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Comprehensive two-dimensional liquid chromatography×gas chromatography: evaluation of the applicability for the analysis of edible oils and fats

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Abstract

Edible fats and oils are complex mixtures containing a wide range of (classes of) compounds. The most important group of compounds are the triglycerides (triacylglycerides, TAGs). Because of the large number of possible fatty acid combinations, an enormous number of TAGs is possible. In the present feasibility study, the applicability of different modes of comprehensive two-dimensional LC×GC for detailed oil and fat analysis is evaluated. Comprehensive LC×GC was found to be an extremely powerful analytical method for the analysis of complex TAG samples. Using the new comprehensive set-ups, TAGs can be separated according to two independent parameters: carbon number vs. number of double bonds, or fatty acid composition vs. number of double bonds. The information content of comprehensive separations by far exceeds that of the current generation of analytical methods. The quantitative results of the separations show a good agreement with data obtained from standard analytical methods. The comprehensive methods studied can also be used for fingerprinting of oil samples, as well as for the analysis of target compounds or compound groups. Highly detailed separations of olive oil samples were obtained. Zooming in on one region of the chromatogram allowed reliable analysis of wax esters without interferences of sterol esters.

Keywords: Comprehensive chromatography; Liquid chromatography×gas chromatography, comprehensive two-dimensional; Oils; Fats; Triglycerides; Wax esters; Sterol esters

1. Introduction

Edible fats and oils are complex mixtures containing a wide range of component classes. The most important group of compounds are the triglycerides (triacylglycerides, TAGs), a set of compounds consisting of three fatty acids linked to a glycerol backbone. Other groups of compounds naturally occurring in oil and fat samples are mono- and di-acylglycerides, free fatty acids, wax esters, alkanes, sterols, oxidized triglycerides, tocopherols etc. In addition to these naturally present classes, other compounds can be deliberately added, e.g. anti-oxidants or compounds to mask the origin of the oil, or can be inadvertently introduced, e.g. pesticides and PAHs. In the last decades, a suite of (chromatography-based) analytical methods has been developed to quantify each of these groups, or, more

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frequently, as individual compounds within a preselected class.

Returning back to the TAGs, because of the large number of possible fatty acids an enormous number of TAGs is possible. Information on the exact composition of the TAG mixture is crucial for understanding e.g. the behaviour of the fat during processing, the crystallisation behaviour, or the ''mouth-feel'' properties of a margarine etc. The full characterisation of the TAG fraction of an oil/fat would require the separation of all individual TAGs followed by quantification of the separated species. To date no single analytical tool exists that has the separation capacity to do this. Combinations of different analytical methods generally allow detailed analysis. Especially the combination of chromatographic methods with mass spectrometry is very powerful. Combination of chromatography and mass spectrometry has been shown to give highly detailed information on different parameters [1–5]. Unfortunately, combined LC–, GC– or SFC–MS systems are expensive and are likely to suffer from problems in quantification. Off-line two dimensional chromatographic separations are widely used for the analysis of various classes of compounds in edible oils and fats. In such approaches an LC or TLC fractionation is applied to the sample and a certain fraction of the mixture is transferred to a GC instrument for further separation. As an example, the DGF official method F-III 1 [6] describes the use of TLC to isolate the desmethylsterols from animal and vegetable fats and oils with subsequent GC analysis of the individual sterols. Following the pioneering work by Grob and co-workers in the late 1980s [7–9], on-line LC–GC has received significant attention from oil and fat researchers mainly in academia [10–12]. Many industrial laboratories, however, consider the on-line combination of LC and GC as too complex and, probably even more important, as too expensive and inflexible. A high investment is needed for an instrument that can only run one application.

Comprehensive chromatography is a new approach towards chromatography that allows a sample to be separated according to two independent axes. In a comprehensive two-dimensional separation, each of the components or fractions from the first column is subjected to a second separating system with different separation characteristics [13,14]. This in contrast to standard two-dimensional chromatography where only one or just a few fractions are transferred to a second column. The resulting comprehensive chromatogram consists of a plane in which each spot represents one (or more) compounds. Because the separation is now performed in two, more or less independent dimensions, the peak capacity, and hence the likelihood of separating compounds, is significantly larger. Moreover, the information content of comprehensive 2D chromatography (here denoted as chrom×chrom) is dramatically increased in comparison with standard two-dimensional chromatography where only one fraction is transferred. The total peak capacity of a comprehensive system can be estimated by multiplying the peak capacity of the first and the second dimension separation [15].

Recently a number of applications of comprehensive two-dimensional GC×GC have been shown [16–19] and instrumentation for this technique is commercially available. For triglyceride analysis, the applicability of GC×GC is limited. There are currently no high temperature GC columns available with a sufficiently different selectivity to make comprehensive GC×GC feasible for this group of analytes. For oil and fat applications, comprehensive LC×GC is a much more obvious method than is GC×GC. As an example, using comprehensive LC×GC it should in principle be possible to first separate a TAG mixture according to the number of double bonds present in the TAG molecule, followed by a second separation based on, e.g. the carbon number of the TAGs.

From the instrumental point of view, fully on-line automated comprehensive LC×GC is extremely demanding. A system for comprehensive LC×GC has been described in literature for the analysis of very volatile compounds (solvents) in water streams [20]. Unfortunately, the interface developed in that work will only work for volatile analytes and can not be applied for comprehensive LC×GC of high molecular mass TAGs. Fortunately, for the current feasibility study the lack of automated on-line instrumentation for automated LC×GC is not a major obstacle. Off-line experiments can easily be performed, especially if methods for large volume injection and fast GC are in place.

In the present article a feasibility study to identify the possibilities and limitations of comprehensive combinations of different chromatographic methods for TAG and oil/fat characterisation is described. Various comprehensive systems based on different forms of LC in the first dimension and GC in the second dimension are discussed. The applicability for detailed TAG analysis or quantification of other classes of compounds in the edible oil/fat are evaluated. Selected samples are studied to provide an insight in the potentials of the various methods. Keywords in this study will be the level of detail of the analyses, but also the flexibility of the instrumentation, i.e. can information on more classes of compounds be obtained with one instrument in a single analysis.

2. Material and methods

Two LC separation modes were used as the first dimension in the comprehensive LC×GC set-up, i.e. silver phase ''double bond'' separations (AgLC) and normal-phase ''polarity'' separations (NPLC). The fractions collected from the AgLC run were analysed using two GC modes: carbon number GC on a high temperature non-polar GC column and fatty acid methyl ester (FAME) GC. In the latter case an intermediate transesterification was needed. The fractions from the NPLC were only analysed using the carbon number method. Details of the various steps are given below.

2.1. LC separations

All first dimension LC separations were performed on a Gilson ternary LC instrument (Gilson, Villiers-le-Bel, France) consisting of three Gilson 305/306 LC pumps, a Gilson 811B dynamic gradient mixer and an 805S manometric module. The instrument was equipped with a TSP UV2000 UV detector (Thermo Finnigan, San Jose, CA, USA) and an Alltech ELSD 500 evaporative light scattering detector (Alltech Associates, Deerfield, IL, USA) connected in series. All separations were performed at room temperature on different LC columns.

Silver phase separations were performed on a home-made 100×4.6 mm I.D. silica column loaded with 10% $AgNO_3$ [21]. The Nucleosil silica material (3 μm, pore size 100 Å) was obtained from Varian-Chrompack (Middelburg, The Netherlands). The gradient program applied is given in Table 1. The flow-rate was 1.5 ml/min. Polarity separations, or group-type separations, were performed on a Lichrosorb 5 diol-modified silica column (250×4.6 mm I.D., particle size 5 μm) obtained from Varian-Chrompack. The flow-rate was 1 ml/min. The mobile phase flow program is given in Table 2. Fractions for further GC analysis were collected at fixed time intervals of 0.5 or 1 min duration using a Gilson FC204 programmable fraction collector. When collecting fractions the ELSD detector was disconnected and the outlet of the UV detector was directly installed in the fraction collector. All solvents were of HPLC grade and were obtained from Merck (Darmstadt, Germany).

Table 1
Mobile phase flow program AgLC analysis

Gradient step	Time (min)	Eluent (%)		
		A	B	C
1	0	97	3	0
2	2.5	96	4	0
3	8	85	15	0
4	9	75	25	0
5	13	10	90	0
6	13.10	0	0	100
7	17	0	0	100
8	17.10	97	3	0
9	20	97	3	0

Eluent A: toluene/hexane 1:1, v/v. Eluent B: toluene/ethylacetate 3:1, v/v. Eluent C: toluene, 80 μl formic acid added per litre.

Table 2
Mobile phase flow program polarity group-type analysis

Gradient step	Time (min)	Eluent (%)	
		A	B
1	0	100	0
2	5	100	0
3	45	80	20
4	50	80	20
5	51	100	0
6	60	100	0

Eluent A: Hexane. Eluent B: Diethylether.

2.2. GC separations

The second dimension GC separations were either performed on a Thermoquest/CE Instruments Trace GC 2000 Series (Fisons, Milan, Italy) or a Shimadzu GC 17A (Shimadzu, Tokyo, Japan). The Trace 2000 GC was used for carbon number analysis of the intact TAGs and for the wax ester analyses. It was equipped with on-column injection and flame ionisation detection. The column used was a J&W Scientific (Folson, CA, USA) DB-1HT column of 7 m and 320 μm I.D. coated with a film of 0.1 μm of the stationary phase, except for the wax ester analyses and the olive oil fingerprinting where an identical column of 25 m length was used. The carrier gas was hydrogen at a constant flow of 3 ml/min. For the carbon number analysis fractions collected using the fraction collector were injected without further intermediate sample handling. Large sample volumes, 20–50 μl depending on the concentration of the TAGs in the sample, were injected using the direct on-column large volume method described previously [22]. An AS 2000 Autosampler (Fisons) was used at an injection rate of 3 μl/s. The oven was programmed from 95 °C (2 min) to 385 °C (3.5 min) at 20 °C/min. The FID detector was kept at 400 °C. Hydrogen and air settings of the detector were as indicated by the manufacturer.

The FAME analyses were performed on a Shimadzu GC 17A. Prior to the FAME analysis transesterification was performed using trimethylsulphonium hydroxide (TMSH, see below). To allow rapid cooling of the oven an (optional) additional fan was installed on the air exhaust. The column was a CP-WAX 52-CB column of 10 m, 150 μm I.D. and coated with a stationary phase of 0.2 μm. Carrier gas was helium operated at a constant inlet pressure of 500 kPa. The oven was programmed from 120 °C (0 min) at 50 °C/min to 255 °C (1 min). Injection was in the split mode. The split ratio was 1:40. The injection volume was 2 μl. Detection was by FID. The injector and detector were operated at 250 and 275 °C, respectively.

2.3. Sample pre-treatment

For the silver phase AgLC separation edible oil samples were pretreated using solid-phase extraction to remove mono and diglycerides as well as free fatty acids [23]. The resulting solution had a concentration of approximately 1 mg/ml. Of this solution, 10 μl was injected into the AgLC. The fractions collected after the AgLC could be subjected to carbon number analysis without any further pretreatment. For FAME analysis of the fractions, conversion of the TAGs into the FAMEs was required. The transesterification procedure selected was the TMSH method as described by Schulte and Weber [24]. A 50-μl aliquot of a 0.2-*M* TMSH solution (Fluka, Buchs, Switzerland) is added (manually) to each fraction collected after the AgLC column. The reaction starts at room temperature and rapidly proceeds to completion when the sample is injected into the hot injector. For the group-type polarity separations, 100 mg of edible oil was dissolved in 2 ml of hexane. Then, 100 μl of this solution was injected without further sample pretreatment onto the LC column. The fractions collected were directly transferred to the GC for carbon number analysis.

3. Results and discussion

3.1. Analytical difficulties

The technical problems associated with fully automated comprehensive LC×GC are tremendous. As an example, for an LC×GC system to be really comprehensive, the second dimensional GC separation should be fast enough to preserve the information contained in the first dimension separation. This means that at least some 3–4 second dimension GC separations have to be performed for every LC peak [14]. At a typical LC peak width of 1 min this would mean that the GC separation should take less than 15 s. For triglyceride separations, this is absolutely impossible. TAGs cover a wide boiling point range which hence necessitates the use of temperature programming over a broad temperature interval. The fastest GC TAG separation ever published took approximately 3 min [25]. From this it is evident that fully automated on-line comprehensive LC×GC would require major efforts in instrument construction. For this reason it was decided to perform off-line feasibility experiments only. If so desired, fully automated comprehensive LC×GC would in

principle be possible, as has been demonstrated by van Deursen [26] in preliminary work using stopped-flow operation of the LC to analyse subsequent fractions of the LC separation using fast GC. In the off-line feasibility experiments fractions of a fixed time width were collected from the LC. These were transferred to a GC for subsequent off-line analysis. Because a large volume injection method was used, no intermediate preconcentration was required. To minimise the total run time, the analysis speed of the carbon number separation and the FAME analysis were first optimised. By using the highest possible programming rate of the instrument and a short column, the cycle time of a carbon number separation could be reduced to approximately 20 min. For a typical comprehensive AgLC×GC separation of 35 fractions this results in a total analysis time of around 11 h per sample. Although evidently unacceptable for routine use, this was not felt a major obstacle for the current feasibility work. The limiting factor was the maximum attainable heating and cooling rate of the GC oven. The use of an instrument capable of faster programming as used by van Deursen [25] would result in a cycle time of less than 5 min.

To allow faster comprehensive silver phase LC×GC FAME separation, we implemented a new method for fast FAME analysis based on the use of a narrow-bore capillary column [27]. Using this method, the time required for the actual separation of the FAMEs was approximately 3.5 min (up to FAME C24:1). Cooling the instrument to the start temperature initially took an additional 10 min! To speed up this part of the cycle, an optional second fan was installed on the air exhaust. In this way the total cycle time (analysis and cooling) could be reduced to approximately 7 min. A representative example of a fast FAME separation of a calibration standard is shown in Fig. 1.

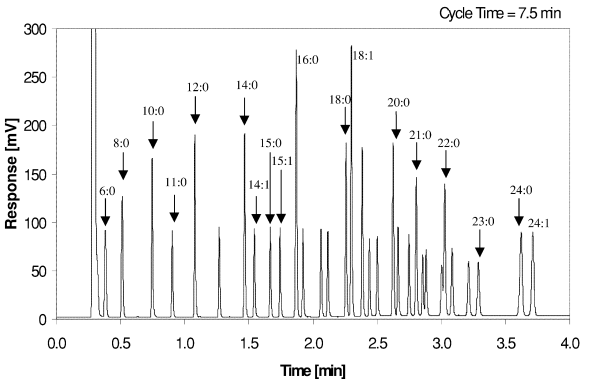

Fig. 1. Representative chromatogram of the new fast FAME separation. Compounds eluting after C18:1 in order of elution: C18:2 (*n*-6,9), C18:3 (*n*-6,9,12), conjugated C18:2, C20:0, C20:1, C20:2 (*n*-6,9), C21:0, C20:3 (*n*-6,9,12), C20:4 (*n*-6,9,12,15), C20:5 (*n*-3,6,9,12,15), C22:0, C22:1, C22:2 (*n*-6,9), C23:0, C24:0, C24:1.

An additional problem that had to be dealt with in the off-line comprehensive LC×GC experiments is the limited sensitivity of the GC systems used in the second dimension. To obtain good chromatography in the first LC dimension, only microgram to milligram amounts can be injected. This mass ends up in a fraction of around 0.5 ml, the typical peak volume in LC. If now a conventional aliquot of this, e.g. 1 μl is injected into the GC at a typical split ratio of 1:100, the actual mass of analyte injected will be below the detection limit of the current GC detectors. This problem could, at least in principle, be solved by off-line manual evaporative preconcentration of the LC fractions. For comprehensive chromatography, where large numbers of fractions are collected, this clearly is not an acceptable option. Using a large volume injection method, the sensitivity problems mentioned above can be solved. A complication here is that the various large volume methods described in literature are very sensitive for changes in the solvent composition. Each solvent composition requires its own set of solvent elimination conditions. Because the first LC dimension in our comprehensive LC×GC analysis is a gradient elution, the solvent composition changes continuously. This will complicate the large volume injection of the collected fractions. Hence, a large volume injection technique is required that is relativity insensible for changes in the solvent properties. In previous work, we have described a direct on-column method that meets this requirement [22]. This method was used throughout the present work for the separations that used carbon number analyses in the second dimension. For the FAME analyses large volume injection can not be applied. The samples have to be injected using hot split injection to obtain rapid and

complete conversion of the TAGs to FAME. By working at a relatively low split ratio an acceptable sensitivity could be obtained.

4. Applications

4.1. Detailed characterisation of intact triacylglycerides

To study the feasibility of comprehensive LC×GC in TAG classification, a number of oils were analysed using comprehensive AgLC×carbon number GC. Examples of comprehensive AgLC×GC are given in so-called ''dot-plots'' in Figs. 2–4. The upper part of the figure gives the silver phase chromatogram. The elution position on the time axis of this chromatogram is related to the number and the accessibility of the double bonds in the TAG molecules. The first peak contains all fully saturated triglycerides irrespective of their carbon number. This peak is called the SSS peak. Here, S is the abbreviation of stearic acid (C18:0). In contrast to what the nomenclature suggests, this peak not only contains triglycerides with three stearic acid chains, but consist of all fully saturated TAGs. The second peak (SOS), consists of the TAGs with one double bond on the 2-position of the glycerol back-bone, the symmetrical mono-unsaturated TAGs. Here, the O represents oleic acid, the most abundantly present fatty acids with one double bond. Again, the O not only refers to oleic acid, but comprises the wider class of all fatty acids with one double bond. The next peak (SSO) contains all triglycerides with one double bond on the one/three position, the asymmetrical mono-unsaturated TAGs. Later peaks represent triglycerides with more than one double bond. The symbol L refers to linoleic acid, a representative of the class of fatty acids with two double bonds. The lower part of figures gives the dot-plot for the comprehensive analysis. Here the *x*-position of the dot represents the elution time in the AgLC separation. The *y*-position is the carbon number. The size of the dot, more specifically the dot area, represents the amount.

Fig. 2 shows the comprehensive AgLC chromatogram of palm kernel oil. In the dot plot, the various classes of TAGs show up as slightly curved, diagonally oriented bands. In the figure, the ovals around the groups of dots represent the compounds with the same number of double bonds. The most pronounced band, the SSS band, comprises the CN range from carbon number 28–52. Within this band, CN36 is the most abundant peak. The second curved band in the dot plot represents the mono-unsaturated TAGs. These compounds span the CN range from approximately 36 to 54. Due to the wide CN distribution of the mono unsaturated TAGs in combination with the limited resolution of the Ag-loaded silica column, no sharp separation according to symmetry can be achieved. For oils with a narrower CN distribution, the SSO and SOS zones are better separated, allowing for detection of symmetry effects. The diagonal orientation of the bands in the plane indicates that some separation based on carbon numbers occurs on the AgLC column as well. TAGs with higher carbon numbers elute slightly before the TAG with the same number of double bonds but shorter total alkyl chain length. In principle this means that the separation of two TAG classes in comprehensive AgLC×GC is better than in stand-alone AgLC. In comprehensive AgLC×GC for example, the last eluting TAG of the SOS class is a short chain SOS TAG. In the LC dimension this component almost co-elutes with the first TAG of the SSO group, which is a long chain SSO TAG. In the second dimension, the GC separation, these two TAGs have the largest possible elution time difference. The resolving power is hence maximised. The two separation modes are largely orthogonal. This improved separation power, however, is still insufficient for providing a full separation between the SOS and SSO band for TAG mixtures containing wide CN distributions. The other mode of silver phase chromatography, columns packed with silver ions loaded onto an ion-exchange resin, might be better suited for this purpose but provide reduced resolution in the poly-unsaturates region [28,29]. Fig. 3 shows the dot plot of an edible oil mixture. Fig. 4 finally shows the comprehensive LC×GC (carbon number) separation of a palm oil, now in the overlay mode. The information shown in the figures is highly valuable for understanding the behaviour of oils and fats. Combination of the bubble graph information with results of an overall fatty acid analysis can further the understanding of the properties of the respective fat even more.

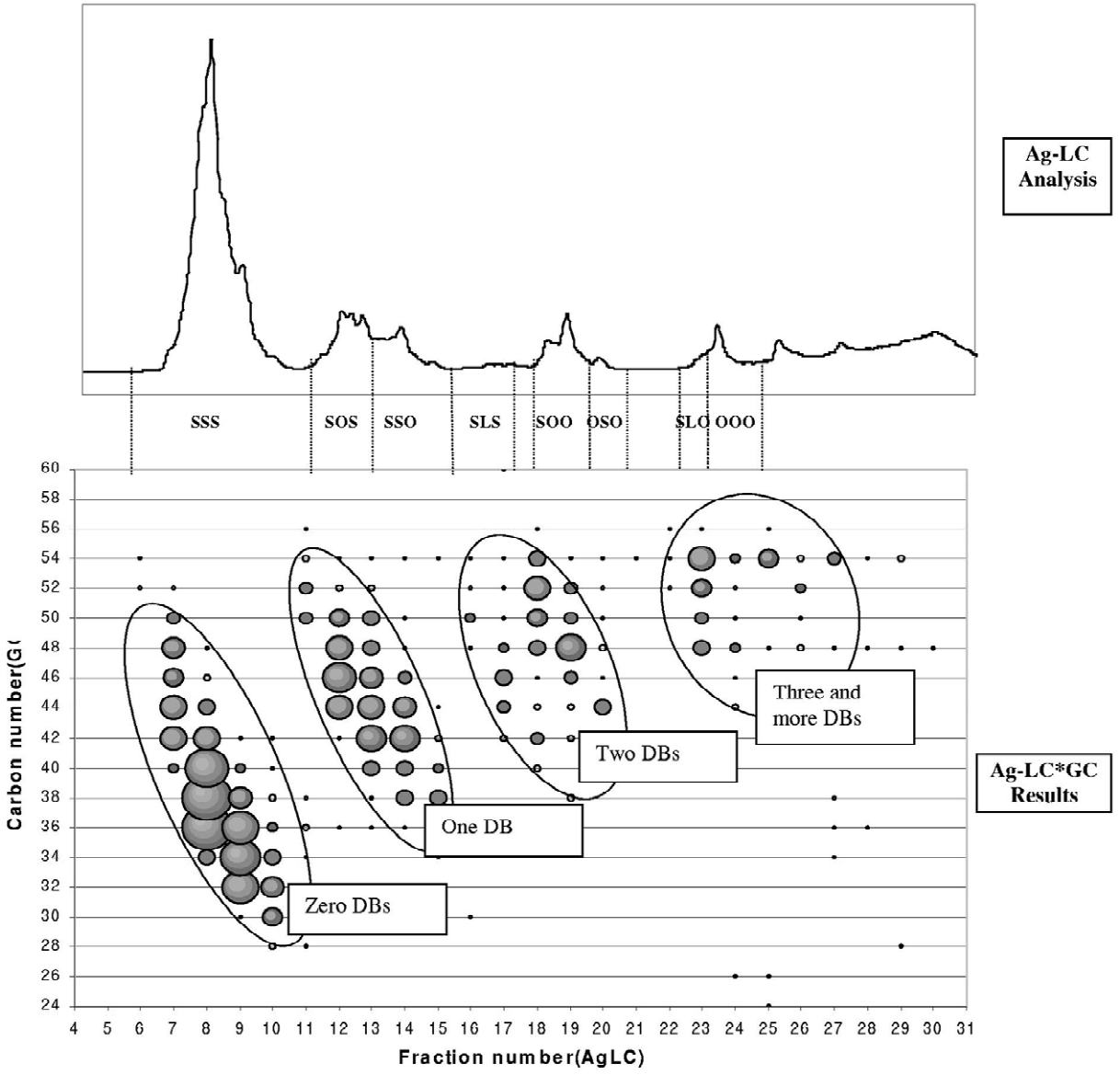

Fig. 2. Comprehensive AgLC×GC (carbon number) separation of a palm kernel oil. For conditions see text.

4.2. Characterisation of triacylglycerides as fatty acid methylesters

In the previous paragraphs, applications of comprehensive AgLC×carbon number GC were shown. This set-up provides information on the carbon numbers of the triglycerides as a function of degree of un-saturation. The second comprehensive LG×GC set-up that was evaluated was AgLC×FAME. Using this technique, detailed information can be obtained on the fatty acid composition of the TAGs as a function of the degree of un-saturation.

An example of a comprehensive AgLC×FAME separation of a palm oil sample is given in Fig. 5. For this same sample the comprehensive AgLC×CN chromatogram was already shown in Fig. 4. In the

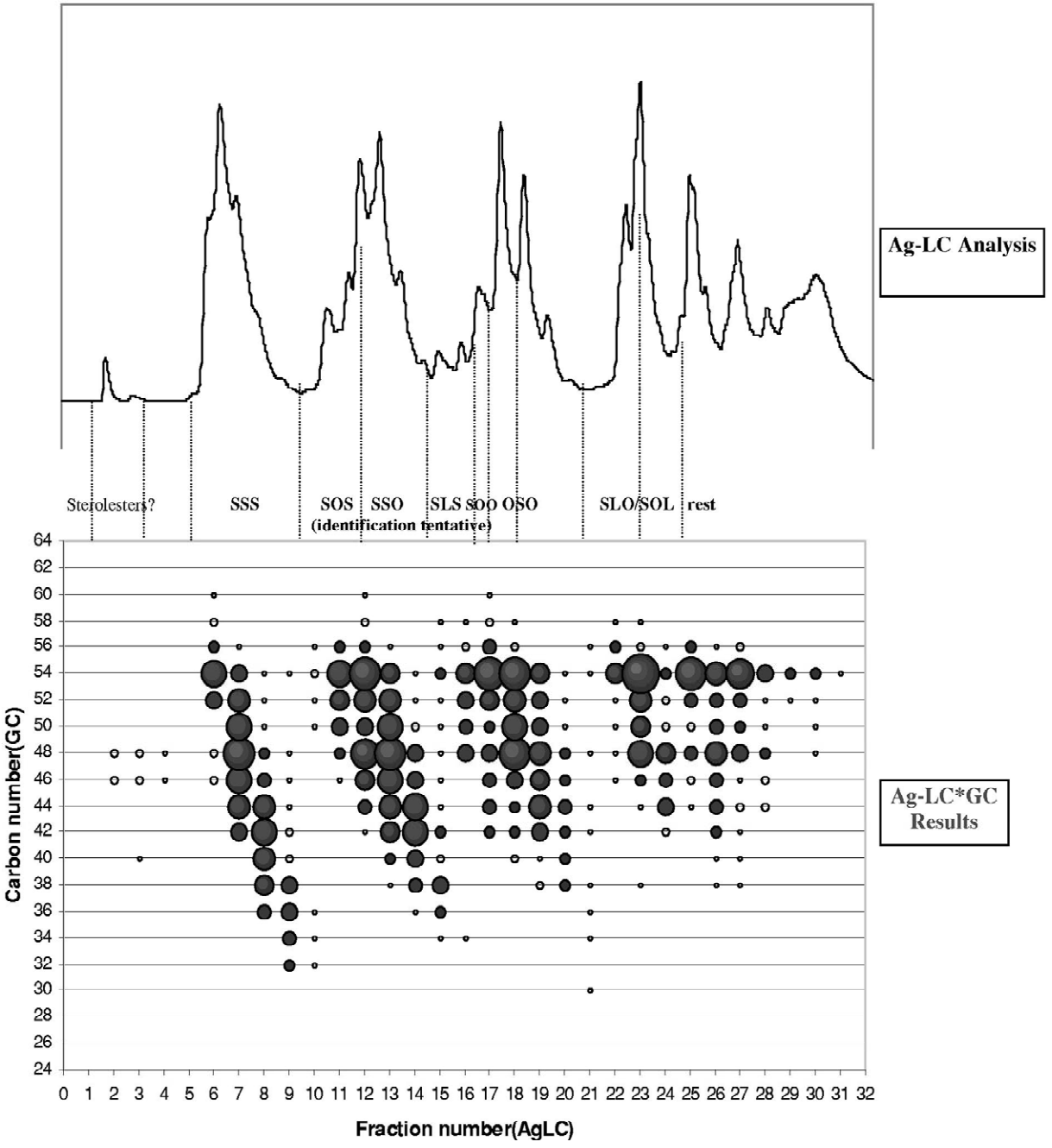

Fig. 3. Comprehensive AgLC×GC (carbon number) separation of a typical complex industrial fat blend.

first dimension of the comprehensive AgLC×FAME set-up the *intact* TAGs are separated based on the number and accessibility of the double bonds. Prior to the FAME analysis in the second dimension, the TAGs are converted to the corresponding FAMEs by transesterification. From the dot plot in Fig. 5 it can

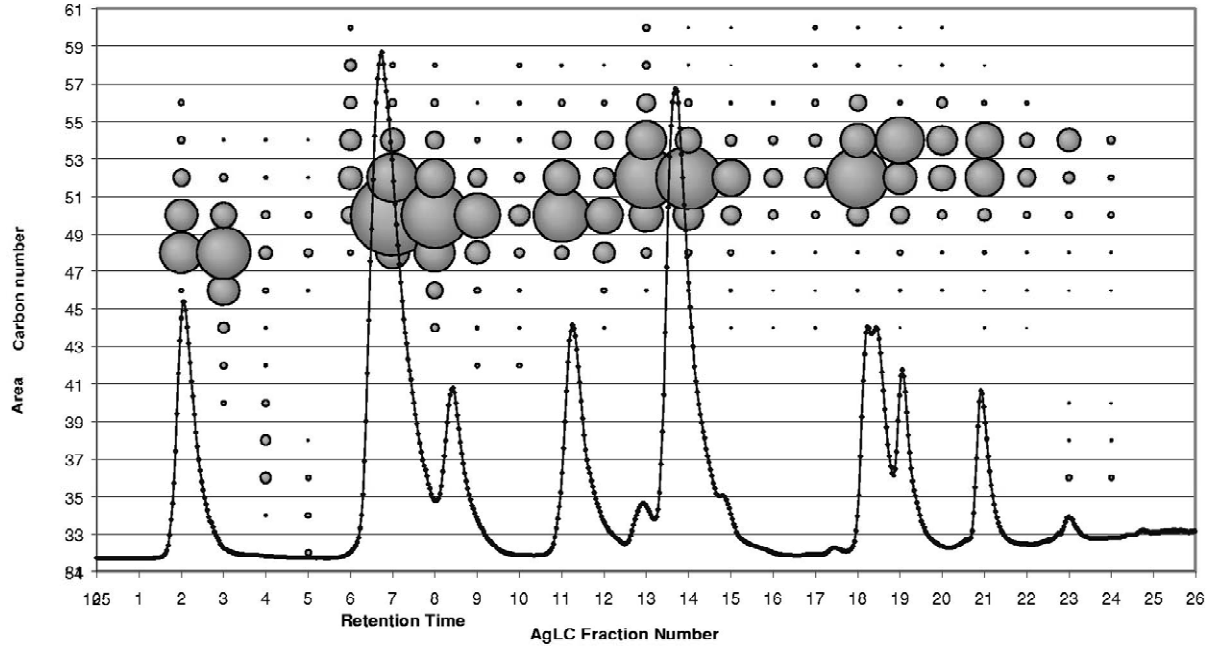

Fig. 4. Comprehensive AgLC×GC (carbon number) separation of a palm oil. Identification of multiple unsaturated TAGs tentative.

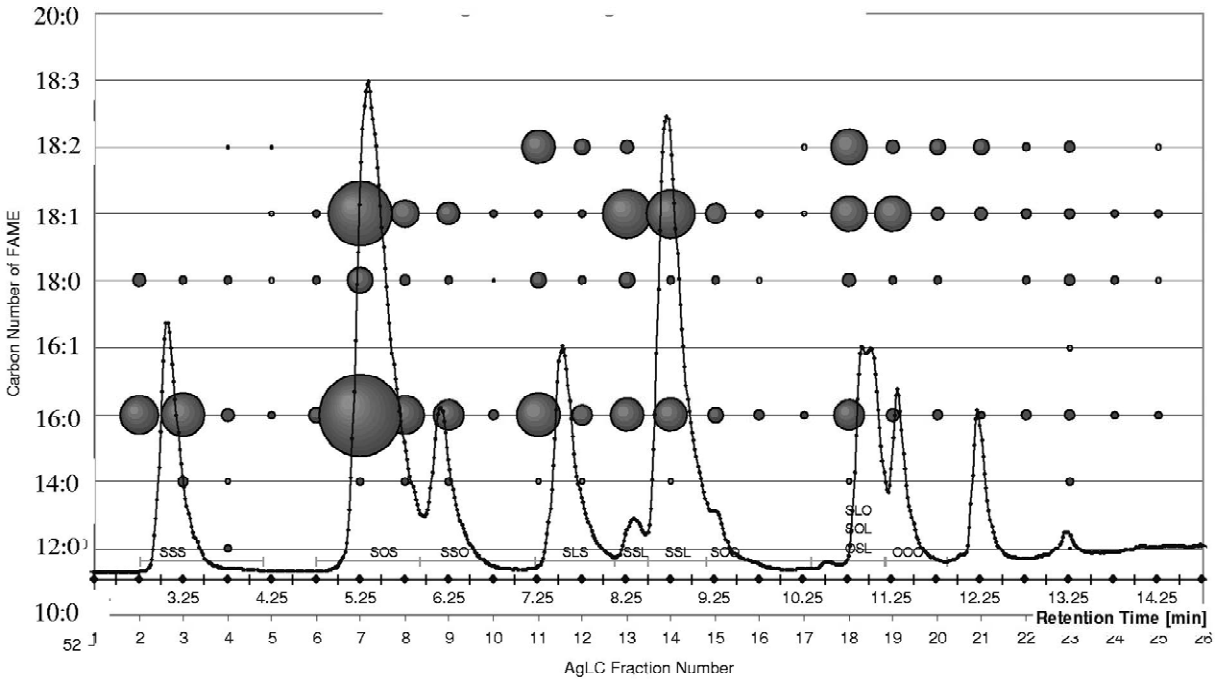

Fig. 5. Comprehensive AgLC×FAME–GC separation of a palm oil. TAG classification for multiple unsaturated TAGs tentative.

be seen that the saturates fraction of the oil consists mainly of C16:0 with small amounts of C18:0, C14:0 and C12:0. The comprehensive AgLC×CN analysis of the same sample shown in Fig. 4 indeed shows the main carbon number present in the SSS peak to be 48, corresponding to three C16:0 fatty acid chains. The SOS band mainly consists of C16:0 and C18:1 in the ratio 2:1. The average carbon number of the SOS molecules hence is 50. This agrees well with the average CN number as found for the SOS peak in comprehensive AgLC×CN GC (see Fig. 4). As expected, in the zone of TAGs with two double bonds, the 18:2 containing TAGs, elute before the TAGs containing two 18:1 fatty acid chains. It should be noted here that due to the limited separation between the SOS and SSO band the typical ratio of symmetrical vs. asymmetrical TAGs of 1–5 is not really evident. As mentioned previously, other modes of silver phase LC will yield a different selectivity allowing accurate determination of this ratio.

4.3. Quantitative aspects of comprehensive LC× GC in TAG characterisation

The discussion of comprehensive AgLC×CN GC and AgLC×FAME presented above is largely qualitative. To be of real practical value, however, the methods should also allow accurate quantitative analysis of complex oils and fats. To study the quantitative aspects of the newly developed comprehensive AgLC×CN GC and AgLC×FAME methods, quantitative data from the new methods were validated against existing methods for characterising oil and fat samples. Comprehensive chromatography as described here basically is the combination of two existing, different methods for TAG analysis. The comprehensive chromatogram contains the multiplied information from each of these two methods. By projecting the dot-plot chromatograms onto either the *x*-axis or the *y*-axis, the results from the single first- or second dimensional separation can be obtained. This than allows for example, a direct comparison of a reconstructed AgLC chromatogram obtained from the projection of a dot-plot onto its *x*-axis on the one hand, with a ''stand-alone'' AgLC chromatogram on the other. This process of projection and the resulting options for cross validation are schematically depicted in Fig. 6.

As can be seen from Fig. 6, comprehensive AgLC×GC can be validated against:

(1) Methods yielding information on the degree of unsaturation of the sample, viz. silver phase LC.

(2) Methods giving the carbon number distribution of the total fat/oil.

(3) Methods giving the overall fatty acid distribution of the total oil/fat.

In this validation study, three samples are analysed using a variety of techniques:

(1) Stand-alone AgLC,

(2) Comprehensive AgLC×GC (25 fractions collected at fixed time intervals),

(3) Comprehensive AgLC×GC on four fractions collected from the AgLC (SSS, SOS, SSO, rest),

(4) Comprehensive AgLC×FAME (25 or 4 fractions).

The sample used in these experiments is a chemically inter-esterified feedstock oil and its oleine and stearine fractions obtained via dry fractionation. For each of the three fractions the carbon number distribution was determined using GC according to an official method [30]. In Table 3, the TAG composition data derived from the comprehensive AgLC×GC are compared with the results of this official method. Additionally, a comparison with theoretical ''random'' data (resulting from the inter-esterification) is given. The random carbon number data is calculated from the overall fatty acid composition assuming random distribution of the fatty acids over the TAGs. To determine the overall CN distribution of the sample from the comprehensive AgLC×GC bubble-graph, the dot-plot chromatograms are basically projected onto the *y*-axis. The peak areas obtained for a specific carbon number in all the fractions are summed giving the total amount of that particular carbon number in the sample. From the data given in Table 3 it is clear that the triglyceride compositions derived from the comprehensive AgLC×GC results are in excellent agreement with the carbon number GC data. Or in other words, projection of the dot-plots onto the *y*-axis yields a correct carbon number distribution chromatogram.

Fig. 7 shows a comparison of an original AgLC chromatogram with a reconstructed chromatogram. The latter chromatogram was obtained by projecting the comprehensive dot-plot chromatogram onto its *x*-axis. The areas of all carbon number peaks in a

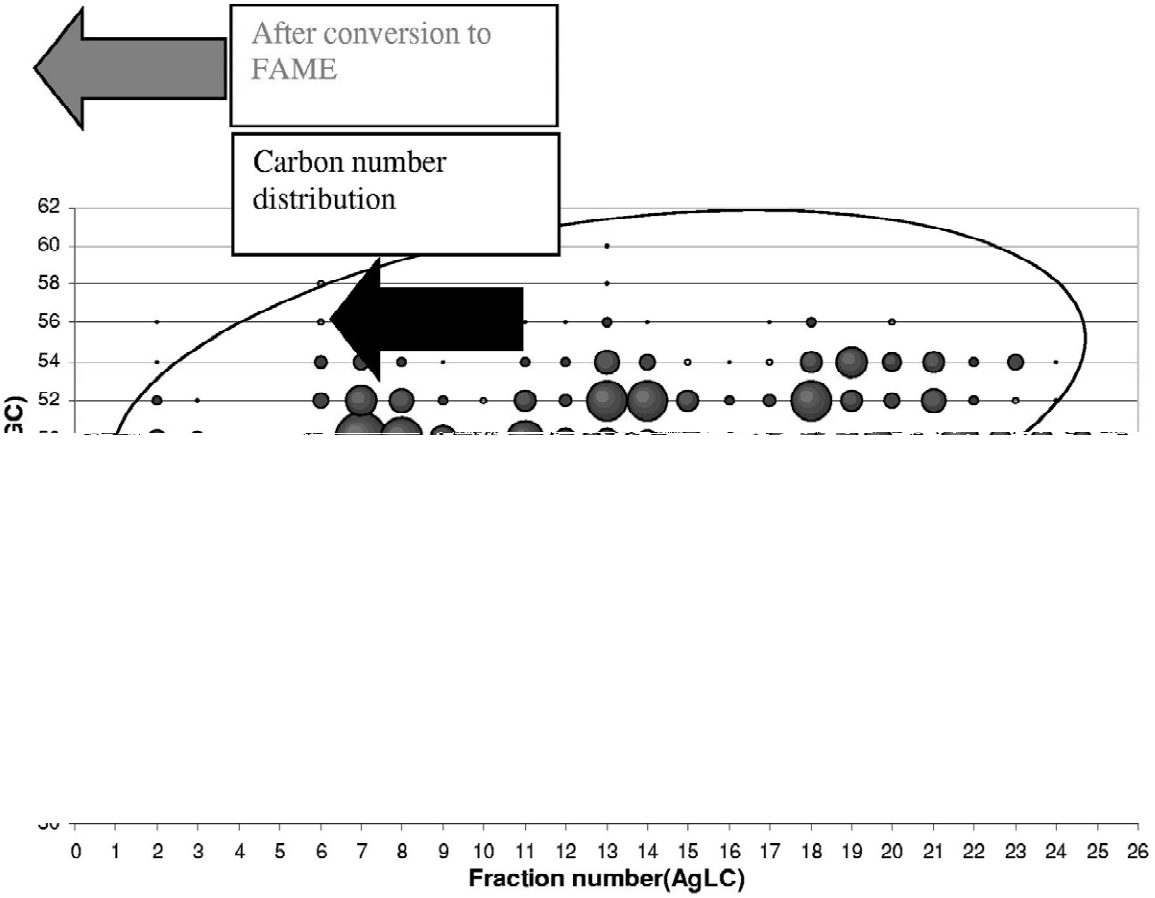

Fig. 6. Schematic representation of the various options for validation of comprehensive AgLC×GC.

certain time fraction were summed to give the overall intensity value at a certain time point. The figure shows an excellent visual agreement between the original and the reconstructed chromatogram. Evidently, the reconstructed chromatogram shows some ''smoothing'' as a result of the relatively low sample collection frequency. The data are also given in tabulated form in Table 4. Combining these two observations results in the final conclusion that the entire dot-plot must be reliable. Additional evidence for this statement is given by the other comparisons.

4.4. Group-type screening of fats and oils

In previous paragraphs, comprehensive LC×GC has been used to study the composition of one group of important oil and fat compounds, the TAGs, in great detail. By using other HPLC and GC columns and separation conditions, it is also possible to zoom in on other classes of compounds present in the oil. In addition to these applications where highly detailed information is obtained on a specific group of compounds, comprehensive LC×GC can also be used to obtain a general overview of an oil sample. In such an analysis the sample is basically separated into classes of compounds. In this way a semi-quantitative impression or fingerprint of the composition of the oil/fat sample can be obtained at one glance. Specific regions of interest can than be selected for further study.

Fig. 8 shows an example of a comprehensive fingerprint analysis of an olive oil. In the separation plane a large number of compound classes can be distinguished. The vast amount of detailed information shown in this graph would be impossible to obtain using any other single chromatographic meth-

Table 3
Comparison results of carbon number data obtained using an official method [30] with those derived from comprehensive AgLC×GC analysis

Sample	Carbon number	Carbon number (%, w/w)		
		Official method	Reconstructed from comprehensive data	Calculated from FAME composition using random assumption
Feed	C32	0.5	0.5	0.6
	C34	1.0	1.2	1.0
	C36	4.5	4.8	4.9
	C38	5.4	5.8	5.8
	C40	14.6	15.7	15.3
	C42	14.0	15.0	14.2
	C44	21.0	21.9	20.9
	C46	16.4	16.3	16.0
	C48	12.6	11.7	12.0
	C50	7.3	6.1	6.9
	C52	2.1	1.0	2.0
	C54	0.3	0.2	0.2
Oleine fraction	C34	1.1	1.2	
	C36	5.0	5.4	
	C38	6.0	6.5	
	C40	15.9	17.2	
	C42	14.7	15.2	
	C44	20.6	22.0	
	C46	15.9	16.8	
	C48	10.3	8.5	
	C50	7.1	5.7	
	C52	2.2	0.6	
Stearine fraction	C32	0.3	0.2	
	C34	0.5	0.5	
	C36	2.2	2.2	
	C38	2.9	3.2	
	C40	9.1	9.8	
	C42	10.5	10.8	
	C44	22.6	23.9	
	C46	18.4	18.2	
	C48	23.3	22.3	
	C50	8.2	6.4	
	C52	1.6	0.6	
	C54	0.2	–	

od. Typical application areas for this type of analyses would be patent infringement detection and oil-origin analysis. The particular chromatographic set-up used for the analysis consisted of a diol modified silica HPLC column in the first dimension and a non-polar GC column in the second dimension. This means the HPLC separation is more or less performed based on the polarity of the compounds, whereas in the second dimension the compounds are eluted on the basis of their boiling point/molecular mass. The first component groups to elute in the HPLC chromatogram (*x*-axis) are the non-polar alkanes and the hydrocarbons squalane/squalene. The slightly more polar wax esters elute as the second group of compounds, followed by the sterol esters. Around the same LC retention time also another group of slightly smaller compounds elutes. Using GC–MS these were tentatively identified to be fatty acid methyl esters. When

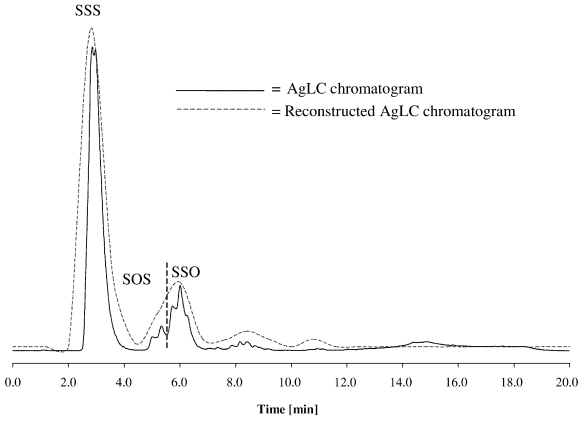

Fig. 7. Comparison of a reconstructed AgLC chromatogram and a stand-alone AgLC separation for the stearine sample. For the comprehensive separation, fractions of 0.5 min were collected.

looking to this area of the chromatogram, the superior resolution power of comprehensive LC×GC immediately becomes apparent. In case that only an LC separation would have been applied, the FAMEs could never have been separated from the sterol esters. These compounds coelute in LC, but are well separated in the second dimension because of their large difference in boiling point or molecular mass. Similarly, squalane would have co-eluted with the wax esters and steradienes. In fact the LC region where these groups of compounds elute would have shown a number of overlapping peaks that could never be assigned unambiguously to certain compound classes. In the comprehensive LC×GC chromatogram the various classes of compounds are very well separated.

4.5. Improved target compound analysis

An example on how the enhanced resolution of comprehensive LC×GC can be used for improved target compound analysis is given in Fig. 9. The aim of the analysis was to determine the wax ester content of an olive oil sample. Wax esters, especially the higher ones, are held responsible for the turbidity often seen for olive oil if stored at lower temperatures. Conventional methods for wax ester analysis are based on either on-line or off-line isolation of the wax esters followed by GC quantification [31]. This isolation is by no means easy because sterol esters elute very close to the wax esters in the LC run. Moreover, the sterol esters overlap with the higher wax esters in the GC separation. From the various subsequent GC runs of the comprehensive separation shown in Fig. 9, it is nicely seen that the higher molecular mass wax esters elute first, followed by the lower wax esters together with the highest sterol esters. The first fractions of the wax esters region are pure. The last fractions contain the last eluting wax esters, the lower wax esters, together with the first eluting sterol esters, the highest sterol esters. Separation of the lower wax esters from the higher sterol esters in the second dimensional GC separation is straightforward. If, on the other hand, all wax esters and some of the higher sterol esters would be in one fraction, GC separation would be impossible. This

Table 4
Comparison of the results of stand-alone AgLC analysis and reconstructed comprehensive AgLC×GC data

Sample	TAG fraction	Standard AgLC [%, w/w]	AgLC reconstructed from AgLC×GC [%, w/w]
Feed	SSS	77.7	80.9
	SOS/SSO	18.8	15.8
	Rest	3.5	3.3
Oleine fraction	SSS	75.4	73.6
	SOS/SSO	20.8	21.5
	Rest	3.8	5.0
Stearine fraction	SSS	89.1	88.5
	SOS/SSO	9.3	9.6
	Rest	1.6	1.9

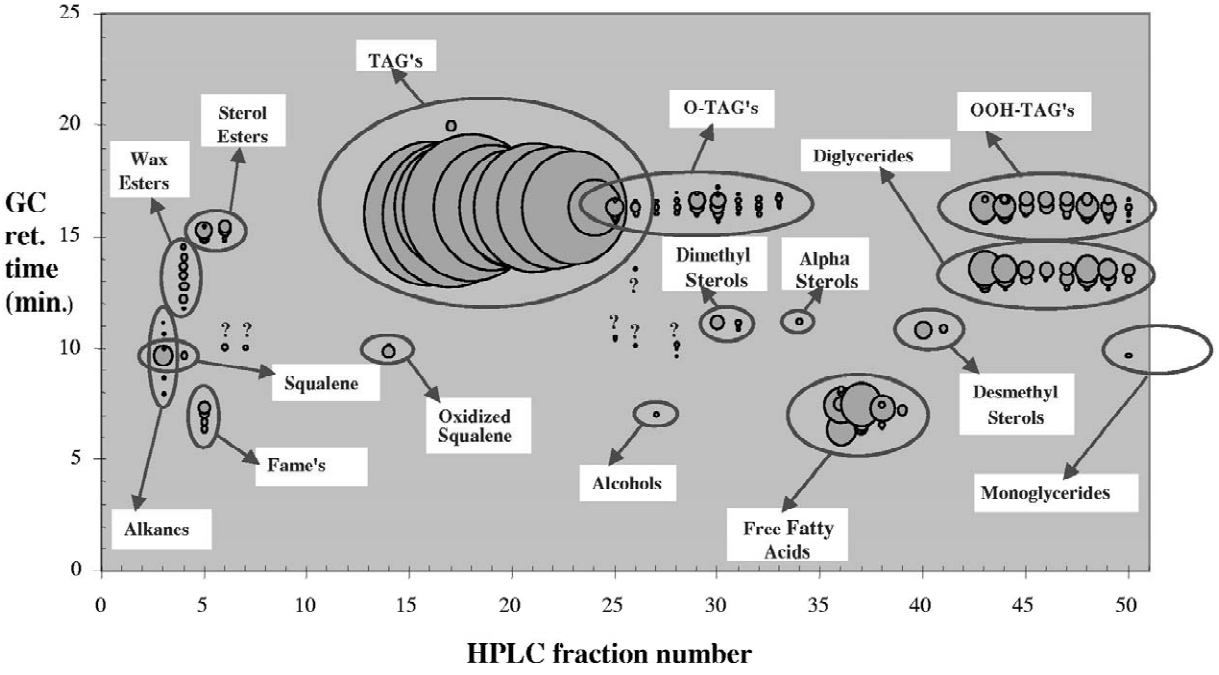

Fig. 8. Comprehensive two-dimensional group-type separation (fingerprinting) of an olive oil sample. Class assignments are tentative.

situation will be very difficult to avoid in single heart-cut LC–GC techniques. Comprehensively coupled LC×GC clearly is capable of providing improved resolution between compounds and compound groups.

5. Conclusions

Comprehensive two-dimensional chromatography is an extremely powerful analytical method for the analysis of complex triglyceride samples. The most promising combinations are (i) comprehensively coupled silver phase liquid chromatography×carbon number GC, or (ii) coupled silver phase×FAME GC. Using the new comprehensive set-ups, three dimensional graphs are obtained that represent the separation of triglycerides according to two independent parameters: carbon number vs. number of double bonds, or fatty acid composition vs. number of double bonds. The information content of such graphs by far exceeds that of the current generation of analytical methods. Using the new comprehensively coupled systems, the composition of TAG mixtures can be analysed with a very high degree of detail, allowing accurate compositional analysis of the samples. Comparison of quantitative data obtained using the novel methods with that of classical analytical methods shows a good agreement between these two methods. Other applications of comprehensive LC×GC include fingerprinting analysis and quantification of target (groups of) compounds that can not be resolved using standard chromatographic systems or simple coupled methods.

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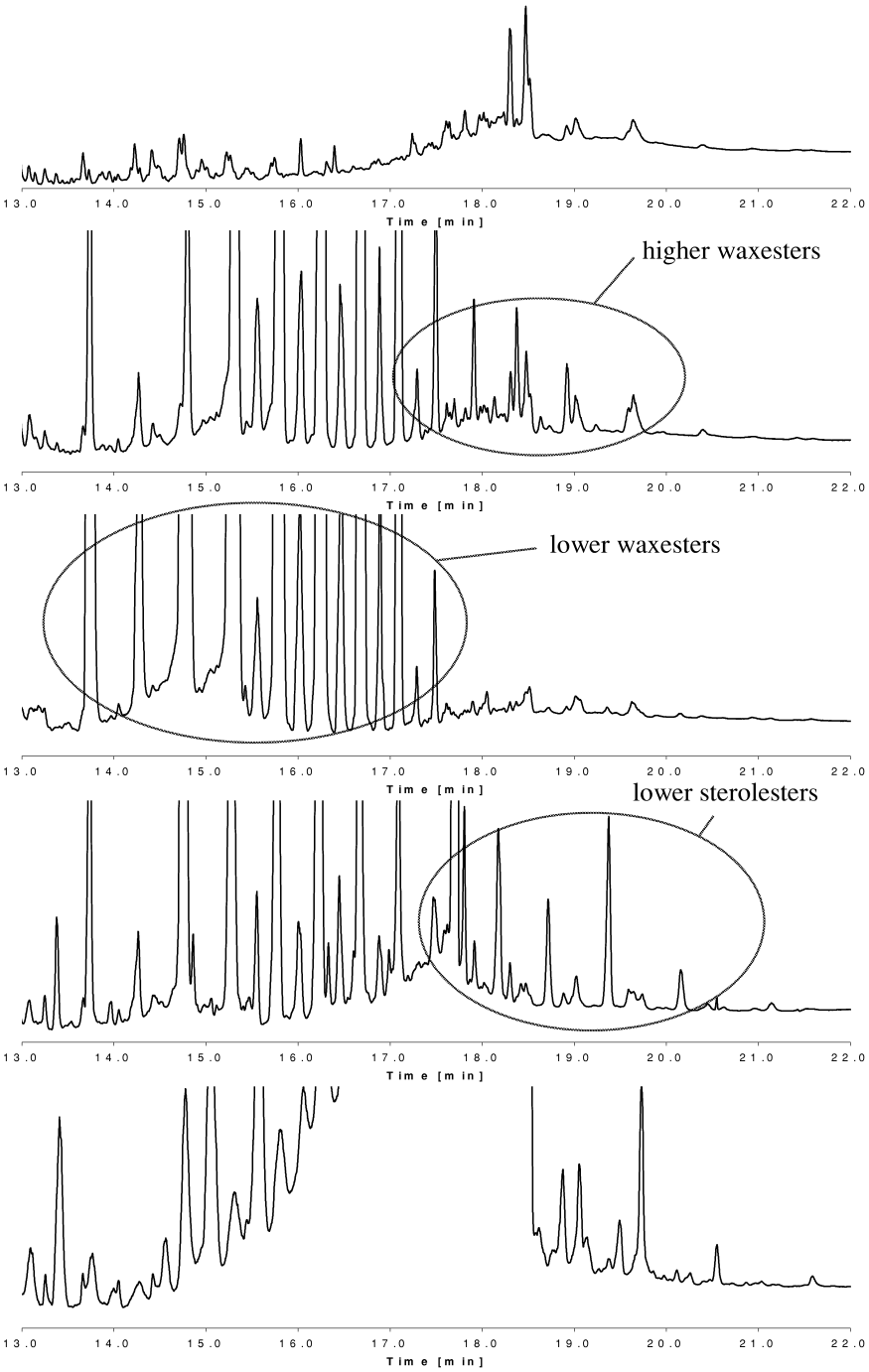

Fig. 9. Subsequent fractions of the comprehensive NPLC×GC separation of an olive oil sample. The first fractions show the wax esters. In the later fractions, sterol esters are seen as well.

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