17	5.67	0.00	0.08	0.01	0.10	0.06	0.12	0.02	2.06	0.00	38.50	0.02	0.66	0.01	0.28	0.01	0.50	0.01
18	7.17	0.01	0.06	0.07	0.09	0.04	0.10	0.11	2.76	0.02	46.08	0.01	0.51	0.01	0.55	0.03	0.60	0.02
19	5.87	0.11	0.03	0.01	0.15	0.03	0.10	0.02	3.73	0.03	36.50	0.02	0.47	0.02	0.12	0.02	0.63	0.04
21	4.78	0.02	0.04	0.02	0.07	0.00	0.06	0.00	2.23	0.01	25.84	0.02	0.48	0.11	2.33	0.02	0.37	0.03
24	4.40	0.01	0.03	0.01	0.76	0.04	0.60	0.03	2.04	0.01	24.13	0.01	0.45	0.10	2.36	0.01	0.37	0.00
25	4.53	0.03	0.04	0.01	0.08	0.06	0.06	0.04	2.12	0.03	23.63	0.01	0.44	0.00	2.42	0.03	0.35	0.01
26	5.88	0.10	0.06	0.08	0.05	0.02	0.05	0.02	2.67	0.06	23.54	0.02	0.53	0.06	2.99	0.00	0.35	0.00
35	5.41	0.02	0.05	0.01	0.04	0.01	0.04	0.03	2.36	0.02	24.68	0.01	0.53	0.03	3.20	0.00	0.37	0.04
42	4.89	0.00	0.06	0.01	0.09	0.00	0.05	0.01	2.74	0.02	22.74	0.02	0.49	0.02	2.57	0.03	0.40	0.02
sample	9,12-18:2	SD	5,9,12-18:3	SD	20:1	SD	5,11-20:2	SD	9,12,15-18:3	SD	22:0	SD	11,14-20:2	SD	5,11,14-20:3	SD	5,9,12,15-18:4	SD
M1	45.21	0.04	16.21	0.01	1.22	0.00	0.14	0.02	0.20	0.01	0.10	0.01	0.65	0.00	1.11	0.03	0.09	0.01
M2	44.99	0.02	17.14	0.06	1.17	0.00	0.14	0.00	0.20	0.03	0.09	0.06	0.60	0.00	1.04	0.03	0.10	0.01
M3	45.34	0.00	17.80	0.02	1.07	0.01	0.15	0.02	0.23	0.01	0.11	0.02	0.63	0.01	1.22	0.01	0.12	0.00
16	42.17	0.01	0.43	0.00	0.61	0.04	0.05	0.01	0.19	0.00	0.12	0.10	0.24	0.04	0.30	0.02	0.01	0.03
17	49.45	0.03	0.48	0.02	0.68	0.03	0.28	0.07	0.09	0.00	0.52	0.00	0.19	0.03	0.30	0.02		
18	39.42	0.12	0.91	0.03	0.60	0.02	0.05	0.03	0.32	0.02	0.18	0.01	0.21	0.02	0.34	0.02	0.04	0.00
19	48.22	0.09	0.37	0.00	0.70	0.01	0.14	0.00	0.64	0.04	0.15	0.02	0.54	0.02	1.62	0.03	0.04	0.00
21	45.93	0.05	14.16	0.02	1.32	0.01	0.15	0.02	0.17	0.01	0.11	0.02	0.73	0.04	1.22	0.00	0.97	0.03
24	46.81	0.02	15.44	0.01	1.21	0.00	0.16	0.01	0.19	0.02	0.13	0.07	0.70	0.01	1.32	0.00	0.11	0.02
25	46.54	0.01	15.96	0.03	1.19	0.03	0.16	0.00	0.19	0.00	0.12	0.00	0.70	0.01	1.33	0.01	0.11	0.01
26	43.76	0.02	16.62	0.02	0.93	0.01	0.66	0.03	0.15	0.01	0.25	0.00	1.22	0.06	1.22	0.01	—	—
35	43.96	0.00	14.93	0.01	1.02	0.02	0.70	0.02	0.15	0.00	0.21	0.01	0.75	0.04	1.26	0.01	0.11	0.02
42	44.41	0.03	17.35	0.01	1.08	0.00	0.68	0.02	0.25	0.01	0.09	0.01	0.62	0.02	1.18	0.03	0.14	0.00
sample		M1		M2		M3		16		17		18		19		21		24		25		26		35		42
DI		2.76		2.90		3.09		0.10		0.12		0.21		0.25		2.44		2.67		2.78		3.04		2.79		3.10
SD		0.03		0.03		0.05		0.03		0.07		0.02		0.03		0.02		0.02		0.03		0.10		0.02		0.03

[a]Fatty acid composition is expressed as wt % of total fatty acids. Values are the mean ± SD of results of triplicate injections of each of three fatty acid methyl ester preparations per sample. M1, M2, and M3 are 50% by weight (w/w) mixtures of *P. koraiensis* (A) + *P. armandii* (A); *P. koraiensis* (A) + *P. sibirica* (A) and *P. armandii* (A) + *P. sibirica* (A), respectively. Samples 16−19, 21, 24−26, 35, and 42 are samples from the U.S. market. Samples 35 and 42 were associated with "pine mouth". DI = [(5,9-18:2 + 5,9,12-18:3 + 5,11,14-20:3)/(9-18:1 + 9,12-18:2 + 11,14-20:2)] × 10.

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Figure 2. Morphological characteristics of reference pine nuts: (1) *P. gerardiana*; (2) *P. pinea* L.; (3) *P. sibirica*; (4) *P. armandii*; (5) *P. koraiensis*.

There are many varieties of Coniferophytinae,[19,26,27] and the size, shape, and length of their seeds can vary significantly. In the work of Destaillats et al.,[7] when the FA composition and the DI value of a particular pine nut sample did not match those of reference *Pinus* specimens, a visual examination of the nuts was undertaken. In several cases, physical separation or sorting of pine nuts on the basis of their morphology (length, diameter, etc.), followed by reanalysis, revealed the presence of more than one *Pinus* species. One sample was found to consist of a mixture of nuts from *P. koraiensis* and *P. armandii*. Another sample with a DI value close to those of *P. sibirica* and *P. massoniana* was determined to be *P. massoniana* on the basis of its overall FA profile and country of origin stated on the package.[7] Neither *P. armandii* nor *P. massoniana* is listed by the FAO[3] as among the species of pine nuts traditionally used for human consumption. In a further study, Destaillats et al.[28] analyzed 17 samples from consumers who reported dysgeusia following consumption of pine nuts. *P. armandii* nuts were found in all samples, either in pure form or mixed with nuts of *P. koraiensis*.

The preselection of pine nuts on the basis of their morphological characteristics[7] may be neither efficient nor accurate. In Figure 2, we compared the morphological characteristics of five species of reference pine nuts (*P. gerardiana*, *P. pinea* L., *P. sibirica*, *P. armandii*, and *P. koraiensis*). On the basis of the overall length and shape, we observed that seeds of *P. gerardiana* and *P. pinea* L. (Figure 2, seed groups 1 and 2, respectively) were difficult to distinguish, as were seeds of *P. sibirica* and *P. armandii* (Figure 2, seed groups 3 and 4, respectively). The *P. koraiensis* nut is larger and has some differences in shape that distinguish it from the other four species (Figure 2, seed group 5).

Apparent morphological differences become increasingly difficult to discern when large quantities of pine nuts are mixed in unknown ratios (e.g., hundreds or thousands of pounds). A determination of whether mixtures of seeds should be visually sorted becomes even more problematic because in the bulk commercial trade, pine nuts from the same species are marketed on the basis of their size,[29] and pine nut sellers from overseas usually grade pine nuts by the number of kernels per 100 g of seeds (e.g., 650−750 pieces/100 g to 1500−1700 pieces/100 g).[30] On the basis of such considerations, we conclude that selection or sorting prior to the GC analysis is likely to be of little value, and we did not attempt to make a selection of seeds based on morphology prior to analysis.

Characterization by a DNA-Based Method. DNA sequence analysis is a powerful biological tool to identify different species of plants.[31] Because of the complexities associated with identifying pine nut mixtures based on DI values, Handy et al.[17] developed a DNA-based method that more clearly identifies the presence of different *Pinus* species in samples associated with pine mouth and other commercially available pine nut samples. Handy et al.'s[17] objective was to develop a more definitive method for differentiating pine nuts in response to reports that *P. armandii* was associated with pine mouth syndrome.[9,25,28]

On the basis of the DNA analysis, all of the pine nut samples associated with pine mouth collected by FDA inspectors contained *P. armandii*, and five of them appeared to be pure *P. armandii* with DI values ranging from 2.91 to 3.16. The reference DI value for this species in our work is 2.89 ± 0.10. Twelve of the 17 samples associated with pine mouth were found, by genetic analysis and FA analysis to be mixed samples, with *P. armandii*, *P. gerardiana*, *P. cembra/sibirica*, *P. pumila*, and *P. koraensis* among the species identified. Similarly, FA analysis and genetic analysis of four of the market samples, which were not to our knowledge associated with pine mouth, also showed them to be mixtures of *P. koraiensis* and *P. armandii* or *P. cembra/sibirica*, and *P. armandii*.

Our studies on the FA composition and DI values of market samples and results of DNA-based analyses indicate that many of the pine nuts available in the United States are mixtures of several species of pine nuts. To our knowledge, this is the first work to provide information on FA composition, DI values, and species identification of pine nuts available in the U.S. market.

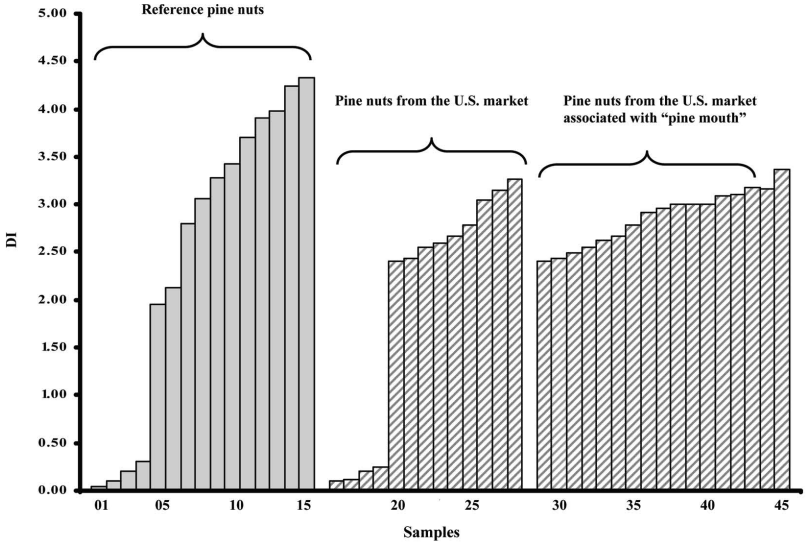

Figure 3. Diagnostic index (DI) of reference pine nuts (samples 1−15) and pine nuts from the U.S. market (samples 16−28), including those from the U.S. market associated with pine mouth (samples 29−43). Samples 44 and 45 are pine nut samples from the Danish market that were associated with pine mouth.

This finding is presented graphically in Figure 3, which shows that there is considerable overlap between the DI values of market samples not associated with pine mouth and those associated with pine mouth. This observation regarding the commercial pine nuts in the U.S. market is in agreement with Sharashkin and Gold,[2] who reported, on the basis of economic rather than analytical considerations, that commercial pine nuts are usually mixtures of different pine nut species.

Of interest is the finding of pine nuts with very low DI values (<0.5) in the U.S. market samples (samples 16−19). On the basis of examination of its FA composition, sample 17, identified by the vendor as originating in Pakistan, may be *P. gerardiana* nuts, which are commonly exported from Pakistan. Sample 19, labeled as Mediterranean pine nuts, may represent a sample of *P. pinea* nuts. Its overall FA composition and high level of 5,11,14-20:3 are consistent with this. The other two samples (16 and 18) may represent pine nuts harvested in the western United States, where *P. monophylla* and *P. edulis* are widely consumed but not yet produced in sufficient quantities for global trade.

Currently Available Methods. Each of the methods we used to determine that pine nuts in the U.S. market consist in general of mixtures has advantages and disadvantages. The FA profile/DI calculation analysis, which can be performed with relatively large sample portions of seeds and does not require presorting before analysis, can clearly show whether DI values match or do not match DI values of reference pine nuts. However, although capable of indicating that a sample of pine nuts is a mixture, the results of the FA profile and calculation of DI values cannot identify the components of the mix. The DNA method, in contrast, provides some species identification but is dependent on the sorting of pine nuts before analysis. We have shown that sorting can be a problematic procedure if morphologically similar species are mixed. Because of the current capabilities of the DNA assay, mixtures may also present a problem when closely genetically related species are mixed. For example, whereas *P. armandii* can be easily resolved from closely related species such as *P. lambertiana*, *P. cembra*, *P. sibirica*, and *P. koraiensis* using the C-D section of the ycfl gene, some other species of pine nuts cannot be differentiated using this region (e.g., *P. yunnanensis/tabuliformis* and *P. cembra/sibirica*).[17] Use of the DNA method would become a problem if, for example, nuts of the edible species *P. sibirica* and *P. cembra* were mixed. In addition, sampling issues due to the use of only one or several seeds would quickly arise if bulk quantities of pine nuts needed to be analyzed.

Köbler et al.[25] recently reported the use of nontargeted 400 MHz ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy to identify *Pinus* species producing nuts that cause taste disturbances. They showed that three groups of pine nuts could be distinguished using principal component analysis. *P. armandii* nuts that were associated with taste disturbances were found in only one of the groups, which, however, also included some *P. armandii* nuts that were not, on the basis of taste testing, associated with taste disturbances.[25] The botanical identification of the pine nuts was based solely on determination of the DI value according to the method of Destaillats et al.[7] Samples with DI values from 2.80 to 3.13 were said to be *P. armandii*. Köbler et al.[25] suggested that their procedure might be used as a means of importation control that would allow the identification of samples suitable for direct clearance while redirecting others for sensory analysis (i.e., organoleptic testing by qualified assessors).

Although the methods currently available, including morphological examination, FA analysis, DNA testing, or NMR analysis, can each provide some useful information about the identity of pine nuts, none have identified a cause of pine mouth syndrome. Recent data from French poison centers covering more than 3000 cases of bitterness following consumption of pine nuts

showed that a rapid increase occurred in May 2009 and peaked in August 2009. The number of reported cases declined sharply after the peak of almost 700 cases reported per month in August 2009.[9] Currently, there is no information about specific samples that were associated with these 3000 cases (e.g., species identification). An etiological agent for pine mouth has not been identified either for the cases reported in Europe or for those reported in the United States. Suggestions regarding causality range from the possible presence of an unidentified toxin (e.g., a contaminant or a natural constituent) resulting from importation of nonedible *Pinus* species to individual susceptibilities possibly related to polymorphism in the genetic expression of taste function. Until the time that the physiological mechanism of pine mouth is understood, monitoring of cases as well as botanical, biological, and chemical characterization of pine nuts will continue to be important.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

FA(s), fatty acid(s); DI, diagnostic index; Δ5-UPIFA, Δ5-unsaturated polymethylene-interrupted fatty acids; methanol, MeOH; ai, anteiso.

REFERENCES

(1) Evaristo, I.; Batisda, D.; Correia, I.; Correia, P.; Costa, R. Chemical profiling of Portuguese *Pinus pinea* L. nuts. *J. Sci. Food Agric.* **2010**, *90*, 1041−1049.

(2) Sharashkin, L.; Gold, M. Pine nuts: species, products, markets, and potential for U.S. production. *Northern Nut Growers Association 95th Annual Report*; Columbia, MO, Aug 2004; pp 16−19.

(3) Food and Agriculture Organization (FAO) of the United Nations, Rome, Italy.Seeds, fruits and cones. In *Non-Wood Forest Products from Conifers*; 1995; available at www.fao.org/docrep/X0453E/X0453e12.htm (accessed Oct 27, 2011).

(4) Nergiz, C.; Dönmez, I. Chemical composition and nutritive value of *Pinus pinea* L. seeds. *Food Chem.* **2004**, *86*, 365−368.

(5) Mostin, M. Taste disturbances after pine nut ingestion. *Eur. J. Emerg. Med.* **2001**, *8* (1), 76.

(6) Munk, M. D. "Pine mouth" syndrome: cacogeusia following ingestion of pine nuts (genus: *Pinus*). An emerging problem? *J. Med. Toxicol.* **2010**, *6* (2), 158−159.

(7) Destaillats, F.; Cruz-Hernandez, C.; Giuffrida, F.; Dionisi, F. Identification of the botanical origin of pine nuts found in food products by gas−liquid chromatography analysis of fatty acid profile. *J. Agric. Food Chem.* **2010**, *58*, 2082−2087.

(8) Agence Française de Sécurité Sanitaire de Aliments (AFSAA). Eléments d'information relatifs à la notification d'amertume liée à la consommation de pignons de pin; 2009; available at http://www.afssa.fr/Documents/RCCP2009sa0166.pdf (accessed Oct 25, 2010).

(9) Flesch, F.; Rigaux-Barry, F.; Saviuc, P.; Garnier, R.; Daoudi, J.; Blanc, I.; Tellier, S. S.; Lasbeur, L. Dysgeusia following consumption of pine nuts: more than 3000 cases in France. *Clin. Toxicol.* **2011**, *49* (7), 1−3.

(10) Kwegyir-Afful, E.; DeJager, L.; Begley, T.; Luccioli, D. Pine mouth syndrome − an emerging food hypersensitivity? *Clin. Transl. Allergy* **2011**, *1*, 25.

(11) Wolff, R. L.; Pédrono, F.; Pasquier, E.; Marpeau, A. M. General characterisitics of *Pinus* ssp seed fatty acid compositions, and importance of Δ5-olefinic acids in the taxonomy and phylogeny of the genus. *Lipids* **2000**, *35* (1), 1−22.

(12) Wolff, R. L.; Dareville, E.; Martin, J. C. Positional distribution of Δ5-olefinic acids in triacylglycerols from conifer seed oils: general and specific enrichment in the sn-3 position. *J. Am. Oil Chem. Soc.* **1997**, *74* (5), 515−523.

(13) Lee, J. W.; Lee, K. W.; Lee, S. W.; Kim, I. H.; Rhee, C. Selective increase in pinolenic acid (all-cis-5,9,12-18:3) in Korean pine nut oil by crystallization and its effect on LDL-receptor activity. *Lipids* **2004**, *39* (4), 383−387.

(14) Wolff, R. L. Discussion of the term "unusual" when discussing Δ5-olefinic acids in plant lipids. *J. Am. Oil Chem. Soc.* **1997**, *74*, 619.

(15) Wolff, R. L.; Christie, W. W.; Pedrono, F.; Marpeau, A. M. Δ5-Olefinic acids in the seed lipids from four ephedra species and their distribution between the α and β positions of triacylglycerols. characteristics common to coniferophytes and cycadophytes. *Lipids* **1999**, *34*, 855−864.

(16) Wolff, R. L. The phylogenetic significance of sciadonic (all-cis 5,11,14−20:3) acid in gymnosperms and its quantitative significance in land plants. *J. Am. Oil Chem. Soc.* **1999**, *76*, 1515−1516.

(17) Handy, S.; Parks, M. B.; Deeds, J. R.; Liston, A.; De Jager, L. S.; Luccioli, S.; Kwegyir-Afful, E.; Fardin-Kia, A. R.; Begley, T. H.; Rader, J. I.; Diachenko, G. W. Use of the chloroplast gene ycf1 for the genetic differentiation of pine nuts obtained from consumers experiencing dysgeusia. *J. Agric. Food Chem.* **2011**, *59*, 10995−11002.

(18) Wolff, R. L.; Lavialle, O.; Pédrono, F.; Destaillats, F.; Marpeau, A. M.; Angers, P.; Aitzemüller, K. Abietoid seed fatty acid compositions − a review of the genera *Abies, Cedrus, Hesperopeuce, Keteleeria, Pseudolarix*, and *Tsuga* and preliminary inference on the taxonomy of Pinaceae. *Lipids* **2002**, *37*, 17−26.

(19) Wolff, R. L.; Lavialle, O.; Pédrono, F.; Pasquier, E.; Deluc, L. G.; Marpeau, A. M.; Aitzetmüller, K. Fatty acid composition of Pinaceae as taxonomic markers. *Lipids* **2001**, *36* (5), 439−451.

(20) Wolff, R. L.; Deluc, L. G.; Marpeau, A. M. Conifer seeds: oil content fatty acid composition. *J. Am. Oil Chem. Soc.* **1996**, *73* (6), 765−771.

(21) Wolff, R. L.; Christie, W. W.; Pedrono, F.; Marpeau, A. M. Arachidonic, eicosapentaenoic, and biosynthetically related fatty acids in the seed lipids from a primitive gymnosperm, *Agathis robusta*. *Lipids* **1999**, *34* (10), 1083−1097.

(22) Delmonte, P. L.; Fardin Kia, A. R.; Kramer, J. K. G.; Mossoba, M.; Sidisky, L.; Rader, J. I. Separation characteristics of fatty acid methyl esters using SLB-IL111, a new ionic liquid coated capillary gas chromatographic column. *J. Chromatogr., A* **2010**, *1218*, 545−554.

(23) Wolff, R. L.; Bayard, C. C. Fatty acid composition of some pine seed oils. *J. Am. Oil Chem. Soc.* **1995**, *72* (9), 1043−1046.

(24) Nasri, N.; Khaldi, A.; Fady, B.; Triki, S. Fatty acids from seeds of *Pinus pinea* L. Composition and population profiling. *Phytochemistry* **2005**, *66*, 1729−1735.

(25) Köbler, H.; Monakhova, Y. B.; Kuballa, T.; Tschiersch, C.; Vancutsem, J.; Thielert, G.; Mohring, A.; Lachenmeier, D. W. Nuclear magnetic resonance spectroscopy and chemometrics to identify pine nuts that cause taste disturbance. *J. Agric. Food Chem.* **2011**, *59* (13), 6877−6881.

(26) Page, C. N. Gymnosperms: Coniferophytina (conifers and ginkgoids). In *The Families and Genera of Vascular Plants*; Kubitski, K., Ed.; Springer: Berlin, Germany, **1990**; Vol. *1*, pp 279−361.

(27) Page, C. N. New and maintained genera in the conifer families Podocarpaceae: an immunological comparison. *Syst. Bot.* **1988**, *12*, 91−97.

(28) Destaillats, F.; Cruz-Hernandez, C.; Giuffrida, F.; Dionisi, F.; Mostin, M.; Verstegen, G. Identification of the botanical origin of commercial pine nuts responsible for dysgeusia by gas−liquid chromatography

analysis of fatty acid profile. *J. Toxicol.* **2011**, DOI: 10.1155/2011/316789.

(29) www.alibaba.com/trade/search?SearchText=pine+nuts+china (accessed Aug 9, 2011).

(30) http://pinenut.com/blog/blog1.php?title=extended-post&more=1&c=1&tb=1&pb=1 (accessed Nov 15, 2011).

(31) Fazekas, A. J.; Burgess, K. S.; Kesanakurti, P. R.; Graham, S. W.; Newmaster, S. G.; Husband, B. C.; Percy, D. M.; Hajibabaei, M.; Barrett, S. C. H. Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. *PLoS One* **2008**, 3 (7), e2802.

CHAPTER 9

FINAL DISCUSSION

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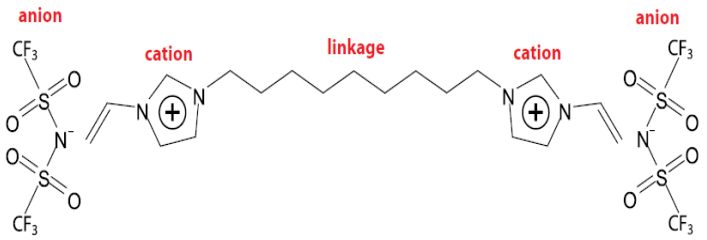
Separation and quantification of *trans* fatty acids using ionic liquid capillary GC column

Room temperature ionic liquids (RTIL) are generally composed of a cation (e.g., alkyl imidazolium, alkyl phosphonium, alkyl pyrrolidium, etc.) compensated by an anion of organic (e.g., NTf_2^-, TfO^-, etc.) or inorganic (e.g., X^-, PF_6^-, BF_4^-, etc.) nature (FIGURE 13) [Qiao et al., 2013]. Their low volatility, relative high thermal stability and high polarity make them attractive stationary phases for GC. The development of dicationic, trigonal tricationic, and cross-linked RTILs led to the novel IL capillary columns currently being developed for the separation of FAME. The unique separation capabilities of SLB-IL111 columns that are novel capillary columns commercialized by Supelco were evaluated and coated with the most polar liquid phase currently available for the gas chromatographic separation of FAME.



1,9-di(3-vinylimidazolium) nonane bis(trifluoromethyl) sulfonyl imidate)

FIGURE 13 Structure of the ionic liquid phase of the SLB-IL100 capillary column.

Methodologies for FAME separations evolved with the availability of columns with increasing polarity of the stationary phases and number of theoretical plates. For more than two decades, 100% cyanopropyl siloxane (CPS) have been the most polar and thermally stable phase available. CPS columns, in the commercial format of 100 m length (0.25 mm ID), provide the greatest number of theoretical plates and have been used to develop methods requiring the most refined separation of geometric and positional isomers of FAME. The AOCS Official Methods Ce-1h 05 and Ce-1j 07 were developed using these columns to measure the *trans* FA content of fats and oils from non-marine origin [Mossoba et al., 2009 (J

AOAC Int. 92, 1284-1300)]. The 100 m x 0.25 mm CPS columns SP-2560 (from Supelco Inc.) and CP-Sil 88 (from Varian Inc.) operated isothermally at 175-180 °C maximized the separation of geometric and positional isomers of unsaturated FAME contained in PHVOs and ruminant fats [Chapter 3].

Despite the high number of FAME separated by SP-2560 and CP-Sil 88 columns, there are still several FAME of interest co-eluting in the 16:1, 18:1, 18:2, 20:1/18:3, and CLA isomers chromatographic regions. Some of these co-elutions can be overcome by applying Ag^+ ion fractionation (Ag^+-HPLC, Ag^+-TLC or Ag^+-SPE) prior to GC quantification, such as the separation of *t*9-16:1 that co-elutes with iso-17:0 [Precht and Molkentin, 2000; Cruz-Hernandez et al., 2006], or by applying two temperature programs to resolve many of the *trans*-and *cis*-16:1, 18:1 and 20:1 isomers [Kramer et al., 2008].

The recent availability of the SLB-IL111, a novel capillary column coated with a proprietary phase that is markedly more polar than 100% CPS, provided a separation tool characterized by significantly higher selectivity toward the position and geometric configuration of FA double bonds. A 100 m SLB-IL111 column operated at 169 °C showed the ability of separate a greater number of FAME compared to 100% CPS column of the same length, including the separation of the main two naturally occurring CLA isomers *t*7,*c*9- and *c*9,*t*11-18:2. Among other benefits, the SLB-IL111 operated at 169 °C provides the elution of all *trans*-18:1 FAME before oleic acid (*c*9-18:1) and *t*16-18:1 between *c*13- and *c*14-18:1. While these chromatographic conditions provided the separation of *t*10-, *t*11-, and *t*12-18:1 when they were present in similar amounts, as in the case of most PHVOs, this resolution did not occur for samples such as milk fat in which these FAME were present in unequal quantities. The separation of these FAME isomers can be significantly improved by doubling the length of the separation column to 200 m, thus taking advantage of the gain in resolution that is proportional to the square root of the number of theoretical plates.

A 200 m SLB-IL111 column was used in this study to develop a GC method that would provide a significantly improved separation of the FAME prepared from vegetable fats and oils and dairy products [Chapter 4], and marine oils [Chapter 7] in a single operation in about 100 min. The time of analysis was similar to that of most other established GC procedures. The method, which aimed to maximize the separation of the 18:1, 18:2 and 18:3 FAME, relied on a combined flow and temperature gradient: the flow rate was increased from 1.6 to

3.0 ml/min after the elution of the 18:2 FAME, while the temperature was raised from 170 °C to 185 °C after the elution of the 18:3 FAME. Figure 14 shows the separation of FAME prepared from milk fat that can be achieved by this method in 90 min.

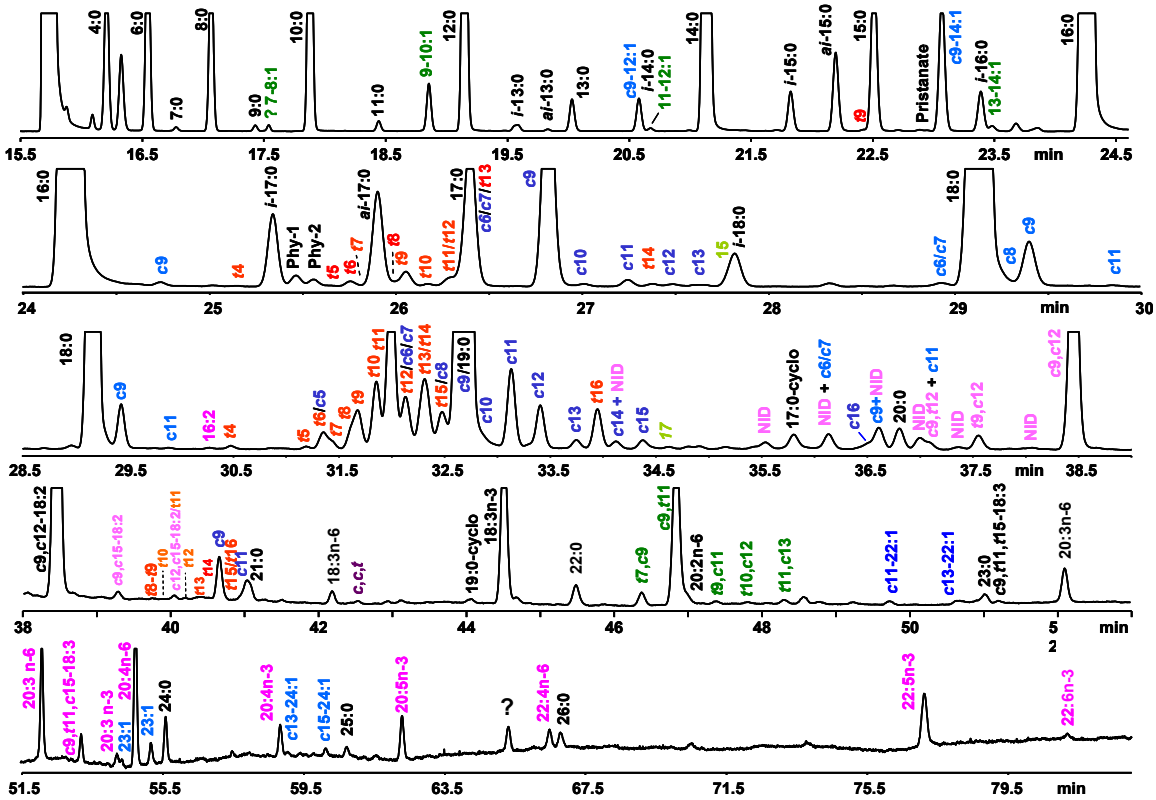


FIGURE 14 Separation of milk fat FAME using a 200 m SLB-IL 111

In addition to separating the C18 FAME which are the primary constituents of most vegetable fats and oils, the selected conditions provided the separation of the short chain FAME including C4:0 contained in milk fat and the long chain PUFA up to docosahexaenoic acid (DHA) contained in marine products such as menhaden oil (FIGURE 15). The separations of all CLA isomers occurring in ruminant fats, including *c*9,*t*11- from *t*7,*c*9-18:2, and of the most geometric and positional isomers from 6,8 - 13,15-18:2 were among the most relevant improvements provided by this separation method. The separation of many of these FAME was not possible using 100 m CPS columns at any isothermal temperature or temperature program conditions [Cruz-Hernandez et al.,2006; Yurawecz et al., 1998]. Thus, the use of this method eliminated the need for a complimentary Ag^{+}-HPLC technique to resolve these two major CLA isomers [Cruz-Hernandez et al., 2004]. The *c*9,*t*11-18:2 content in ruminant fats was overestimated using 100% CPS columns, unless an additional Ag^{+}-HPLC step was included to measure these two CLA isomers. The novel methodology described here for FAME separation and quantification will significantly 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.

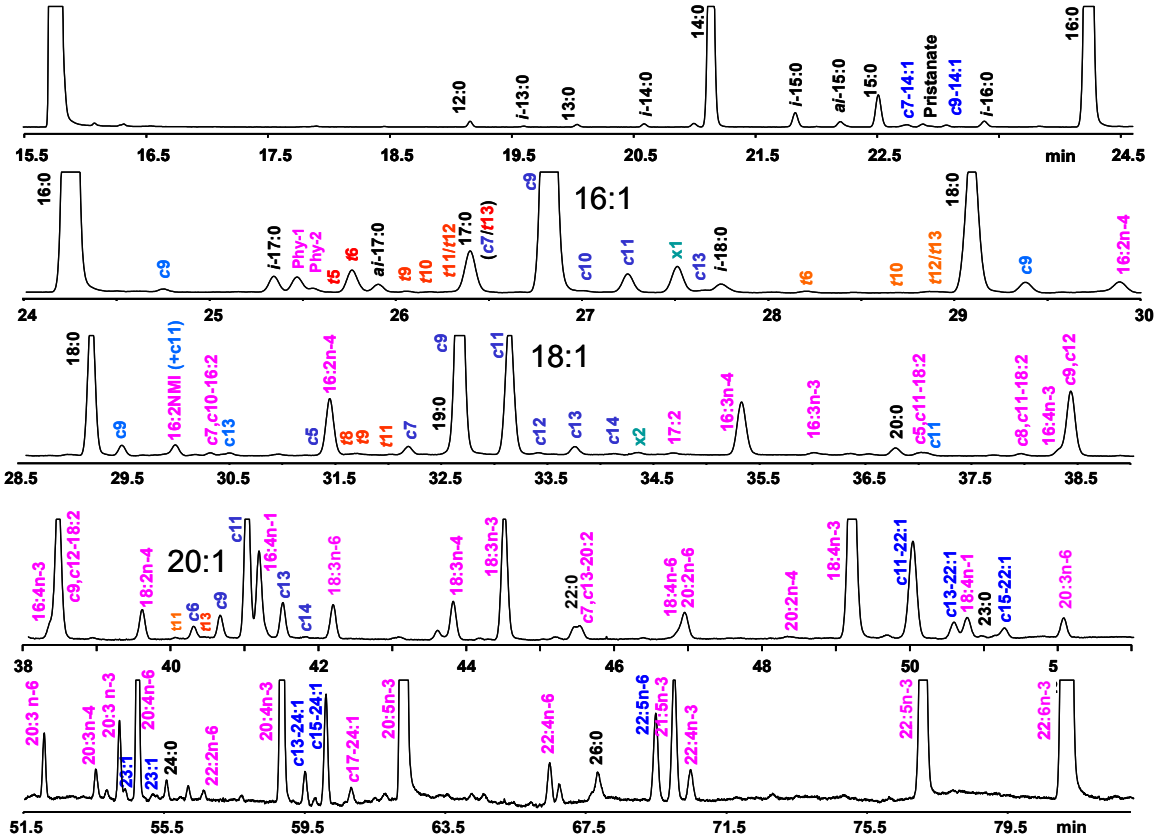


FIGURE 15 Separation of menhaden oil FAME using a 200 m SLB-IL 111

Figure 16 shows the comparison between a 100 m SP-2560 operated at 180 °C isothermal and a 200 m SLB-IL111 operated under the flow and temperature gradient conditions described above for the separation of 14:0–16:0 milk fat FAME. The higher polarity of SLB-IL111 results in a higher retention of FAME with double bonds, which was evident by the observed reversal of the elution order of *c*9-16:1 and 17:0, or *c*9-17:1 and 18:0. The experimental conditions selected for the separation using the SLB-IL111 provided a major improvement in the separation of FAME present in the chromatographic region from 16:0 to 18:0, allowing the quantification of most of the 16:1 FA.

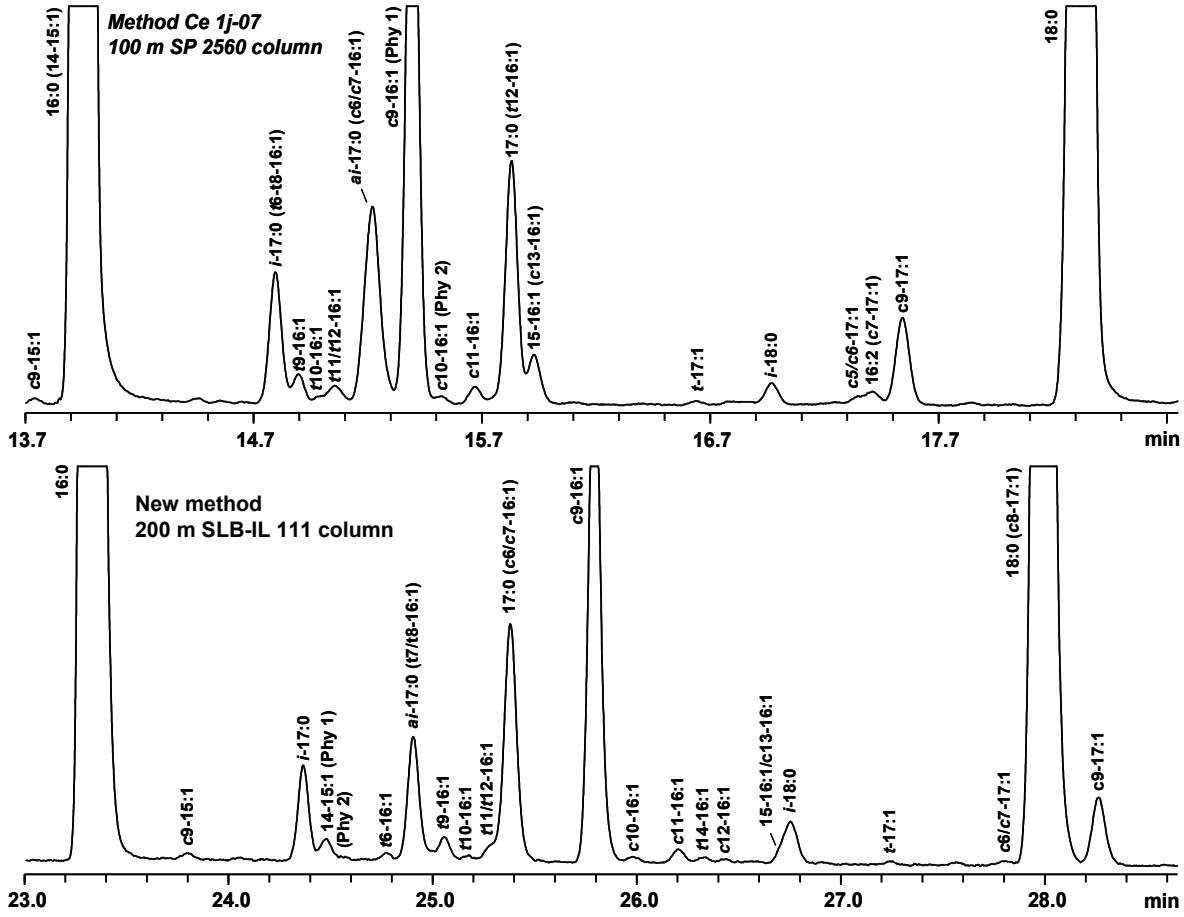


FIGURE 16 Comparison of the 16:0 to 18:0 FAME region of a pizza sample analyzed according to AOCS Official Method Ce 1j-07 (top chromatogram) and the new developed GC method (bottom chromatogram)

Figure 17 shows the same comparison of the two GC columns (SP-2560 and SLB-IL111), but of the separation of milk fat FAME from 18:0 to linoleic acid (*c*9,*c*12-18:2). The new method provided separation of most *trans*-18:1 contained in milk fat including vaccenic acid, and several 18:2 FAME that co-eluted when using the AOCS Official Method with a SP-2560. Milk fat contains among others both the *t*10-18:1 and the *t*11-18:1 (vaccenic acid) isomers. Unlike *t*11-18:1, *t*10-18:1 is not known to provide any beneficial health effects in animals or humans (Field et al., 2009). Several reports have shown that high amounts on *t*10-18:1 are an indicator of ruminants fed diets containing high levels of digestible carbohydrates or high in PUFA [Griinari et al., 1998; Cruz-Hernandez et al., 2007].

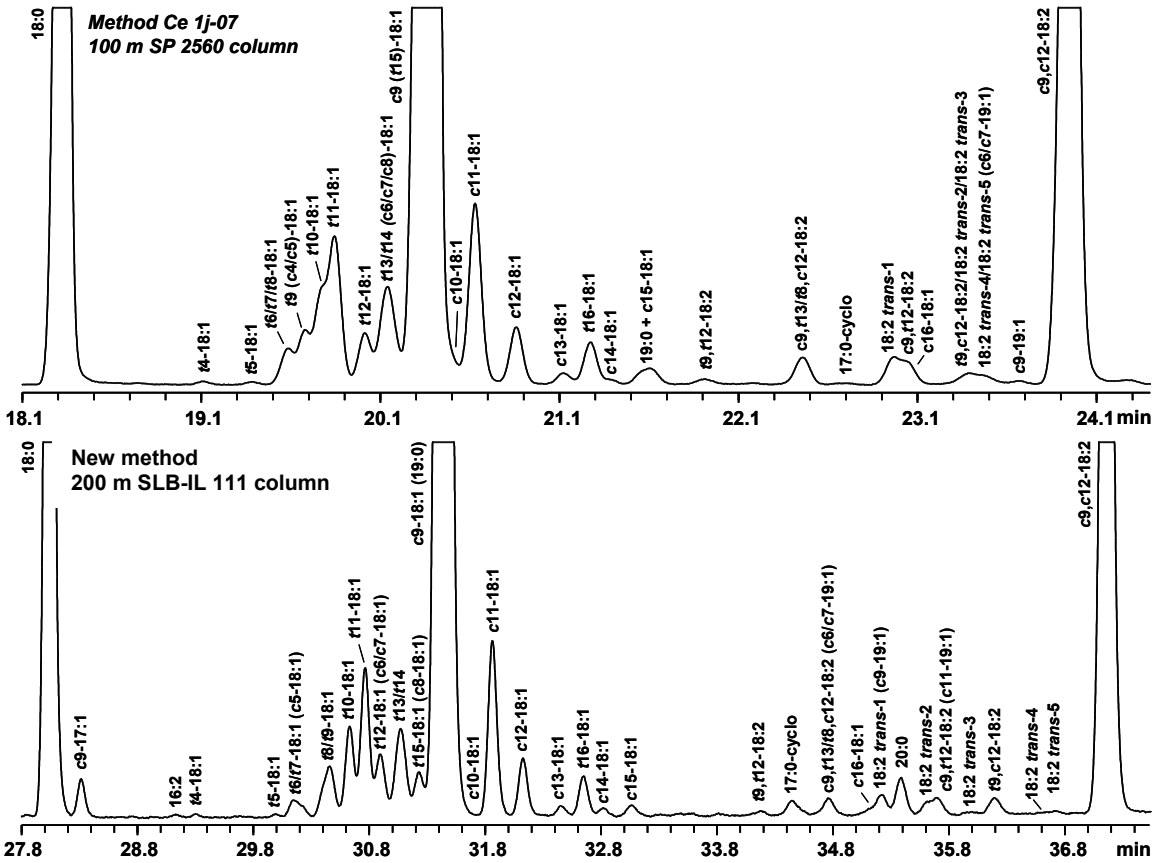


FIGURE 17 Comparison of the 18:0 to *cis*9,*cis*12-18:2 FAME region of a pizza sample analyzed according to AOCS Official Method Ce 1j-07 (top chromatogram) and the new developed GC method (bottom chromatogram). The lower chromatogram contains several unknown *trans* containing 18:2 isomers.

The major advantage of the new method, compared to the AOCS methods Ce 1j-07 and Ce 1h-05, was to enable the resolution of *t*10- and *t*11-18:1 and to identify samples containing significant amounts of *t*10-18:1. In addition, the method made it possible to resolve a number of *trans* containing 18:2 isomers.

Based on the improved chromatographic separation of FAME provided by the SLB-IL111 column, we speculated that the quantitation of total *trans* fat would be more accurate than that provided by the Official Methods Ce 1h-05 or Ce 1j-07 using 100% CPS columns. However, a comparison of selected samples investigated showed that the quantitative analysis of total *trans* fat was similar using the 200 m SLB-IL111 column compared to the 100 m SP-2560 column according to Method Ce 1j-07 [Tyburczy et al., 2012].

The chromatographic separation of mono-*trans* 18:2 and mono-*trans* 18:3 FAME is a critical factor affecting the measurement of the total *trans* FA content of oils, foods and fats. The deodorization step during processing of vegetable oils has been shown to cause isomerization of double bonds of all-*cis* PUFA, the natural form of most PUFA in vegetable oils. This process results in the formation of mono-*trans* and di-*trans* PUFA. The oil content in these *trans* PUFA may reach up to 3.5% of total FA in vegetable oils [Wolff, 1992]. In a current study of 30 commercial vegetable oils we found that the total *trans* content ranged from 0.03% to 2.18% [Mossoba et al., 2013].

The content of total *trans* fat of 32 representative fast food samples was determined in another study. The primary objective of the study was to analyze the current levels of *trans* fat, including the content of *trans* PUFA, in representative fast food samples from U.S.-based restaurants using the AOAC 996.06 extraction and transmethylation procedure with AOCS Official Method Ce 1j-07 for the quantitation of FAME [Tyburczy et al., 2012]. The content of *trans* MUFA, mono-*trans* 18:2 and mono-*trans* 18:3 FAME differed by food category. For ruminant-derived foods, *trans* MUFA consisted primarily of *trans* 18:1 FAME, which were the predominant *trans* FA class (4.4% of total FA). In contrast, the combined content of *trans* 18:2 and *trans* 18:3 FAME was observed to be relatively minor (<1% of total FA). For many of the chicken tenders/nuggets, French fries and apple pie/turnover samples considered in the study, the ratio of *trans* MUFA to the sum of *trans* 18:2 plus *trans* 18:3 FA was less than 1.0, reflecting the typical content of *trans* PUFA and low levels of *trans* MUFA in oils that have undergone deodorization but not partial hydrogenation [Wolff, 1992]. In other samples, the ratio of *trans* MUFA to the sum of *trans* 18:2 plus *trans* 18:3 FA was greater than 1.0, and could be explained by the use of partially hydrogenated oil in the preparation of these foods. Therefore, these results reveal the importance of the ratio of *trans* MUFA to *trans* 18:2 plus *trans* 18:3 FA for identifying the presence of partially hydrogenated oils in

non-ruminant-derived foods. They also emphasize the advantage of use of an SLB-IL111 column in making these determinations.

Preparation of Reference Materials to Support Identification of Fatty Acids

The first step in the development of a chromatographic method, including quantification, is the correct identification of the analytes whose separations were observed. Among other available techniques for identification (e.g. mass spectrometry) of separated compounds pure reference materials are needed to compare with separated compounds. Reference materials are also needed for the process of method validation. Analysis of the FA composition of fats and oils requires the quantification of a large number of FA, many of which are not available as reference materials. Jensen [2002] reported the presence of at least 400 FA in milk fat. In this study (Chapter 5) more than 150 FA in milk fat, and over 100 FA in menhaden oil (Chapter 7) using the 200 m SLB-IL111 column were separated and identified

The limited availability of commercial *trans* fatty acid reference materials for use in the development of gas chromatographic separation methods played a critical role in this research project. *Trans* monounsaturated FA (*t*MUFA), the primary source of TFA in food, are of limited availability as reference materials from commercial suppliers. With the exception of data regarding the separation of 16:1 FAME positional/geometrical isomers [Kramer et al. 2008; Precht and Molkentin, 2000], identification of the GC elution profile of MUFA, other than 18:1 FAME, is limited to that of the available reference materials. Data regarding non-conjugated mono-*trans* dienes (present in milk fat) or mono-*trans* PUFA isomers are also rarely available.

To overcome this limitation, a project was undertaken to synthesize most of the geometric and positional isomers from C14 to C20. Saturated straight-chain FA are most commonly synthesized from olefins isolated from petroleum by applying:

A) Continuous air oxidation of aliphatic hydrocarbons catalyzed by permanganate;
B) Oxidation of straight-chain terminal olefins;
C) Oxidation of straight-chain alcohols;

The unsaturated FA are usually synthesized by applying a coupling reaction of alkynes according to a general method that contains three distinctive steps:

1) Construction of the alkynes;
2) Introduction of acid functionality;
3) Partial reduction of the acetylenes;

These processes can produce large quantities of FA at low cost but are not practical as reference materials for GC analyses because they lack purity. Therefore, many synthetic procedures from the literature for the preparation of the reference materials were reviewed and needed for the quantification of FA (including *trans* FA) in fats and oils of non marine origin. The reactions were adapted for small scale application using glassware and equipments available in most laboratories (Chapter 3).
Mixtures containing geometric and positional isomers of MUFA were obtained by repeated addition and elimination of HBr to commercially available *cis*-MUFA. Subsequent fractionation of the synthetic geometric mixture into *cis*- and *trans*-MUFA by Ag^+SPE or Ag^+-HPLC produces reference mixtures that were used “as is” for the identification of these FAME in fats and oils. If pure MUFA were needed, they were obtained by a combination of RP- and Ag^+-HPLC fractionation.

The entire preparation can be summarized in 5 steps:

1) Addition of HBr to a commercially available carboxylic acid with at least one double bond;
2) Elimination of HBr using hot alkali;
3) Methylation using BF_3/Methanol;
4) Purification (e.g. HPLC);
5) Isomerization with para-toluene sulfinic acid (PTSA);

Using this preparation scheme (FIGURE 18), it was possible to prepare and purify the positional and geometric isomers of MUFA from 14:1 to 20:1.

The addition/elimination of HBr to monoenoic FA was repeated until the targeted isomerization was reached. After obtaining the desired MUFA isomerization, the products of reaction were re-converted to methyl esters before being used as reference materials for GC analysis or fractionation by HPLC.

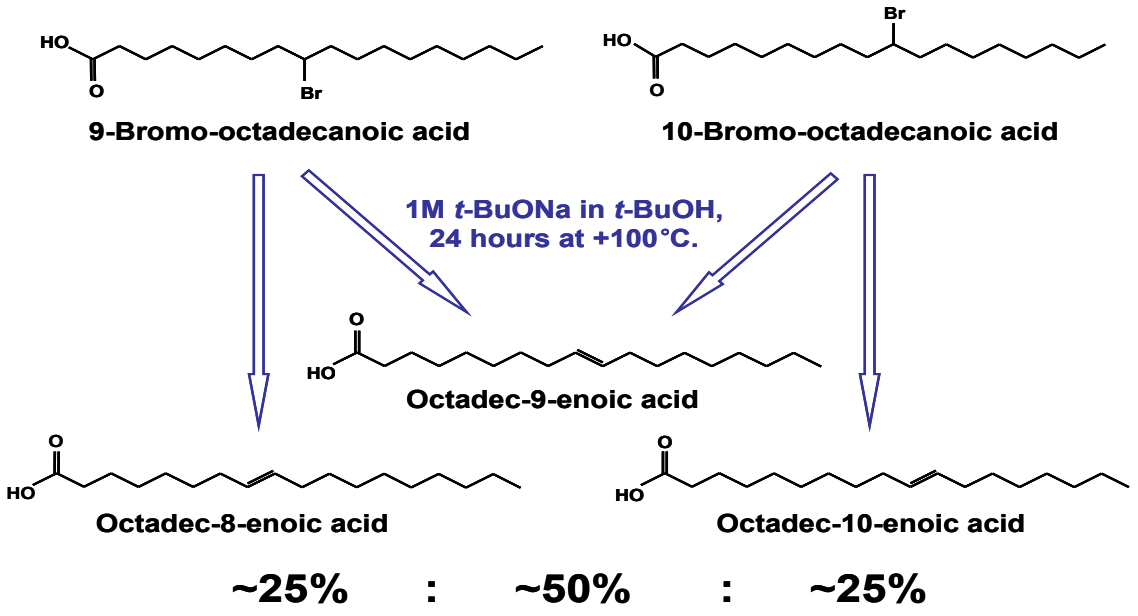


FIGURE 18 Scheme for the preparation of MUFA positional isomers. In each case the geometric isomers were subsequently resolved using Ag^+-HPLC.

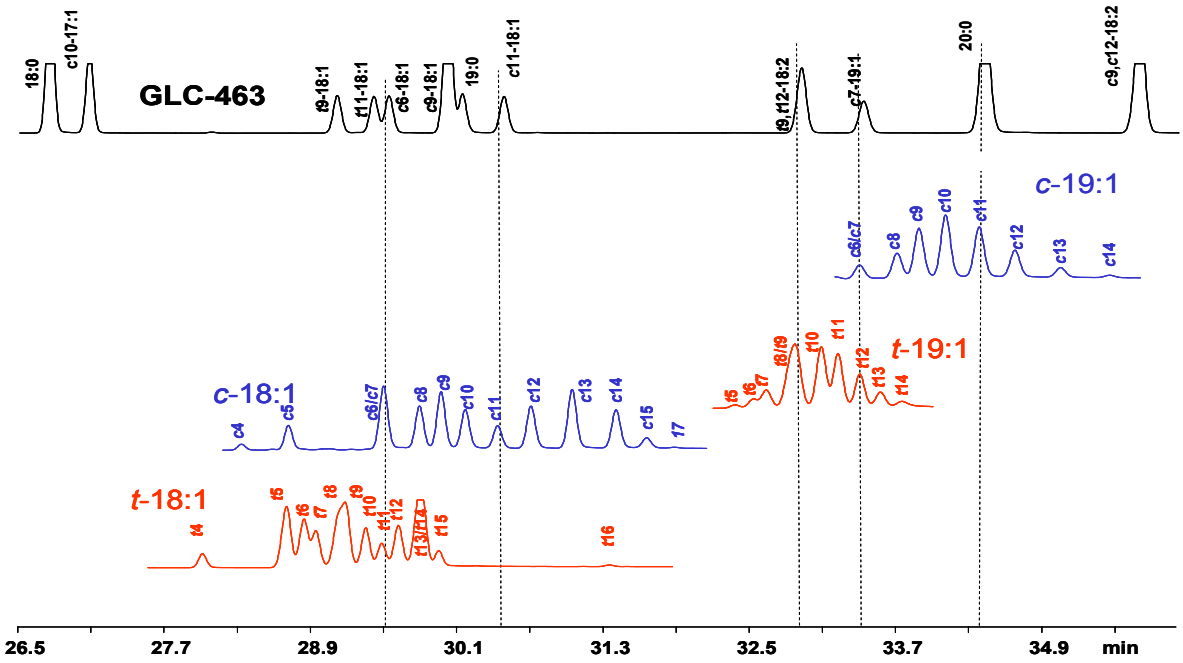


FIGURE 19 GC-FID chromatogram of 18:1 and 19:1 positional and geometric FAME mixture, prepared according to presented procedure.

Ag^+-HPLC provided selectivity toward the *cis* and *trans* double bonds of FAME, and allowed the fractionation of geometrical isomers of MUFA. The thermodynamically favored *trans* isomers were produced by the de-hydrobromination reaction in about twice the amount as the *cis* isomers. Figure 19 shows the GC-FID separation of the reaction products of 18:1 and 19:1 isomers using a 200 m SLB-IL111 capillary column. The fractionation of the mixtures into the *cis* and *trans* groups of positional isomers was obtained by applying Ag^+-HPLC.

In this study, positional/geometrical and non-conjugated isomers of linoleic acid were also prepared (FIGURE 20). These FAME were prepared by conducting a single addition/elimination with HBr to linoleic acid. A second repetition of the addition/elimination process could have led to a more interesting set of linoleic acid isomers, but significant side reactions, which reduced the yield of the expected FAME, were observed. The positional/geometrical isomers of linoleic acids are of special interest, due to their presence in milk fat and dairy products, as well as IP-TFA. The co-elution of mono *trans*-18:2 with *cis*-18:1 and 19:1 FAME isomers makes their identification very challenging and there is an urgent need for additional reference materials.

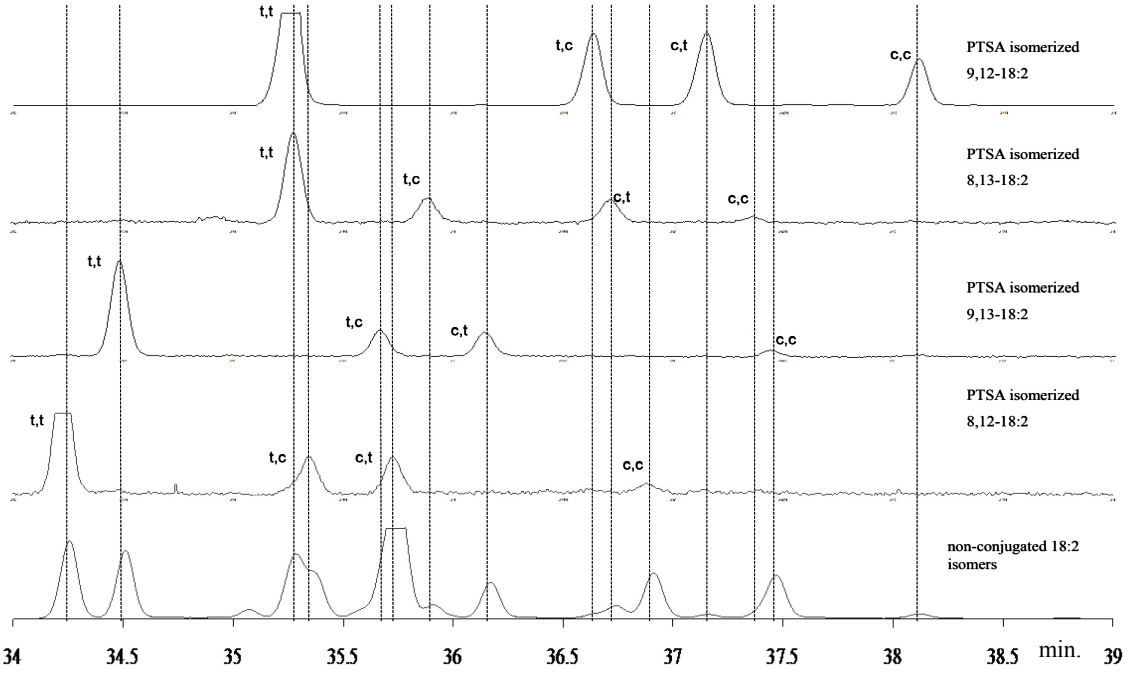


FIGURE 20 GC-FID chromatogram of positional and geometric isomers FAME mixture (bottom chromatogram) prepared after a single addition and bromination of HBr according to the presented procedure. PTSA, *para*-toluene sulfonic acid.

The preparation of isolated non-conjugated 18:2 isomers relied on well known chemical principles and low-cost supplies available in most laboratories. It allowed the preparation of the reference materials needed for the quantification of the *trans*-FA in dairy products and vegetable oils. The starting material can be either commercially available FAME such as linoleic acid, or an FA isolated from a natural source (e.g. seed oils). The synthesis of FAME using addition/elimination reactions, mentioned above, was used to produce larger quantities of relatively pure unsaturated FAME such as 8,13-, 9,13-, and 8,12-18:2 (FIGURE 20). However, the equipment and skills needed to carry out these reactions make them unsuitable for laboratories not dedicated to synthetic organic chemistry.

Purified MUFA positional isomers can be isomerized by *para*-toluene sulfonic acid (PTSA) to obtain mixtures of geometrical isomers. Reversible addition of PTSA provided geometrical isomerization of double bonds without causing their migration on the alkyl chain (FIGURE 20 & FIGURE 21).

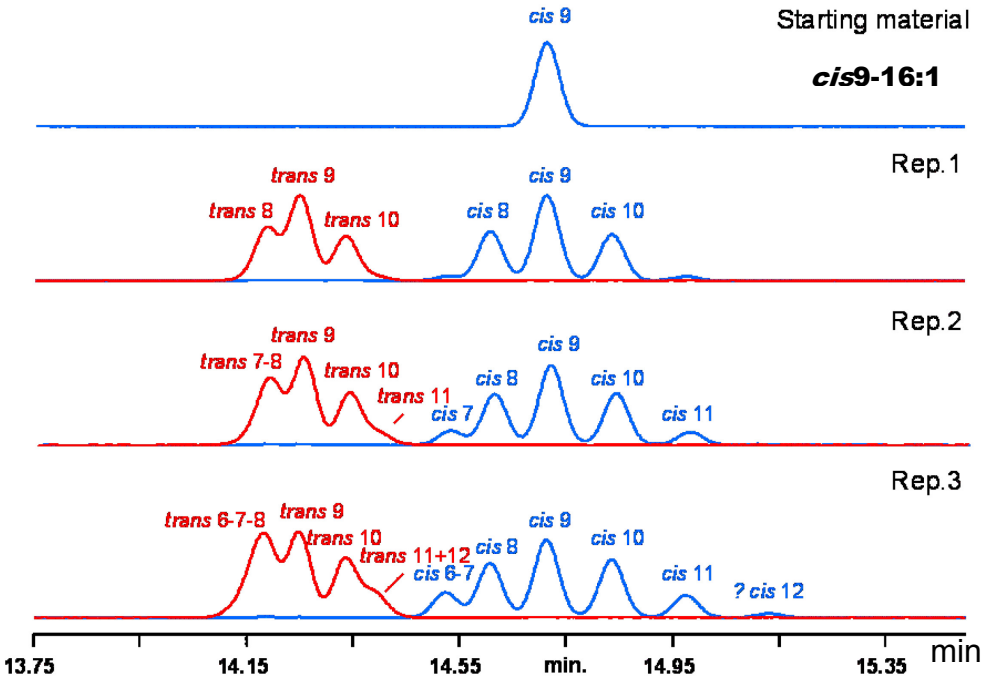


FIGURE 21 GC-FID of purified and PTSA isomerized mixture of 16:1 FAME after three repetitions (Rep.) of addition/elimination reaction of HBr, starting with *cis*9-16:1

Multidimensional Gas Chromatography

For several decades, capillary GC has been the most frequently used technique for the separation of compounds having the volatility needed to repartition between a gas and a liquid phase. Fatty acids are generally derivatized to their FAME prior to GC analysis. To evaluate the new ionic liquid capillary column (SLB-IL111), a 200 m x 0.25 mm column was selected to resolve complex mixtures of FAME prepared from milk fat and fish oil (menhaden oil). These two matrices represent the most complex FA profiles of natural fats that challenges the current GC technology to separate these FAME. Compared to other available methodologies, this method significantly improved the separation of several closely eluting FAME in complex lipid mixtures that include geometrical and positional isomers. For example, a total of more than 150 FAME prepared from milk fat [Chapter 5] and 109 FAME prepared from menhaden oil [Chapter 7] were separated in a single GC analysis.

The official AOCS methodologies optimized for the separation of FAME prepared from vegetable oils and animal fats employs long (100 m) 100% CSP capillary columns to maximize the separation of unsaturated FA positional/geometric isomers. In contrast, the official AOCS methodology developed for the separation of marine oil FAME employ a much shorter CARBOWAX type capillary column of lower polarity that focused on the separation of unsaturated FAME within different chain lengths. Columns coated with CARBOWAX phases are preferred for the separation of marine oil FAME based on the principle that all the PUFA with an *n* carbon alkyl chain elute before the saturated *n*+2 FAME. This approach avoids the overlap of PUFA differing in their chain lengths but jeopardizes the separation of the geometric isomers of unsaturated FAME.

These two different separation strategies applied to the analysis of vegetable, animal and marine oil FAME can be combined in a comprehensive two dimensional GC separation (GC X GC). FAME can be separated first using a highly polar GC column that provides high selectivity toward the position and geometric configuration of double bonds, followed by a separation using a lower polarity short column (1-3 m) that primarily serves to resolve the FAME based on their chain lengths. However, for practical reasons, most of the GC X GC applications reported in the literature for FAME used a low polarity column for the first dimension separation (1D) and a short polar column for the second dimension separation (2D) Despite the higher theoretical peak capacity of these GC X GC systems compared to those of

classic mono-dimensional GC (1D-GC) systems, the selected conditions resulted in a lower number of separated FAME compared to well optimized 1D-GC.

In the current study, a novel approach for GC X GC analysis of FAME was developed that allowed the use of a highly polar column for 1D, and followed by a 2D separation based uniquely on the carbon skeleton of FAME. In some cases, a catalyzer or a reducer was added between the two dimensions of separation before the thermal modulator. The reducer, consisting of a capillary tube coated with palladium on its inner surface, catalyzed the reduction of double bonds, in the presence of hydrogen carrier gas, to their corresponding saturated FAME. The concept of online hydrogenation has been applied in an earlier stage of lipid research primarily to assist in the identification of FAME, including mass spectrometry [Ackman 1989]. The same chromatographic conditions were selected for the 1D separation as were used
for the 1D-GC analyses,

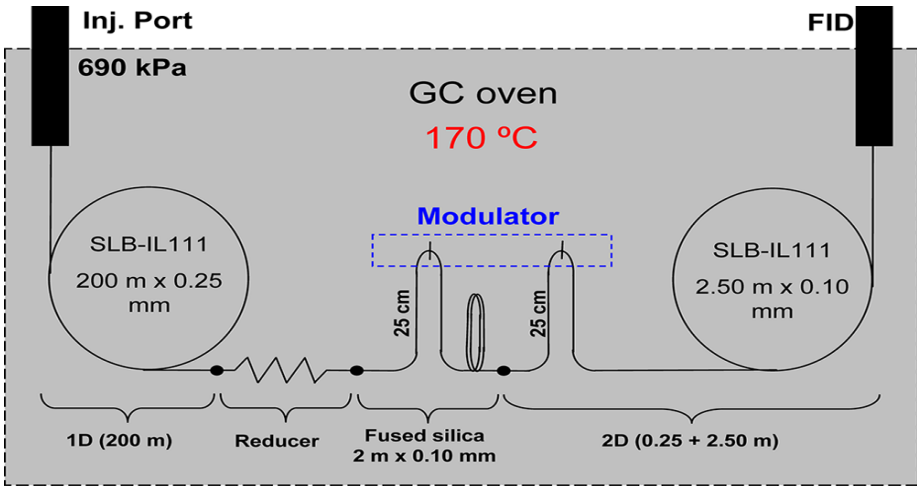


FIGURE 22 Scheme of GC × GC apparatus

which insured the same resolution previously established (Chapter 5). The highly polar 200 m SLB-IL111 column was maintained at 170 °C. After separation on the 1D column, the U-FAME were reduced to their carbon skeleton by the capillary reactor, and the reaction products were subsequently separated using a 2.5 m × 0.10 mm SLB-IL111 maintained at the same temperature as 1D. The preparation of the capillary reactor is described in chapter 6. The scheme of the described GC X GC apparatus is shown in Figure 22.

The GC X GC system used for these separations required joining two capillary columns on each end of the catalytic reducer and the whole system was maintained in the same oven. Figure 23 shows the separation of FAME prepared from menhaden oil, which consists of a

complex mixture of FA. The results of the two dimensional separation shows the elution of all the saturated FAME on a straight diagonal line bisecting the separation plane, while the FAME with the same carbon skeleton but differing in number, geometric configuration or position of double bonds lie on lines parallel to the 1D time axis. The separation of FAME on 2D with different carbon skeletons (i.e. chain length, branches) appear on different lines and avoids the coelution of PUFA with different chain lengths, which presents a challenge when peaks co-elute even when using high polarity GC columns. In the marine oil sample, the 16:1 FAME were separated from 17:0 (*n*-, *i*- and *ai*-) and from methyl phytanate. The 16:2, 16:3 and 16:4 FAME were separated from the C17, C18, C19 and C20 FAME. However, the 17:1 and 19:1 FAME, which are often ignored because of their co-elution and low abundance, were clearly identified and quantified. A particular challenge for *trans* fat quantitation has been the resolution and identification of the 18:3 and 20:1 FAME. Now there was no interference of the two sets of FA isomers and all the individual isomers were well resolved on different lines. The current GC-online reduction X GC system described took advantage of the far superior separation reported using a 200 m SLB-IL111 by 1D-GC [Chapter 7], and made use of the GC X GC technique [Chapter 6] provided a means to resolve FAME based on differences in chain length, thus avoiding several inescapable overlaps.

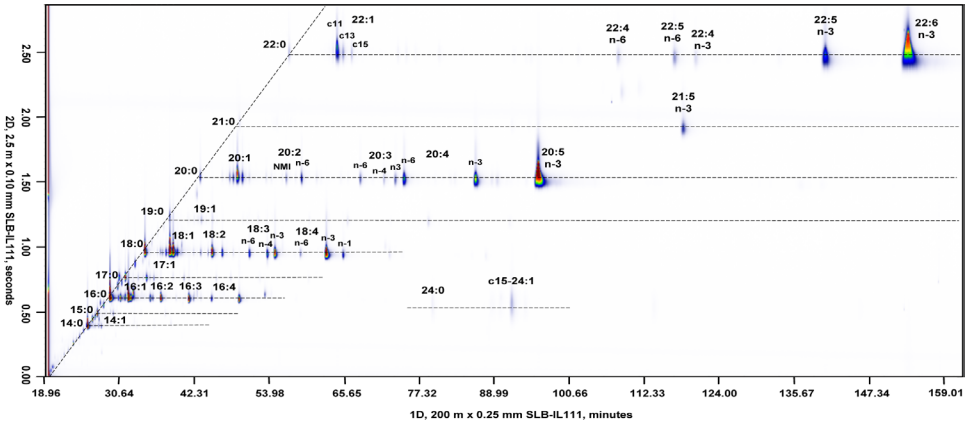


FIGURE 23 GC × GC separation of the FAME prepared from menhaden fish oil sample

While the 170 °C isothermal GC-online reduction x GC elution of FAME using a 200 m x 0.25 mm SLB-IL111 for 1D allowed for direct comparison of elution patterns and quantitative results, the experimental conditions were further optimized to take greater advance of the GC x GC capabilities. The convenience of a single oven design was maintained, but a 100 m column was selected in the 1D separation and an elution temperature program [Chapter 6] was carried out. Figure 24 shows the separation of the FAME of menhaden oil utilizing the modified system. The separation time of the second dimension was shortened to 2 s, which resulted in the late eluting long chain PUFA eluting as sharper 1D peaks that lowered the limits of quantification. The saturated FAME with at least 14 carbons now eluted in an almost straight line parallel to the 1D time axis, and FAME with the same chain lengths eluted on crescent curves. The separation space was now better utilized, and the analytes (FAME) were distributed over most of the plane. The short 1D separation time of ~90 min made this set of experimental conditions more suitable for routine analysis. This is the first reported application of GC – online reduction x GC, but further optimization is expected. The unique FAME separations reported in this study provided confidence that this technique will become the new standard in the analysis of complex lipid mixtures.

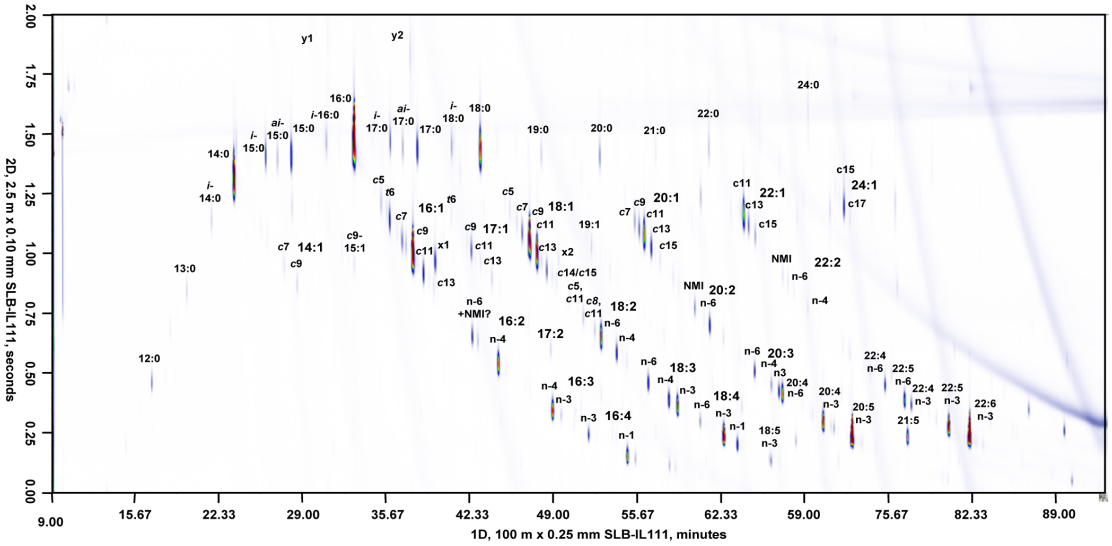


FIGURE 24 GC × GC separation of the FAME prepared from menhaden fish oil sample. Instrument configuration as in Figure 23, with modifications: ^{1}D column 100 m x 0.25 mm SLB-IL111, and temperature program (130 ˚C per 23 min, then increased 1.3 ˚C to 220 ˚C)

Pine Nut Species in the U.S. Market

The nutritious nature of nuts from certain *Pinus* species and their use as a dietary staple has been recognized for hundreds or thousands of years [Evaristo et al., 2010]. However, lately some consumers report taste disturbances (i.e., dysguesia) following the consumption of pine nuts. Pine nut dysguesia, often referred to as "pine mouth" or "pine nut syndrome (PNS)", was first described in 2001 [Mostin, 2001]. Consumer complaints include a bitter or metallic taste in the mouth which is often intensified by the ingestion with certain foods. These symptoms appear 1-2 days after ingestion and may persist from 8 days to 2 weeks after consumption of the pine nuts [Mostin, 2001]. To date, no specific chemical compound in consumer complaint samples has been identified as a potentially causative agent for the taste disturbances.

The identification of the species of pine nuts in commercial samples became an area of intense investigation on the basis of a report by the French Food Safety Agency that nuts from the species *P. armandii* were associated with consumer complaints [AFSAA, 2009]. They hypothesized that consumption of pine nuts from *P. armandii*, a species not previously consumed in some markets, might be linked to the taste disturbances.

Use of gas chromatographic analysis of the FA in pine nuts was a possible strategy for identification of pine nut species. A review of the FA content of 144 *Pinus* species [Flesch et al., 2011] showed species-specific FA profiles. These species-specific differences in individual FA made it possible to establish a "diagnostic index" (DI) capable of differentiating among different *Pinus* species [Destaillats et al., 2010]. However, the DI values and the content of some of the individual FA were shown to overlap in several species [Chapter 8]. These overlaps occurred in part because of differences in the content of individual FA within species, genetic variations, the choice of the chromatographic method (including GC column) and the problems with identification of individual FA. It was fundamental to use gas chromatographic techniques to correctly identify specific FA in species identification. This proved challenging since standards for some of the unique FA were not commercially available.

In this study, the FA composition of a series of reference pine nuts and samples from the US market, including samples associated with pine mouth were evaluated, including the DI calculations used to differentiate among pine nut species. For example, the content of

5,9,12,15-18:4 in several species were: *P. armandii*, 0.08-0.12%; *P.tabuliformis*, 0.40%; *P. wallichiana*, 0.15-0.18%; *P. yunnanensis*, 0.50%. Another study reported the following contents of 5,9,12,15-18:4: *P. armandii*, 0.0-0.04%; *P. tabuliformis*, 0.06%; *P. wallichiana*, 0.09 %; *P. yunnanensis*, 0.0-0.1% [Chapter 8],.

These observations indicate that the DI is strongly dependent on the chromatographic method used and the authentification of the pine species. An DI value of a mixed sample of pine nuts is insufficient for certain identification. However, it was found out that the DIs of homogeneous samples (samples containing only one pine species) were reproducible and a valuable indicator.

The genetic variation in *P. armandii* within a limited geographic area in China [Chen and Chen, 2011] suggests that different subspecies or varieties might be included in commodities for international trade. According to The International Plant Names Index [IPNI.org, 2012], the recognized subspecies and varieties of interest in this regard include *P. armandii var. amamiana, P.armandii* Franch (*P. armandii var. armandii)*, *P. armandii var. dabeshanensis, P.armandii var. farjonii, P. armandii var. fenzeliana, P. armandii var. mastersiana*, *P.armandii subsp. mastersiana*, and *P. armandii subsp. yuana*.

Currently, pine nuts in international trade are not required to be identified at the species or varietal level. It is also difficult to obtain authentic reference pine nut samples from the varieties of interest. In spite of such difficulties, DNA analysis proved to be a potentially powerful method for identifying species and subspecies of *P. armandii.*

Because of the complexities associated with identifying pine nut mixtures based on DI values alone, a DNA-based method was developed that more clearly identified the presence of different *Pinus* species in samples associated with “pine mouth” and other commercially available pine nuts samples [Handy et al., 2011 & Chapter 8]. The genetic assessment of 17 samples associated with pine mouth confirmed that 70% of these samples were a mixture of *P. armandii* and other pine species such as *P. koraiensis*.

Previous attempts to differentiate among pine nuts prior to their analysis were based on their morphological attributes (e.g., length of nuts, diameter, color, etc.). It was found that pre-selection of pine nuts based on their morphological characteristics may be neither efficient nor

accurate. Based on overall length and shape, it was observed that seeds of *P. gerardiana* and *P. pinea* L. were difficult to distinguish, as were seeds of *P. sibirica* and *P. armandii* [Chapter 8].

As a result of these studies, it was possible to calculate more accurate DI values that were based on the separation of the two 18:1 isomers (*c*9-18:1 and *c*11-18:1) that were not resolved in a previous report [Destaillats et al., 2010]. According to Wolff et al., [1997], the isomer *c*9-18:1 is the precursor of *c*5,*c*9-18:2 (taxoleic acid) and only this isomer should be, and was, included in the present DI calculations. It was demonstrated that a combination of DNA analysis and proper GC analysis was a very useful approach for identification and characterization of *Pinus* species. This work also clearly demonstrated that the majority of pine nuts available in the U.S. market, including those associated with pine mouth, were mixtures of nuts from different *Pinus* species.

REFERENCES

Ackman, R. G. (1967). "Calculation of ECL values in the gas-liquid chromatography of multiple-branched fatty acids." J Chromatogr 28(2): 225-231.
Ackman, R. G. (1967). "The chain-length overlap problem in gas-liquid chromatography with polyester liquid phases." Lipids 2(6): 502-505.
Ackman, R. G. and R. P. Hansen (1967). "The occurrence of diastereomers of phytanic and pristanic acids and their determination by gas-liquid chromatography." Lipids 2(5): 357-362.
Ackman, R. G. and S. N. Hooper (1968). Examination of Isoprenoid Fatty Acids as Distinguishing Characteristics of Specific Marine Oils with Particular Reference to Whale Oils, Pergamon Press.
Ackman, R. G., S. N. Hooper and D. L. Hooper (1974). "Linolenic acid artifacts from the deodorization of oils." Journal of the American Oil Chemists Society 51(3): 42-49.
Ackman, R. G., L. Safe, S. N. Hooper, M. Paradis and S. Safe (1973). "7-Methyl-7-hexadecenoic acid: Isolation from lipids of the ocean sunfishMola mola (Linnaeus) 1758." Lipids 8(1): 21-24.
Adahchour, M., J. Beens, R. J. J. Vreuls and U. A. T. Brinkman (2006). "Recent developments in comprehensive two-dimensional gas chromatography (GC × GC): I. Introduction and instrumental set-up." TrAC Trends in Analytical Chemistry 25(5): 438-454.
Adahchour, M., E. Jover, J. Beens, R. J. Vreuls and U. A. Brinkman (2005). "Twin comprehensive two-dimensional gas chromatographic system: concept and applications." J Chromatogr A 1086(1-2): 128-134.
Adam, F., F. Bertoncini, V. Coupard, N. Charon, D. Thiebaut, D. Espinat and M. C. Hennion (2008). "Using comprehensive two-dimensional gas chromatography for the analysis of oxygenates in middle distillates I. Determination of the nature of biodiesels blend in diesel fuel." J Chromatogr A 1186(1-2): 236-244.
Adlof, R. O., L. C. Copes and E. A. Emken (1995). "Analysis of the monoenoic fatty acid distribution in hydrogenated vegetable oils by silver-ion high-performance liquid chromatography." Journal of the American Oil Chemists' Society 72(5): 571-574.
Aldai, N., M. E. Dugan, J. K. Kramer, P. S. Mir and T. A. McAllister (2008). "Nonionophore antibiotics do not affect the trans-18:1 and conjugated linoleic acid composition in beef adipose tissue." J Anim Sci 86(12): 3522-3532.
Aldai, N., M. E. R. Dugan, D. C. Rolland and J. K. G. Kramer (2009). "Survey of the fatty acid composition of Canadian beef: Backfat and longissimus lumborum muscle." Canadian Journal of Animal Science 89(3): 315-329.
Aldai, N., G. Hervas, A. Belenguer, P. Frutos, A. R. Mantecon and J. K. Kramer (2012). "Evaluating the in vitro metabolism of docosahexaenoic acid in sheep rumen fluid." Lipids 47(8): 821-825.
Alves, S. P. and R. J. Bessa (2009). "Comparison of two gas-liquid chromatograph columns for the analysis of fatty acids in ruminant meat." J Chromatogr A 1216(26): 5130-5139.
Anderson, J. L. and D. W. Armstrong (2003). "High-Stability Ionic Liquids. A New Class of Stationary Phases for Gas Chromatography." Analytical Chemistry 75(18): 4851-4858.
Anderson, J. L. and D. W. Armstrong (2003). "High-stability ionic liquids. A new class of stationary phases for gas chromatography." Anal Chem 75(18): 4851-4858.
Anderson, J. L. and D. W. Armstrong (2005). "Immobilized ionic liquids as high-selectivity/high-temperature/high-stability gas chromatography stationary phases." Anal Chem 77(19): 6453-6462.

Anderson, J. L. and D. W. Armstrong (2005). "Immobilized Ionic Liquids as High-Selectivity/High-Temperature/High-Stability Gas Chromatography Stationary Phases." Analytical Chemistry 77(19): 6453-6462.
Anderson, J. L., J. Ding, T. Welton and D. W. Armstrong (2002). "Characterizing Ionic Liquids On the Basis of Multiple Solvation Interactions." Journal of the American Chemical Society 124(47): 14247-14254.
Anderson, J. L., J. Ding, T. Welton and D. W. Armstrong (2002). "Characterizing ionic liquids on the basis of multiple solvation interactions." J Am Chem Soc 124(47): 14247-14254.
Anderson, J. L., R. Ding, A. Ellern and D. W. Armstrong (2004). "Structure and Properties of High Stability Geminal Dicationic Ionic Liquids." Journal of the American Chemical Society 127(2): 593-604.
Anderson, J. L., R. Ding, A. Ellern and D. W. Armstrong (2005). "Structure and properties of high stability geminal dicationic ionic liquids." J Am Chem Soc 127(2): 593-604.
Armstrong, D. W., L. He and Y. S. Liu (1999). "Examination of ionic liquids and their interaction with molecules, when used as stationary phases in gas chromatography." Anal Chem 71(17): 3873-3876.
Aro, A., W. Becker and J. I. Pederssen (2006). Trans fatty acids in the Nordic countries.
Azizian, H., J. K. G. Kramer, S. Ehler and J. M. Curtis (2010). "Rapid quantitation of fish oil fatty acids and their ethyl esters by FT-NIR models." European Journal of Lipid Science and Technology 112(4): 452-462.
Banni, S., G. Carta, E. Angioni, E. Murru, P. Scanu, M. P. Melis, D. E. Bauman, S. M. Fischer and C. Ip (2001). "Distribution of conjugated linoleic acid and metabolites in different lipid fractions in the rat liver." Journal of Lipid Research 42(7): 1056-1061.
Bannon, C. D., J. D. Craske and L. M. Norman (1988). "Effect of overload of capillary gas-liquid chromatographic columns on the equivalent chain lengths of C18 unsaturated fatty acid methyl esters." Journal of Chromatography A 447(0): 43-52.
Bauman, D. E., D. M. Barbano, D. A. Dwyer and J. M. Griinari (2000). "Technical note: production of butter with enhanced conjugated linoleic acid for use in biomedical studies with animal models." J Dairy Sci 83(11): 2422-2425.
Belury, M. A. (2002). "Dietary conjugated linoleic acid in health: physiological effects and mechanisms of action." Annu Rev Nutr 22: 505-531.
Bertsch, W. (1999). "Two-Dimensional Gas Chromatography. Concepts, Instrumentation, and Applications – Part 1: Fundamentals, Conventional Two-Dimensional Gas Chromatography, Selected Applications." Journal of High Resolution Chromatography 22(12): 647-665.
Beyers, E. C. and E. A. Emken (1991). "Metabolites of cis,trans, and trans,cis isomers of linoleic acid in mice and incorporation into tissue lipids." Biochim Biophys Acta 1082(3): 275-284.
Bousquet, O. and F. Le Goffic (1995). "Counter-current chromatographic separation of polyunsaturated fatty acids." Journal of Chromatography A 704(1): 211-216.
Breitbach, Z. and D. Armstrong (2008). "Characterization of phosphonium ionic liquids through a linear solvation energy relationship and their use as GLC stationary phases." Analytical and Bioanalytical Chemistry 390(6): 1605-1617.
Breitbach, Z. S. and D. W. Armstrong (2008). "Characterization of phosphonium ionic liquids through a linear solvation energy relationship and their use as GLC stationary phases." Anal Bioanal Chem 390(6): 1605-1617.
Burr, G. O. and M. M. Burr (1929). "A NEW DEFICIENCY DISEASE PRODUCED BY THE RIGID EXCLUSION OF FAT FROM THE DIET." Journal of Biological Chemistry 82(2): 345-367.
Burr, G. O. and M. M. Burr (1930). "ON THE NATURE AND RÔLE OF THE FATTY ACIDS ESSENTIAL IN NUTRITION." Journal of Biological Chemistry 86(2): 587-621.

Burr, G. O., M. M. Burr and E. S. Miller (1932). "ON THE FATTY ACIDS ESSENTIAL IN NUTRITION. III." Journal of Biological Chemistry 97(1): 1-9.
Cao, X. and Y. Ito (2003). "Supercritical fluid extraction of grape seed oil and subsequent separation of free fatty acids by high-speed counter-current chromatography." J Chromatogr A 1021(1-2): 117-124.
Carballeira, N. and M. Maldonado (1988). "6-methyl-8-hexadecenoic acid: A novel fatty acid from the marine spongeDesmapsama anchorata." Lipids 23(7): 690-693.
Castello, G., S. Vezzani and G. D'Amato (1997). "Effect of temperature on the polarity of some stationary phases for gas chromatography." Journal of Chromatography A 779(1–2): 275-286.
Christiansen, E., S. Schnider, B. Palmvig, E. Tauber-Lassen and O. Pedersen (1997). "Intake of a diet high in trans monounsaturated fatty acids or saturated fatty acids. Effects on postprandial insulinemia and glycemia in obese patients with NIDDM." Diabetes Care 20(5): 881-887.
Christie, W. W. (1988). "Separation of molecular species of triacylglycerols by high-performance liquid chromatography with a silver ion column." Journal of Chromatography A 454(0): 273-284.
Christie, W. W. (1993). Advances in Lipid Methodology, 2nd edition, The Oily Press.
Christie, W. W. (2003). Lipid Analysis: Isolation, Separation, Identification, and Structural Analysis of Lipids, Amer Oil Chemists Society.
Christie, W. W., E. Y. Brechany and V. K. Shukla (1989). "Analysis of seed oils containing cyclopentenyl fatty acids by combined chromatographic procedures." Lipids 24(2): 116-120.
Christie, W. W. and G. H. M. Breckenridge (1989b). "Separation of cis and trans isomers of unsaturated fatty acids by high-performance liquid chromatography in the silver ion mode." Journal of Chromatography A 469(0): 261-269.
Christie, W. W., F. D. Gunstone, I. A. Ismail and L. Wade (1968). "Fatty acids. 17 The synthesis and chromatographic and spectroscopic properties of the cyclopropane esters derived from all the methyl octadecenoates (delta 2-delta 17)." Chem Phys Lipids 2(2): 196-202.
Clandinin, M. T., S. Cheema, C. J. Field, M. L. Garg, J. Venkatraman and T. R. Clandinin (1991). "Dietary fat: exogenous determination of membrane structure and cell function." The FAEB Journal 5(13): 2761-2769.
Clement, L., H. Poirier, I. Niot, V. Bocher, M. Guerre-Millo, S. Krief, B. Staels and P. Besnard (2002). "Dietary trans-10,cis-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse." J Lipid Res 43(9): 1400-1409.
Cruz-Hernandez, C., Z. Deng, J. Zhou, A. R. Hill, M. P. Yurawecz, P. Delmonte, M. M. Mossoba, M. E. Dugan and J. K. Kramer (2004). "Methods for analysis of conjugated linoleic acids and trans-18:1 isomers in dairy fats by using a combination of gas chromatography, silver-ion thin-layer chromatography/gas chromatography, and silver-ion liquid chromatography." J AOAC Int 87(2): 545-562.
Cruz-Hernandez, C., J. K. G., Kramer, J. J. Kennelly, D. R. Glimm, B. M. Sorensen, E. K. Okine, L. A. Goonewardene and R. J. Weselake (2007). "Evaluating the conjugated linoleic acid and trans 18:1 isomers in milk fat of dairy cows fed increasing amounts of sunflower oil and a constant level of fish oil." J Dairy Sci 90(8): 3786-3801.
Cruz-Hernandez, C., J. K. G., Kramer, J. Kraft, V. Santercole, Or-Rashid, Z. Deng, M. E. R. Dugan, P. Delmonte, M. P. Yurawecz (2006). " Systematic analysis of *trans* and conjugated linoleic acids in milk and meat of ruminants. In: Yurawecz, M. P., J. K. G., Kramer, O., Gudmundsen, M. W., Pariza, S., Banni (eds). Advances in Conjugated Linoleic Acid Research, Volume 3. AOCS Press, Champaign, IL, 2006; pp 45-93.
Dannenberger, D., G. Nuernberg, N. Scollan, W. Schabbel, H. Steinhart, K. Ender and K. Nuernberg (2004). "Effect of diet on the deposition of n-3 fatty acids, conjugated linoleic and

C18:1trans fatty acid isomers in muscle lipids of German Holstein bulls." J Agric Food Chem 52(21): 6607-6615.
De Geus, H. J., I. Aidos, J. de Boer, J. B. Luten and U. A. Brinkman (2001). "Characterisation of fatty acids in biological oil samples using comprehensive multidimensional gas chromatography." J Chromatogr A 910(1): 95-103.
De Roos, N. M., M. L. Bots and M. B. Katan (2001). "Replacement of Dietary Saturated Fatty Acids by Trans Fatty Acids Lowers Serum HDL Cholesterol and Impairs Endothelial Function in Healthy Men and Women." Arteriosclerosis, Thrombosis, and Vascular Biology 21(7): 1233-1237.
Deans, D. R. (1968). "A new technique for heart cutting in gas chromatography [1]." Chromatographia 1(1-2): 18-22.
Degen, C., J. Ecker, S. Piegholdt, G. Liebisch, G. Schmitz and G. Jahreis (2011). "Metabolic and growth inhibitory effects of conjugated fatty acids in the cell line HT-29 with special regard to the conversion of t11,t13-CLA." Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1811(12): 1070-1080.
Delmonte, P., A. R. Fardin-Kia, J. K. Kramer, M. M. Mossoba, L. Sidisky, C. Tyburczy and J. I. Rader (2012). "Evaluation of highly polar ionic liquid gas chromatographic column for the determination of the fatty acids in milk fat." J Chromatogr A 1233: 137-146.
Delmonte, P., A. R. Fardin-Kia and J. I. Rader (2013). "Separation of fatty acid methyl esters by GC-online hydrogenation x GC." Anal Chem 85(3): 1517-1524.
Delmonte, P., A. R. Fardin Kia, J. K. Kramer, M. M. Mossoba, L. Sidisky and J. I. Rader (2011). "Separation characteristics of fatty acid methyl esters using SLB-IL111, a new ionic liquid coated capillary gas chromatographic column." J Chromatogr A 1218(3): 545-554.
Delmonte, P., Q. Hu, A. R. Kia and J. I. Rader (2008). "Preparation, chromatographic separation and relative retention times of cis/trans heptadecaenoic (17:1) fatty acids." J Chromatogr A 1214(1-2): 30-36.
Delmonte, P., A. Kataok, B. A. Corl, D. E. Bauman and M. P. Yurawecz (2005). "Relative retention order of all isomers of cis/trans conjugated linoleic acid FAME from the 6,8- to 13,15-positions using silver ion HPLC with two elution systems." Lipids 40(5): 509-514.
Delmonte, P., A. Kataoka, B. A. Corl, D. E. Bauman and M. Yurawecz (2005). "Relative retention order of all isomers of cis/trans conjugated linoleic acid FAME from the 6,8- to 13,15-positions using silver ion HPLC with two elution systems." Lipids 40(5): 509-514.
Delmonte, P., A. R. Kia, Q. Hu and J. I. Rader (2009). "Review of methods for preparation and gas chromatographic separation of trans and cis reference fatty acids." J AOAC Int 92(5): 1310-1326.
Delmonte, P. and J. I. Rader (2007). "Evaluation of gas chromatographic methods for the determination of trans fat." Anal Bioanal Chem 389(1): 77-85.
Delmonte, P., J. A. Roach, M. M. Mossoba, K. M. Morehouse, L. Lehmann and M. P. Yurawecz (2003). "Synthesis and isolation of trans-7,cis-9 octadecadienoic acid and other CLA isomers by base conjugation of partially hydrogenated gamma-linolenic acid." Lipids 38(5): 579-583.
Delmonte, P., M. P. Yurawecz, M. M. Mossoba, C. Cruz-Hernandez and J. K. Kramer (2004). "Improved identification of conjugated linoleic acid isomers using silver-ion HPLC separations." J AOAC Int 87(2): 563-568.
Destaillats, F., C. Cruz-Hernandez, F. Giuffrida and F. Dionisi (2010). "Identification of the botanical origin of pine nuts found in food products by gas-liquid chromatography analysis of fatty acid profile." J Agric Food Chem 58(4): 2082-2087.
Destaillats, F., C. Cruz-Hernandez, F. Giuffrida, F. Dionisi, M. Mostin and G. Verstegen (2011). "Identification of the Botanical Origin of Commercial Pine Nuts Responsible for Dysgeusia by Gas-Liquid Chromatography Analysis of Fatty Acid Profile." Journal of Toxicology 2011.

Destaillats, F., P. A. Golay, F. Joffre, M. de Wispelaere, B. Hug, F. Giuffrida, L. Fauconnot and F. Dionisi (2007). "Comparison of available analytical methods to measure trans-octadecenoic acid isomeric profile and content by gas-liquid chromatography in milk fat." J Chromatogr A 1145(1-2): 222-228.
Destaillats, F., M. Guitard and C. Cruz-Hernandez (2011). "Identification of Delta6-monounsaturated fatty acids in human hair and nail samples by gas-chromatography-mass-spectrometry using ionic-liquid coated capillary column." J Chromatogr A 1218(52): 9384-9389.
Destaillats, F., R. L. Wolff, D. Precht and J. Molkentin (2000). "Study of individual trans- and cis-16:1 isomers in cow, goat, and ewe cheese fats by gas-liquid chromatography with emphasis on the trans-delta3 isomer." Lipids 35(9): 1027-1032.
Destaillats, F. d. r., C. Cruz-Hernandez, F. Giuffrida and F. Dionisi (2010). "Identification of the Botanical Origin of Pine Nuts Found in Food Products by Gas−Liquid Chromatography Analysis of Fatty Acid Profile." Journal of Agricultural and Food Chemistry 58(4): 2082-2087.
Dijkstra, A. J. (2006). "Revisiting the formation of trans isomers during partial hydrogenation of triacylglycerol oils." European Journal of Lipid Science and Technology 108(3): 249-264.
Doell, D., D. Folmer, H. Lee, M. Honigfort and S. Carberry (2012). "Updated estimate of trans fat intake by the US population." Food Addit Contam Part A Chem Anal Control Expo Risk Assess 29(6): 861-874.
Dole, V. P., A. T. James, J. P. Webb, M. A. Rizack and M. F. Sturman (1959). "The fatty acid patterns of plasma lipids during alimentary lipemia." J Clin Invest 38: 1544-1554.
Duhem, K. (2009). “Legislation relating to *trans* fatty acids”. In: Destaillats, F. (ed), Sebedio, J. L., Dionisi, F., Chardigny J. M., *Trans* fatty acids in human nutrition, second edition, The Oily Press, Bridgwater, England (2009), pp 381-393
Dutriez, T., J. Borras, M. Courtiade, D. Thiebaut, H. Dulot, F. Bertoncini and M. C. Hennion (2011). "Challenge in the speciation of nitrogen-containing compounds in heavy petroleum fractions by high temperature comprehensive two-dimensional gas chromatography." J Chromatogr A 1218(21): 3190-3199.
Eifert, E. d. C., R. d. P. Lana, D. P. D. Lanna, P. B. Arcuri, M. I. Leão, S. d. C. Valadares Filho, W. M. Leopoldino and J. H. S. d. Silva (2005). "Efeitos do fornecimento de monensina e óleo de soja na dieta sobre o desempenho de vacas leiteiras na FAe inicial da lactação." Revista Brasileira de Zootecnia 34: 2123-2132.
Eulitz, K., M. P. Yurawecz, N. Sehat, J. Fritsche, J. A. Roach, M. M. Mossoba, J. K. Kramer, R. O. Adlof and Y. Ku (1999). "Preparation, separation, and confirmation of the eight geometrical cis/trans conjugated linoleic acid isomers 8,10- through 11,13-18:2." Lipids 34(8): 873-877.
Evaristo, I., D. Batista, I. Correia, P. Correia and R. Costa (2010). "Chemical profiling of Portuguese Pinus pinea L. nuts." J Sci Food Agric 90(6): 1041-1049.
Fardin-Kia, A. R., S. M. Handy and J. I. Rader (2012). "Characterization of pine nuts in the U.S. market, including those associated with "pine mouth", by GC-FID." J Agric Food Chem 60(10): 2701-2711.
Farmer, E. H. and D. A. Sutton (1943). "37. The course of autoxidation reactions in polyisoprenes and allied compounds. Part IV. The isolation and constitution of photochemically-formed methyl oleate peroxide." Journal of the Chemical Society (Resumed) 0(0): 119-122.
Fazekas, A. J., K. S. Burgess, P. R. Kesanakurti, S. W. Graham, S. G. Newmaster, B. C. Husband, D. M. Percy, M. Hajibabaei and S. C. Barrett (2008). "Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well." PLoS One 3(7): e2802.

Feller, S. E. and K. Gawrisch (2005). "Properties of docosahexaenoic-acid-containing lipids and their influence on the function of rhodopsin." Curr Opin Struct Biol 15(4): 416-422.
Ferdinandusse, S., H. Rusch, A. E. M. van Lint, G. Dacremont, R. J. A. Wanders and P. Vreken (2002). "Stereochemistry of the peroxisomal branched-chain fatty acid α- and β-oxidation systems in patients suffering from different peroxisomal disorders." Journal of Lipid Research 43(3): 438-444.
Field, C. J., H. H. Blewett, S. Proctor and D. Vine (2009). "Human health benefits of vaccenic acid." Applied Physiology, Nutrition, and Metabolism 34(5): 979-991.
Firestone, S., A. Sheppard (1992). "Determination of *trans* fatty acids. In: Christie WW 9ed), Advances in Lipid Methodology-One, The Oily Press, Ayr, UK, 1992; pp 273-256
Flesch, F., F. Rigaux-Barry, P. Saviuc, R. Garnier, J. Daoudi, I. Blanc, S. S. Tellier and L. Lasbeur (2011). "Dysgeusia following consumption of pine nuts: More than 3000 cases in France." Clinical Toxicology 49(7): 668-670.
Fournier, V., F. Destaillats, P. Juanéda, F. Dionisi, P. Lambelet, J.-L. Sébédio and O. Berdeaux (2006a). "Thermal degradation of long-chain polyunsaturated fatty acids during deodorization of fish oil." European Journal of Lipid Science and Technology 108(1): 33-42.
Fournier, V., P. Juaneda, F. Destaillats, F. Dionisi, P. Lambelet, J. L. Sebedio and O. Berdeaux (2006). "Analysis of eicosapentaenoic and docosahexaenoic acid geometrical isomers formed during fish oil deodorization." J Chromatogr A 1129(1): 21-28.
Freeman Allen, C., P. Good, H. Davis, P. Chisum and S. Fowler (1966). "Methodology for the separation o plant lipids and application to spinach leaf and chloroplast lamellae." Journal of the American Oil Chemists' Society 43(4): 223-231.
Fritsche, J. and H. Steinhart (1997). "Contents of trans fatty acids (TFA) in German foods and estimation of daily intake." Lipid / Fett 99(9): 314-318.
Fritsche, S., T. Rumsey, M. Yurawecz, Y. Ku and J. Fritsche (2001). "Influence of growth promoting implants on fatty acid composition including conjugated linoleic acid isomers in beef fat." European Food Research and Technology 212(6): 621-629.
Gardner, H. W. (1989). "Oxygen radical chemistry of polyunsaturated fatty acids." Free Radic Biol Med 7(1): 65-86.
Gebauer, S. K., J. M. Chardigny, M. U. Jakobsen, B. Lamarche, A. L. Lock, S. D. Proctor and D. J. Baer (2011). "Effects of ruminant trans fatty acids on cardiovascular disease and cancer: a comprehensive review of epidemiological, clinical, and mechanistic studies." Adv Nutr 2(4): 332-354.
Giddings, J. C. (1995). "Sample dimensionality: a predictor of order-disorder in component peak distribution in multidimensional separation." J Chromatogr A 703(1-2): 3-15.
Glass, R. L., T. P. Krick, D. M. Sand, C. H. Rahn and H. Schlenk (1975). "Furanoid fatty acids from fish lipids." Lipids 10(11): 695-702.
Golay, P. A., F. Giuffrida, F. Dionisi and F. Destaillats (2009). "Streamlined methods for the resolution and quantification of fatty acids including trans fatty acid isomers in food products by gas chromatography." J AOAC Int 92(5): 1301-1309.
Gómez-Cortés, P., C. Tyburczy, J. T. Brenna, M. Juárez and M. A. de la Fuente (2009). "Characterization of cis-9 trans-11 trans-15 C18:3 in milk fat by GC and covalent adduct chemical ionization tandem MS." Journal of Lipid Research 50(12): 2412-2420.
Griinari, J. M., D. A. Dwyer, M. A. McGuire, D. E. Bauman, D. L. Palmquist and K. V. Nurmela (1998). "Trans-octadecenoic acids and milk fat depression in lactating dairy cows." J Dairy Sci 81(5): 1251-1261.
Gu, Q., F. David, F. Lynen, P. Vanormelingen, W. Vyverman, K. Rumpel, G. Xu and P. Sandra (2011). "Evaluation of ionic liquid stationary phases for one dimensional gas chromatography-mass spectrometry and comprehensive two dimensional gas chromatographic analyses of fatty acids in marine biota." J Chromatogr A 1218(20): 3056-3063.

Gunstone, F. D., I. A. Ismail and M. Lie ken jie (1967). "Fatty acids, part 16. Thin layer and gas—liquid chromatographic properties of the cis and trans methyl octadecenoates and of some acetylenic esters." Chemistry and Physics of Lipids 1(4): 376-385.
Han, S. N., L. S. Leka, A. H. Lichtenstein, L. M. Ausman, E. J. Schaefer and S. N. Meydani (2002). "Effect of hydrogenated and saturated, relative to polyunsaturated, fat on immune and inflammatory responses of adults with moderate hypercholesterolemia." J Lipid Res 43(3): 445-452.
Handy, S. M., M. B. Parks, J. R. Deeds, A. Liston, L. S. de Jager, S. Luccioli, E. Kwegyir-Afful, A. R. Fardin-Kia, T. H. Begley, J. I. Rader and G. W. Diachenko (2011). "Use of the chloroplast gene ycf1 for the genetic differentiation of pine nuts obtained from consumers experiencing dysgeusia." J Agric Food Chem 59(20): 10995-11002.
Harfoot, C. G. and G. P. Hazlewood (1997). Lipid metabolism in the rumen. The Rumen Microbial Ecosystem. P. N. Hobson and C. S. Stewart, Springer Netherlands: 382-426.
Harynuk, J., B. Vlaeminck, P. Zaher and P. J. Marriott (2006). "Projection of multidimensional GC data into alternative dimensions-exploiting sample dimensionality and structured retention patterns." Anal Bioanal Chem 386(3): 602-613.
Henneberg, D., U. Henrichs and G. Schomburg (1975). "Special techniques in the combination of gas chromatography and mass spectrometry." Journal of Chromatography A 112(0): 343-352.
Herb, S. F., P. Magidman, F. Luddy and R. W. Riemenschneider (1962). "Fatty acids of cows' milk. B. Composition by gas-liquid chromatography aided by other methods of fractionation." Journal of the American Oil Chemists Society 39(3): 142-146.
Hobson, P. N. and C. S. Stewart (1997). Rumen Microbial Ecosystem, Springer.
Hotamisligil, G. S. (2005). "Role of endoplasmic reticulum stress and c-Jun NH2-terminal kinase pathways in inflammation and origin of obesity and diabetes." Diabetes 54 Suppl 2: S73-78.
Huang, K., X. Han, X. Zhang and D. Armstrong (2007). "PEG-linked geminal dicationic ionic liquids as selective, high-stability gas chromatographic stationary phases." Analytical and Bioanalytical Chemistry 389(7-8): 2265-2275.
Huang, K., X. Han, X. Zhang and D. W. Armstrong (2007). "PEG-linked geminal dicationic ionic liquids as selective, high-stability gas chromatographic stationary phases." Anal Bioanal Chem 389(7-8): 2265-2275.
Huebschmann, H. J. (2008). Handbook of GC/MS, Wiley.
Hulshof, K. F., M. A. van Erp-Baart, M. Anttolainen, W. Becker, S. M. Church, C. Couet, E. Hermann-Kunz, H. Kesteloot, T. Leth, I. Martins, O. Moreiras, J. Moschandreas, L. Pizzoferrato, A. H. Rimestad, H. Thorgeirsdottir, J. M. van Amelsvoort, A. Aro, A. G. Kafatos, D. Lanzmann-Petithory and G. van Poppel (1999). "Intake of fatty acids in western Europe with emphasis on trans fatty acids: the TRANSFAIR Study." Eur J Clin Nutr 53(2): 143-157.
Ishii, K., H. Okajima, T. Koyamatsu, Y. Okada and H. Watanabe (1988). "The composition of furan fatty acids in the crayfish." Lipids 23(7): 694-700.
Ito, Y. (2005). "Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography." J Chromatogr A 1065(2): 145-168.
James, A. T. and A. J. Martin (1956). "Gas-liquid chromatography: the separation and identification of the methyl esters of saturated and unsaturated acids from formic acid to n-octadecanoic acid." Biochem J 63(1): 144-152.
Jensen, R. G. (2002). "The Composition of Bovine Milk Lipids: January 1995 to December 2000." Journal of Dairy Science 85(2): 295-350.
Juaneda, P. (2002). "Utilisation of reversed-phase high-performance liquid chromatography as an alternative to silver-ion chromatography for the separation of cis- and trans-C18:1 fatty acid isomers." J Chromatogr A 954(1-2): 285-289.

Kairenius, P., V. Toivonen and K. Shingfield (2011). "Identification and Ruminal Outflow of Long-Chain Fatty Acid Biohydrogenation Intermediates in Cows Fed Diets Containing Fish Oil." Lipids 46(7): 587-606.
Kairenius, P., V. Toivonen and K. J. Shingfield (2011). "Identification and ruminal outflow of long-chain fatty acid biohydrogenation intermediates in cows fed diets containing fish oil." Lipids 46(7): 587-606.
Köbler, H., Y. B. Monakhova, T. Kuballa, C. Tschiersch, J. Vancutsem, G. Thielert, A. Mohring and D. W. Lachenmeier (2011). "Nuclear Magnetic Resonance Spectroscopy and Chemometrics to Identify Pine Nuts That Cause Taste Disturbance." Journal of Agricultural and Food Chemistry 59(13): 6877-6881.
Kodali, D. R. and G. R. List (2005). Trans fats alternatives, AOCS Press [Imprint].
Kolanowski, W. and G. Laufenberg (2006). "Enrichment of food products with polyunsaturated fatty acids by fish oil addition." European Food Research and Technology 222(3-4): 472-477.
Kraft, J., M. Collomb, P. Möckel, R. Sieber and G. Jahreis (2003). "Differences in CLA isomer distribution of cow's milk lipids." Lipids 38(6): 657-664.
Kramer, J. G., C. B. Blackadar and J. Zhou (2002). "Evaluation of two GC columns (60-m SUPELCOWAX 10 and 100-m CP sil 88) for analysis of milkfat with emphasis on CLA, 18 -and long-chain 18 FA." Lipids 37(8): 823-835.
Kramer, J. G., C. Cruz-Hernandez, M. Or-Rashid and M. R. Dugan (2004). "The use of total trans-11 containing FA, rather than total "n−7" FA, Is recommended to assess the content of FA with a positive health image in ruminant fats." Lipids 39(7): 693-695.
Kramer, J. G., V. Fellner, M. R. Dugan, F. Sauer, M. Mossoba and M. Yurawecz (1997). "Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids." Lipids 32(11): 1219-1228.
Kramer, J. G., M. Hernandez, C. Cruz-Hernandez, J. Kraft and M. R. Dugan (2008). "Combining Results of Two GC Separations Partly Achieves Determination of All cis and trans 16:1, 18:1, 18:2 and 18:3 Except CLA Isomers of Milk Fat as Demonstrated Using Ag-Ion SPE Fractionation." Lipids 43(3): 259-273.
Kramer, J. G., P. Parodi, R. Jensen, M. Mossoba, M. Yurawecz and R. Adlof (1998). "Rumenic acid: A proposed common name for the major conjugated linoleic acid isomer found in natural products." Lipids 33(8): 835-835.
Kramer, J. K., M. Hernandez, C. Cruz-Hernandez, J. Kraft and M. E. Dugan (2008). "Combining results of two GC separations partly achieves determination of all cis and trans 16:1, 18:1, 18:2 and 18:3 except CLA isomers of milk fat as demonstrated using Ag-ion SPE fractionation." Lipids 43(3): 259-273.
Kramer, J. K. G., C. Cruz-Hernandez and J. Zhou (2001). "Conjugated linoleic acids and octadecenoic acids: Analysis by GC." European Journal of Lipid Science and Technology 103(9): 600-609.
Kramer, J. K. G., R. C. Fouchard and K. J. Jenkins (1985). "Differences in Chromatographic Properties of Fused Silica Capillary Columns, Coated, Crosslinked, Bonded, or Crosslinked and Bonded with Polyethylene Glycols (Carbowax 20M) Using Complex Fatty Acid Methyl Ester Mixtures." Journal of Chromatographic Science 23(2): 54-56.
Kramer, J. K. G. and J. Zhou (2001). "Conjugated linoleic acid and octadecenoic acids: Extraction and isolation of lipids." European Journal of Lipid Science and Technology 103(9): 594-600.
Kramer, J. K. G., M. M., Mossoba (eds) (2009). "Official Methods for the Determination of *Trans* Fat." 2nd edition, AOCS Press, Urbana, IL, USA.
Kuhnt, K., M. Baehr, C. Rohrer and G. Jahreis (2011). "Trans fatty acid isomers and the trans-9/trans-11 index in fat containing foods." Eur J Lipid Sci Technol 113(10): 1281-1292.

Kwegyir-Afful, E., L. DeJager, T. Begley and S. Luccioli (2011). "Pine mouth syndrome - an emerging food hypersensitivity?" Clinical and Translational Allergy 1(Suppl 1): P25.
Lawrence, P. and J. T. Brenna (2006). "Acetonitrile covalent adduct chemical ionization mass spectrometry for double bond localization in non-methylene-interrupted polyene fatty acid methyl esters." Anal Chem 78(4): 1312-1317.
Lee, J.-W., K.-W. Lee, S.-W. Lee, I.-H. Kim and C. Rhee (2004). "Selective increase in pinolenic acid (all-cis-5,9,12–18 : 3) in Korean LDL-receptor activity." Lipids 39(4): 383-387.
Leheska, J. M., L. D. Thompson, J. C. Howe, E. Hentges, J. Boyce, J. C. Brooks, B. Shriver, L. Hoover and M. F. Miller (2008). "Effects of conventional and grass-feeding systems on the nutrient composition of beef." J Anim Sci 86(12): 3575-3585.
Lichtenstein, A. H., L. M. Ausman, S. M. Jalbert and E. J. Schaefer (1999). "Effects of Different Forms of Dietary Hydrogenated Fats on Serum Lipoprotein Cholesterol Levels." New England Journal of Medicine 340(25): 1933-1940.
Luke, L. L. and J. V. Brunnock (1968). “Separation of naphtenic and parafinic hydrocarbons up to C11 from hydrocarbon mixtures by gas chromatography on faujasite molecular sieves.” Ger. Offen., 1, 908418
Mahe, L., T. Dutriez, M. Courtiade, D. Thiebaut, H. Dulot and F. Bertoncini (2011). "Global approach for the selection of high temperature comprehensive two-dimensional gas chromatography experimental conditions and quantitative analysis in regards to sulfur-containing compounds in heavy petroleum cuts." J Chromatogr A 1218(3): 534-544.
Mathur, G. P. and V. M. Gandhi (1972). "PROSTAGLANDIN IN HUMAN AND ALBINO RAT SKIN." J Investig Dermatol 58(5): 291-295.
Michaud, A. L., P. Lawrence, R. Adlof and J. T. Brenna (2005). "On the formation of conjugated linoleic acid diagnostic ions with acetonitrile chemical ionization tandem mass spectrometry." Rapid Commun Mass Spectrom 19(3): 363-368.
Michaud, A. L., M. P. Yurawecz, P. Delmonte, B. A. Corl, D. E. Bauman and J. T. Brenna (2003). "Identification and characterization of conjugated fatty acid methyl esters of mixed double bond geometry by acetonitrile chemical ionization tandem mass spectrometry." Anal Chem 75(18): 4925-4930.
Milosevic, M., V. Milosevic, J. K.G., Kramer, H., Azizian, M. M., Mossoba. (2004). “Determining low levels of trans fatty acids in foods by an improved ATR-FTIR procedure.” Lipid Technol., 16, 229-231
Mjos, S. A. (2003). "Identification of fatty acids in gas chromatography by application of different temperature and pressure programs on a single capillary column." J Chromatogr A 1015(1-2): 151-161.
Mjøs, S. A. (2006b). "Prediction of equivalent chain lengths from two-dimensional fatty acid retention indices." Journal of Chromatography A 1122(1–2): 249-254.
Mjøs, S. A. and M. Solvang (2006a). "Geometrical isomerisation of eicosapentaenoic and docosahexaenoic acid at high temperatures." European Journal of Lipid Science and Technology 108(7): 589-597.
Molkentin, J. and D. Precht (1995). "Optimized analysis oftrans-octadecenoic acids in edible fats." Chromatographia 41(5-6): 267-272.
Momchilova, S., B. Nikolova-Damyanova and W. W. Christie (1998). "Silver ion high-performance liquid chromatography of isomeric cis- and trans-octadecenoic acids: Effect of the ester moiety and mobile phase composition." Journal of Chromatography A 793(2): 275-282.
Mondello, L., A. Casilli, P. Q. Tranchida, P. Dugo and G. Dugo (2003). "Detailed analysis and group-type separation of natural fats and oils using comprehensive two-dimensional gas chromatography." J Chromatogr A 1019(1-2): 187-196.

Mondello, L., A. C. Lewis and K. D. Bartle (2002). Multidimensional Chromatography, Wiley.
Mossoba, M., A. Seiler, J. K. G. Kramer, V. Milosevic, M. Milosevic, H. Azizian and H. Steinhart (2009). "Nutrition Labeling: Rapid Determination of Total trans Fats by Using Internal Reflection Infrared Spectroscopy and a Second Derivative Procedure." Journal of the American Oil Chemists' Society 86(11): 1037-1045.
Mossoba, M. M., R. E. McDonald, J. A. G. Roach, D. D. Fingerhut, M. P. Yurawecz and N. Sehat (1997). "Spectral confirmation of trans monounsaturated C18 fatty acid positional isomers." Journal of the American Oil Chemists' Society 74(2): 125-130.
Mossoba, M. M., J. Moss and J. K. Kramer (2009). "Trans fat labeling and levels in U.S. foods: assessment of gas chromatographic and infrared spectroscopic techniques for regulatory compliance." J AOAC Int 92(5): 1284-1300.
Mozaffarian, D. (2006). "Trans fatty acids - effects on systemic inflammation and endothelial function." Atheroscler Suppl 7(2): 29-32.
Mozaffarian, D., A. Aro and W. C. Willett (2009). "Health effects of trans-fatty acids: experimental and observational evidence." Eur J Clin Nutr 63 Suppl 2: S5-21.
Mozaffarian, D., H. Cao, I. B. King, R. N. Lemaitre, X. Song, D. S. Siscovick and G. S. Hotamisligil (2010). "Trans-palmitoleic acid, metabolic risk factors, and new-onset diabetes in U.S. adults: a cohort study." Ann Intern Med 153(12): 790-799.
Mozaffarian, D. and R. Clarke (2009). "Quantitative effects on cardiovascular risk factors and coronary heart disease risk of replacing partially hydrogenated vegetable oils with other fats and oils." Eur J Clin Nutr 63 Suppl 2: S22-33.
Munk, M.-D. (2010). "“Pine Mouth” Syndrome: Cacogeusia Following Ingestion of Pine Nuts (Genus: Pinus). An Emerging Problem?" Journal of Medical Toxicology 6(2): 158-159.
Nasri, N., A. Khaldi, B. Fady and S. Triki (2005). "Fatty acids from seeds of Pinus pinea L.: composition and population profiling." Phytochemistry 66(14): 1729-1735.
Nergiz, C. and İ. Dönmez (2004). "Chemical composition and nutritive value of Pinus pinea L. seeds." Food Chemistry 86(3): 365-368.
Nimal Ratnayake, W. M., S. Hansen and M. Kennedy (2006). "Evaluation of the CP-Sil 88 and SP-2560 GC columns used in the recently approved 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 method." Journal of the American Oil Chemists' Society 83(6): 475-488.
Pariza, M. W., Y. Park and M. E. Cook (2001). "The biologically active isomers of conjugated linoleic acid." Prog Lipid Res 40(4): 283-298.
Parodi, P. W. (1977). "Conjugated Octadecadienoic Acids of Milk Fat." Journal of dairy science 60(10): 1550-1553.
Pascal, J. C. and R. G. Ackman (1975). "Occurrence of 7-methyl-7-hexadecenoic acid, the corresponding alcohol, 7-methyl-6-hexadecenoic acid, and 5-methyl-4hexadecenoic acid in sperm whale oils." Lipids 10(8): 478-482.
Payagala, T., Y. Zhang, E. Wanigasekara, K. Huang, Z. S. Breitbach, P. S. Sharma, L. M. Sidisky and D. W. Armstrong (2008). "Trigonal Tricationic Ionic Liquids: A Generation of Gas Chromatographic Stationary Phases." Analytical Chemistry 81(1): 160-173.
Payagala, T., Y. Zhang, E. Wanigasekara, K. Huang, Z. S. Breitbach, P. S. Sharma, L. M. Sidisky and D. W. Armstrong (2009). "Trigonal tricationic ionic liquids: a generation of gas chromatographic stationary phases." Anal Chem 81(1): 160-173.
Piperova, L. S., B. B. Teter, I. Bruckental, J. Sampugna, S. E. Mills, M. P. Yurawecz, J. Fritsche, K. Ku and R. A. Erdman (2000). "Mammary lipogenic enzyme activity, trans fatty acids and conjugated linoleic acids are altered in lactating dairy cows fed a milk fat-depressing diet." J Nutr 130(10): 2568-2574.

PRECHT, #160, D., MOLKENTIN, #160 and J. (2003). Overestimation of linoleic acid and trans-C18 :2 isomers in milk fats with emphasis on trans Δ9,trans Δ12-octadecadienoic acid. Kempten, ALLEMAGNE, Agrar-Verlag Allgaü.
Precht, D. and J. Molkentin (1996). "Rapid analysis of the isomers of trans-octadecenoic acid in milk fat." International Dairy Journal 6(8–9): 791-809.
Precht, D. and J. Molkentin (1997). "Trans-geometrical and positional isomers of linoleic acid including conjugated linoleic acid (CLA) in German milk and vegetable fats." Lipid / Fett 99(9): 319-326.
Precht, D. and J. Molkentin (1999). "C18:1, C18:2 and C18:3 trans and cis fatty acid isomers including conjugated cis delta 9, trans delta 11 linoleic acid (CLA) as well as total fat composition of German human milk lipids." Nahrung 43(4): 233-244.
Precht, D. and J. Molkentin (2000). "Recent trends in the fatty acid composition of German sunflower margarines, shortenings and cooking fats with emphasis on individual C16 : 1, C18 : 1, C18 : 2, C18 : 3 and C20 : 1trans isomers." Food / Nahrung 44(4): 222-228.
Precht, D. and J. Molkentin (2000). "Trans fatty acids in human milk." European Journal of Lipid Science and Technology 102(10): 635-640.
Precht, D. and J. Molkentin (2000a). "Identification and quantitation of cis/trans C16:1 and C17:1 fatty acid positional isomers in German human milk lipids by thin-layer chromatography and gas chromatography/mass spectrometry." European Journal of Lipid Science and Technology 102(2): 102-113.
Precht, D. and J. Molkentin (2003). "Overestimation of linoleic acid and trans-C18 :2 isomers in milk fats with emphasis on trans ?9,trans ?12-octadecadienoic acid." Milchwissenschaft 58(1-2): 30-34.
Precht, D., J. Molkentin, F. Destaillats and R. Wolff (2001). "Comparative studies on individual isomeric 18：1 acids in cow, goat, and ewe milk fats by low-temperature high-resolution capillary gas-liquid chromatography." Lipids 36(8): 827-832.
Purcaro, G., P. Quinto Tranchida, L. Conte, A. Obiedzinska, P. Dugo, G. Dugo and L. Mondello (2011). "Performance evaluation of a rapid-scanning quadrupole mass spectrometer in the comprehensive two-dimensional gas chromatography analysis of pesticides in water." J Sep Sci 34(18): 2411-2417.
Purcaro, G., P. Q. Tranchida, C. Ragonese, L. Conte, P. Dugo, G. Dugo and L. Mondello (2010). "Evaluation of a rapid-scanning quadrupole mass spectrometer in an apolar x ionic-liquid comprehensive two-dimensional gas chromatography system." Anal Chem 82(20): 8583-8590.
Qiao, L., K. Lu, M. Qi and R. Fu (2013). "Separation performance of guanidinium-based ionic liquids as stationary phases for gas chromatography." J Chromatogr A 1276: 112-119.
Ragonese, C., D. Sciarrone, P. Q. Tranchida, P. Dugo and L. Mondello (2012). "Use of ionic liquids as stationary phases in hyphenated gas chromatography techniques." J Chromatogr A 1255: 130-144.
Ragonese, C., P. Q. Tranchida, P. Dugo, G. Dugo, L. M. Sidisky, M. V. Robillard and L. Mondello (2009). "Evaluation of use of a dicationic liquid stationary phase in the FAt and conventional gas chromatographic analysis of health-hazardous C18 cis/trans fatty acids." Anal Chem 81(13): 5561-5568.
Ragonese, C., P. Q. Tranchida, P. Dugo, G. Dugo, L. M. Sidisky, M. V. Robillard and L. Mondello (2009). "Evaluation of Use of a Dicationic Liquid Stationary Phase in the FAt and Conventional Gas Chromatographic Analysis of Health-Hazardous C18 Cis/Trans Fatty Acids." Analytical Chemistry 81(13): 5561-5568.
Rajakangas, J., S. Basu, I. Salminen and M. Mutanen (2003). "Adenoma growth stimulation by the trans-10, cis-12 isomer of conjugated linoleic acid (CLA) is associated with changes in mucosal NF-kappaB and cyclin D1 protein levels in the Min mouse." J Nutr 133(6): 1943-1948.

Ratnayake, N., #160, W. M., Hansen, #160, S. L., Kennedy, #160 and M. P. (2006). Evaluation of the CP-Sil 88 and SP-2560 GC columns used in the recently approved 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 method. Heidelberg, ALLEMAGNE, Springer.
Ratnayake, W. M. (2004). "Overview of methods for the determination of trans fatty acids by gas chromatography, silver-ion thin-layer chromatography, silver-ion liquid chromatography, and gas chromatography/mass spectrometry." J AOAC Int 87(2): 523-539.
Ratnayake, W. M., B. Olsson and R. G. Ackman (1989). "Novel branched-chain fatty acids in certain fish oils." Lipids 24(7): 630-637.
Ratnayake, W. M., L. J. Plouffe, E. Pasquier and C. Gagnon (2002). "Temperature-sensitive resolution of cis- and trans-fatty acid isomers of partially hydrogenated vegetable oils on SP-2560 and CP-Sil 88 capillary columns." J AOAC Int 85(5): 1112-1118.
Reedy, G. and Mossoba, M.M. (1999). " Matrix isolation GC-FTIR". In: Mossoba MM (ed), Spectral Methods in Food Analysis, Marcel Dekker Inc., New York, NY, 1999; pp 325-396
Reid, V. R., J. A. Crank, D. W. Armstrong and R. E. Synovec (2008). "Characterization and utilization of a novel triflate ionic liquid stationary phase for use in comprehensive two-dimensional gas chromatography." J Sep Sci 31(19): 3429-3436.
Roach, J. G., M. Yurawecz, J. G. Kramer, M. Mossoba, K. Eulitz and Y. Ku (2000). "Gas chromatography—High resolution selected-ion mass spectrometric identification of trace 21 2 fatty acids eluting with conjugated linoleic acid isomers." Lipids 35(7): 797-802.
Santercole, V., P. Delmonte and J. K. Kramer (2012). "Comparison of separations of fatty acids from fish products using a 30-m Supelcowax-10 and a 100-m SP-2560 column." Lipids 47(3): 329-344.
Scholfield, C. R., R. O. Butterfield and H. J. Dutton (1966). "Preparation of pure methyl esters by counter double current distribution." Lipids 1(3): 163-165.
Schomburg, G., E. Hübinger, H. Husmann and F. Weeke (1982). "On-line hydrogenation of unsaturated and saturated sample components in capillary reactors coupled to either inlet or outlet of capillary columns." Chromatographia 16(1): 228-232.
Sciotto, C. and S. A. Mjos (2012). "Trans isomers of EPA and DHA in omega-3 products on the European market." Lipids 47(7): 659-667.
Sebedio, J. L., M. Catte, M. A. Boudier, J. Prevost and A. Grandgirard (1996). "Formation of fatty acid geometrical isomers and of cyclic fatty acid monomers during the finish frying of frozen prefried potatoes." Food Research International 29(2): 109-116.
Sebedio, J. L., W. W. Christie and R. O. Adlof (2003). Advances in conjugated linoleic acid research, Amer Oil Chemists Society.
Sebedio, J. L., W. M. N. Ratnayake, R. G. Ackman and J. Prevost (1993). "Stability of polyunsaturated omega-3 fatty acids during deep fat frying of Atlantic mackerel (Scomber scombrus L.)." Food Research International 26(3): 163-172.
Seeley, J. V., S. K. Seeley, E. K. Libby, Z. S. Breitbach and D. W. Armstrong (2008). "Comprehensive two-dimensional gas chromatography using a high-temperature phosphonium ionic liquid column." Anal Bioanal Chem 390(1): 323-332.
Seeley, J. V., S. K. Seeley, E. K. Libby and J. D. McCurry (2007). "Analysis of biodiesel/petroleum diesel blends with comprehensive two-dimensional gas chromatography." J Chromatogr Sci 45(10): 650-656.
Sehat, N., R. Rickert, M. Mossoba, J. G. Kramer, M. Yurawecz, J. G. Roach, R. Adlof, K. Morehouse, J. Fritsche, K. Eulitz, H. Steinhart and Y. Ku (1999). "Improved separation of conjugated fatty acid methyl esters by silver ion-high-performance liquid chromatography." Lipids 34(4): 407-413.

Sen Gupta, A. K. and H. Peters (1966). "Isolierung und Strukturaufklärung polyverzweigter Fettsäuren aus Fischöl." Fette, Seifen, Anstrichmittel 68(5): 349-360.
Shingfield, K. J., C. K. Reynolds, B. Lupoli, V. Toivonen, M. P. Yurawecz, P. Delmonte, J. M. Griinari, A. S. Grandison and D. E. Beever (2005). "Effect of forage type and proportion of concentrate in the diet on milk fatty acid composition in cows given sunflower oil and fish oil." Animal Science 80(02): 225-238.
Siegler, W. C., J. A. Crank, D. W. Armstrong and R. E. Synovec (2010). "Increasing selectivity in comprehensive three-dimensional gas chromatography via an ionic liquid stationary phase column in one dimension." J Chromatogr A 1217(18): 3144-3149.
Singh, D., M. E. Rezac and P. H. Pfromm (2009). "Partial Hydrogenation of Soybean Oil with Minimal Trans Fat Production Using a Pt-Decorated Polymeric Membrane Reactor." Journal of the American Oil Chemists' Society 86(1): 93-101.
Spitzer, V. (1996). "Structure analysis of fatty acids by gas chromatography--low resolution electron impact mass spectrometry of their 4,4-dimethyloxazoline derivatives--a review." Prog Lipid Res 35(4): 387-408.
Stubbs, C. D. and A. D. Smith (1984). "The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function." Biochim Biophys Acta 779(1): 89-137.
Thompson, R. H. (1997). "Direct Measurement of Total trans- and cis-Octadecenoic Fatty Acids Based on a Gas—Liquid Chromatographic Class Separation of trans-18:1 and cis-18:1 Fatty Acid Methyl Esters." Journal of Chromatographic Science 35(11): 536-544.
Tiyapongpattana, W., P. Wilairat and P. J. Marriott (2008). "Characterization of biodiesel and biodiesel blends using comprehensive two-dimensional gas chromatography." J Sep Sci 31(14): 2640-2649.
Tranchida, P. Q., F. A. Franchina, P. Dugo and L. Mondello (2012). "A flow-modulated comprehensive gas chromatography-mass spectrometry method for the analysis of fatty acid profiles in marine and biological samples." J Chromatogr A 1255: 171-176.
Tricon, S., G. C. Burdge, E. L. Jones, J. J. Russell, S. El-Khazen, E. Moretti, W. L. Hall, A. B. Gerry, D. S. Leake, R. F. Grimble, C. M. Williams, P. C. Calder and P. Yaqoob (2006). "Effects of dairy products naturally enriched with cis-9,trans-11 conjugated linoleic acid on the blood lipid profile in healthy middle-aged men." The American Journal of Clinical Nutrition 83(4): 744-753.
Tsuzuki, W. and K. Ushida (2009). "Preparative separation of cis- and trans-isomers of unsaturated fatty acid methyl esters contained in edible oils by reversed-phase high-performance liquid chromatography." Lipids 44(4): 373-379.
Tyburczy, C., P. Delmonte, A. R. Fardin-Kia, M. M. Mossoba, J. K. Kramer and J. I. Rader (2012). "Profile of trans fatty acids (FA) including trans polyunsaturated FA in representative FAt food samples." J Agric Food Chem 60(18): 4567-4577.
Tyburczy, C., M. M. Mossoba, A. R. Fardin-Kia and J. I. Rader (2012). "Evaluation of low trans fat edible oils by attenuated total reflection-Fourier transform infrared spectroscopy and gas chromatography: a comparison of analytical approaches." Anal Bioanal Chem 404(3): 809-819.
Ulberth, F., R. Gabernig and F. Schrammel (1999). "Flame-ionization detector response to methyl, ethyl, propyl, and butyl esters of fatty acids." Journal of the American Oil Chemists' Society 76(2): 263-266.
Van Pelt, C. K. and J. T. Brenna (1999). "Acetonitrile chemical ionization tandem mass spectrometry to locate double bonds in polyunsaturated fatty acid methyl esters." Anal Chem 71(10): 1981-1989.
Van Tol, A., P. L. Zock, T. van Gent, L. M. Scheek and M. B. Katan (1995). "Dietary trans fatty acids increase serum cholesterylester transfer protein activity in man." Atherosclerosis 115(1): 129-134.

Veldsink, J. W., M. J. Bouma, N. H. Schön and A. A. C. M. Beenackers (1997). "Heterogeneous Hydrogenation of Vegetable Oils: A Literature Review." Catalysis Reviews 39(3): 253-318.
Villegas, C., Y. Zhao and J. M. Curtis (2010). "Two methods for the separation of monounsaturated octadecenoic acid isomers." J Chromatogr A 1217(5): 775-784.
Western, R. J., S. S. Lau, P. J. Marriott and P. D. Nichols (2002). "Positional and geometric isomer separation of FAME by comprehensive 2-D GC." Lipids 37(7): 715-724.
Willett, W. C., M. J. Stampfer, J. E. Manson, G. A. Colditz, F. E. Speizer, B. A. Rosner, L. A. Sampson and C. H. Hennekens (1993). "Intake of trans fatty acids and risk of coronary heart disease among women." Lancet 341(8845): 581-585.
Wolff, R. (1992). "trans-Polyunsaturated fatty acids in French edible rapeseed and soybean oils." Journal of the American Oil Chemists' Society 69(2): 106-110.
Wolff, R. (1997). "Discussion of the term "unusual" when discussing Δ5-olefinic acids in plant lipids." Journal of the American Oil Chemists' Society 74(5): 619-619.
Wolff, R. (1999). "The phylogenetic significance of sciadonic (all-cis 5,11,14–20:3) acid in gymnosperms and its quantitative significance in land plants." Journal of the American Oil Chemists' Society 76(12): 1515-1516.
Wolff, R. and C. Bayard (1995). "Fatty acid composition of some pine seed oils." Journal of the American Oil Chemists' Society 72(9): 1043-1046.
Wolff, R., W. Christie, F. Pédrono and A. Marpeau (1999). "Arachidonic, eicosapentaenoic, and biosynthetically related fatty acids in the seed lipids from a primitive gymnosperm, Agathis robusta." Lipids 34(10): 1083-1097.
Wolff, R., E. Dareville and J.-C. Martin (1997). "Positional distribution of Δ5-olefinic acids in triacylglycerols from conifer seed oils: General and specific enrichment in the sn-3 position." Journal of the American Oil Chemists' Society 74(5): 515-523.
Wolff, R., L. Deluc and A. Marpeau (1996). "Conifer seeds: Oil content and fatty acid composition." Journal of the American Oil Chemists' Society 73(6): 765-771.
Wolff, R., O. Lavialle, F. Pédrono, E. Pasquier, L. Deluc, A. Marpeau and K. Aitzetmüller (2001). "Fatty acid composition of pinaceae as taxonomic markers." Lipids 36(5): 439-451.
Wolff, R., F. Pédrono, E. Pasquier and A. Marpeau (2000). "General characteristics of Pinus spp. Seed fatty acid compositions, and importance of Δ5-olefinic acids in the taxonomy and phylogeny of the genus." Lipids 35(1): 1-22.
Wolff, R. and D. Precht (1998). "Comments on the resolution of individual trans-18:1 isomers by gas-liquid chromatography." Journal of the American Oil Chemists' Society 75(3): 421-422.
Wolff, R. L., W. W. Christie, F. Pedrono, A. M. Marpeau, N. Tsevegsuren, K. Aitzetmuller and F. D. Gunstone (1999). "Delta5-olefinic acids in the seed lipids from four Ephedra species and their distribution between the alpha and beta positions of triacylglycerols. Characteristics common to coniferophytes and cycadophytes." Lipids 34(8): 855-864.
Wolff, R. L., O. Lavialle, F. Pedrono, E. Pasquier, F. Destaillats, A. M. Marpeau, P. Angers and K. Aitzetmuller (2002). "Abietoid seed fatty acid compositions--a review of the genera Abies, Cedrus, Hesperopeuce, Keteleeria, Pseudolarix, and Tsuga and preliminary inferences on the taxonomy of Pinaceae." Lipids 37(1): 17-26.
Wolff, R.L., D. Precht, J. Molkentin (1998). "Occurance and distribution profiles of *trans*-18:1 acids in edible fats of natural origin". In: Sebedio J-L, Christie WW (eds), Trans Fatty Acids in Human Nutrition, The Oily Press, Dundee, Scotland, 1998; pp 1-34
Wonsil, B. J., J. H. Herbein and B. A. Watkins (1994). "Dietary and ruminally derived trans-18:1 fatty acids alter bovine milk lipids." J Nutr 124(4): 556-565.
Yu, Q. T., B. N. Liu, J. Y. Zhang and Z. H. Huang (1989). "Location of double bonds in fatty acids of fish oil and rat testis lipids. Gas chromatography-mass spectrometry of the oxazoline derivatives." Lipids 24(1): 79-83.

Yurawecz, M., J. G. Roach, N. Sehat, M. Mossoba, J. G. Kramer, J. Fritsche, H. Steinhart and Y. Ku (1998). "A new conjugated linoleic acid isomer, 7 trans, 9 cis-octadecadienoic acid, in cow milk, cheese, beef and human milk and adipose tissue." Lipids 33(8): 803-809.
Yurawecz, M. P. and J. K. G. Kramer (2006). Advances in conjugated linoleic acid research, American Oil Chemists' Society.
Zapadlo, M., J. Krupcik, T. Kovalczuk, P. Majek, I. Spanik, D. W. Armstrong and P. Sandra (2011). "Enhanced comprehensive two-dimensional gas chromatographic resolution of polychlorinated biphenyls on a non-polar polysiloxane and an ionic liquid column series." J Chromatogr A 1218(5): 746-751.
Zapadlo, M., J. Krupcik, P. Majek, D. W. Armstrong and P. Sandra (2010). "Use of a polar ionic liquid as second column for the comprehensive two-dimensional GC separation of PCBs." J Chromatogr A 1217(37): 5859-5867.

SUMMARY

The negative health impacts of dietary *trans* fatty acids have been documented in many scientific publications in the last decade. Numerous studies have also examined the relationship between different positional and geometric isomers of unsaturated fatty acids with double bonds in the *trans* configuration and coronary heart disease. The analysis of and accurate chromatographic separation of, various fatty acid isomers are critical factors in all these studies.

The objectives of this work were to develop and improve analytical methods, including synthesis of reference FA, for the identification and quantification of positional/geometric isomers of *trans* fatty acids.

1) A new gas chromatographic method was developed by utilizing a novel ionic liquid capillary GC column (200 m SLB-IL111). This method has several advantages compared to methods using cyanopropyl siloxane columns, which are currently recommended for the challenging analysis of mixtures containing geometric and positional isomers of FAME. The new GC method when operated isothermally at 168 °C, improved the separation of *c*/*t*-CLA isomers and the separation of *c*9,*t*11- from *t*7,*c*9-CLA. This was not possible using cyanopropyl siloxane columns. The selected chromatographic conditions provided the separation of co-eluting fatty acid isomeric pairs. It eliminated the concern raised in most reports that *c*9,*t*11-18:2 is overestimated by the contribution of *t*7,*c*9-CLA. It provided for the separation of *t*15-18:1, which generally co-elutes 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. This new method was then successfully applied to the analysis of milk fat [Chapter 5] and fish oil [Chapter 7].

2) Significant improvements in the analysis of FAME were achieved by preparing small amounts of reference materials using simple synthetic procedures.

Positional/geometric isomers of C14:1 – C20:1 FAME, positional/geometric isomers of linoleic acid (*c*9,*c*12-18:2), positional/geometric isomers of alpha-linolenic, gamma-linolenic acid methyl esters and conjugated linoleic acid methyl esters were prepared, isolated and purified.

3) Multi dimensional chromatography is one of the most rapidly growing techniques in separation science. A new GC-online reduction × GC chromatographic system was developed that provided a significant enhancement of previously developed single dimensional GC methods for FAME separation. Using this new methodology, the FAME could be easily identified based on a simple interpretation model: under isothermal conditions, saturated FAME lay on a diagonal line bisecting the separation plane and FAME with the same carbon skeleton lay on lines parallel to the ^{1}D time axis. The chromatographic conditions described have been shown to allow the separation of the FAME prepared from the most complex samples, including fish oil and biological extracts. The introduction of a palladium reducer between the two dimensions of separation, thus reducing the unsaturated FAME to their saturated form, provided an enhanced separation compared to GC × GC systems based solely on the orthogonality of the two separations. By separating FAME with different chain lengths on different lines, this method allowed the analysis of samples containing complex mixtures of polyunsaturated fatty acid methyl esters with various chain lengths.

Zusammenfassung

Die negativen Auswirkungen der erhöhten Aufnahme von trans-Fettsäuren (*t*FA) und deren Positionsisomeren wurden in zahlreichen wissenschaftlichen Studien mit einer Risikoerhöhung für koronare Herzerkrankungen dokumentiert und bestätigt. Die korrekte, chromatografische Trennung der verschiedenen Fettsäuren (FS) und deren chemischen Analyse sind in allen durchgeführten Studien von großer Bedeutung. Die Ziele der vorliegenden Arbeit lagen in der Neuentwicklung und Verbesserung von analytischen Methoden für die Identifizierung und Quantifizierung der Positionsisomeren der *t*FA und betreffen die Synthese von Standards für *t*FS.

1) Es wurde eine gaschromatografische Methode unter Verwendung einer neuartigen „ionic liquid" GC-Kapillarsäule (200 m SLB-IL111) entwickelt. Im Vergleich zu den existierenden GC-Methoden, die Cyanopropyl-Kapillarsäulen (CPS) für die Trennung der Fettsäurenisomere empfehlen, erwies sich die neu entwickelte Methode, speziell für die Trennung des Isomerenpaares *c*9,*t*11-CLA/*t*7,*c*9-CLA, sehr vorteilhaft. Die in der Vergangenheit angewendeten GC-Methoden (GC mit CPS) waren nicht in der Lage das obengenannte Isomerenpaar ausreichend zu trennen und des öfteren wurde der Gehalt an *c*9/*t*11-18:2 überschätzt. Gleichzeitig wurde auch die chromatografische Trennung von *t*15-18:1 und *c*9-18:1 und partiell des Isomerenpaares *t*13/*t*14-18:1, *c*8/*c*6/*c*7-18:1, sowie mehreren *t,t*-CLA Isomeren verbessert. Für die Trennung der FS im Milchfett [Chapter 5] und Fischöl [Chapter 7] wurde die neu entwickelte Methode erfolgreich eingesetzt.

2) Die synthetische Herstellung von ungesättigten Fettsäuremethylestern (FSME) im Labormaßstab verbesserte erheblich die gaschromatografische Bestimmung und Identifizierung der Fettsäureisomeren. Positions- und geometrische Isomere von C14:1-C20:1, Linolsäure, alpha-Linolensäure, gamma-Linolensäure und CLAs wurden synthetisch hergestellt und in reiner Form isoliert.

3) Der Bereich der multidimensionalen Chromatografie gehört zu den am schnellsten wachsenden Bereichen der chromatografischen Trennungstechniken. Ein innovatives, chromatografisches GC-System (GC-online Reduktion × GC) wurde entwickelt, welches im Vergleich zu allen vorherigen monodimensionalen Methoden eine erhebliche Verbesserung der chromatografischen Trennung von FSME lieferte. Die neue Methodik machte die Identifizierung und die Bestimmung von FSME gemäß den folgenden Prinzipien erheblich einfacher. Unter isothermen Bedingungen lagen die gesättigten FSME auf einer Diagonalen im zweidimensionalen chromatografischen Trennungsraum, wobei die FSME mit gleicher Anzahl an Kohlenstoffatomen parallel zur 1D Zeitachse angeordnet waren. Die neuen chromatografischen Bedingungen verbesserten die Trennung von komplexen FSME in Milchfett und Fischöl. Der Einsatz von einem Kapillar-Katalysator zwischen den zwei Dimensionen (1D und 2D), der mit metallischem Palladium beladen war und alle vorhandenen Doppelbindungen der ungesättigten FSME reduzierte, lieferte sogar eine erweiterte chromatografische Trennung im Vergleich zu konventionellem GC×GC, welches nur auf Orthogonalität der 1D und 2D basiert. Das neu entwickelte System erlaubte die Trennung von komplexen und oft co-eluierenden, mehrfach ungesättigten FSME mit unterschiedlichen Kohlenstoffkettenlängen.

LIST OF PUBLICATIONS

Delmonte, P.; Hu, Qing.; Fardin-Kia, Ali R. and Rader, Jeanne I., "Preparation, chromatographic separation and relative retention times of cis/trans heptadecaenoic (17:1) fatty acids". *J. Chrom. A.*; 1214(1-2), 30-36 (2008)

Delmonte, P.; Fardin-Kia, Ali R.; Hu, Qing and Rader, Jeanne I., "Review of Methods for Preparation and Gas Chromatographic Separation of trans and cis Reference Fatty Acids". *J. AOAC Int.*, 92 (5), 1310-1325 (2009)

Delmonte, P.; Fardin-Kia Ali R.; Kramer, John K.G.; Mossoba, Magdi M.; Sidisky, Len and Rader, Jeanne I., "Separation characteristics of fatty acid methyl esters using SLB-IL111, a new ionic liquid coated capillary gas chromatographic column". *J. Chrom. A.*; 1218, 545-554 (2011)

Handy, Sara M.; Parks, Matthew B.; Deeds, Jonathan R.; Liston, Aaron; Lowri S. de Jager, Luccioli, Stefano; Kwegyir-Afful, Ernest; Fardin-Kia Ali R., Begley, Timothy H.; Rader, Jeanne I. and Diachenko, Gregory W., "Use of the Chloroplast Gene ycf1 for the Genetic Differentiation of Pine Nuts Obtained from Consumers Experiencing Dysgeusia". *J. Agric. Food Chem.*; 59, 10995-11002 (2011)

Fardin-Kia, Ali R.; Handy Sara M. and Rader, Jeanne I., "Characterization of Pine Nuts in the U.S. Market, Including Those Associated with "Pine Mouth", by GC-FID". *J. Agric. Food Chem.*; 60, 2701-2711 (2012)

Tyburczy, C.; Delmonte, P.; Fardin-Kia, Ali R.; Mossoba, Magdi M; Kramer, JK.; Rader, Jeanne I., "Profile of trans fatty acids (FA) including trans polyunsaturated FA in representative FAt food samples". *J. Agric. Food. Chem.*; 60(18), 4567-77 (2012)

Tyburczy, C.; Mossoba, Magdi M; Fardin-Kia, Ali R; Rader, Jeanne I., "Evaluation of low trans fat edible oils by attenuated total reflection-Fourier transform infrared spectroscopy and gas chromatography: a comparison of analytical approaches", *Anal. Bioanal. Chem.*; 404, 809-819 (2012)

Mossoba, Magdi M.; Kramer, John K. G.; Azizian, Hormoz; Kraft, Jana; Delmonte, Pierluigi; Fardin-Kia Ali R.; Bueso, Francisco J.; Rader, Jeanne I. and Lee, Jung K., "Application of a Novel, Heated, Nine-Reflection ATR Crystal and a Portable FTIR Spectrometer to the Rapid Determination of Total Trans Fat". *J. Am. Oil Chem.*; 89, 419-429 (2012)

Delmonte, P.; Fardin-Kia Ali R.; Kramer, John K.G.; Mossoba, Magdi M.; Sidisky, Len; Tyburczy, C. and Rader, Jeanne I., "Evaluation of highly polar ionic liquid gas chromatographic column for the determination of the fatty acids in milk fat". J. Chrom. A.; 1233, 137-146 (2012)

Delmonte, P; Fardin-Kia, Ali R.; Rader, Jeanne I., "Separation of Fatty Acid Methyl Esters by GC-Online Hydrogenation × GC". *Anal. Chem.* 85(3), 1517-1524 (2013)

Vyas, D.; Moallem, U.; Teter, B.B.; Fardin-Kia, A.R.; Erdman, R.A., "Milk fat responses to butterfat infusion during conjugated linoleic acid-induced milk fat depression in lactating dairy cows". *Journal of Dairy Science,* 96, 2387-2399 (2013)

Mosoba, Magdi M.; Azizian, Hormoz; Tyburczy, Cynthia; Kramer, John K. G.; Delmonte, Pierluigi; Fardin-Kia, Ali R.; Rader, Jeanne I., " Rapid FT-NIR Analysis of Edible Oils for Total SFA, MUFA, PUFA, and Trans FA with Comparison to GC". *J. Am. Oil Chem.,* DOI 10.1007/s11746-013-2234-z (2013)

Fadin-Kia, Ali R.; Delmonte, P.; Kramer, John K.G; Jahreis, G.; Kuhnt, K.; Santercole, V.; Rader, Jeanne I., "Separation of fatty acids in menhaden oil using highly polar ionic liquid gas chromatographic column and confirmation of their identities using silver ion fraction and mass spectrometry";, *Lipids* (2013) (just accepted)

LIST OF LECTURES, POSTERS AND ABSTRACS

Fardin-Kia, Ali R., "Preparation, Isolation and Determination of Non-conjugated Geometric/positional Isomers of Linoleic Acid". AOCS, Phoenix, AZ, May 17th, 2010

Fardin-Kia, Ali R., "Separation of Fatty Acids in Marine Oils With Highly PolarIonic Liquid gas Chromatographic Columns". AOCS, Montreal, Canada, April 30th, 2013

Fardin-Kia, Ali R.; Delmonte, Pierluigi. and Rader, Jeanne. I., "Preparation and relative retention times of non-conjugated 18:2 Fatty acid isomers" , AOAC, Orlando, FL, May (2009)

Mossoba, Magdi. M.; Azizian, Hormuz; Kramer, John K.G.; Delmonte, Pierluigi; Fardin-Kia, Ali R., "Rapid Determination of Total Saturated Fat and Total trans Fat Contents by FT-NIR for Regulatory Compliance", AOCS, Cincinnati, OH, May, (2011)

Delmonte, Pierluigi; Fardin-Kia, Ali R.; Kramer John K.G.; Mossoba, Magdi M.; Rader, Jeanne I., "Separation of fatty acid methyl esters utilizing the novel ionic Liquid SLB-111", AOCS, Cincinnati, OH, May, (2011)

Mossoba, Magdi. M.; Azizian, Hormuz; Kramer, John K.G.; J. Kraft; Delmonte, Pierluigi; Fardin-Kia, Ali R., F.J. Bueso; J.I. Rader, "Performance Evaluation of a Heated 9-Bounce Internal Reflection Portable Mid-FTIR Spectrometer for the Rapid Determination of Total trans Fat", AOCS, Cincinnati, OH, May, (2011)

Delmonte, P.; Fardin-Kia, Ali R.; Rahul, P.; Tyburczy, C.; Rader, Jeanne I., "A Comprehensive LCxLC Approach to Separation of Phytochemicals in Dietary Supplements", Abstract #2220-4, PITTCON, Orlando, FL, (2012)

Ledford, Edward B.; Spear, William; Wu, Zhanpin; Delmonte, Pierluigi; Fardin-Kia Ali R.; Rader, Jeanne I., "GCxGC Separation of Fatty Acid Methyl-Esters: Using Giddings Dimensionality to Aid the Interpretation of Comprehensive Two-Dimensional Gas Chromatograms", Abstract #490-2, PITTCON, Philadelphia, PA, (2013)

Delmonte, Pierluigi; Fardin-Kia, Ali R.; Ledford, Edward B; Wu, Zhanpin; Rader, Jeanne I., " Separation of Fatty Acid Methyl Esters by Comprehensive GC-Online Hydrogenation x GC", Abstract #1120-4, PITTCON, Philadelphia, PA, (2013)

Pawar, Rahul.; Grundel, Erich.; Rader, Jeanne I.; Krynitsky, Alex.; Fardin-Kia, Ali R.; Knolhoff, Ann M.; Croleyl, Timothy R.; Eason, Michael, "Determination of biogenic amines in Acacia rigidula and its dietary supplements", Abstract #AGFD107, ACS, New Orleans, LA, (2013)

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Ali Reza Fardin-Kia