Column Selection for the Analysis of Fatty Acid Methyl Esters

Application

Food Analysis

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Abstract

The analysis of fatty acid methyl esters (FAMEs), derived from food, is a very important food characterization procedure. These 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 their carbon number, the degree of unsaturation, the *cis-trans* configuration, and the location of the double bonds.

In this application note, three different stationary phases are compared for the separation of FAMEs. Polyethylene glycol columns gave good separation for the less complex samples, but they did not separate *cis-trans* isomers. A medium polar cyanopropyl column (DB23) provided excellent separation for complex FAME mixtures and also achieved some *cis-trans* separation. For more detailed *cis-trans* separation, the highly polar HP-88 cyanopropyl column is preferred.

Introduction

The analysis of FAMEs is used for the characterization of the lipid fraction in foods, and is one of the most important analyses for food. Lipids mainly consist of triglycerides, being esters of one glycerol molecule and three fatty acid molecules. Most edible fats and oils contain mainly fatty acids ranging from lauric acid (dodecanoic acid) to arachidic acid (eicosanoic acid). Besides the linear saturated fatty acids, branched fatty acids, mono-unsaturated, di-unsaturated, and higher unsaturated fatty acids can also occur. An overview of the most important fatty acids and their abbreviations appears in Table 1.

Table 1. Fatty Acids, Common Names, and Abbreviations

Fatty acid	Common Name	Abbreviation
Butanoic acid	Butyric acid	C4:0
Decanoic acid	Caproic acid	C10:0
Dodecanoic acid	Lauric acid	C12:0
Tetradecanoic acid	Myristic acid	C14:0
Hexadecanoic acid	Palmitic acid	C16:0
Hexadecenoic acid	Palmitoleic acid	C16:1
Octadecanoic acid	Stearic acid	C18:0
cis-9-Octadecenoic acid	Oleic acid	C18:1- *cis* (n9)
trans-9-Octadecenoic acid	Elaidic acid	C18:1- *trans* (n9)
all *cis*-9,12-Octadecadienoic acid	Linoleic acid	C18:2 - *cis* (n6)
all *trans*-9,12-Octadecadienoic acid	Linolelaidic acid	C18:2 - *trans* (n6)
all *cis*-9,12,15-Octadecatrienoic acid	α-Linolenic acid	C18:3 (n3)
all *cis*-6,9,12-Octadecatrienoic acid	γ-Linolenic acid	C18:3 (n6)
Eicosanoic acid	Arachidic acid	C20:0
cis-11-Eicosenoic acid		C20:1 (n9)
all *cis*-11,14-Eicosadienoic acid		C20:2 (n6)
all *cis*-11,14,17-Eicosatrienoic acid		C20:3 (n3)
all *cis*-8,11,14-Eicosatrienoic acid	Dihomogammalinolenic acid	C20:3 (n6)
all *cis*-5,8,11,14-Eicosatetraenic acid	Arachidonic acid	C20:4 (n6)
all *cis* 5,8,11,14,17-Eicosapentenoic acid	EPA	C20:5 (n3)
Docosanoic acid	Behenic acid	C22:0
cis-13-Docosenoic acid	Erucic acid	C22:1 (n9)
all *cis*-7,10,13,16-Docosatetraenoic acid		C22:4 (n6)
all *cis* 4,7,10,13,16,19-Docosahexenoic acid	DHA	C22:6 (n3)
Tetracosanoic acid	Lignoceric acid	C24:0
cis-15-tetracosenoic acid	Nervonic acid	C24:1 (n9)

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 an HP-FFAP column), more robust and reproducible chromatographic data are obtained if the fatty acids are derivatized to the methyl esters. For the derivatization, including hydrolysis and methylation, different methods are available [1]. These methods are easy to use and do not require expensive reagents or equipment. A typical sample preparation method is described in the sample preparation section.

After preparation of the FAMEs, they are separated according to the carbon number (number of carbon atoms in the fatty acid chain, excluding the methyl ester carbon) and 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, three stationary phases are compared for the separation of FAMEs. The first method uses DB-Wax, a polyethylene glycol column, where FAMEs from C4 (butyric acid) to C24 (lignoceric acid) can be separated according to carbon number and degree of unsaturation. On these columns, no separation of *cis*- and *trans*-isomers is obtained, and for complex mixtures, such as fish oils, some FAMEs are difficult to separate. However, the separation of FAMEs on polyethylene glycol columns is widely used and are applied to the characterization of "classical" samples, such as vegetable oils from corn, maize, olive, and soybean. Moreover, animal fats can also be

analyzed. One important application is the analysis of butyric acid in milk fat. The concentration of butyric acid in milk is an important indicator of milk quality, and its analysis is therefore very important in milk, dairy, and chocolate products.

For the analysis of complex samples, such as fish oils, additional resolution of the FAMEs is needed, and is obtained using a capillary column coated with a cyanopropyl-stationary phase, such as a DB-23. On this column, highly unsaturated fatty acids, such as all *cis* 5, 8, 11, 14, 17-eicosapentenoic acid methyl ester (EPA, C20:5 ω3) and all *cis* 4,7,10,13,16,19-docosahexenoic acid methyl ester (DHA, C22:6 ω3) are separated from other FAMEs. This analysis is very important in the framework of recent interest in omega-3 fatty acid determination. On the cyanopropyl column, separation of the *cis*- and *trans*-isomers is also possible. Due to stronger interaction of the *cis*-isomer with the cyano-dipole, the *trans*-isomers elute before the *cis*-isomers. In this way, the determination of *trans*-fatty acids is also performed, however, the polarity of the stationary phase is not sufficient to fully separate complex *cis-trans* mixtures.

For the separation of a complex FAME mixture containing a relatively large amount of *trans*-fatty acids, a highly polar HP-88 column is preferred. On this highly polar column excellent separation between different *cis*- and *trans*-isomers is obtained, however, some higher molecular weight fatty acids are more difficult to separate.

An overview of columns and their application area is summarized in Figure 1.

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%–0.1% (w/v) concentration.

For column check-out, a 37-component mixture (Supelco #18919) was used. The mixture is available as a 100-mg neat mixture, containing C4–C24 FAMEs (2%–4% relative concentration). The whole sample was diluted in 10-mL hexane (final concentration = 0.2–0.4 mg/mL per FAME) before use. Oil and fat samples can be prepared using different methods [1–5].

Sample Preparation Method [5]

Weigh 100-mg sample in a 20-mL test tube (with screw cap) or reaction vial. Dissolve the sample in 10-mL hexane. Add 100-µL 2 N potassium hydroxide in methanol (11.2 g in 100 mL). Close the tube or vial and vortex for 30 s. Centrifuge. Transfer the clear supernatent into a 2-mL autosampler vial.

Analytical Conditions

The analyses were performed on an Agilent 6890 GC equipped with a flame ionization detector (FID). Automated split injection was performed using an Agilent 7683 autosampler. The instrumental configuration and analytical conditions are summarized in Table 2 (DB-Wax column), Table 3 (DB-23 column) and Table 4 (HP-88 column).

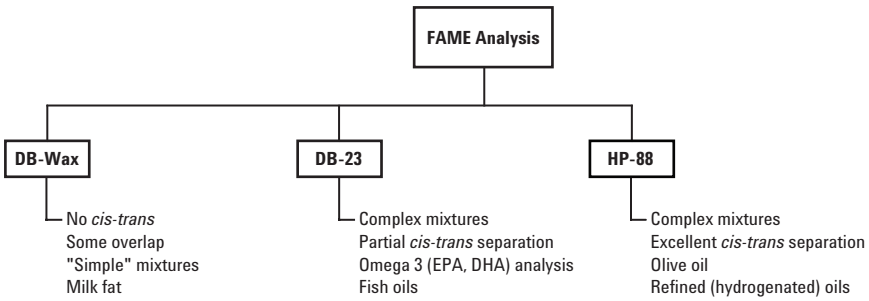

Figure 1. Overview of column selection for FAMEs analysis.

Table 2. DB-Wax Method 1

Instrumentation	
Chromatographic system:	Agilent 6890 GC
Inlet	Split/Splitless
Detector	FID or Agilent 5973 MSD
Automatic Sampler	Agilent 7683
Liner	Split liner (p/n 5183-4647)
Column	30 m × 0.25 mm ID, 0.25 µm DB-Wax (J&W 122-7032)
Experimental Conditions GC-FID	
Inlet temperature	250 °C
Injection volume	1 µL
Split ratio	1/50
Carrier gas	Hydrogen
Head pressure	53 kPa constant pressure (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: 40 mL/min; Air: 450 mL/min; Helium make-up gas: 30 mL/min.

Table 3. DB-23 Method 2

Instrumentation	
Chromatographic system:	Agilent 6890 GC
Inlet	Split/Splitless
Detector	FID or Agilent 5973 MSD
Automatic Sampler	Agilent 7683
Liner	Split liner (p/n 5183-4647)
Column	60 m × 0.25 mm ID, 0.15 µm DB-23 (J&W 122-2361)
Experimental Conditions GC-FID	
Inlet temperature	250 °C
Injection volume	1 µL
Split ratio	1/50
Carrier gas	Helium
Head pressure	230 kPa constant pressure (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: 40 mL/min; Air: 450 mL/min; Helium make-up gas: 30 mL/min.

Table 4. HP-88 Methods 3A and 3B

Instrumentation	
Chromatographic system:	Agilent 6890 GC
Inlet	Split/Splitless
Detector	FID or Agilent 5973 MSD
Automatic Sampler	Agilent 7683
Liner	Split liner (p/n 5183-4647)
Column A	100 m × 0.25 mm ID, 0.2 µm HP-88 (J&W 112-88A7)
Column B	60 m × 0.25 mm ID, 0.2 µm HP-88 (J&W 122-8867)
Experimental Conditions GC-FID	
Inlet temperature	250 °C
Injection volume	1 µL
Split ratio	1/50
Carrier gas A	Hydrogen
Carrier gas B	Helium
Head pressure	2 mL/min constant flow
Oven temperature A	120 °C, 1 min, 10 °C/min to 175 °C, 10 min, 5 °C/min to 210 °C, 5 min 5 °C/min to 230 °C, 5 min
Oven temperature B	175 °C, 10 min, 3 °C/min, 220 °C, 5 min
Detector temperature	280 °C
Detector gases	Hydrogen: 40 mL/min; Air: 450 mL/min; Helium make-up gas: 30 mL/min.

Results

A typical chromatogram for the analysis of the 37-compound FAMEs reference standard, obtained on the DB-Wax column is shown in Figure 2.

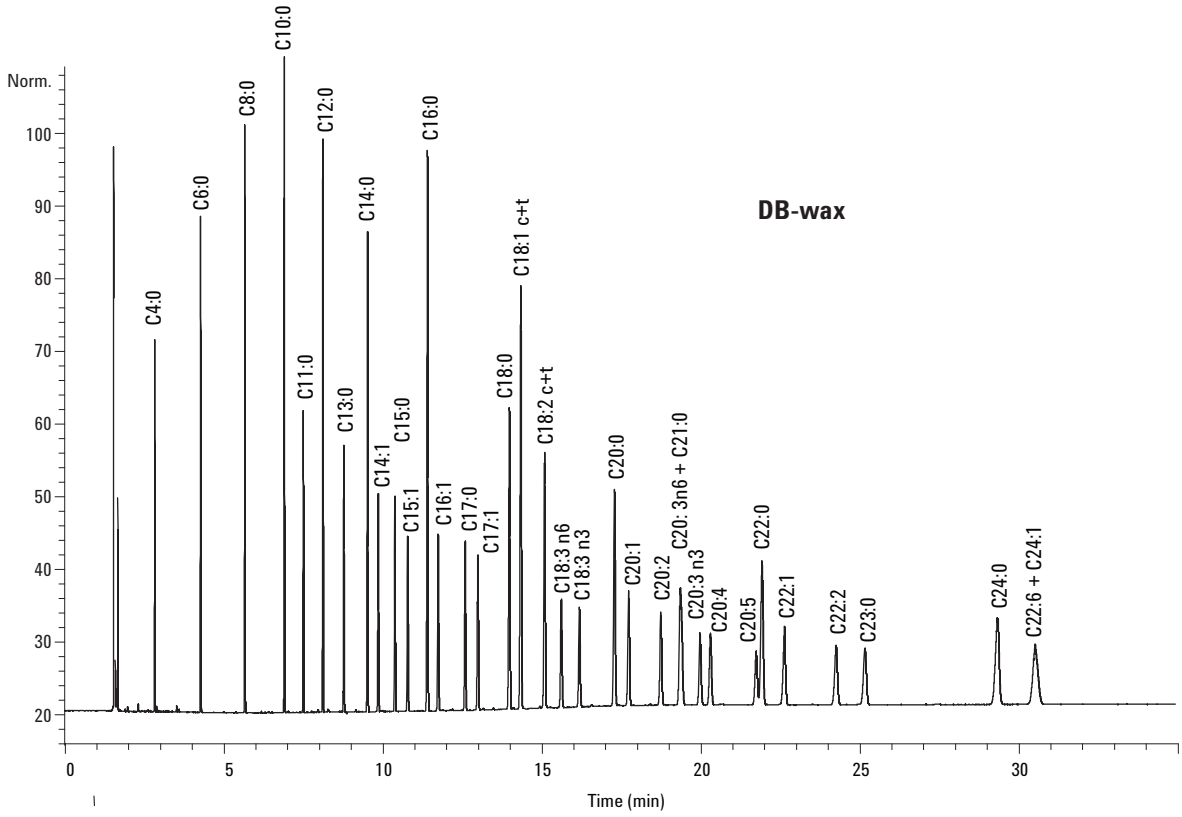

Figure 2. GC-FID analysis of 37-component FAMEs standard mixture on a 30 m × 0.25 mm ID, 0.25 µm DB-Wax column using Method 1. (See Table 2).

A good separation is obtained, except for the following compounds: *cis*- and *trans*-C18:1 coelute at 14.38 min, *cis*- and *trans*-C18:2 coelute at 15.13 min, C20:3 n6 and C21:0 coelute at 19.44 min, and C22:6 and C24:1 coelute at 30.73 min. However, this separation is sufficient for some classical oil and fat characterization methods. Butyric acid elutes at 4.28 min and can be determined in milk fat using this method. This is demonstrated in Figure 3, showing the analysis of a certified reference sample of milk fat (CRM 164, [6]).

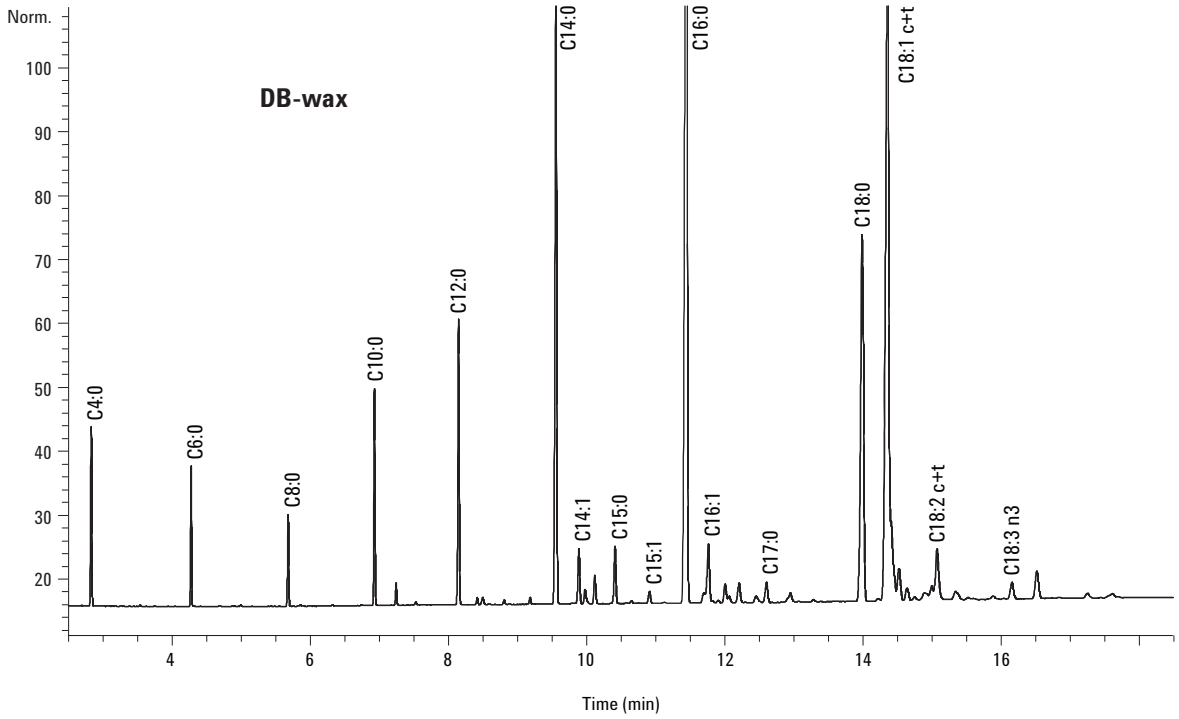

Figure 3. GC-FID analysis of milk fat (CRM 164) fatty acids on a 30 m × 0.25 mm ID, 0.25 µm DB-Wax column using Method 1, Table 2.

The separation of the 37-compound FAME standard mixture on the 60 m × 0.25 mm ID, 0.15 µm DB-23 column is shown in Figure 4.

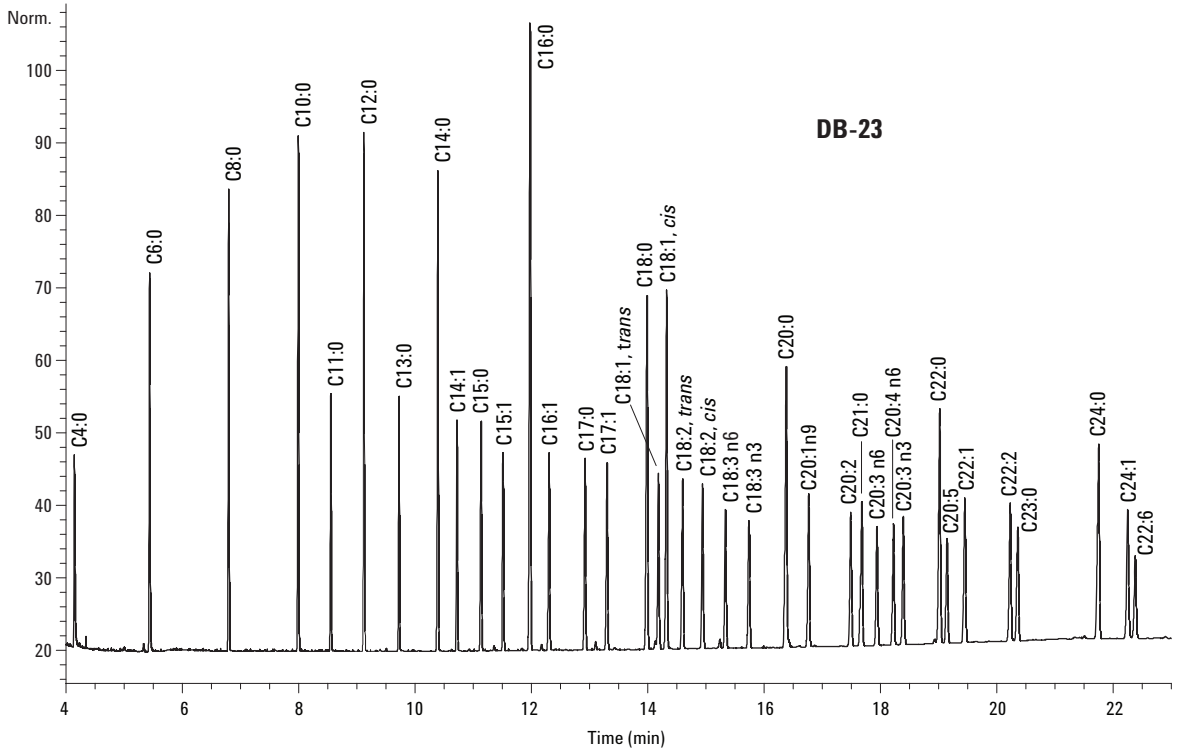

Figure 4. GC-FID analysis of FAMEs standard mixture on a 60 m × 0.25 mm ID, 0.15 µm DB-23 column using Method 2, Table 3.

Using these conditions, all compounds in the standard mixture are well resolved. Important is the separation of the *cis/trans* isomers and the separation of EPA (19.15 min) and DHA (22.38 min) components. This method is very useful for the analysis of fatty acid in complex mixtures, and especially for the determination of omega-3 fatty acids (such as EPA and DHA). An example of the separation obtained for a mixture of polyunsaturated fatty acids from a marine source is given in Figure 5. EPA and DHA can easily be detected and quantified.

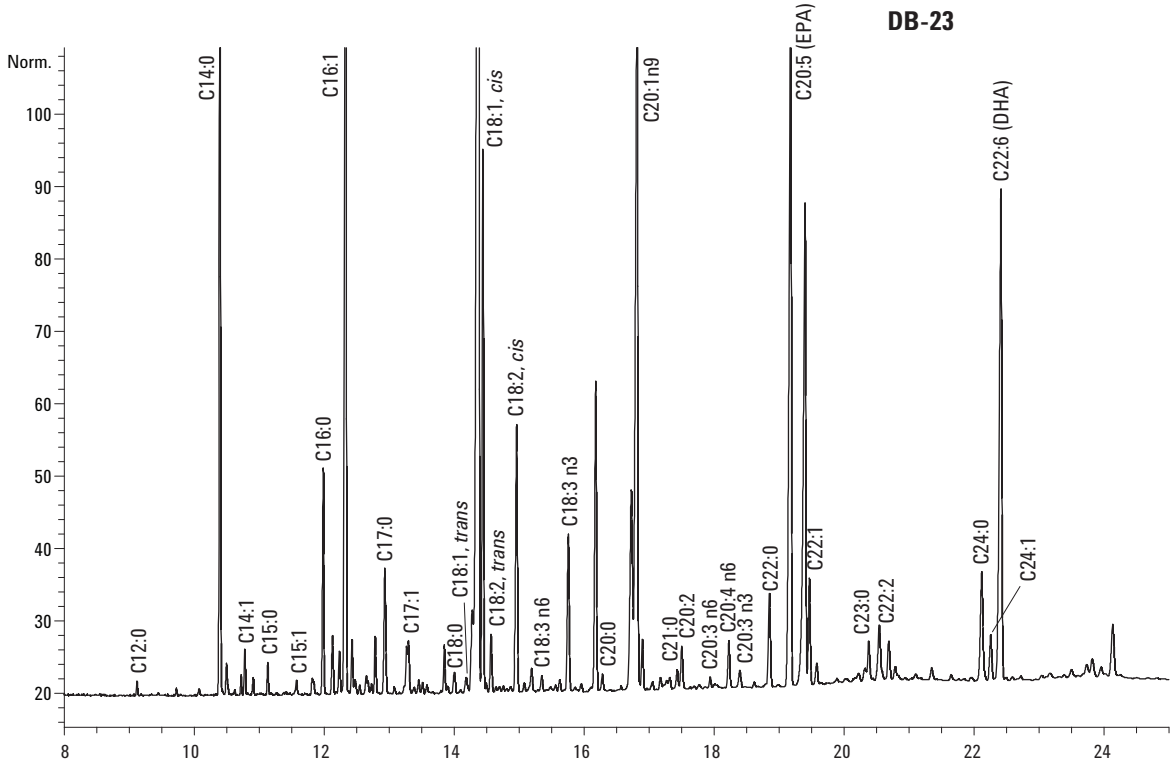

Figure 5. GC-FID analysis of unsaturated fatty acid mixture from marine origin on a 60 m × 0.25 mm ID, 0.15 µm DB-23 column using Method 2, Table 3.

The separation of the 37-compound mixture on the highly polar HP-88 column is shown in Figure 6.

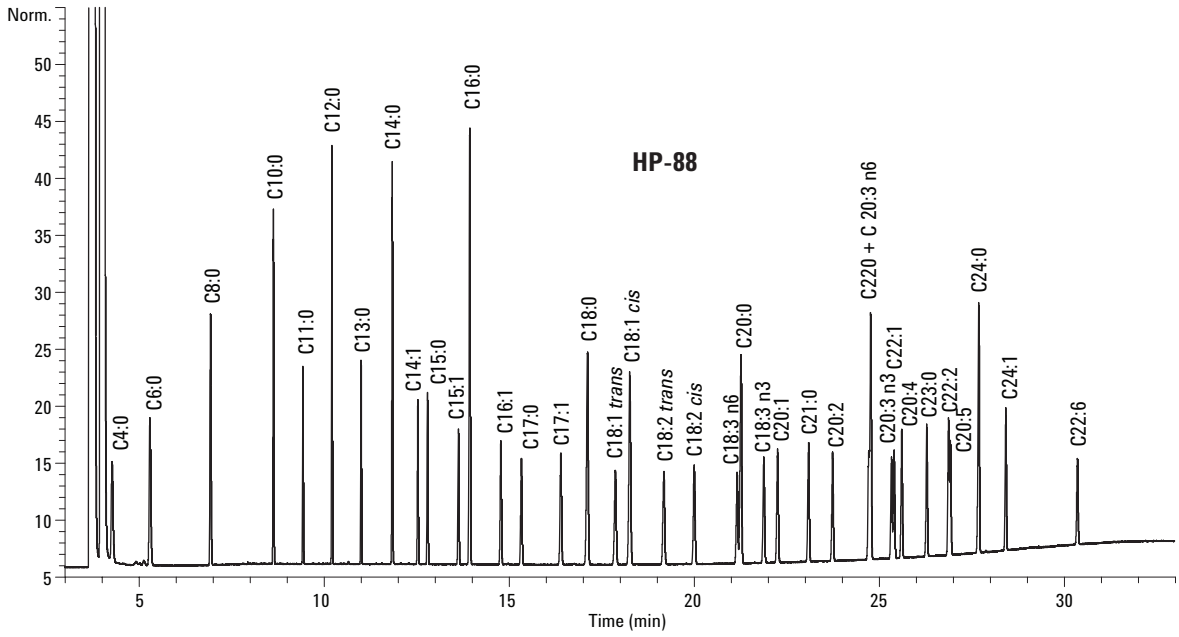

Figure 6. GC-FID analysis of 37-component FAMEs standard mixture on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4A.

Again a quite good separation is obtained, except for the separation of C22:0 and C20:3 (n-6) that coelute at 24.7 min. Using this column, however, the separation of *cis*- and *trans*-isomers is excellent. This is illustrated by the separation of a standard mixture containing five C18:1 isomers. The *cis*- and *trans*- positional isomers are well separated, as shown in Figure 7.

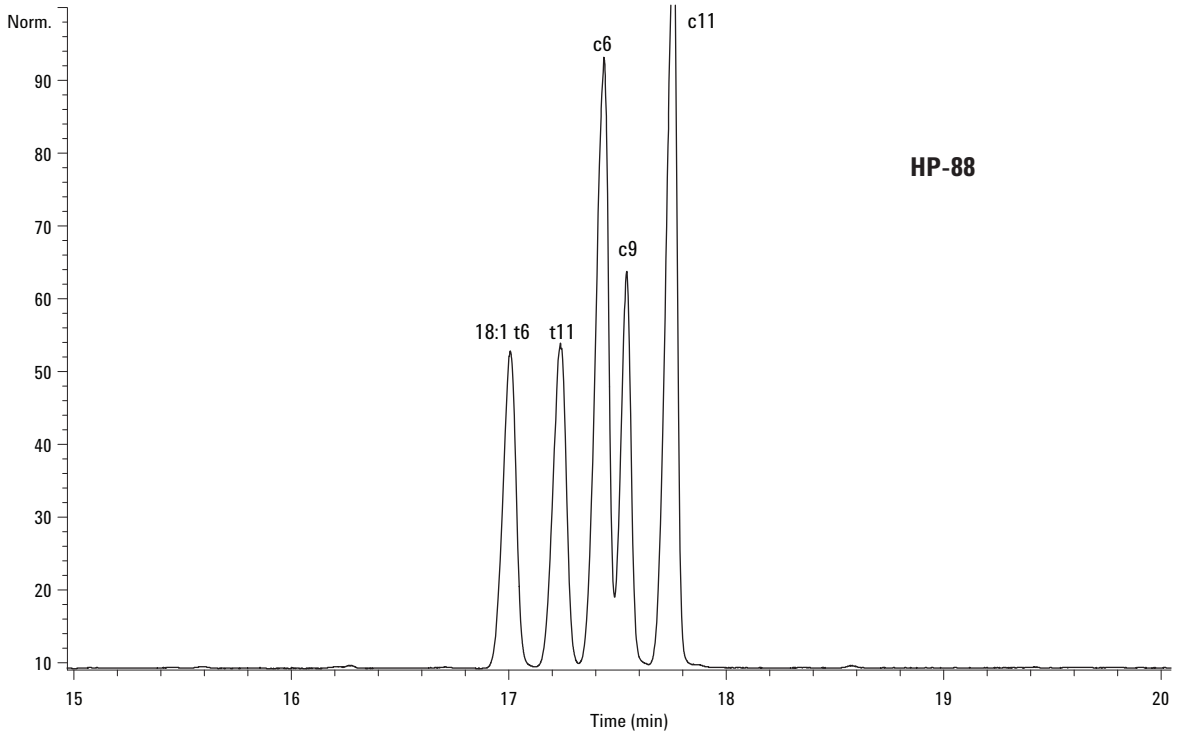

Figure 7. GC-FID analysis of C18:1 isomers on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4A.

Equally good separation is obtained for four C18:2 isomers (*trans-trans, cis-trans, trans-cis* and *cis-cis*), as shown in Figure 8.

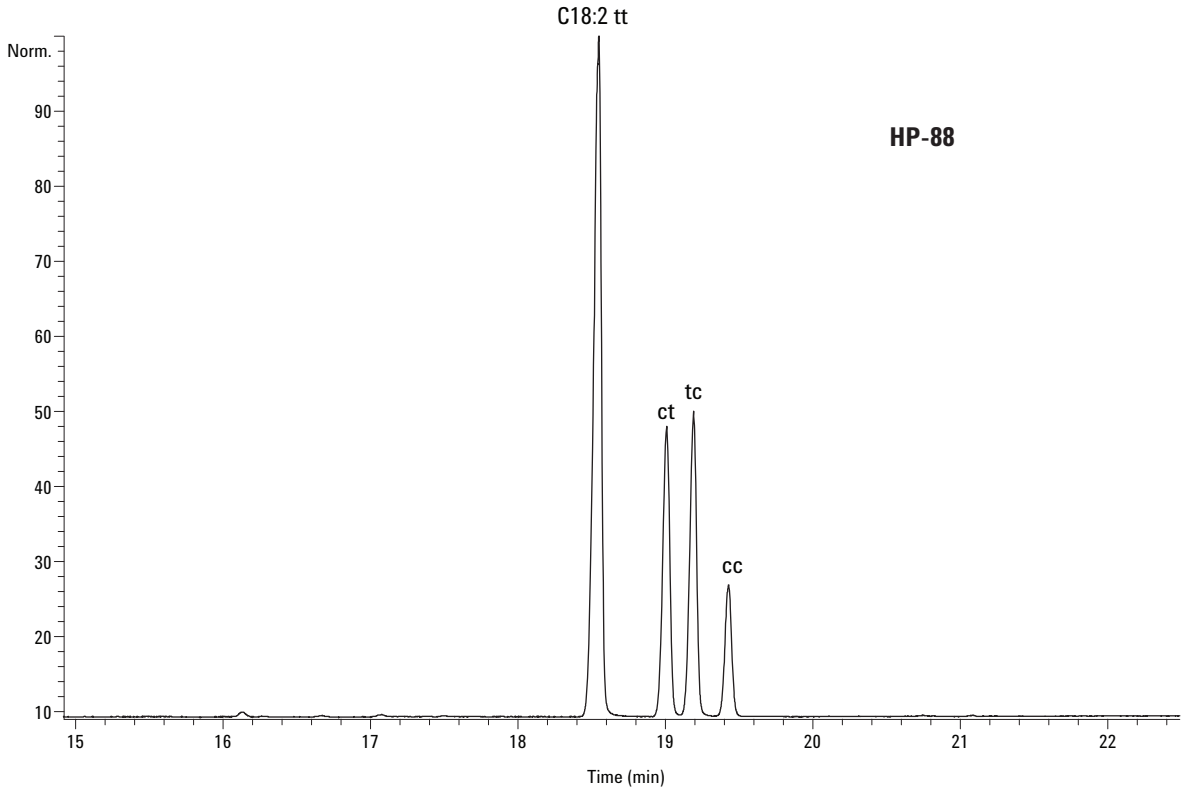

Figure 8. GC-FID analysis of C18:2 isomers on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A, Table 4.

A comparison between a DB-23 and a HP-88 column was made for the separation of a highly hydrogenated oil. Due to the hydrogenation process, all possible positional and geometrical (*cis-trans*) isomers are formed. The sample was analyzed on a DB-23 column and an HP-88 column respectively, both isothermally at 180 °C. the chromatograms are compared in Figure 9 (details of C18:1 elution window). Although Figure 4 shows baseline separation of *trans*-C18:1(n9) and *cis*-C18:1 (n9) in the 37-component standard, Figure 9 demonstrates that for real samples containing several C18:1 isomers, the *cis-trans* separation with the HP-88 is a preferred column choice.

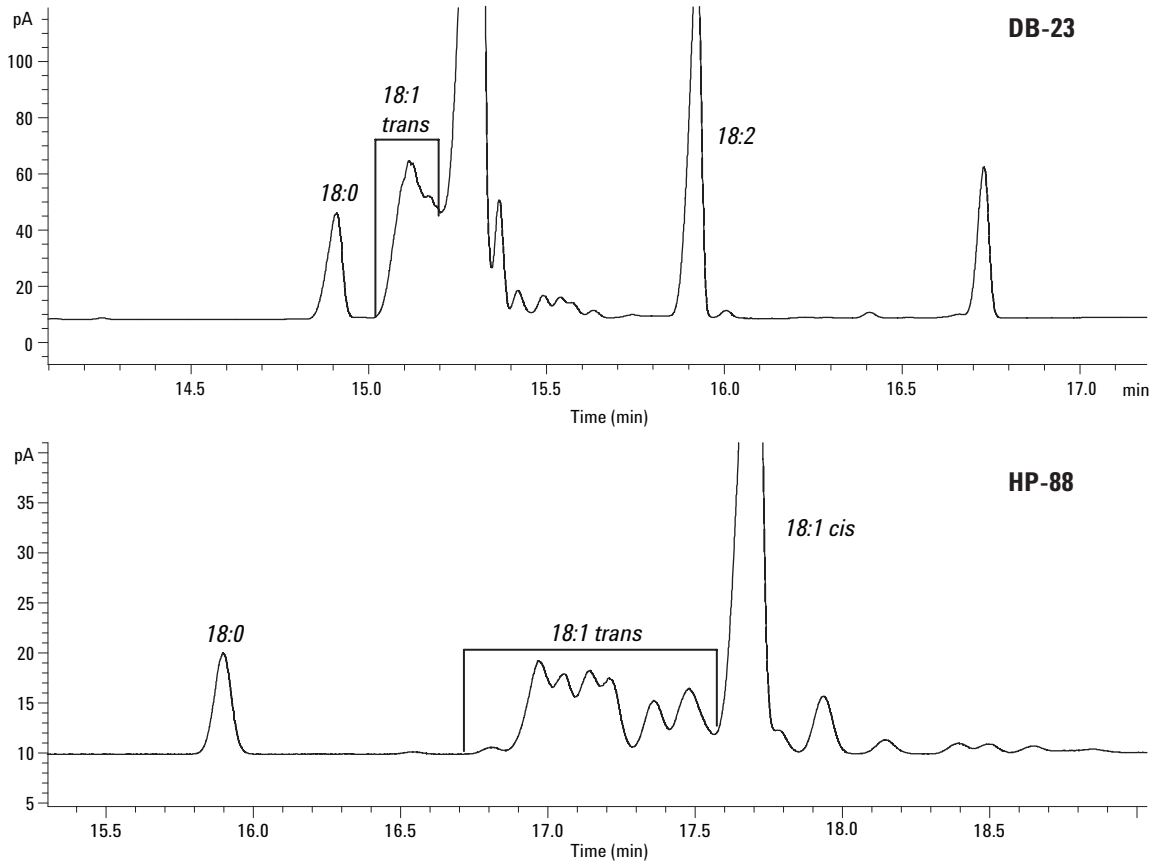

Figure 9. Comparison of the separation of C18:1 isomers from a hydrogenated oil obtained on a 60 m x 0.25 mm ID, 0.15 µm DB-23 column (upper window) and on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column. Both analyses were performed at 180 °C isothermally.

The application of the HP-88 is demonstrated by the analysis of a partially hydrogenated rapeseed oil. The separation of the *trans* fatty acids is clearly illustrated in Figure 10. The valley between *trans*- and *cis*-isomers can easily be determined. Also the other *trans*-isomers (C18:2 and C18:3) can be detected.

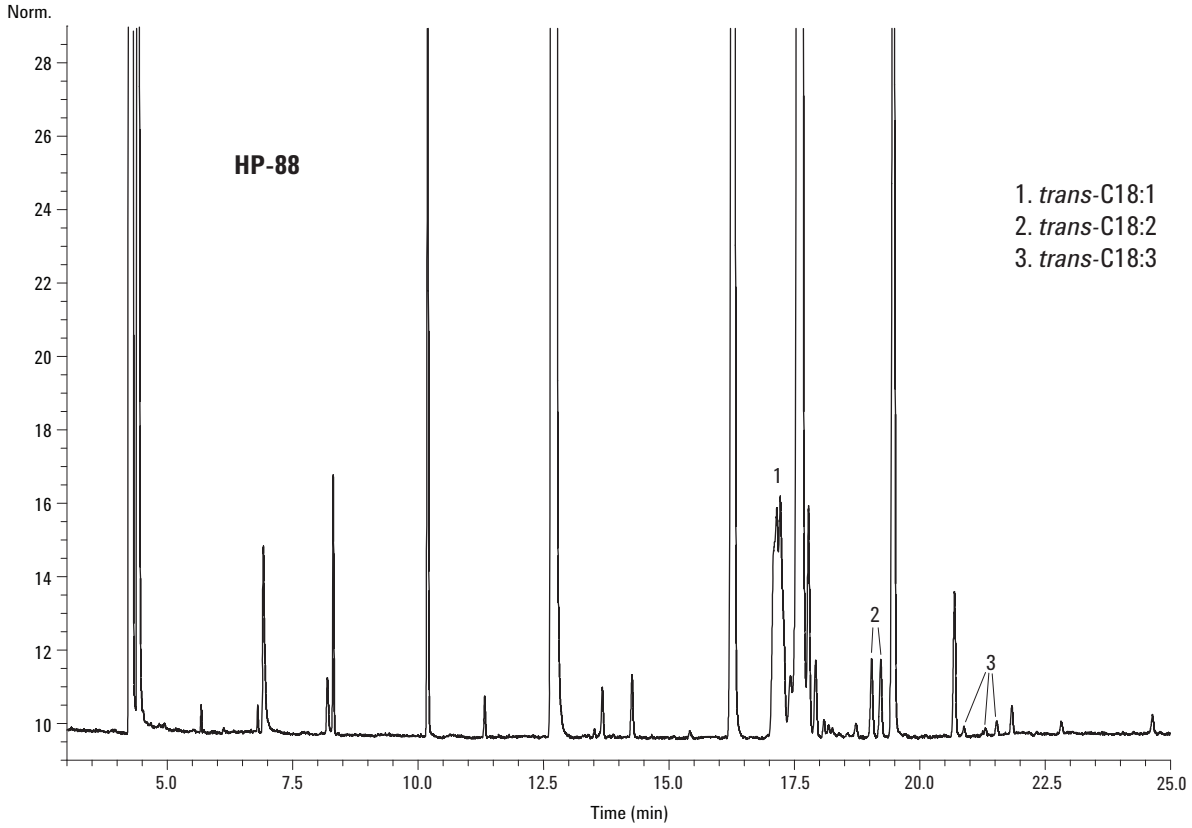

Figure 10. GC-FID analysis of FAMEs from a partially hydrogenated rapeseed oil on a 100 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3A. (See Table 4).

The same column can also be used for quality control of olive oil according to EC regulation 2568/91 [5]. Using the method described in Table 4 (Column A – Method A), the analysis time for the 100-m column is approximately 35 min using hydrogen as carrier gas. For the QC analysis of olive oil, a 60-m column can also be used (Table 4 – column B). Using helium as carrier gas and a different temperature program (that is, oven temperature B, Table 4), an excellent separation is obtained in less than 20 min, as shown in Figure 11. The obtained separation fully conforms to the EC regulation [5].

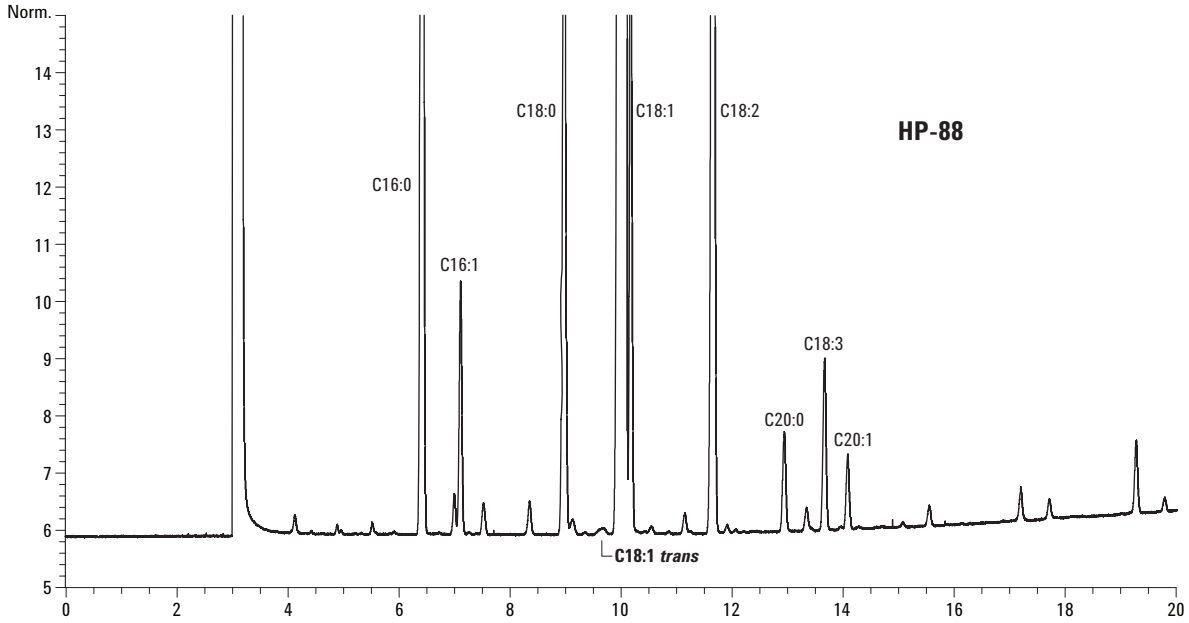

Figure 11. GC-FID analysis of olive oil FAMEs on a 60 m × 0.25 mm ID, 0.2 µm HP-88 column using Method 3B, Table 4.

Conclusions

Three types of stationary phases can be used for the analysis of FAMEs.

1. A DB-Wax column, is useful for the analysis of classical edible oils and fats, including the determination of butyric acid in milk fat. Using this column, however, no separation of *cis-trans* isomers is obtained.
2. A medium polar DB-23 cyanopropyl column is excellent for the analysis of complex FAME mixtures, including fish oils, allowing the determination of omega 3 fatty acids such as EPA and DHA. Partial *cis-trans* separation is obtained.
3. For the most demanding separation of *cis-trans* separation, an HP-88 column is recommended. This column is also the column of choice for olive oil QC analysis.

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