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Detailed analysis and group-type separation of natural fats and oils using comprehensive two-dimensional gas chromatography

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Abstract

Comprehensive gas chromatography (GC×GC) is an adequate methodology for the separation and identification of very complex samples. It is based on the coupling of two capillary columns that each give a different but substantial contribution to the unprecedented resolving power of this technique. The 2D space chromatograms that derive from GC×GC analysis have great potential for identification. This is due to the fact that the contour plot positions, pinpointed by two retention time coordinates, give characteristic patterns for specific families of compounds that can be mathematically translated. This investigation concerned the application of this principle to fatty acid methyl esters that were grouped on an equal double bond number basis. The ester samples were derived from various lipids and all underwent bidimensional analysis on two sets of columns. Peak attribution was supported by mass spectra, linear retention indices and information reported in the literature.

Keywords: Linear retention indices; Food analysis; Gas chromatography, comprehensive two-dimensional; Retention indices; Gas chromatography, orthogonal; Fatty acid methyl esters; Lipids

1. Introduction

Fatty acids (FA), the building blocks of fats and oils, play a fundamental role in the maintenance of good health [1,2]. The majority are present in lipids as esters (mainly triacylglycerols, phospholipids, glycolipids, sphingolipids, etc.) and in some minor groups in other forms. The nutritive/physiological importance of these compounds is based on their dual role as fuel suppliers and as building blocks in biological membranes. This structural function is due to surface-active properties since they are amphiphilic molecules. Saturated and mono- or polyunsaturated aliphatic carboxylic acids are the two great groups into which FA are divided. The saturates are present mainly in animal fats while the mono- and polyunsaturates (PUFA) are dominant in vegetable and fish oils. PUFA are generally characterized by allylic double bonds that are situated in various positions along the carbon chain. The ω3 and ω6 members of this group, such as linoleic and linolenic acid families, cannot be synthesized by our body and

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have to be introduced with food. As such they are called essential fatty acids. A well-balanced consumption of fatty acids is widely encouraged as an excessive intake of saturates and/or a low intake of unsaturates can be prime factors in the cause of widely spread illnesses such as atherosclerosis and coronary heart disease [3]. Natural fats and oils differ greatly in their qualitative and quantitative fatty acid composition. Fish oils, for example, are complex matrices characterized by the presence of various high molecular mass PUFA, while butter, on the other hand, has a specific low molecular mass saturated fatty acid fraction. The improvement and/or introduction of analytical methods capable of achieving the thorough identification of these natural matrices is, therefore, important.

Several techniques have been developed for the analysis of lipids with varying results. Although GC methods have always been the prime analytical choice, applications regarding HPLC can be briefly mentioned. These have mostly concerned regioselective analyses such as the qualitative and quantitative determination of the fatty acid composition in the *sn*-2 position of triacylglycerols via enzymatic hydrolysis [4]. Although this approach has high time costs, this particular analytical aspect is interesting. As regards GC techniques, these can only be applied to lipids that undergo methylic transesterification and can be considered the most effective for total fatty acid determination [5]. Two main GC techniques have been used in the past for the identification of fatty acid methyl esters. The earliest concerned the use of glass or stainless steel columns (3–4 m length and 2–4 mm I.D.) packed with a solid support coated by a polar liquid phase. The quality of separation was low and chromatograms were characterized by several overlapping peaks. Their use rapidly decreased with the revolutionary introduction of open-tubular capillary columns that achieved high resolution standards as the effects that led to band broadening in packed columns were greatly reduced. Conventional OTC GC methods produce effective separations and are employed in official lipidic determinations. In the case of very complex samples, higher resolution power is required. This is achieved through the coupling of different chromatographic techniques leading to multidimensional chromatography (LC–GC, GC–GC, LC–LC etc.). In these specific methods one or more fractions of the initial sample is transferred from the

Table 1
Peak identifications and LRI on both column sets

No.	Compound	LRI	
		Apol/pol	Pol/apol
1	$C_{11:0}$	1437	1685
2	$C_{12:0}$	1534	1841
3	$C_{13:0}$	1631	1937
4	$C_{14:1}$	1701	–
5	$C_{14:0}$	1737	2035
6	$C_{15:0iso}$	1787	2105
7	$C_{15:0anteiso}$	1806	2088
8	$C_{15:1}$	1814	–
9	$C_{15:0}$	1839	2134
10	$C_{16:0iso}$	1896	2187
11	$C_{16:4\omega1}$	1910	2418
12	$C_{16:3\omega4}$	1914	2360
13	$C_{16:1\omega7}$	1922	2265
14	$C_{16:1\omega5}$	–	2277
15	$C_{16:2\omega4}$	1926	2325
16	$C_{16:0}$	1938	2235
17	?	1995	–
18	$C_{17:0iso}$	2003	2325
19	$C_{17:0anteiso}$	2012	2325
20	$C_{17:1}$	2020	2371
21	$C_{17:0}$	2040	2334
22–23	$C_{18:0iso} + C_{18:3\omega6}$	2102	2403/2540
24–25	$C_{18:4\omega3} + C_{18:5\omega3}$	2109	2608/–
26	$C_{18:4\omega1}$	–	2619
27–28	$C_{18:2\omega6} + C_{18:3\omega4}$	2116	2509/2560
29	$C_{18:1\omega9}$	2121	2460
30–31	$C_{18:1\omega7} + C_{18:3\omega3}$	2126	2467/2574
32	$C_{18:1\omega5}$	–	2480
33	$C_{18:2\omega4}$	2131	2528
34	$C_{18:0}$	2144	2436
35	$C_{19:0}$	2247	–
36	$C_{20:4\omega6}$	2285	2766
37	$C_{20:5\omega3}$	2294	2871
38	$C_{20:3\omega6}$	2303	2741
39–40	$C_{20:2\omega6}+C_{20:4\omega3}$	2311	2696/2819
41	$C_{20:3\omega4}$	2315	–
42	$C_{20:2\omega4}$	2320	2713
43	$C_{20:1\omega9}$	2324	2663
44	$C_{20:3\omega3}$	2328	2780
45	$C_{20:1\omega7}$	2330	2671
46	$C_{20:2\omega3}$	2335	–
47	$C_{20:0}$	2347	2640
48	$C_{21:5\omega3}$	2391	3091
49	$C_{21:0}$	2445	–
50	$C_{22:5\omega6}$	2465	3208
51	$C_{22:6\omega3}$	2473	3351
52	$C_{22:4\omega6}$	2477	–
53	$C_{22:5\omega3}$	2487	3286
54	$C_{22:3\omega4}$	2497	–
55	$C_{22:2\omega6}$	2502	–
56	$C_{22:4\omega3}$	2506	3230
57	$C_{22:1\omega9}$	2521	–
58	$C_{22:1\omega7}$	2528	–
59	$C_{22:0}$	2545	3008

first column to the second. As a consequence of this, multidimensional separation is performed only on a portion of the matrix.

Comprehensive two-dimensional gas chromatography (GC × GC) is a multidimensional separation technique where the initial sample is separated on two different GC columns connected in series with a modulator located between them [6]. This sort of application achieves the near complete characterization of the initial sample. There have been various approaches as regards the type of modulator employed. The first commercial instrument employed the thermal sweeper type modulator which was based on a thick stationary phase trap and the subsequent pulsed reinjection of the retained solutes via a rotating heater [7]. The elution of the reinjected solutes from the second column is achieved before the following introduction. Another later approach was the longitudinally modulated cryogenic system (LMCS) that achieved low temperature trapping and refocusing, exploiting the freezing effect of a CO_2 stream passing through a moving modulator. The oscillations of the modulator at the head of the second column meant that solutes underwent a process of trapping, refocusing and then release [8]. Comprehensive 2-D GC has been applied successfully in many fields such as environmental [9,10], petrochemical [11,12], flavour and fragrances [13,14], pesticides [15] and foods [16]. For investigations concerning fatty acids there have been only two publications [17,18]. The authors of these works mentioned the potential of GC×GC for the identification of compounds in the absence of commercially available standards.

In this research, which can be considered a further development of the aforementioned papers, two different sets of columns were employed. The analyses concerned animal, vegetable and fish oils. Linear retention indices (LRI) were calculated for all substances

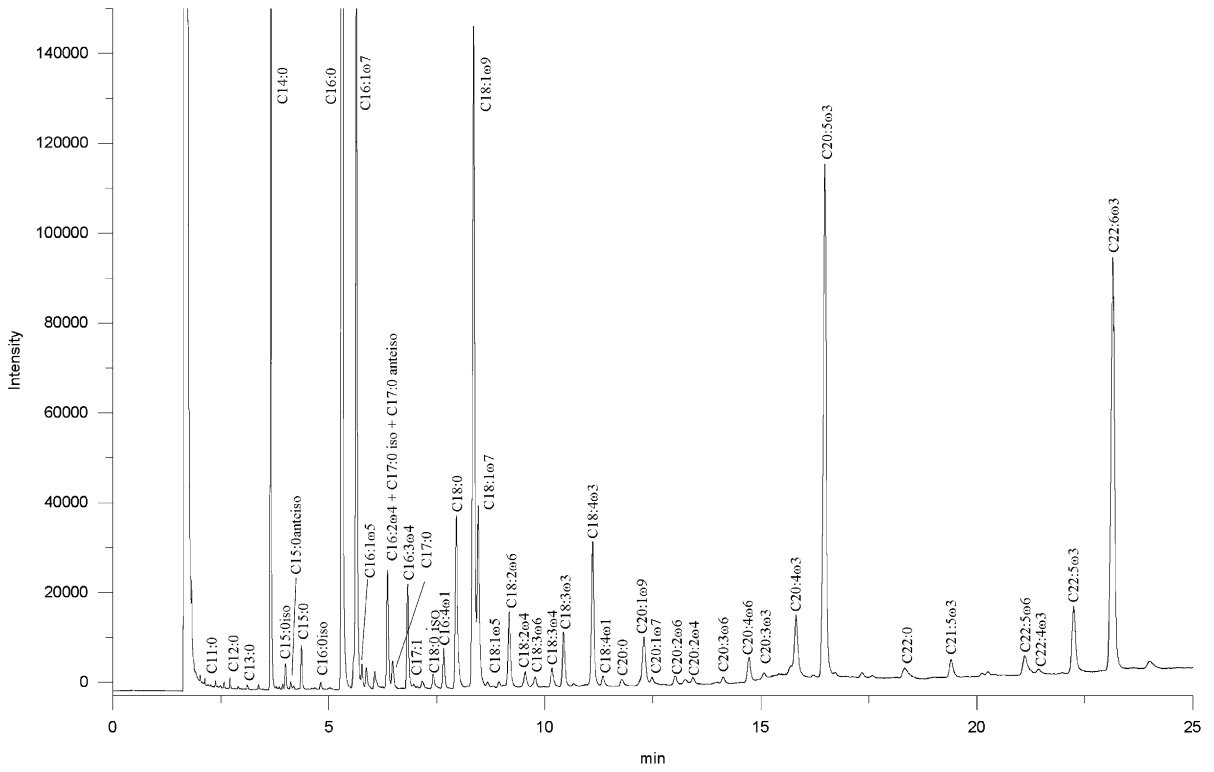

Fig. 1. Conventional GC–FID chromatogram of the menhaden oil FAMEs sample obtained with column set1 (polar–apolar) without use of the cryogenic modulator. For quantitative results see Table 2.

on both of the column sets. This information in combination with mass spectra was employed for reliable peak identification in all samples). All identified compounds in the first dimension were subsequently attributed in the 2D space chromatograms. As suggested by Brinkman et al. in recent work [18], curves were extrapolated from the 2D space chromatograms for families of FAMEs regarding analysis on both column sets. Each family was formed by groups of FAMEs with the same double bond number.

2. Experimental

2.1. Standard

Solution: 100 ppm of normal alkanes ($C_{14:0}$–$C_{34:0}$) in hexane (Supelco, Milan, Italy).

2.2. Samples and sample preparation

A total of nine commercial different fats and oils (menhaden, cod liver, tallow, lard, extra virgin olive, peanut, margarine, sunflower, butter) were used. Prior to injection, all samples were transesterified and then diluted 1:10 (v/v) with *n*-hexane.

Fatty acid methyl esters were obtained by simultaneous transesterification of all samples as follows: the fat was melted (40 °C) and dehydrated by filtering on a paper filter with Na_2SO_4 (for oils this procedure was skipped). One gram or milliliter of the fat or oil was added with 1 ml of a solution of 10% H_2SO_4 in methanol, and put at 110 °C for 2 h in a closed vial.

2.3. GC × GC analysis

GC × GC analyses were performed using a Shimadzu 2010 model gas chromatograph (Shimadzu,

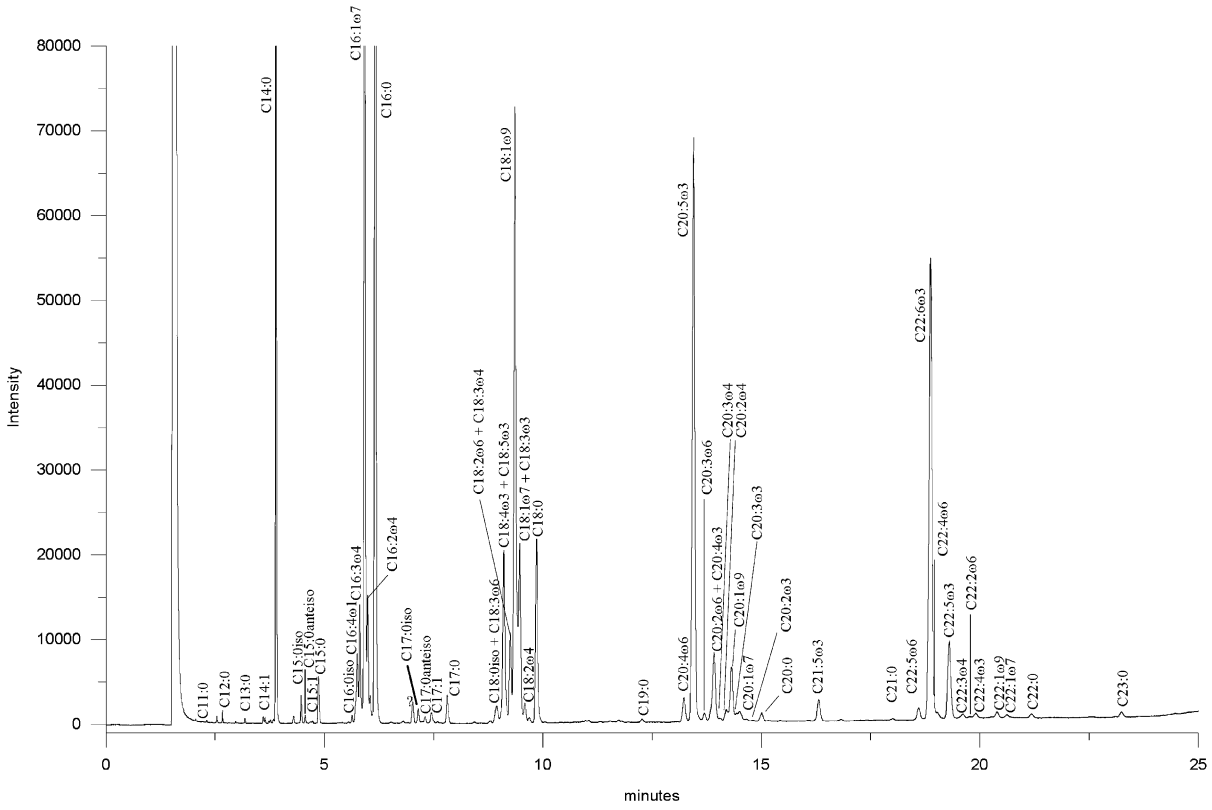

Fig. 2. Conventional GC–FID chromatogram of the menhaden oil FAMEs sample obtained with column set2 (apolar–polar) without use of the cryogenic modulator. For quantitative results see Table 2.

Milan, Italy) equipped with a flame ionization detection (FID) system (operated at 50 Hz data acquisition frequency), AOC-20is series auto sampler (Shimadzu), and GC Solution software for data acquisition. The GC was equipped with an LMCS Everest longitudinally modulated cryogenic system (LMCS; Chromatography Concepts, Doncaster, Australia), with a mechanical stepper motor drive for movement of the cryotrap. A modulation frequency of 0.1666 Hz (6 s cycle) was applied in all analyses and initiated by the GC Solution programmed external events that via the electronic controller also starts the motor operation. CO_2 is supplied to the trap, and its expansion cools the trap that is thermostatically regulated at about 0 °C. A small internal flow of nitrogen gas (about 10 ml/min) prevents ice formation inside the trap. Data are collected by the GC Solution software and, by using its export function, the ASCI data were converted into a matrix with rows corresponding to a 6-s duration, and data columns covering all successive second dimension 6-s chromatograms using the 2D GC Converter 2.0 (Chromatography Concepts). Contour representation of the 2D chromatograms was through Transform version 3.3 software (Fortner Software, VA, USA). The columns set for GC × GC analysis consisted of two columns, which were serially connected by a zero-dead-volume glass press-fit (Mega, Legnano, Italy). In this study we used two sets of columns. Set 1: the conventional first dimension column was a BPX5 (5% diphenyl+ 95% dimethyl

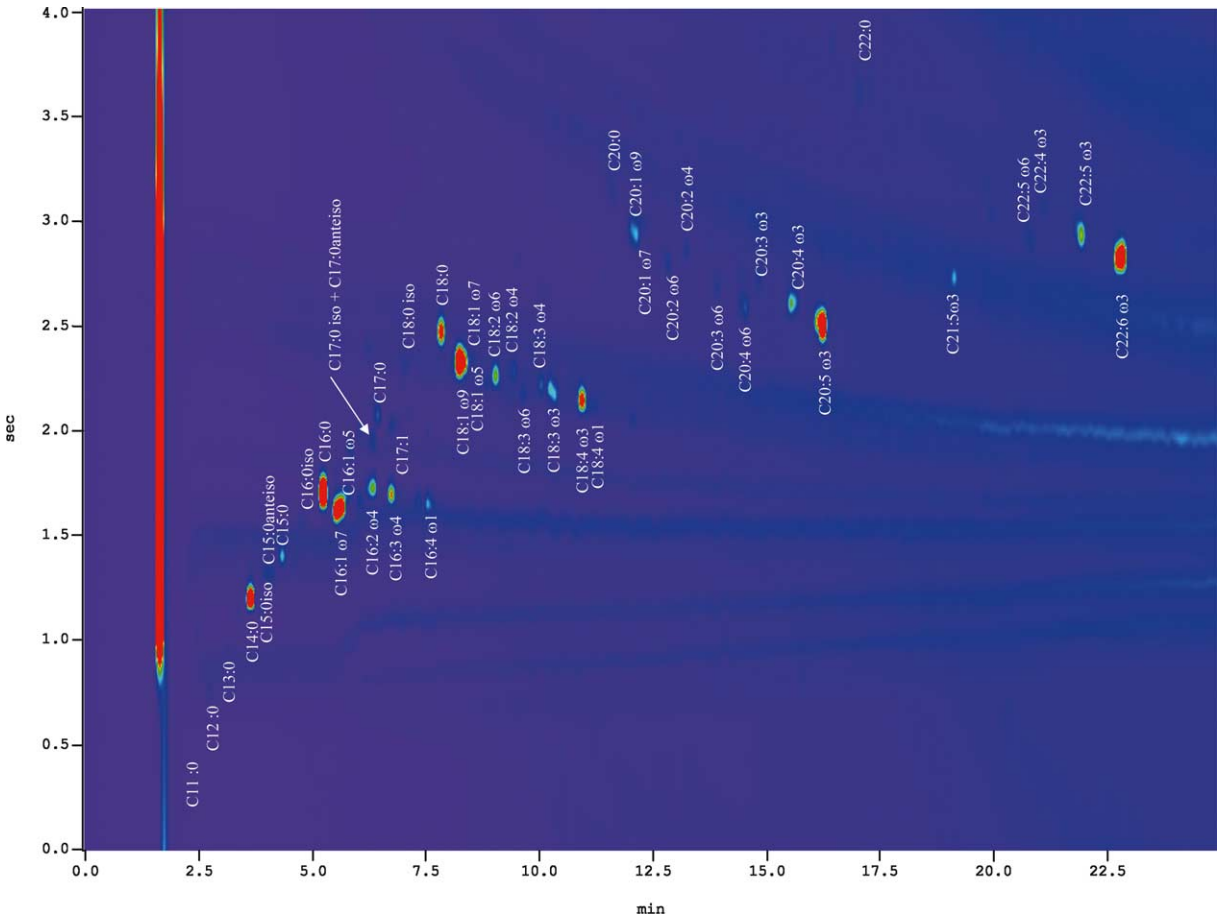

Fig. 3. GC × GC–FID chromatogram of the menhaden oil FAMEs sample obtained through the activation of the cryogenic modulator with column set1 (polar–apolar).

polysiloxane) 30 m × 0.25 mm I.D., 0.25 μm film thickness (SGE International, Rinhwood, Australia) and the secondary fast column was a Supelcowax-10 (polyethylene glycol) 1 m × 0.10 mm I.D., 0.10 μm film thickness (Supelco). Set 2: the conventional first dimension was a Supelcowax-10 (polyethylene glycol) column 30 m × 0.25 mm I.D., 0.25 μm film thickness and the secondary fast column was an SPB-5 (5% diphenyl+95% dimethyl polysiloxane) 1 m × 0.10 mm I.D., 0.10 μm film thickness (Supelco). The operational conditions for both sets of columns were the same. Temperature programmed conditions from 200 to 250 °C at 2 °C/min. The GC was equipped with a split/splitless injector (260 °C); an injection volume of 1.0 μl was employed using the autosampler, and a split ratio of 100:1 was used. The carrier gas was hydrogen, and the column head pressure was 200 kPa at constant pressure. FID, 280 °C; H_2, 50 ml/min; air, 400 ml/min; make-up, 50 ml/min (N_2).

2.4. GC–MS analysis

The analyses were carried out by GC–MS on a Shimadzu QP5050 (Shimadzu) system equipped with commercial mass spectral libraries and a laboratory-made library containing linear retention indices calculated on the two sets of columns: BPX5 and Supelcowax-10 and Supelcowax-10 and SPB-5. Temperature program, 200–250 °C at 2 °C/min; carrier gas, He delivered at constant pressure of 400 kPa;

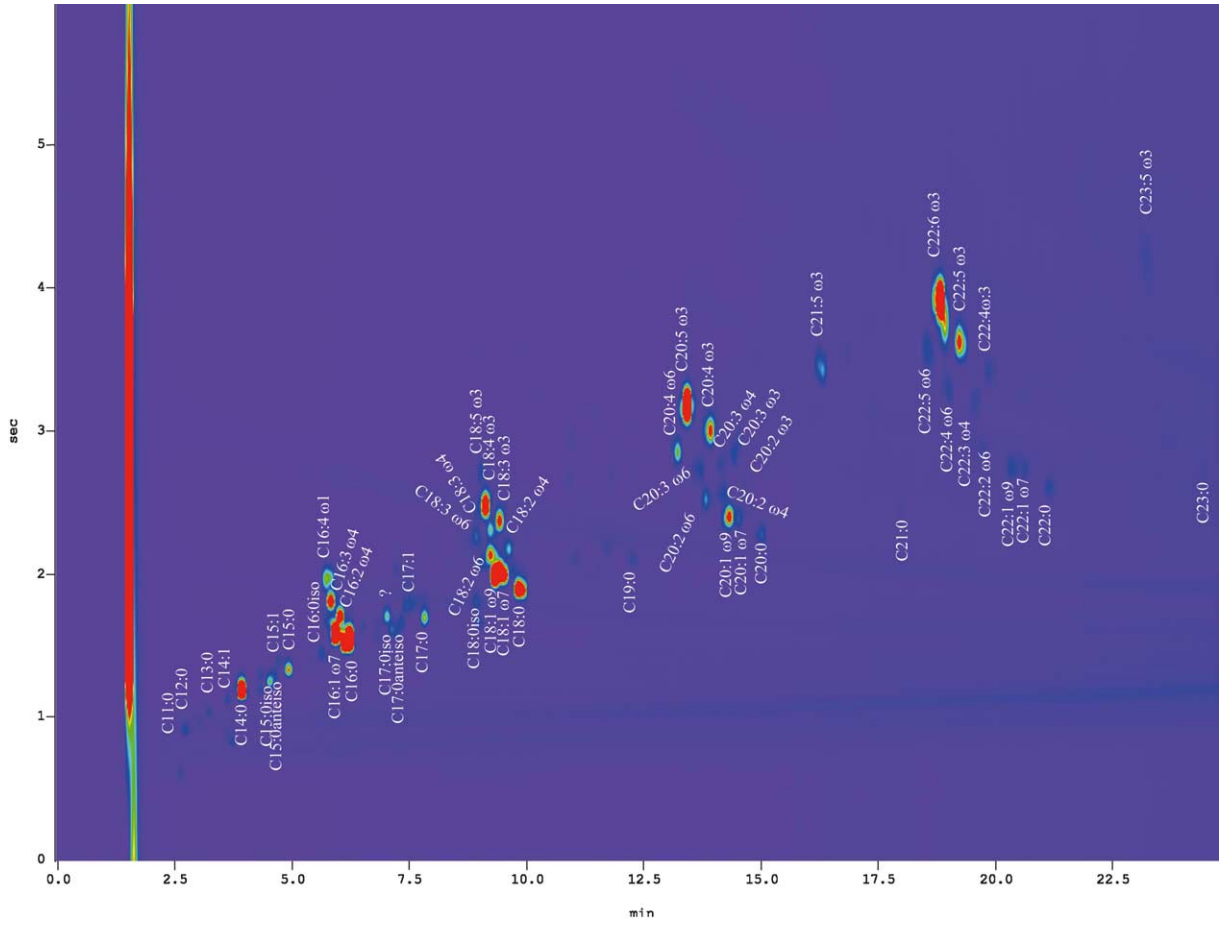

Fig. 4. GC × GC–FID chromatogram of the menhaden oil FAMEs sample obtained through the activation of the cryogenic modulator with column set2 (apolar–polar).

injection volume, 1.0 μl; split ratio, 100:1 (250 °C); interface temperature, 230 °C; ionization energy, 70 eV; multiplier at 1.5 kV; acquisition mass range 40–400; solvent delay, 3 min.

3. Results and discussion

In this research, the aim of the comprehensive 2D-GC application on FAME samples is to evaluate the predictive potential of this technique for the identification of compounds when no commercial standards are available. The determination of substances through prediction is achieved through the definition of specific zones in the 2D-space chromatogram in which families of compounds fall. Patterns formed by groups of molecules in the 2D plane are related to their chemical structures. The first analytical step in this work was the monodimensional separation and identification of all FAME samples, first with a polar and then with an apolar column. This was carried out by GC–FID and GC–MS. Nearly all compounds where identified through the use of mass spectra and LRI combined information [19] contained in a laboratory-constructed library (Table 1). The next step regarded the formation of two different sets of columns; the first set was formed by a BPX-5 30 m × 0.25 mm I.D., 0.25 μm film thickness coupled with a Supelcowax-10 1 m × 0.10 mm I.D., 0.10 μm film thickness. The second set was formed by a Supelcowax-10 30 m × 0.25 mm I.D., 0.25 μm film thickness combined with an SPB-5 1 m × 0.10 mm I.D., 0.10 μm film thickness. GC analyses carried out on all samples with both column sets were primarily achieved without the activation of the cryogenic modulator as can be seen in Figs. 1 and 2 for two consecutive separations of a menhaden oil matrix, respectively, with sets 1 and 2. In this and in all analyses, the contribution towards separation

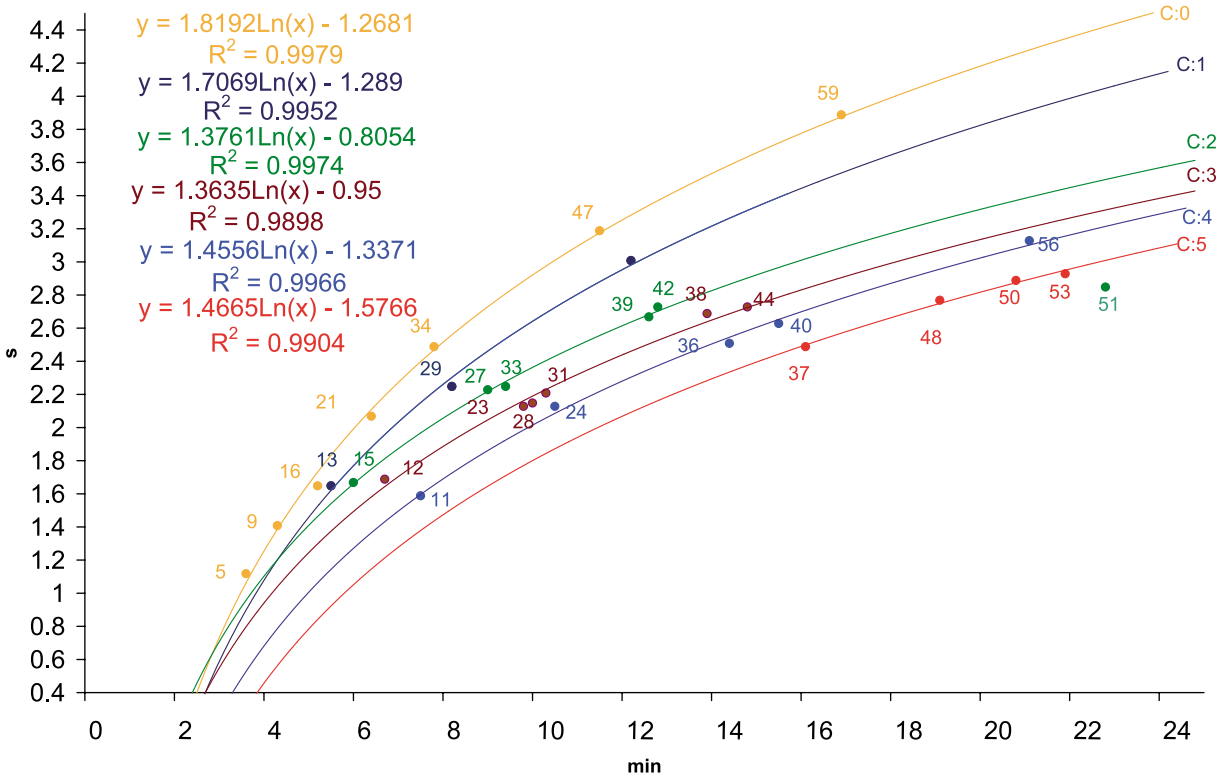

Fig. 5. Six exponential curves corresponding to FAMEs families grouped on an equal double bond number basis (0 to 5 range) derived from column set1 (polar–apolar) GC × GC–FID chromatogram of the menhaden oil FAMEs sample. For conditions see Section 2.

given by the shorter column in respect to the longer one, in both sets, is negligible. From this preliminary application and observation of the FAMEs' eluting order the following conclusions can be drawn: as regards to the polar column whose separating capacity is based on the different polarities of solutes it can be affirmed that (1) saturated esters elute before the correspondent mono-unsaturated esters; (2) homologous saturated group retention times decrease as we pass from the linear molecules to the anteiso and onto the iso isomer; (3) in homologous unsaturated families the compound with the highest ω number elutes first (e.g. $C_{18:1\omega9}$ and $C_{18:1\omega7}$, $C_{20:2\omega6}$ and $C_{20:2\omega4}$); (4) in groups containing compounds with the same carbon number and ω value the first ester eluted is the one with the lowest number of double bonds; (5) in families of unsaturated esters with the same carbon number, retention will be stronger for the fatty acid methyl esters with the minor number of double bonds but with the ω3 value (e.g. $C_{22:5\omega6}$ and $C_{22:4\omega3}$).

As regards to the second apolar column set whose separating power is based mainly on solute vapour pressures it can be affirmed that: (1) saturated esters elute after unsaturated esters with the same carbon number. Retention times, in this case, decrease with the number of double bonds; (2) homologous saturated esters follow this elution pattern: iso, anteiso and then the linear compound; (3) in homologous unsaturated families the elution order is the same as seen in the polar column; (4) in groups containing esters that have the same carbon number and ω position but a different double bond number, a lower double bond presence coincides with a stronger retention.

It must be pointed out that all peak identifications reported in Figs. 1 and 2 were not only achieved through the combination of GC–MS and LRI information but also through the careful study of the contour plots obtained with the activation of the cryogenic system. In fact, the following construction of the exponential curves achieved through first and second dimension

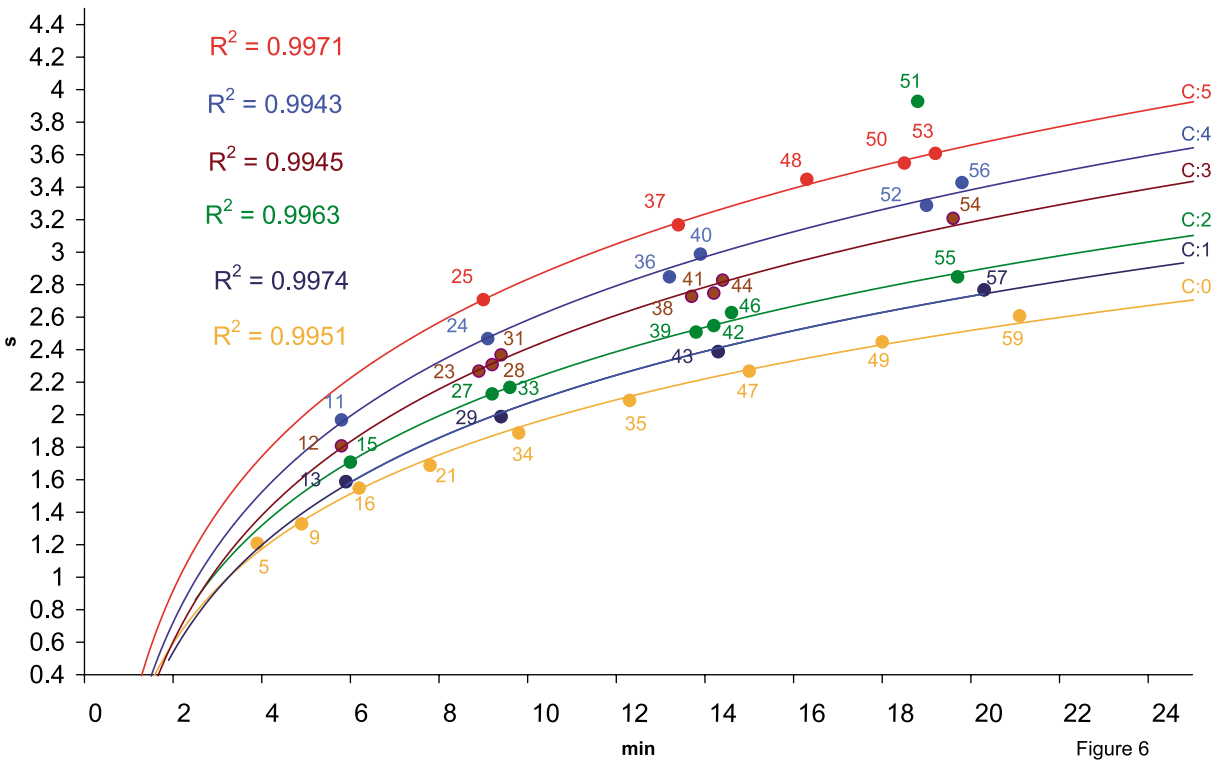

Fig. 6. Six exponential curves corresponding to FAMEs families grouped on an equal double bond number basis (0 to 5 range) derived from column set2 (apolar–polar) GC × GC–FID chromatogram of the menhaden oil FAMEs sample. For conditions see Section 2.

Table 2
FAMEs: relative quantitative results on both column sets

Compound	% Apol/pol	% Pol/apol
$C_{11:0}$	0.01	0.01
$C_{12:0}$	0.07	0.07
$C_{13:0}$	0.04	0.03
$C_{14:1}$	0.05	–
$C_{14:0}$	6.98	6.86
$C_{15:0iso}$	0.29	0.23
$C_{15:0anteiso}$	0.09	0.07
$C_{15:1}$	0.09	–
$C_{15:0}$	0.52	0.43
$C_{16:0iso}$	0.01	0.01
$C_{16:4\omega1}$	0.98	0.75
$C_{16:3\omega4}$	1.42	1.37
$C_{16:1\omega7}$	9.66	9.64
$C_{16:2\omega4}$	1.33	1.50[a]
$C_{16:0}$	0.30	–
$C_{17:0iso}$	0.20	–
$C_{17:0anteiso}$	0.11	–
$C_{17:1}$	21.04	19.82
$C_{17:0}$	0.21	0.17
$C_{18:0iso}+C_{18:3\omega6}$	0.43	0.42
$C_{18:4\omega3}+C_{18:5\omega3}$	0.43	0.48
$C_{18:2\omega4}+C_{18:3\omega4}$	3.18	2.61[b]
$C_{18:1\omega9}$	1.44	1.62
$C_{18:1\omega7}+C_{18:3\omega3}$	10.14	9.86
$C_{18:2\omega3}$	4.16	4.12
$C_{18:0}$	0.35	–
$C_{18:1\omega5}$	3.15	3.05
$C_{18:2\omega6}$	–	0.05
$C_{19:0}$	–	1.32
$C_{18:4\omega1}$	0.08	–
$C_{20:4\omega6}$	–	0.21
$C_{20:5\omega3}$	0.55	0.64
$C_{20:3\omega6}$	11.50	10.92
$C_{20:2\omega6}+C_{20:4\omega3}$	0.21	0.16
$C_{20:3\omega4}$	1.75	2.41
$C_{20:2\omega4}$	0.01	–
$C_{20:1\omega9}$	0.28	0.24
$C_{20:3\omega3}$	1.25	1.20
$C_{20:1\omega7}$	0.11	–
$C_{20:2\omega3}$	0.31	0.22
$C_{20:0}$	0.07	0.06
$C_{21:5\omega3}$	0.23	0.16
$C_{21:0}$	0.53	0.52
$C_{22:5\omega6}$	0.05	–
$C_{22:6\omega3}$	0.32	0.42
$C_{22:5\omega3}$	11.46	10.97
$C_{22:3\omega4}$	1.99	1.84
$C_{22:2\omega6}$	0.21	–
$C_{22:4\omega3}$	0.09	–
$C_{22:1\omega9}$	0.21	0.19
$C_{22:1\omega7}$	0.33	–
$C_{22:0}$	0.20	–
$C_{23:0}$	0.16	–

[a] Co-eluted in set1.
[b] Only $C_{18:5\omega3}$.

retention time values concerning families of compounds characterized by the same double bond value proved to be a valuable source of information.

In fact, Figs. 3 and 4 illustrate the GC × GC–FID chromatograms belonging to a menhaden oil sample analyzed on both column sets. Also reported in the figures is the attribution of all the peaks identified in the first dimension analysis. Figs. 5 and 6 show the curves that were obtained through the 2D-elution coordinates extrapolated from Figs. 3 and 4. As previously foreseen by Brinkman et al. [18], exponential functions were derived for FAMEs groups characterized by the same number of double bonds and, in all cases, with a good fit.

The same analytical procedure was applied to all other animal, vegetable and fish oils.

After all this qualitative information that was obtained through a deductive procedure that concerned all analytical elements (monodimensional GC chromatograms, bidimensional GC chromatograms and exponential curves), this investigation was completed with the determination of relative quantities regarding all compounds in the monodimensional analysis for both sets. In fact, as reported in Table 2, for the main part of the components, quantitative data were comparable. This was a further confirmation of correct compound attribution.

Acknowledgements

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