Improving the Analysis of Fatty Acid Methyl Esters Using Retention Time Locked Methods and Retention Time Databases

Application

Food

Authors

Frank David
Research Institute for Chromatography
Pres. Kennedypark 20, B-8500 Kortrijk, Belgium

Pat Sandra
University of Gent
Krijgslaan 281 S4, B-9000 Gent, Belgium

Philip L. Wylie
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

The analysis of fatty acid methyl esters is a very important application in food analysis. Fatty acid methyl esters are normally analyzed on columns coated with polar stationary phases such as polyethylene glycols or cyanopropyl silicones, allowing separation of fatty acids according to carbon number and according to the degree of unsaturation.

Two retention time locked methods are presented in this application note. In the first method, the analysis of fatty acid methyl esters is performed on a DB-Wax column using gas chromatography/flame ionization detector or gas chromatography/mass spectrometry. In the second method, a DB-23 column is used. Retention time and mass spectral libraries are available for both methods. Retention time locking allows easy peak identification, easy exchange of data between instruments (gas chromatography/flame ionization detector, gas chromatography/mass spectrometry, different labs), and avoids the need to modify the retention times in calibration tables after column maintenance or column change.

Introduction

The analysis of fatty acid methyl esters (FAMEs) is used for the characterization of the lipid fraction in foods and is one of the most important applications in food analysis. Lipids mainly consist of triglycerides, which are esters of one glycerol molecule and three fatty acids. Most edible fats and oils are composed largely of 12- to 20-carbon fatty acids [lauric acid (dodecanoic acid) to arachidic acid (eicosanoic acid)]. Besides linear saturated fatty acids, branched, mono-unsaturated, di-unsaturated, and higher unsaturated fatty acids can occur. An overview of the most important fatty acids and their common abbreviations appears in Table 1.

Table 1. Fatty Acids, Common Names and Abbreviation Used

Fatty acid	Common Name	Simplified Abbreviation[1]	Abbreviation specifying *cis* and *trans* bonds[1]
Butanoic acid	Butyric acid	4:0	4:0
Decanoic acid	Caproic acid	10:0	10:0
Dodecanoic acid	Lauric acid	12:0	12:0
Tetradecanoic acid	Myristic acid	14:0	14:0
Hexadecanoic acid	Palmitic acid	16:0	16:0
Hexadecenoic acid	Palmitoleic acid	16:1 n-7	9*c*-16:1
Octadecanoic acid	Stearic acid	18:0	18:0
cis-9-Octadecenoic acid	Oleic acid	18:1 n-9	9*c*-18:1
trans-9-Octadecenoic acid	Elaidic acid	*t*18:1 n-9	9*t*-18:1
all *cis*-9,12-Octadecadienoic acid	Linoleic acid	18:2 n-6	9*c*12*c*-18:2
ll *trans*-9,12-Octadecadienoic acid	Linolelaidic acid	*t*18:2 n-6	9*t*12*t*-18:2
all *cis*-9,12,15-Octadecatrienoic acid	alpha-Linolenic acid	18:3 n-3	9*c*12*c*15*c*-18:3
all *cis* -6,9,12-Octadecatrienoic acid	gamma-Linolenic acid	18:3 n-6	6*c*9*c*12*c*-18:3
Eicosanoic acid	Arachidic acid	20:0	20:0
cis-11-Eicosenoic acid		20:1 n-9	11*c*-20:1
all *cis* -11,14-Eicosadienoic acid		20:2 n-6	11*c*14*c*-20:2
all *cis* -11,14,17-Eicosatrienoic acid		20:3 n-3	11*c*14*c*17*c*-20:3
all *cis* -8,11,14-Eicosatrienoic acid	Dihomogammalinolenic acid	20:3 n-6	8*c*11*c*14*c*-20:3
all *cis* -5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4 n-6	5*c*8*c*11*c*14*c*-20:4
all *cis* 5,8,11,14,17-Eicosapentaenoic acid	EPA	20:5 n-3	5*c*8*c*11*c*14*c*17*c*-20:5
Docosanoic acid	Behenic acid	22:0	22:0
cis -13-Docosenoic acid	Erucic acid	22:1 n-9	13*c*-22:1
all *cis* -7,10,13,16-Docosatetraenoic acid		22:4 n-6	7*c*10*c*13*c*16*c*-22:4
all *cis* 4,7,10,13,16,19-Docosahexaenoic acid	DHA	22:6 n-3	4*c*7*c*10*c*13*c*16c19*c*-22:6
Tetracosanoic acid	Lignoceric acid	24:0	24:0
cis -15-tetracosenoic acid	Nervonic acid	24:1 n-9	15*c*-24:1

[1]Several different versions of fatty acid nomenclature and structural abbreviation have been used in the past. For discussions of past and currently-accepted nomenclature, the following web sites are recommended:
http://www.ajcn.org/misc/lipid.shtml
http://www.chem.qmul.ac.uk/iupac/lipid/
http://www.aocs.org/member/division/analytic/fanames.htm
http://www.cyberlipid.org/index.htm

For the characterization of the lipid fraction, the triglycerides are hydrolyzed (saponified) into glycerol and free fatty acids. Although the free fatty acids can be analyzed directly on polar stationary phases (such as a FFAP column), more robust and reproducible chromatographic data are obtained if the fatty acids are derivatized to the FAMEs. Several methods are available for derivatization, which requires hydrolysis of the glycerides and methylation of the resulting fatty acids [1]. These easy-to-use methods do not require expensive reagents or equipment. Two useful methods are described in the Experimental section.

After preparation of the FAMEs, the FAMEs are separated according to carbon number (number of carbon atoms in the fatty acid chain, not including the methyl ester carbon) and according to the degree of unsaturation. Moreover, the position of the double bond(s) and the geometric configuration (*cis*/*trans*) are also important parameters and their determination adds additional information to the characterization of the lipid fraction in food.

In this application note, two methods are described for the GC analysis of FAMEs. The method choice depends both on sample complexity and the degree of fatty acid characterization that is required (Figure 1). Method 1 uses a DB-Wax column that separates FAMEs from C4 (butyric acid) to C24 (lignoceric acid) according to carbon number and unsaturation. On this column, no separation of *cis* and *trans* isomers is obtained, and for complex mixtures (such as fish oils), some FAMEs are not resolved. However, the separation of FAMEs on polyethylene glycol columns is widely used and can be applied to the characterization of "classical" samples such as vegetable oils (corn oil, maize oil, olive oil, soybean oil, and so on). For certain applications, animal fats can also be analyzed using the Agilent DB-Wax column. An important application, for instance, is the analysis of butyric

acid in milk fat. The concentration of butyric acid in milk is an important indicator of its quality. This determination is very important in milk and dairy analysis and in the analysis of chocolate products. All these applications can be performed on the Agilent DB-Wax column using method 1.

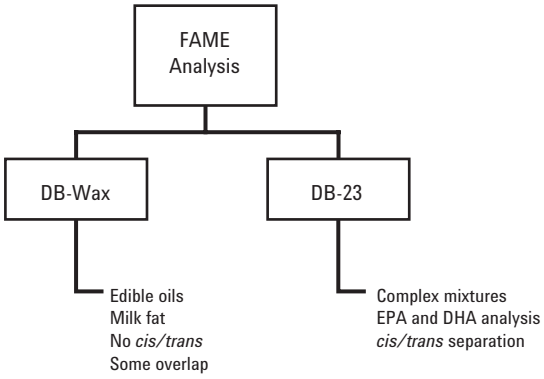

Figure 1. Overview of method selection for FAME analysis.

For the analysis of more complex samples, such as fish oils, additional resolution of FAMEs is obtained using a capillary column coated with a cyanopropyl stationary phase (method 2). On an Agilent DB-23, highly unsaturated fatty acids, such as all *cis* 5,8,11,14,17-eicosapentenoic acid methyl ester (EPA, 20:5 n-3) and all *cis* 4,7,10,13,16, 19-Docosahexenoic acid methyl ester (DHA, 22:6 n-3), are separated from other FAMEs. Separation of *cis* and *trans* isomers is also possible on the cyanopropyl column due to the stronger interaction of *cis* isomers with the cyano-dipole. This causes the *trans* isomers to elute before the *cis* isomers. An increasingly important food analysis is the determination of *trans* fatty acids, which can be performed using the DB-23 column with the conditions described in method 2.

Both methods are retention time locked using methyl stearate as the locking compound. Retention time locking (RTL) allows the analyst to obtain virtually identical retention times on any GC, independent of the inlet, injection technique, or detector used [2, 3, 4]. Because RTL reproduces retention times so accurately, FAME identification can be done based on absolute retention times. It is unnecessary to have all of the FAME standards available because peak identification is possible using Agilent's published retention time database. An additional benefit of RTL is that retention times in the calibration table remain unchanged even after column maintenance or column change (after re-locking the method). Moreover, gas chromatography/flame ionization detection (GC/FID) and gas chromatography/mass spectrometry (GC/MS) methods can be scaled [2, 3], so that retention times obtained on the two different systems match very closely. For GC/MS, a custom spectral library was created so that data files can be screened using spectra together with their locked retention times [4].

Experimental

Samples

Reference standards of FAMEs can be obtained from different sources as solutions or as neat compounds. For analysis, the standards are typically dissolved in hexane at a 0.01 to 0.1 % (w/v) concentration. For method and instrument check-out, a 37-component mixture (Supelco number 18919) was used. The mixture was purchased as a 100-mg neat mixture, containing C4 to C24 FAMEs (2 to 4% relative concentration). The whole sample was diluted in 10 mL of hexane (final concentration = 0.2 to 0.4 mg/mL per FAME).

Oil and fat samples can be prepared using any one of several different methods [1]. The following sample preparation methods were tested.

Sample preparation method 1 [5] : Weigh 100-mg sample in a 20-mL test tube (with screw cap) or a reaction vial. Dissolve the sample in 10 mL of hexane. Add 100 µL of 2N potassium hydroxide in methanol (11.2 g in 100 mL). Close the tube or vial, and vortex for 30 seconds. Centrifuge. Transfer the clear supernatant to a 2-mL autosampler vial.

Sample preparation method 2 [6]: Weigh 50-mg sample in a 20-mL test tube (with screw cap) or a reaction vial. Add 2 mL of 2N sodium hydroxide in methanol (8 g in 100 mL). Close the tube or vial, and heat at 80 °C for 1 hour. Allow to cool. Add 2 mL of a 25% borontrifluoride solution in methanol (Sigma-Aldrich, cat. no. 13,482-1, 50% solution in methanol, to be diluted to 25% in methanol). Close the tube or vial, and heat again for 1 hour at 80 °C. Allow to cool. Add 5 mL of water and 5 mL of hexane. Shake well. Allow the phases to separate (or centrifuge). Transfer the clear supernatant to a 2-mL autosampler vial.

Both methods performed equally well.

Analytical Conditions

The analyses were performed on an Agilent 6890 GC equipped with an FID or on an Agilent 6890/5973 GC/MSD system. Automated split injection was performed using an Agilent 7683 automatic sampler. The instrumental configuration and analytical conditions are summarized in Table 2 (DB-Wax column) and Table 3 (DB-23 column). For both methods, methyl stearate was used as the locking standard. The retention time for methyl stearate was locked at 14.000 min. When duplicating this method, the column head pressure can be set to the pressures indicated in Tables 2 and 3 (nominal pressure). Then the RTL calibration runs can be performed (at -20%, -10%, +10% and +20% of the nominal pressure)[4]. The retention time versus head pressure curve is then automatically calculated and stored in the method.

Table 2. DB-Wax Method

Instrumentation		
Chromatographic system	Agilent 6890 GC	
Inlet	Split/splitless	
Detector	FID or Agilent 5973 MSD	
Automatic sampler	Agilent 7683	
Liner	Split liner (part no. 5183-4647)	
Column	30-m × 0.25-mm id × 0.25-µm DB-Wax (part no. 122-7032)	
Experimental conditions GC/FID		
Inlet temperature	250 °C	
Injection volume	1 µL	
Split ratio	1/50	
Carrier gas	Hydrogen	
Head pressure	Methyl stearate is retention time locked to 14.000 min Constant Pressure mode (pressure approximately 53 kPa at 50 °C, 36 cm/s at 50 °C)	
Oven temperature	50 °C, 1 min, 25 °C/min to 200 °C, 3 °C/min to 230 °C, 18 min	
Detector temperature	280 °C	
Detector gases	Hydrogen: Air: Helium make-up gas:	40 mL/min 450 mL/min 30 mL/min
Experimental conditions GC/MS		
Inlet temperature	250 °C	
Injection volume	1 µL	
Split ratio	1/50	
Carrier gas	Helium	
Head pressure	Methyl stearate is retention time locked to 14.000 min Constant Pressure mode (pressure approximately 55 kPa at 50 °C, 36 cm/s at 50 °C)	
Oven temperature	50 °C, 1 min, 25 °C/min to 200 °C, 3 °C/min to 230 °C, 18 min	
Detector temperature	280 °C	
MSD Parameters	Scan (40 to 500 amu), threshold 100 MS quad 150 °C MS source 230 °C Solvent delay: 2 min	

Table 3. DB-23 Method

Instrumentation		
Chromatographic system	Agilent 6890 GC	
Inlet	Split/splitless	
Detector	FID or Agilent 5973 MSD	
Automatic sampler	Agilent 7683	
Liner	Split liner (part no. 5183-4647)	
Column	60-m × 0.25-mm id × 0.15-µm DB-23 (part no. 122-2361)	
Experimental conditions GC/FID		
Inlet temperature	250 °C	
Injection volume	1 µL	
Split ratio	1/50	
Carrier gas	Helium	
Head pressure	Methyl stearate is retention time locked to 14.000 min Constant Pressure mode (pressure approximately 230 kPa at 50 °C, 33 cm/s at 50 °C)	
Oven temperature	50 °C, 1 min, 25 °C/min to 175 °C, 4 °C/min to 230 °C, 5 min	
Detector temperature	280 °C	
Detector gases	Hydrogen: Air: Helium make-up gas:	40 mL/min 450 mL/min 30 mL/min
Experimental conditions GC/MS		
Inlet temperature	250 °C	
Injection volume	1 µL	
Split ratio	1/50	
Carrier gas	Helium	
Head pressure	Methyl stearate is retention time locked to 14.000 min Constant Pressure mode (pressure approximately 180 kPa at 50 °C, 33 cm/s at 50 °C)	
Oven temperature	50 °C, 1 min, 25 °C/min to 175 °C,4 °C/min to 235 °C, 5 min	
Transfer line	250 °C	
MSD parameters	Scan (40 to 500 amu), threshold 100 MS quad 150 °C, MS source 230 °C Solvent delay: 3.5 min	

Results and Discussion

A typical chromatogram for the analysis of the FAME reference standard (obtained on the DB-Wax column using method 1) is shown in Figure 2. A good separation is obtained, except for the following compounds: *cis* and *trans* 18:1 co-elute at 14.38 min, *cis* and *trans* 18:2 co-elute at 15.13 min, 20:3 n-6 and 21:0 co-elute at 19.44 min, and 22:6 and 24:1 co-elute at 30.73 min. However, this separation is sufficient for most classical oil and fat characterization. Butyric acid elutes at 2.85 min and can be determined in milk fat using the same method. Because the GC/MS and GC/FID instruments were locked, virtually identical chromatograms were obtained on both systems. A comparison between the GC/FID and GC/MS profiles is shown in Figure 3. Although different outlet pressures (ambient versus vacuum) and different carrier gases (hydrogen in GC/FID and helium for GC/MS) were used, the correspondence between the two chromatograms was very good. Peaks detected in the GC/FID trace can easily be located in the GC/MS trace and identification is possible based on retention times alone or in combination with mass spectra.

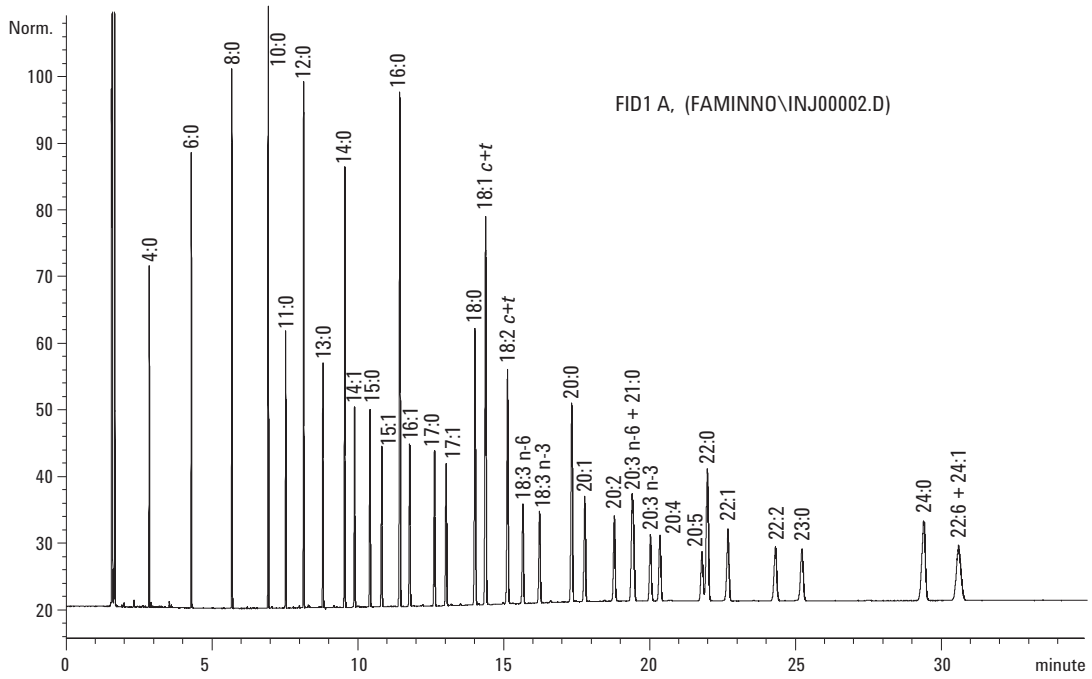

Figure 2. GC/FID analysis of FAMEs standard mixture on a 30-m × 0.25-mm id 0.25-µm DB-Wax column (part no. 122-7032) using method 1 (see Table 2).

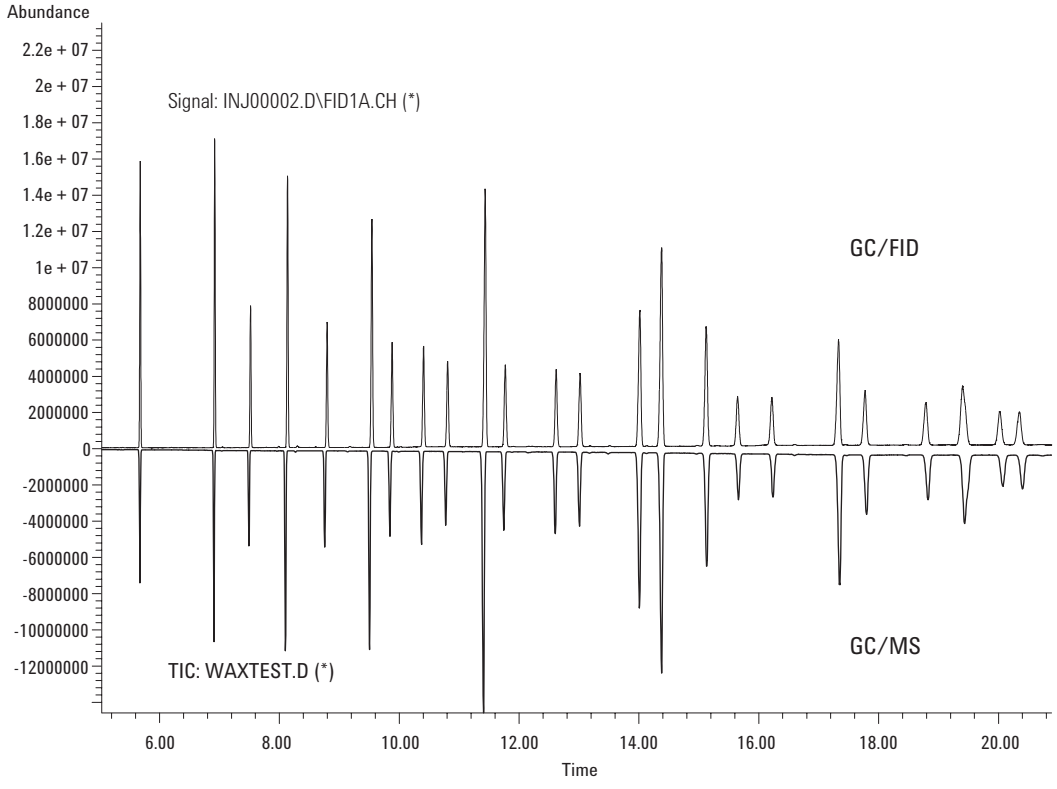

Figure 3. Comparison of GC/FID and GC/MS chromatograms obtained on a 30-m × 0.25-mm id × 0.25-µm DB-Wax column (part no. 122-7032) using method 1 (see Table 2).

The retention time locked method on the DB-Wax column was applied to the analysis of two certified reference samples (CRM 162, a soy-maize blend oil and CRM 164, a milk fat)[7]. Both samples were prepared according to sample preparation method 2. The resulting chromatograms are shown in Figure 4 (soy-maize oil blend) and in Figure 5 (milk fat). The peaks were automatically identified using the RTL FAMEs retention time database. The quantitative results are summarized in Tables 4 and 5.

Figure 4 shows a classical fatty acid profile normally obtained for edible oils. Figure 5 shows the fatty acid profile typical for milk fat. Butyric acid elutes at 2.85 min and is easily detected. Very good reproducibility is obtained. The standard deviation of the relative areas is smaller than 1% in all cases. Also the correspondence between the measured fatty acid composition and the certified values is good.

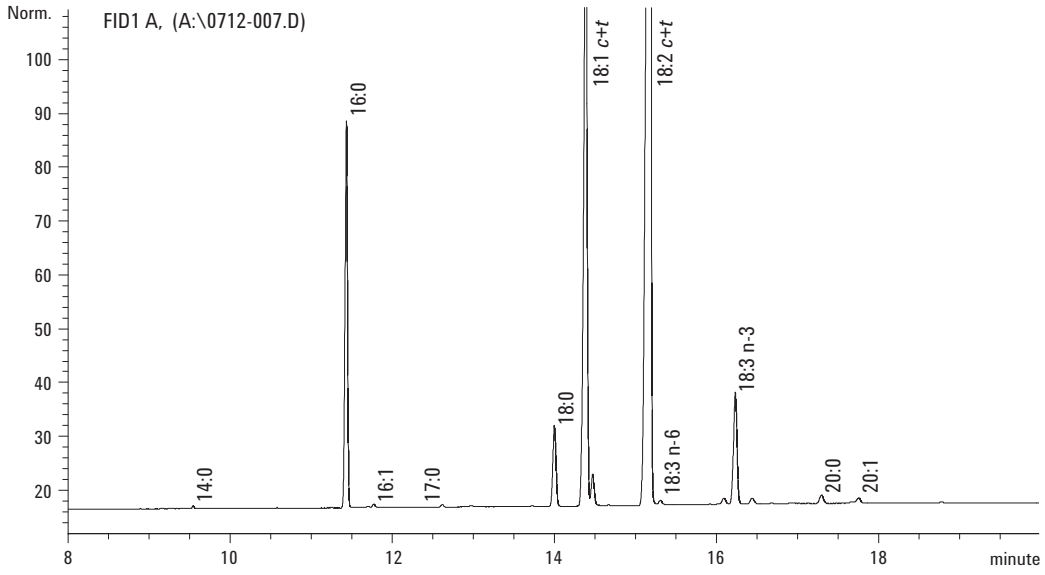

Figure 4. GC/FID analysis of soy-maize oil (CRM 162) FAMEs on a 30-m × 0.25-mm id × 0.25-µm DB-Wax column (part no. 122-7032) using method 1 (see Table 2).

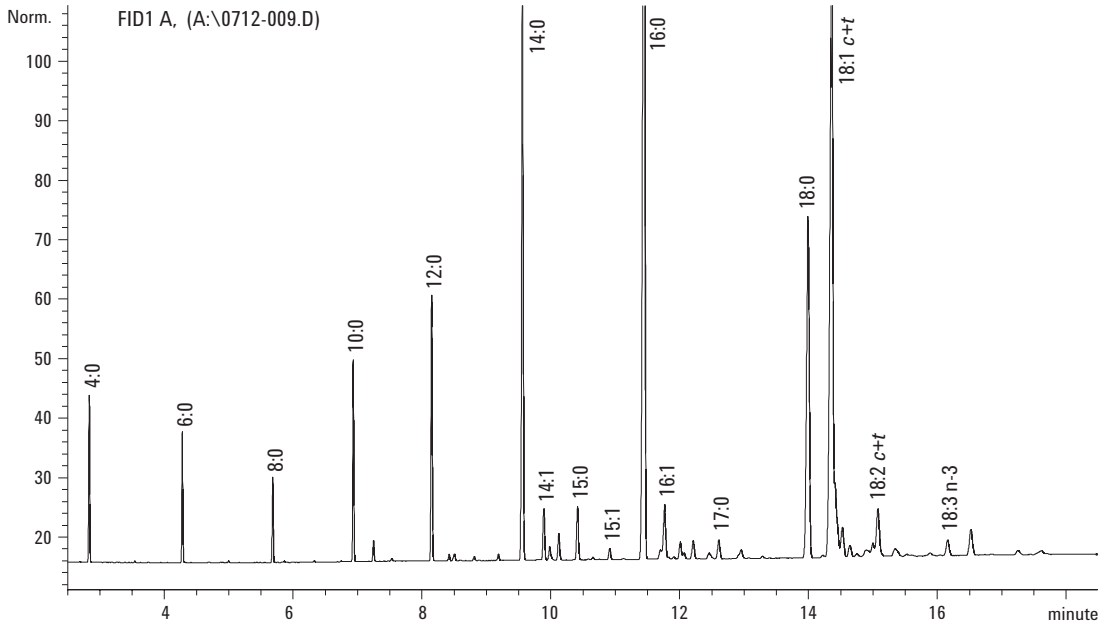

Figure 5. GC/FID analysis of milk fat (CRM 164) FAMEs on a 30-m × 0.25-mm id × 0.25-µm DB-Wax column (part no. 122-7032) using method 1 (see Table 2).

Table 4: Quantitative Data Obtained Using RTL Method 1 for CRM 162

Fatty acid	Measured concentration (g/100 g)	Standard deviation	Certified concentration (g/100 g) [7]	Uncertainty (**)
16:0	10.607	0.003	10.65	0.17
18:0	2.917	0.005	2.87	0.07
18:1	24.461	0.013	24.14	0.28
18:2	57.051	0.017	56.66	0.54
18:3	4.286	0.017	4.68	0.21
20:0	0.368	0.003	(0.3) (*)	
20:1	0.246	0.003	(0.2) (*)	

(*) Indicative values, not certified

(**) Uncertainty is taken as half-width of the 95% confidence interval of the certified mean value.

Table 5: Quantitative Data Obtained Using RTL Method 1 for CRM 164

Fatty acid	Measured concentration (g/100 g)	Standard deviation	Certified concentration (g/100 g) [7]	Uncertainty (*)
4:0	3.522	0.012	3.49	0.06
6:0	2.318	0.003	2.36	0.19
8:0	1.420	0.002	1.36	0.10
10:0	3.010	0.006	2.89	0.12
12:0	3.907	0.008	4.03	0.10
14:0	11.383	0.014	10.79	0.35
16:0	27.693	0.005	26.91	0.84
18:0	10.882	0.009	10.51	0.40
18:1	24.832	0.009	24.82	0.61
18:2 (Σ)	2.844	0.012	2.68	0.40
18:3	0.604	0.005	0.51	0.04

(*) Uncertainty is taken as half-width of the 95% confidence interval of the certified mean value.

The separation for the 37-component standard mixture on the 60-m × 0.25-mm id 0.15-µm DB-23 column using method 2 is shown in Figure 6. Using these conditions, all compounds in the standard mixture are resolved. Important is the separation of the *cis*/*trans* isomers and the separation of EPA (20:5, 19.15 min) and DHA (22:6, 22.38 min). This method is very useful for the analysis of fatty acid profiles in complex mixtures. An example of the separation obtained for a mixture of polyunsaturated FAMEs from a marine source appears in Figure 7. The identifications shown on the chromatogram were done by using a classical calibration table with a ±5% retention time window for identification and quantitation (the default setting). Using this setting, the last three peaks (at 22.122, 22.262, and 22.414 min) were identified as 24:0, 24:1, and 22:6, respectively. The result from the peak identification using the locked retention time database appears in Table 6. RTL library searching shows that the initial identification (using the calibration table) was wrong. The correct identification was: 22.122 min = unknown (no fatty acid methyl ester), 22.262 min = 24:1, and 22.414 min = 22:6. These identifications could be confirmed easily by GC/MS. The profiles obtained by GC/FID and GC/MS for the same sample of marine FAMEs appears in Figure 8. An excellent retention time correlation was obtained.

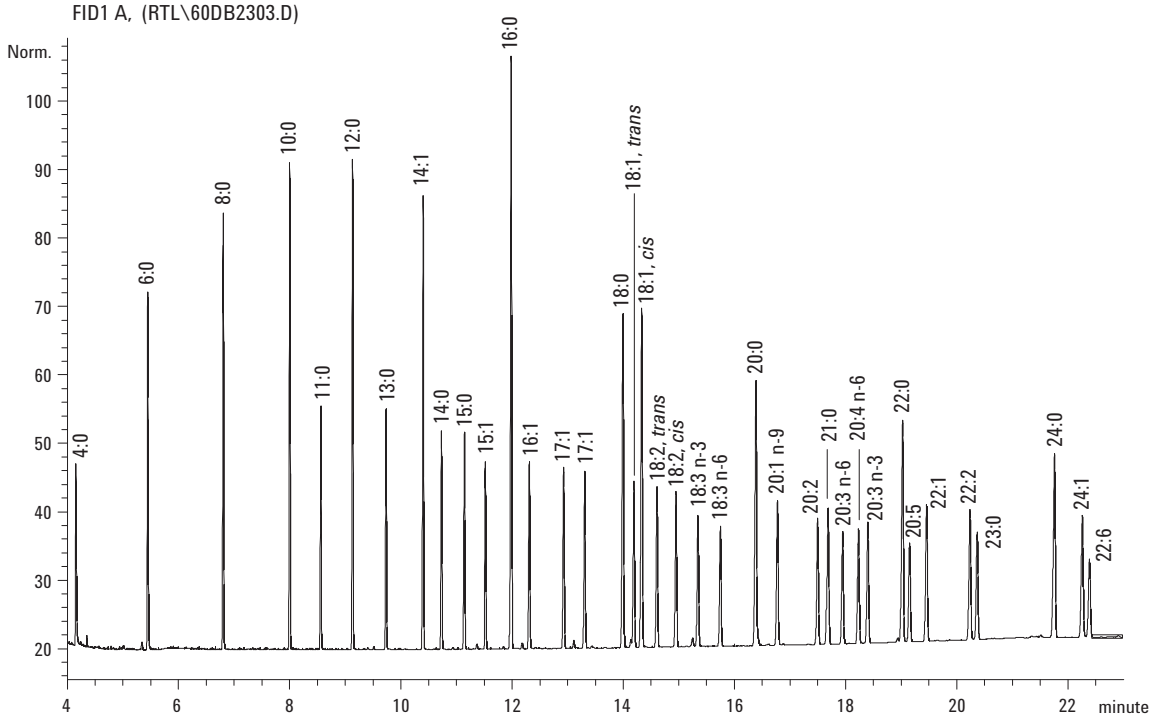

Figure 6. GC/FID analysis of FAMEs standard mixture on a 60-m × 0.25-mm id × 0.15-µm DB-23 column (part no. 122-2361) using method 2 (see Table 3).

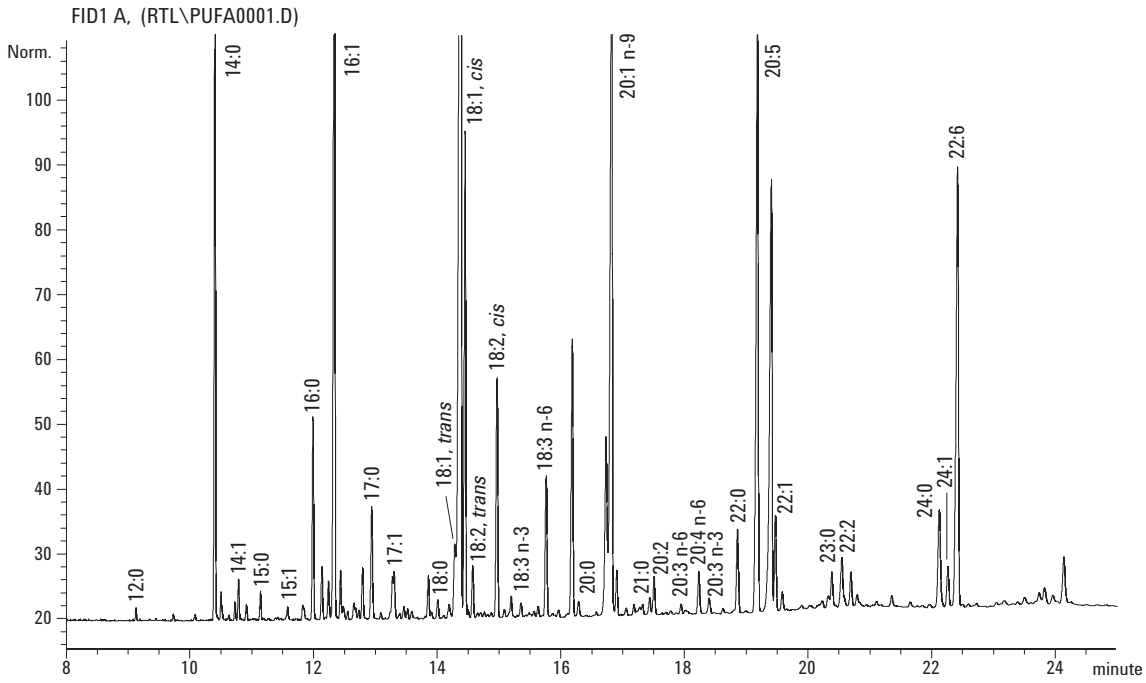

Figure 7. GC/FID analysis of unsaturated FAME mixture of marine origin on a 60-m × 0.25-mm id × 0.15-µm DB-23 column (part no. 122-2361) using method 2 (see Table 3). The peak at 22.122 min was initially identified as 24:0 using a normal calibration table with the default retention time window. However, RTL library searching proved that this compound was, instead, an unknown.

Table 6. GC/FID Peak Identifications Using the FAMEs Retention Time Database

Data file:	D:\HPCHEM\2\DATA\RTL\PUFA0001.D		
Sample name:	PUFA 1		
Instrument 2:	2/15/02 2:40:50 PM		
Injection date:	2/14/02 5:04:32 PM	Seq. Line :	1
Sample name:	PUFA 1	Vial:	01
Acq. operator:	VH	Inj:	1
Acq. method:	RTLDB23.M		

Results of RT table search

Search results for 22.122 ±0.100 minutes	
Contains elements:	{No restriction}
Does not contain elements:	{No restriction}
RTT file searched:	D:\HPCHEM\RTL\FAMDB23.RTT
No matches found.	

Search results for 22.262 ±0.100 minutes	
Contains elements:	{No restriction}
Does not contain elements:	{No restriction}
RTT file searched:	D:\HPCHEM\RTL\FAMDB23.RTT
RT	Compound
22.254	Nervonic acid methyl ester
	Code 24:1

Search results for 22.414 ±0.100 minutes	
Contains elements:	{No restriction}
Does not contain elements:	{No restriction}
RTT file searched:	D:\HPCHEM\RTL\FAMDB23.RTT
RT	Compound
22.382	*cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid methyl ester
	Code 22:6

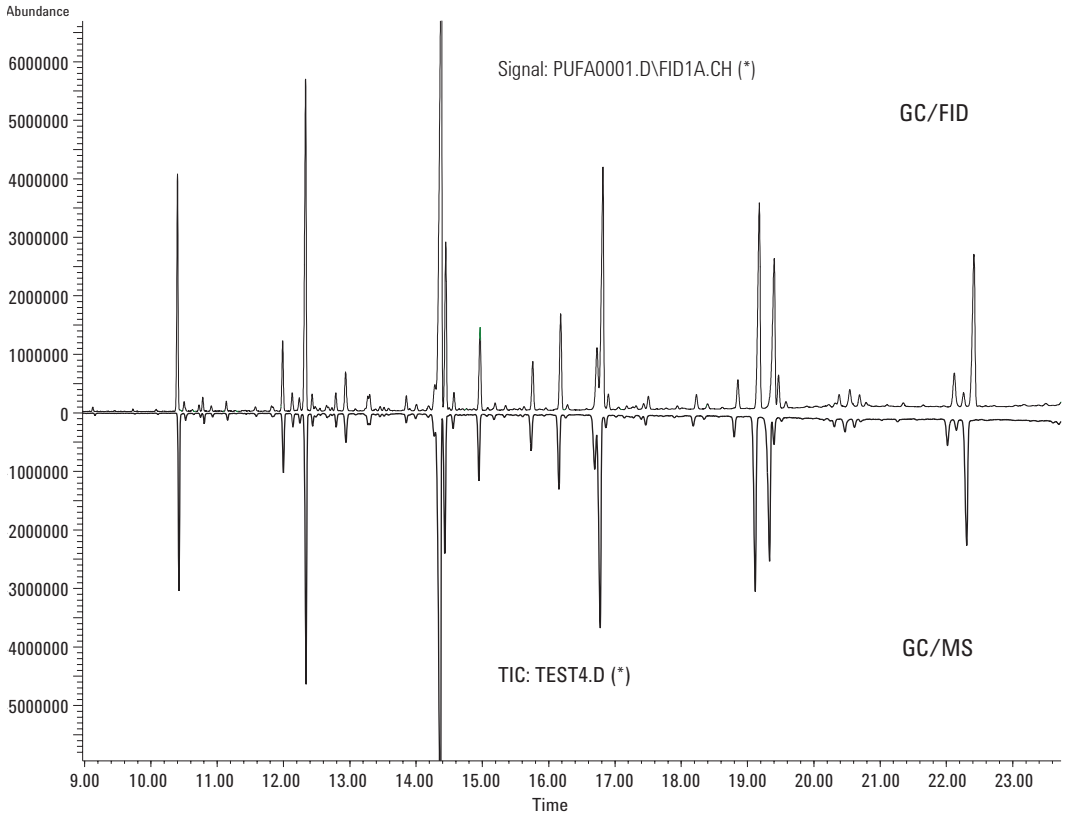

Figure 8. Comparison of GC/FID and GC/MS chromatograms obtained for the unsaturated FAME mixture of marine origin on a 60-m × 0.25-mm id × 0.15-µm DB-23 column (part no. 122-2361) using method 2 (see Table 3).

Conclusions

Two methods are described for the analysis of FAMEs. Method 1, using an Agilent DB-Wax column, is useful for the analysis of classical edible oils and fats, including the determination of butyric acid in milk fat. Method 2, applying an Agilent DB-23 cyanopropyl column, can be used for the analysis of more complex samples, including fish oils and hydrogenated fats, for the determination of EPA and DHA and for *cis*/*trans* determination. Using retention time locking, GC/FID and GC/MS retention times can be closely matched for easy correlation of chromatograms between the instruments. RTL database searching makes peak identification more accurate.

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