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Characterisation of fatty acids in biological oil samples using comprehensive multidimensional gas chromatography

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Abstract

Comprehensive multidimensional gas chromatography can adequately resolve very complex mixtures of analytes such as the fatty acid mixtures which are contained in, e.g., fish and vegetable oils. Well-ordered patterns are obtained in the two-dimensional separation plane which can be used to tentatively identify peaks when no standard is available. The technique which can also be used for quantification, i.e., quantitative ratio analysis, should be especially useful for fingerprinting purposes. Unravelling the composition of complex mixtures such as fish oils appears to be highly rewarding.

Keywords: Oils; Comprehensive two-dimensional gas chromatography; Gas chromatography; Fatty acids

1. Introduction

Fatty acids are divided into saturated, monounsaturated and polyunsaturated acids (PUFAs), depending on the presence and number of unsaturated double bonds in the fatty acid chain. Fats containing PUFAs are liquid at room temperature and are called oils. Vegetable oils contain PUFAs with two or three double bonds, and in fish oils a large proportion of PUFAs have five or six double bonds. The location of the double bonds in vegetable and fish oils is also different. The vegetable oils mainly contain the ω-6 series of fatty acids, while fish oils primarily contain the ω-3 series of fatty acids. The long-chain (i.e., more than 18 carbons) derivatives of both types of polyunsaturates are classed as essential fatty acids; as the human body is unable to synthesise them, they must be included in any diet [1]. In fish, the most important ω-3 fatty acids are eicosapentaenoic acid (20:5, EPA) and docosahexaenoic acid (22:6, DHA) which play major roles in the cardiovascular system, and also the central nervous, immune and visual systems [2–5]. On the other hand, rats consuming diets containing high levels of erucic acid (22:1n9) present in rapeseed oil show high accumulation of fat in the heart muscle [6], and erucic acid is assumed to be the factor responsible for cardiac lesions [7,8].

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These facts illustrate the importance of detailed knowledge of the fatty acid composition of oils.

Gas chromatography (GC) is mainly used to separate fatty acids, after their transesterification hydrogenation to methyl esters (FAMEs). Special stationary phases such as Sil-88 (100% cyanopropylpolysiloxane) are used which have, however, the disadvantage that they are restricted to a maximum operation temperature of 500 K. In some instances a pre-separation by column liquid chromatography (LC) is applied to fractionate the sample prior to GC. For example, Lie and Lambertsen determined the fatty acid composition of glycerophospholipids in cod tissue by first fractionating the sample into four classes using adsorption LC on silica [9]. Normally, single-column GC is applied; however, for the determination of minor compounds with retention times similar to those of large interfering peaks, this technique will offer insufficient resolution. Wahl et al. used heart-cut multidimensional GC to separate furan fatty acids from fish tissue extracts [10]: the sample is separated on a HP-1 (100% methylpolysiloxane) column after which the fractions of interest are on-line switched to a second Stabilwax (100% bonded polyethylene glycol) column for further separation.

In recent years, comprehensive multidimensional gas chromatography (GC×GC) has been shown to be very useful for unravelling complex samples such as kerosene and other petrochemical mixtures [11,12]. With this new technique, the peaks eluting from the first GC column enter a thermal modulator which traps each subsequent small portion of eluate, focuses these portions and introduces them into a second column for further separation. The second separation is made sufficiently fast (e.g., 5–10 s) to permit the continual introduction of small fractions from the first column without mutual interference; that is, the whole sample can be subjected to the two-dimensional (2D) separation in a single run. The peak capacity that can be achieved is close to the product of those of the two individual GC separations [13,14].

Usually, the stationary phase in the first GC column has a non-polar (e.g., 100% methylpolysiloxane), and the second column a somewhat polar character (e.g., 85% methyl, 7% phenyl, 7% cyanopropyl, 1% vinyl polysiloxane). This results in a first-dimension separation which is mainly based on vapour pressure. Consequently, the compounds contained within one portion of eluate that is introduced into the second column have essentially the same vapour pressure. Therefore, the separation on the second column will be independent of temperature and will depend on specific interactions with the stationary phase. Identification of an analyte in a sample becomes, of course, more reliable since every compound now has two characteristic retention times. The separation of, for example, mixtures of homologous groups results in well-structured bands of these groups more or less parallel to each other in the ultimately constructed 2D plot, as shown by illustrative examples in Refs. [12,14].

The above information on GC×GC, indicates that the separation of FAMEs should be considerably improved by subjecting them to this novel technique. As an example, the benefits of GC×GC for the characterisation of FAMEs in samples from biological origin will be discussed in this paper.

2. Materials and methods

2.1. Chemicals

Boron trifluoride–methanol complex for synthesis, sodium hydroxide and sodium chloride, used for sample preparation (see below), were of analytical-reagent quality and were purchased from Merck (Darmstadt, Germany). Picograde 2,2,4-trimethylpentane and HPLC-grade methanol were from Promochem (C.N. Schmidt, Amsterdam, The Netherlands).

2.2. Standards

The following FAME standards were supplied by Alltech (Applied Science Group, Breda, The Netherlands), the individual FAMEs *cis*-9-hexadecenoic acid (16:1), *cis*-9-octadecenoic acid (18:1), *cis,cis*-9,12-octadecadienoic acid (18:2), all-*cis*-9,12,15-octadecatrienoic acid (18:3), 11,14-eicosadienoic acid (20:2), all-*cis*-8,11,14-eicosatrienoic acid (20:3), 11,14,17-eicosatrienoic acid (20:3), all-*cis*-5,8,11,14-eicosatetrienoic acid (20:4), 5,8,11,14,17-eicosapentaenoic acid (20:5), *cis*-13-docosenoic acid (22:1)

and *cis*-tetracosenoic acid (24:1) and the FAME mixtures K1000 FAME mix, NIH FAME Mix F and L209 FAME Mix.

Two PUFA ester standard mixtures in which the identity of several major peaks is known, PUFA No. 1 which has a marine source, and PUFA No. 3 from menhaden oil, were purchased from Supelco (Sigma–Aldrich, Zwijndrecht, The Netherlands).

2.3. Samples

The vegetable oils used were commercial olive oil and sunflower oil. The fish oil samples were commercial cod liver oil (Möller's tran, Norway), and herring oil that had been processed from herring maatjes by-products produced in the pilot plant at the Netherlands Institute for Fisheries Research, and had been stored for 15 days at 275 K, or for 2 or 35 days at 323 K as described in Ref. [15].

2.4. Sample preparation

Before being analysed by GC×GC, the fatty acids were converted into methyl esters according to the AOCS official method Ce 1b-89 [16]. Since quantification was not the primary purpose of this preliminary study, the step referring to the addition of internal standards to the samples was omitted.

2.5. GC×GC system

The GC×GC system (Fig. 1) consists of a HP 6890 gas chromatograph (Hewlett-Packard, Wilmington, DE, USA) with a thermal modulator assembly (Zoex, Lincoln, NE, USA). The modulator has two main components: a small section of capillary column, the modulator tube, and a rotating slotted heater. The modulator tube is positioned at the interface between the two capillary columns. The slotted heater periodically rotates over the modulator tube to desorb, spatially compress and release, i.e., inject, portions of the first column eluate from the modulator tube into the second column.

A 9.0 m×0.2 mm I.D., 0.33 μm HP-1 (dimethylpolysiloxane) fused-silica column (Hewlett-Packard), was connected to the split–splitless injector and, at the other end, to the modulator tube (8.2 cm×0.1 mm fused-silica coated with 3.5 μm dimethylpolysiloxane; Quadrex, New Haven, CT, USA) using a miniature press fit (Zoex). Using two miniature press fits and a 0.11 m×0.1 mm uncoated capillary, the end of the modulator tube was connected to the second column [0.30 m×0.1 mm I.D., 0.2 μm CP-WAX-52 (polyethylene glycol) (Chrompack, Middelburg, The Netherlands)] which was installed in the second oven chamber. This second oven chamber was isolated from the first oven by a ceramic wall and could be temperature-programmed from the GC control panel. The end of the second column was connected to a flame ionisation detection (FID) system via a 0.40 m×0.1 mm uncoated fused-silica capillary. The carrier gas was 99.999% pure helium at 250 kPa, from Hoek Loos (Schiedam, The Netherlands).

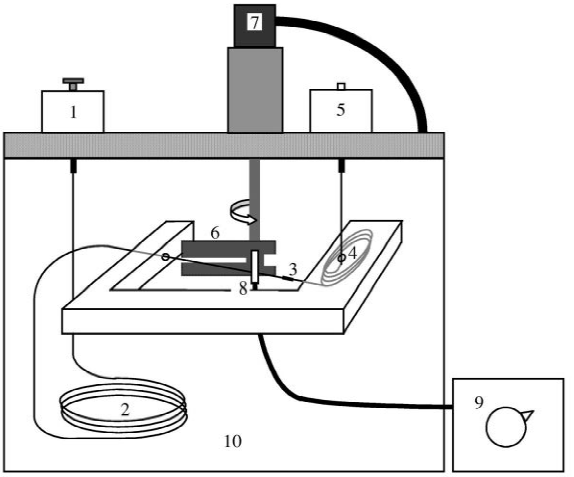

Fig. 1. Comprehensive GC×GC set-up: 1=injector; 2=first column; 3=modulator tube; 4=second column in second oven chamber; 5=detector; 6=rotating slotted heater; 7=stepper motor; 8=cylindrical heater; 9=Variac; 10=GC oven.

A stepper motor mounted on the GC oven roof was used to drive the slotted heater via a rotor shaft. The slotted heater was heated by a cylindrical heater which was also the down-end rotating point of the shaft inside the GC oven. The heater received a constant voltage from a Variac which resulted in a temperature of 100±5 K above the oven temperature (measured in the heater slot).

The modulator tube and the uncoated fused-silica capillary at its downstream end were tightly stretched in the modulator cartridge using small springs which

Table 1
GC×GC experimental conditions

Injection	Split (50:1), 1.0 μl, 543 K
Detection	FID, 573 K
GC oven temperature program	373 K, 2 min; 30 K min^{-1} to 433 K; 0.5 K min^{-1} to 523 K
Second column oven temperature program	453 K, 2 min; 0.75 K min^{-1} to 553 K
Modulation period	5.0 s
Slotted heater velocity	0.15 revolutions s^{-1}
Slotted heater pause time at end modulator tube	0.5 s

were glued to the columns, so that the slotted heater covered the junction between the modulator tube and the uncoated capillary during rotation. The design of the cartridge was such that the slotted heater could make the required full turn. To prevent the heater slot from touching the modulator tube while sweeping over it, the three screws under the cartridge were adjusted. Next, the screws were fixed with check nuts.

Control software for GC×GC (version 2.0z13; Southern Illinois University, Carbondale, IL, USA/ Zoex) was used to control the modulation period, the sweep speed of the slotted heater and the pause time, which is the time the slotted heater is held over the end of the modulator tube after sweeping over it. The optimised experimental conditions are given in Table 1.

All data were acquired using GC×GC (version 2.0z13) software which sampled the FID signal at 100 Hz. For visualisation, Transform 2D 3.3.0 PPC software (Fortner Research LLC, Sterling, VA, USA) and special integration software (Zoex) were used; the latter was also used for data integration.

3. Results and discussion

As was indicated in the Introduction, even sophisticated one-dimensional, and primarily vapour-pressure-based GC cannot provide a (near) complete resolution of all homologues, congeners, etc., contained in many fat and oil samples. The added value of an on-line coupled second-dimension separation on a more polar column is exemplified by the contour plot of Fig. 2. Using a menhaden oil sample as an example, nine baseline-resolved peaks show up instead of the earlier four; they are, in addition, sharper and therefore more easily visualised.

In order to demonstrate that the rapid, i.e., seconds-versus-minutes, second-dimension separation creates beneficial effects across a whole chromatogram, Fig. 3 shows the complete 75 min×5 s GC×GC analysis of a herring oil sample. Next to the considerably improved separation caused by the selective analyte–stationary phase interactions on the second column, distinct patterns are seen to emerge in the GC×GC contour plot. As an attempt to show that peaks of series of homologous compounds indeed elute on parallel lines such as are sketched in Fig. 3, a test was carried out with *n*-alkanes having 16 to 25 carbon atoms. The alkanes eluted with 6–61 min first-dimension, and 3.99–4.88 s second-dimension times. A curve through their 2D elution coordinates resulted in an exponential function:

$$t_{1\text{st dim}} = 0.033 \cdot e^{2.32 \cdot t_{2\text{nd dim}}} \quad (1)$$

with a good fit ($R^2 = 0.9995$). This shows that the pattern can be considered reliable and can also be used for other groups of compounds, with every homologous group having its own specific curvature. In the present instance, the test was done with *n*-alkanes instead of FAMEs since more individual congeners were available to us.

Using the retention times of the limited number of fatty acid standards available, peaks in the chromatogram of Fig. 3 were identified, and 2D retention plots were drawn for various groups of FAMEs. As regards the precision of the plots for the individual analytes, from previous studies with other compounds it is known that for the positions at which peaks elute in the 2D plane, relative to each other, the standard deviation is less than 0.07 s in the

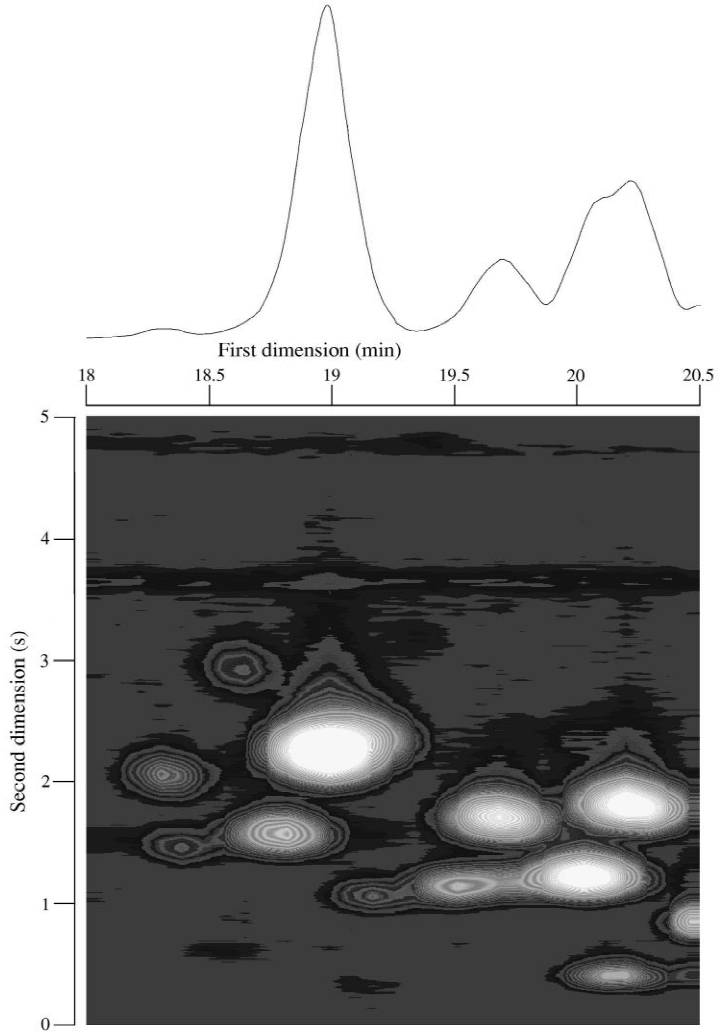

Fig. 2. Part of GC×GC–FID chromatogram of a menhaden oil sample, with the upper trace showing the reconstructed one-dimensional chromatogram.

second dimension, and the first-dimension position is constant to ±1 the second-dimension chromatogram [17]. With regard to the general shape of the retention patterns, one should add that some further improvement – i.e., less curvature – would have been possible by properly adapting the first- and second-dimension temperature programs. This rather time-consuming operation was not performed because the main interest was in the C_{16}–C_{22} fatty acid congeners which are well separated.

Finally, attention should be given to the sharpness of the peaks in Figs. 2 and 3 (as well as in subsequent figures). The second-dimension peak widths were typically 0.3–0.6 s at the base. The slight tailing that can be seen in some cases, was due to overloading, because rather concentrated samples were injected to facilitate the visualisation of trace compounds.

Fig. 3 includes three tentative peak identifications which were based on the interpretation of the retention plots. It is worthwhile to draw attention to the peak appearing between $C_{18:1n9}$ and $C_{22:1}$ in the monounsaturated fatty acid elution band and which was identified as $C_{20:1n9}$. Recently, a pure standard

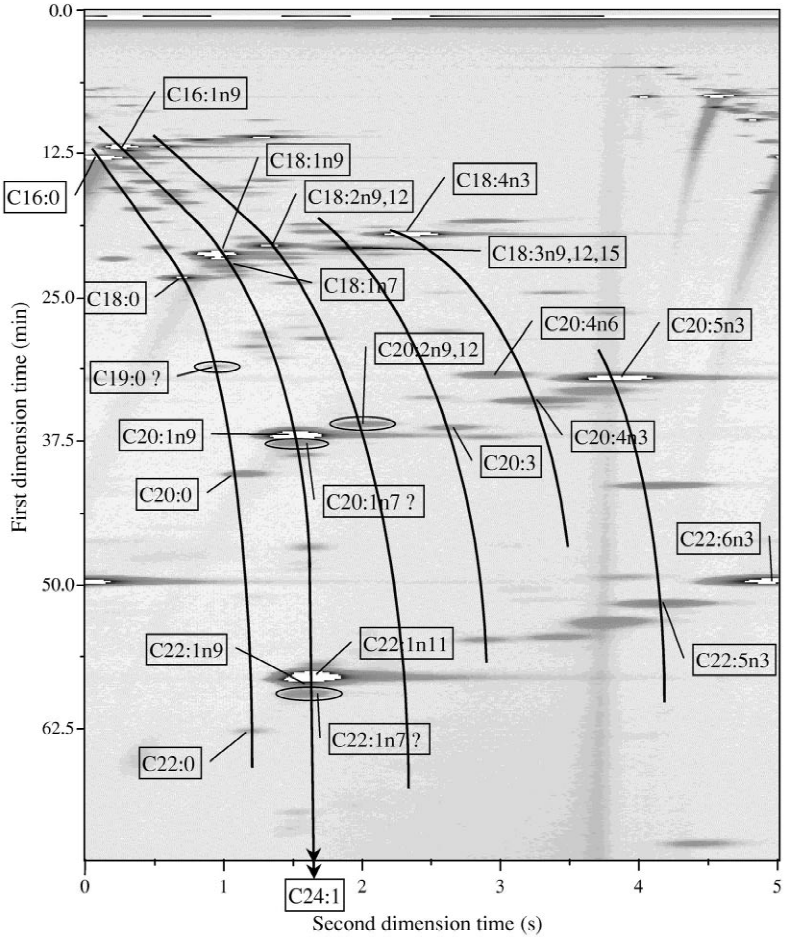

Fig. 3. GC×GC–FID chromatogram of herring oil sample (2 days at 323 K); peaks were identified by using available standards and elution bands of homologous groups, which were sketched as explained in the text.

of $C_{20:1n9}$ became available and its first- and second-dimension retention times were found to match those of the pertinent peak in the herring oil sample as well as in other samples.

3.1. Applications

In oils of biological origin fatty acids with even carbon numbers are dominant, whereas in fish oils relatively large amounts of fatty acids are present with odd carbon numbers, primarily 15, 17 or 19 carbon atoms, compared to vegetable oils [18,19]. In one-dimensional GC the odd-numbered fatty acids can hardly be detected in between their major even-numbered neighbours, and GC×GC is required to improve resolution and focus the (minor) peaks by means of modulation operation. As an example, Fig. 4 shows a blow-up of the area between the C_{18} (18.5–24 min) and C_{20} (31.5–41 min) areas of a menhaden oil sample. Qualitatively, the patterns observed in this part of the GC×GC chromatogram are similar to those found for the adjacent even-carbon-numbered FAMEs in this sample (data not shown) and in Fig. 3. The present group of peaks can therefore tentatively be identified as C_{19} fatty acid congeners having from zero to at least three double bonds. Even if, unfortunately, no standards of odd-carbon-numbered fatty acids were available for

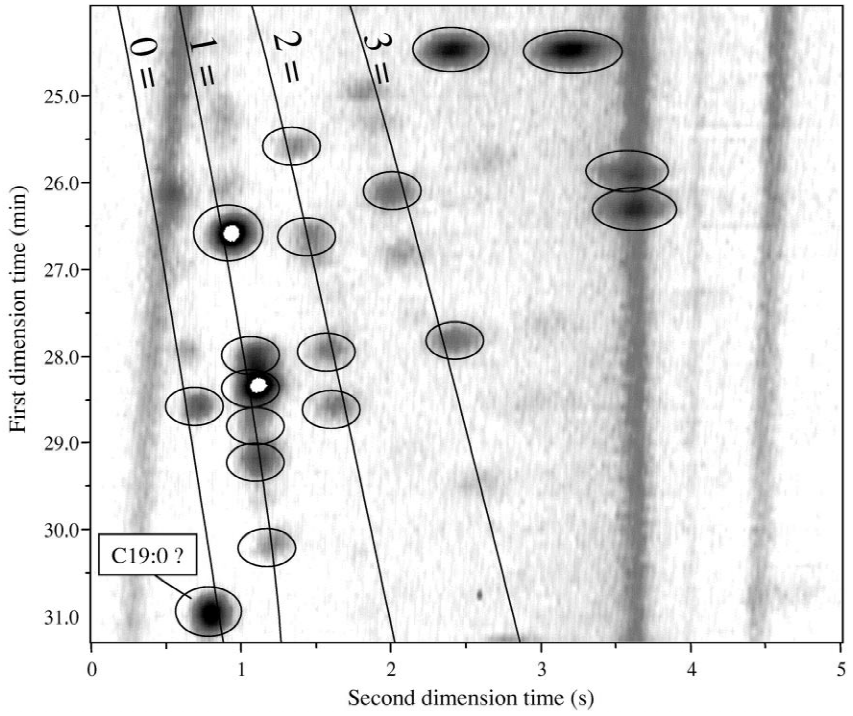

Fig. 4. Blow-up of part of a GC×GC–FID chromatogram of a menhaden oil sample, tentatively identified as the C_{19} fatty acids area. 0–3 indicate the number of double bounds.

conformation purposes, the ease with which a large number of minor components can be distinguished – and therefore subjected to further study – is worth noting.

As a demonstration of the differences between the fatty acid composition of a fish and a vegetable oil, Fig. 5 which shows the GC×GC chromatogram of an olive oil, should be compared with Fig. 3 above. Again, a relatively high concentration was injected to make minor peaks visible. There is, therefore, some overloading and tailing in both dimensions for compounds present in higher concentrations. First-dimension tailing of, for example, $C_{18:1n9}$ is observed as the band curving down towards the first-dimension axis. The explanation is that $C_{18:1n9}$ is tailing while eluting from the first column and is therefore present in several subsequent portions injected into the second column; the second-column temperature slowly increases and consequently the peaks elute at progressively lower second-dimension retention times. That overloading is indeed the cause of the observed tailing, and not the GC×GC operation, is convincingly demonstrated by the analysis of a diluted sample, with the ''C_{18} window'' being shown as an insert of Fig. 5. For the rest, comparison of Figs. 3 and 5 shows the much higher complexity of the fish oil sample. Fig. 5 also shows the absence of highly polyunsaturated fatty acids in the olive oil. The presence of fatty acids with more than four double bonds is a property unique to fish oils [6,20].

In earlier studies, it has been shown that quantification of peaks present in GC×GC chromatograms is perfectly possible [17,21,22]. Such an evaluation was not the main goal of the present study, but the ratios of several selected FAMEs (measured as peak areas) were determined in a variety of samples. The results are shown in Table 2. One should be aware that the FAMEs of Table 2 were selected rather arbitrarily: they should help to illustrate the potential of the present approach and do not include all major, or discriminating, constituents such as, e.g., many C_{18} fatty acids (see Figs. 3 and 5). One interesting observation is that the amounts of the various FAMEs in maatjes herring are essentially constant,

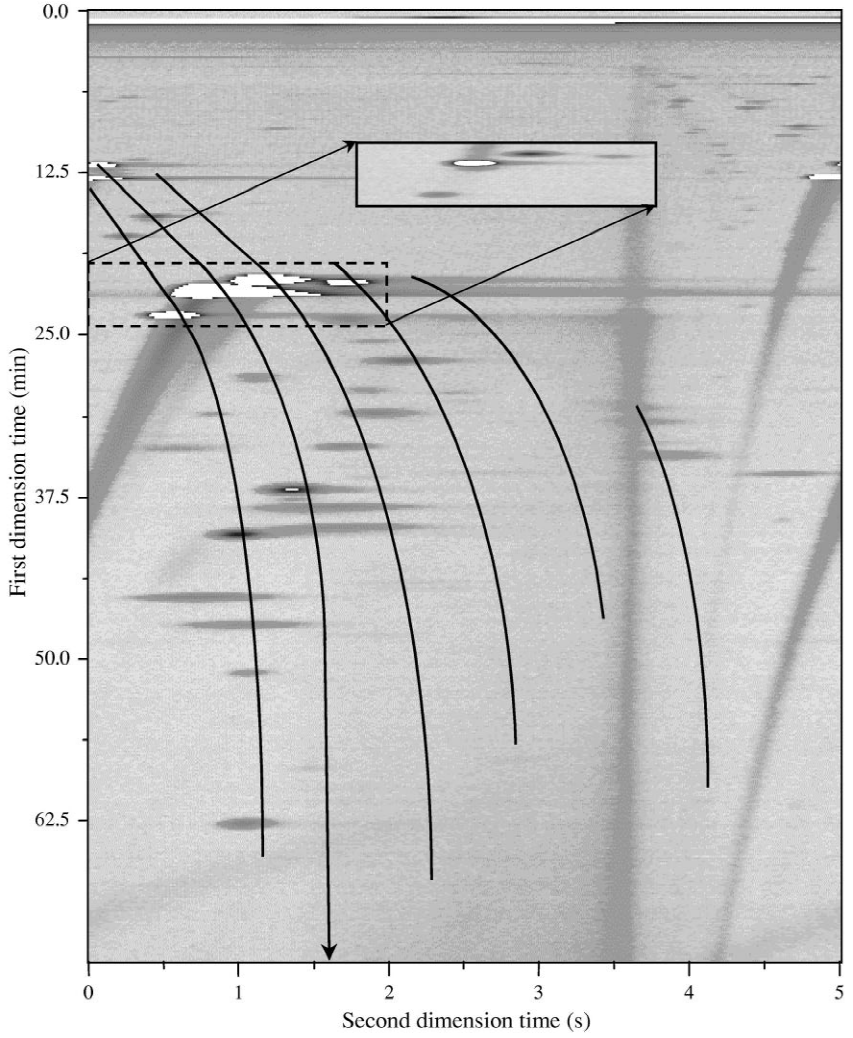

Fig. 5. GC×GC–FID of a commercial olive oil sample. The insert shows the ''C_{18} window'' from the analysis of a diluted sample.

Table 2
Relative peak areas of selected FAMEs in vegetable and fish oil samples (sum = 100%)

Sample	20:0	22:0	22:1n7?	22:1n9	22:1n11	20:5	22:6
Sunflower oil (commercial)	67	29	<LOD	0.57	1.4	2.1	<LOD
Olive oil (commercial)	76	19	<LOD	0.94	<LOD	3.9	<LOD
Maatjes herring							
15 days at 275 K	0.45	0.11	0.50	1.2	51	25	22
2 days at 323 K	0.45	0.12	0.51	2.2	51	24	22
35 days at 323 K	0.45	0.10	0.51	1.8	51	24	22
Cod liver oil (commercial)	0.16	0.05	0.25	1.5	26	28	44
PUFA No. 1, marine source	0.05	0.08	0.65	4.7	27	35	32
PUFA No. 3, menhaden oil	0.40	0.27	0.41	0.86	0.04	57	41

LOD, limit of detection.

irrespective of the pretreatment; this is in accordance with the findings of Aidos et al. [23]. Further, in vegetable oils there is a clear dominance of the saturated over the non-saturated fatty acids while the reverse is true for the fish oil samples. The fish oils show high amounts of 20:5 and 22:6, whereas in the vegetable oils these compounds are present at much lower levels or are even essentially absent. Finally, it is noteworthy that the concentrations of 22:1n9, erucic acid, were rather low in all samples. Still, the levels in the samples of herring, which preferentially feed on copepods [24] are seen to be distinctly higher than those in menhaden oil since, as adults, menhaden feed by filtering phytoplankton [25].

4. Conclusions

Comprehensive multidimensional GC is a powerful technique for unravelling the nature of complicated mixtures of fatty acids (as their methyl esters) which occur, e.g., in fish oils. The on-line and real-time combination of a first conventional-length vapour-pressure-related and an extremely rapid second separation which is based on selective interactions between the stationary phase and the analytes of interest yields well ordered two-dimensional contour plots in which homologous series of FAMEs show up on parallel lines. This enables the provisional identification of unknowns. Next to much improved resolution, analyte focusing during modulation which reduces peak width, enhances the detectability of minor peaks.

Because of the characteristic separation profiles which are obtained, GC×GC can be recommended as a fast fingerprint technique to examine the identity of a sample with, if required, subsequent quantification of individual peaks, which does not appear to create any problems.

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