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Automated on-line comprehensive two-dimensional LC × GC and LC × GC–ToF MS: Instrument design and application to edible oil and fat analysis

After a successful off-line feasibility study, the automation of comprehensively coupled liquid chromatography and gas chromatography (LC × GC) has been studied. Important aspects to consider when developing automated LG × GC include the relative speeds of the two dimensions, the compatibility of the LC eluent (type and flow rate) with the GC dimension, and the column loadabilities. Because the GC separation is relatively slow, the LC instrument has to be operated in the stop-flow mode. Two interfaces for transferring large numbers of subsequent LC fractions to the GC were constructed: one based on a six-port switching valve, and one which uses a dual side-port syringe. Both interfaces were found to perform fully acceptably. The actual transfer of the LC fraction to the GC was realised using a standard split injector to vaporise the compounds and LC eluent. Gas phase splitting was applied to match LC mass load and GC column loadability. The standard deviations of the peak areas obtained in this way were better than 7% ($n = 6$). The reliability of the system was demonstrated by the problem-free analysis of large series of oil and fat samples, with the focus on both intact triglycerides and their fatty acid methyl esters (FAMEs). Finally, the hyphenation of the automated LC × GC system to a sensitive and rapid-scanning time-of-flight mass spectrometer was realised. By using LC × GC–ToF MS, the information density of the chromatograms could be improved even further, which allowed easy identification of individual compounds as well as compound groups.

Key Words: Comprehensive two-dimensional chromatography; Comprehensive LC × GC; LC × GC–ToF MS; Edible fats and oils; Triacylglycerides; Triglycerides; TAG; Fatty acid methyl esters; FAME

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1 Introduction

In many samples that are nowadays analysed using high-resolution chromatographic systems, the number of compounds of interest that is present significantly exceeds the peak capacity of the separation column. Separation of all compounds of interest from one another and from interfering (matrix) analytes is, hence, by definition impossible. Fortunately, several strategies are available to circumvent this problem. To quote an example, by using modern sample pre-treatment techniques, groups of compounds that are of no interest can be eliminated prior to injection [1]. Alternatively, if the analytes of interest share a unique structural property, they can be monitored with a selective detector. Finally, mass spectrometric (MS) detection is also a powerful means for the selective extraction of the peaks of interest from a complex chromatogram.

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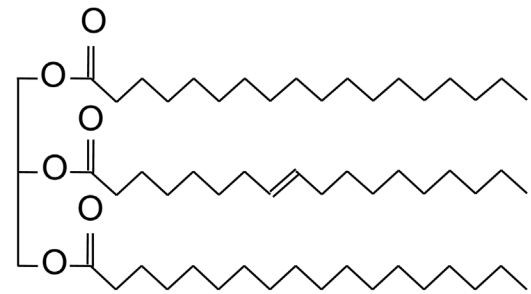
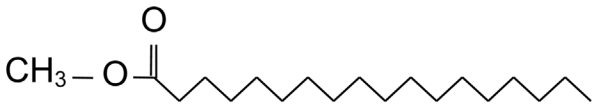
Multidimensional chromatography is another solution to what we here call 'the limited peak capacity problem'. In contrast to the above mentioned approaches, where selectivity is introduced either in the sample-preparation or the detection step, this route is based on increasing the peak capacity of the chromatographic system. Comprehensive two-dimensional (2D) chromatography, the most sophisticated form of this approach, provides an unsurpassed peak capacity. For example, Venkatramani and Phillips detected over 6000 compounds in a kerosene sample using GC × GC [2]. Several authors also demonstrated the use of LC × LC for improved separations [e. g. 3–5].

An application area of comprehensive chromatography that is of particular interest to us is edible oil and fat analysis. The main constituents of an edible fat/oil are the triacylglycerides (TAGs). A TAG consists of a glycerol backbone where each of the three OH groups is esterified with a fatty acid. **Figure 1** shows the molecular structure of a TAG. Because of the large number of fatty acids that occurs in nature, the number of potential TAGs is enor-



TAG



FAME

Figure 1. Molecular structure of a triacylglyceride (TAG) and a fatty acid methyl ester (FAME). Fatty acids can vary in chain length as well as number, orientation and position of the double bonds. Typical fatty acid chain lengths range from 4 to 24 (animal fat) or 10 to 20 (vegetable oil).

mous. Although remarkable separations can be obtained on dedicated stationary phases such as the polarisable liquid phases used in GC [6, 7], separation of all TAGs present in a sample using a single LC or GC column is impossible. In previous work we demonstrated the feasibility of comprehensive LC × GC for detailed TAG analysis. In that work the effluent eluting from the LC column was collected in approx. 50 narrow time-fractions that were subsequently analysed by (fast) GC [8]. In this way a comprehensive 2D separation was obtained. For fat and oil samples comprehensive LC × GC not only provided a significantly higher peak capacity than either LC and GC by itself, it also allowed separation of the sample according to two independent properties: the number of double bonds and the carbon number, respectively. This information cannot be reliably obtained by using any one-dimensional chromatographic analytical method.

The principle of off-line comprehensive LC × GC was also applied to fatty acid methyl esters (FAMEs, see Figure 1) [9]. Because of the multiplicity of chain lengths, different degrees of unsaturation, and the many geometrical and positional isomers present, full separation of the, typically, extremely complex FAME mixture is again impossible using either LC or GC alone, not even if six silver-phase LC (AgLC) columns are connected in series [10, 11], or if a 120 m × 250 μm ID capillary GC column is used [12]. A combination of AgLC and GC has been proposed in the literature for the separation of the most relevant sub-set of FAMEs, the conjugated linoleic acids [13]. In the proposed protocol the linoleic acid (C18:2) sub-fraction of a FAME mixture is isolated by means of preparative AgLC and further analysed on a very long polar GC column. No experimental data on this proposed method are presented in the quoted paper. A 2D approach that has been applied in the literature is silver-phase thin-layer chromatography (AgTLC) combined with GC analysis of the TLC spots [14]. The resolution of this method is extremely high, but the very laborious nature and poor quantitative performance preclude routine use of this 2D technique. A more promising approach is GC × GC, which was successfully used for FAME analysis of fish and vegetable oils. Ordered structures in the 2D plane facilitated analyte identification, and quantitative ratio analysis gave no problems [15].

In our previous AgLC × GC experiments, the degree of separation was very high. It was also evident, however, that the use of comprehensive LC × GC in an industrial environment would require full automation. So far, only one paper on fully automated comprehensive LC × GC has been published. Quigley *et al.* described the automated LC × GC analysis of volatiles in water [16]. However, their interface can be used only for highly volatile compounds, i. e. neither for TAGs nor for FAMEs.

In the present paper we will discuss different approaches for fully automated LC × GC. The basic design criteria such as speed, solvent compatibility and sensitivity will be summarized and used as the starting point for instrument design. The merits of the different interfaces will be briefly discussed and two of these will be evaluated in more detail. The use of rapid-scanning GC–ToF MS in the second dimension to improve information-density even further will also be reported, using edible oils and fats (TAGs) as well as their FAMEs as sample types. However, the main goal of the study is interface design and evaluation, rather than providing detailed compositional information on the fat blends studied.

2 Materials and methods

2.1 LC and GC equipment and conditions

Two LC systems were used, a Waters Alliance 2690 HPLC system (Waters, Milford, MA, USA) and an Agilent 1100 HPLC (Agilent Technologies, Wilmington, DE, USA). The latter instrument contains a Rheodyne six-port valve that can be used for flow switching. All GC separations were performed on an Agilent Model 6890 GC equipped with an OPTIC 2 or OPTIC 3 injector (ATAS GL, Veldhoven, the Netherlands). For the experiments with the syringe-based interface the GC was equipped with a FOCUS injection robot (ATAS GL). GC detection was

Table 1. LC conditions for the various separations of TAGs and FAMEs. All solvents were of p.a. grade and were purchased from Merck (Darmstadt, Germany). Hexane was dried using Molsieve 5 Å prior to use.

Analytes	Column	Conditions
TAG	25 cm × 4.6 mm ID, 5 μm Ag(I)-loaded ion exchange	Dichloromethane/acetone (99.5:0.5) [*conditions adapted from ref. 8*] from $t = 0-15$ min; step to pure acetonitrile ($t = 15-25$ min). Flow rate, 0.5 mL/min.
	25 cm × 2.0 mm ID, 5 μm Ag(I)-loaded ion exchange	Pure dichloromethane from $t = 0-10$ min; step to pure acetonitrile ($t = 10-20$ min)
	10 cm × 4.6 mm ID, 3 μm Nucleosil, 10% $AgNO_3$-loaded silica	Eluent A: toluene/hexane 1:1 (*v/v*). Eluent B: toluene/ethyl acetate 3:1 (*v/v*). Eluent C: toluene, 80 μL formic acid added per litre. From (A/B/C = 97/3/0) to (96/4/0) at $t = 2.5$ min, to (85/15/0) at $t = 8$ min, to (75/25/0) at $t = 9$ min, to (10/190/0) at $t = 13$ min, to (0/0/100) at $t = 13.1$ min; hold to $t = 17$ min (all linear gradients). Flow rate, 1 mL/min.
FAME	25 cm × 2.0 mm ID, 5 μm Ag(I)-loaded ion exchange	Dichloromethane/acetone (99.5:0.5) *(conditions adapted from ref. 8)* for $t = 0-15$ min; next, step gradient to pure acetonitrile ($t = 15-20$ min). Flow rate, 0.2 mL/min.
	25 cm × 4.6 mm ID, 5 μm Ag(I)-loaded ion exchange	Dichloromethane from $t = 0-10$ min. Flow rate, 1.0 mL/min.

either by FID, or by mass spectrometry using a Pegasus III ToF MS (Leco, St. Joseph, MI, USA) in the positive ion EI mode. Helium was used as the carrier gas in all GC experiments. Three dimensional LC × GC-FID chromatograms were constructed using the bubble plot option from Microsoft Excel.

Three columns were used for AgLC, two Chromspher Lipid silver-loaded ion-exchange columns of 25 cm × 4.6 mm ID or 25 cm × 2.0 mm ID, both packed with 5 μm Ag(I)-loaded particles purchased from Varian (Middelburg, the Netherlands), and a 10 cm × 4.6 mm ID 10% $AgNO_3$-loaded silica (3 μm Nucleosil) packed in-house [17, 18]. For the GC separation of the intact TAGs a 5 m × 0.53 mm ID Ultimetal SIMDIST, coated with a film of 0.17 μm was applied. This column was operated in the constant pressure mode (50 kPa) giving a high carrier-gas flow rate of 13 mL/min to minimise the analysis time. All FAME separations were performed on a 25 m × 0.25 mm ID column coated with a film of 0.25 μm of the polar VF-25 ms for FAMEs stationary phase. The gas flow rate here was 1 mL/min. Both columns were from Varian. The split flow was 150 mL/min in all experiments which resulted in a split ratio of approx. 1 : 11 in the TAG analysis and 1 : 150 in the analysis of the FAMEs. When MS detection was applied for the intact TAGs, spectra were acquired over the mass range 50–1000 amu to maximise the information content. For the FAMEs the mass range was 50–500 amu. Spectral acquisition invariably was at 20 spectra per second.

The LC conditions for the various columns and separations are given in **Table 1**. These are all standard conditions. It was one of the requirements of the current project that the LC conditions should not have to be altered upon changing from standard LC separations to automated comprehensive separations.

The GC conditions were selected to obtain the fastest possible separations. For the intact TAGs, ballistic heating of the column oven was applied. This resulted in a heating rate of approximately 140 K/min at the starting temperature, decreasing to approx. 40 K/min at the final temperature. The initial temperature was 130°C (no hold). The final temperature was 380°C (1 min hold). The total cycle time (analysis and cooling) was approx. 10 min. For the FAME separations, the temperature was programmed to 176°C (final time 7.3 min) at a programming rate of 50 K/min. Different initial temperatures were used, depending on the analytes of interest and the method of interfacing (vide infra).

2.2 FAME preparation

To convert fat or oil samples into FAMEs, 50 mg of the fat were dissolved in 5 mL methanol containing 5 vol.% H_2SO_4. This solution was gently shaken for (at least) 12 h at 70°C to effect acid-catalysed trans-esterification of the TAGs. After reaction, 5 mL of ice water were added to the solution and the FAMEs were extracted with 5 mL of *n*-heptane. The heptane phase was isolated and washed (twice) with 5 mL of cold water to remove traces of acid. The sample was then ready for analysis using LC × GC–ToF MS. It must be stressed that acid-catalysed conversion can result in the formation of non-FAME by-products as well as in double-bond isomerisation [19]. Here, acid catalysis was deliberately selected to make sure that all compounds that can be present in the FAME reaction product were indeed present to enable evaluation in the worst-case scenario.

3 Results and discussion

3.1 Design considerations and principles for automated LC × GC

In designing systems for automated LC × GC, two issues require closer consideration: (i) the interfacing of LC and GC and (ii) the sequence of operation of the LC and GC instruments. Below, these issues will be addressed in more detail to arrive at a number of designs for the final automated instrument.

In LC × GC, narrow time fractions of the first-dimension LC run are transferred to a GC column for a second separation. In the off-line mode, fractions are collected and stored in vials for sequential analysis by GC. The preferred mode of injection into the GC depends on two parameters: the nature of the solvent and the concentrations of the analytes. If a normal-phase LC separation is performed using a non-polar organic solvent as the eluent, a wide variety of injection methods is available. Large-volume injection using on-column or programmed temperature vaporising (PTV) approaches [20–22] can be used if the analytes are present in low concentrations. Normal on-column or even split injection of microlitre volumes will suffice for higher levels. If reversed-phase LC separation is performed with an organic/aqueous eluent, transfer to the GC is much more difficult, although several methods for the injection of aqueous samples have been reported [23–25]. Fortunately, in oil and fat research, LC separations are frequently of the normal-phase type which makes injection essentially straightforward. Actually, normal-phase LC is the most logical mode of operation for the first dimension in LC × GC because, at least for the current application, normal-phase LC and GC have a higher degree of orthogonality than reversed-phase LC and GC. To a rough first approximation, both reversed-phase LC and GC separate on the basis of size (alkyl chain length for fats with equal number of double bonds). Normal-phase LC, on the contrary, separates on the basis of polarity.

Here it is also interesting to emphasise the differences between conventional LC–GC and comprehensive LC × GC. In LC–GC only one, or just a few, selected fractions are transferred to the GC for further analysis. Oppositely, in LC × GC more or less the entire chromatogram is further separated in the second dimension column. Relevant is also the difference in application area. Whereas LC–GC as pioneered and advocated by Grob [26] is generally performed to enable sensitive detection of one group of analytes present at a very low level in a complex sample, LC × GC is applied to characterise the complex sample itself! The concentrations of some of the analytes can, hence, be relatively high and, instead of full transfer of a collected LC fraction, splitting will be necessary to prevent overloading of the GC column (unless a very narrow LC column is used). In both our off-line and our on-line LC × GC work we opted for the use of standard-bore LC columns with partial transfer of the collected fraction to the GC. The practical requirements of this mode of operation are much less stringent than when miniaturised LC columns are employed. In the off-line mode, the partial transfer was realised by injecting only a limited percentage, typically 1–10% (2–20 μL) of a collected LC fraction. In the on-line mode, partial transfer of an LC time slice to the GC can be effected by liquid-phase flow splitting or gas-phase splitting after effluent evaporation. We preferred the second option, mainly for reasons of simplicity. Because of the high gas flow the residence times of the compounds in the injector is extremely short, ruling out thermal degradation.

A second issue to consider in automated LC × GC is the sequence of operation of the LC and the GC run. Unlike the situation in GC × GC, where the second-dimension run is only a few seconds long, typical GC runs in LC × GC require at least several minutes. In GC × GC typically some four second-dimension runs are collected 'on-the-fly' as the peak elutes from the first-dimension column. In LC × GC this on-the-fly operation is not possible, as will be outlined below. In GC, the absolute retention time differences for a particular analyte on different GC columns are small. If two compounds co-elute on one GC column, they will generally also elute within a rather narrow time window on another column. The second-dimension separation in GC × GC can therefore be performed isothermally and can, hence, be very fast [27]. On the other hand, in LC × GC compounds can co-elute from the LC column while having extremely large retention time differences in GC, even if the latter separation is performed under temperature-programmed conditions. As an example, in our class-type AgLC separations of triglycerides, compounds with 32 up to 60 carbon atoms were found in the same fraction of the LC run. To elute these compounds in GC, a temperature programme covering a temperature range of at least 150°C is required. Even if very fast heating and cooling are used, separation times will at least be 1–2 min [28] and total cycle times, at least some 2–3 min. With a typical LC peak width of 30 s it is, hence, impossible to record four second-dimension GC runs during the elution of an LC peak. On-the-fly coupling of LC × GC is therefore not feasible, unless the LC separation is slowed down to obtain peak widths of at least 8–12 min! We rather prefer the stop-flow mode. Here a fraction is transferred to the GC and the LC flow is stopped during the GC separation of this fraction. LC × GC can then be realised in a fully automated on-line fashion. Of course, full automation can also be achieved by using an at-line combination of LC and GC. Here fractions can be collected on, e.g., a 96-well plate for further analysis by GC after completion of the LC run. This option is not further explored in this study. Our

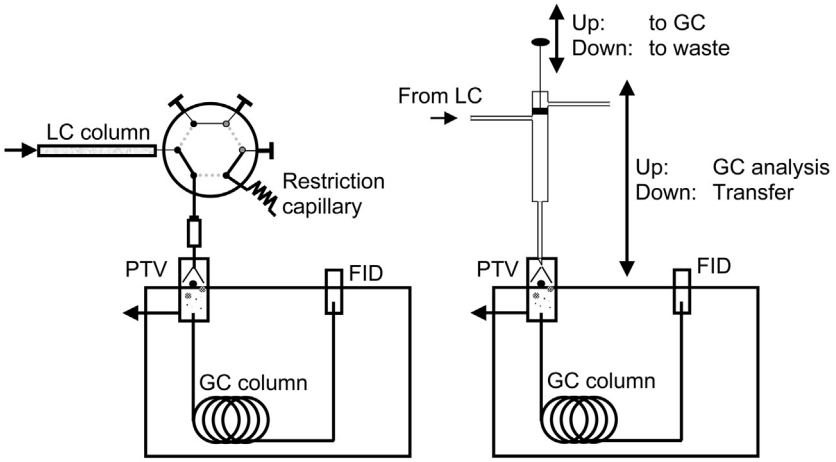


Figure 2. Schematics of the valve-based (left) and the syringe-based (right) interface designed for LC × GC.

automated LC × GC work focuses exclusively on methods based on stop-flow coupling.

3.2 Interfaces for automated LC × GC

In stop-flow LC × GC, the LC flow is stopped after the transfer of a fraction from the LC to the GC is completed. The GC programme is then started and the LC is only started again when the GC has returned to the initial ready status. To keep the total run time as short as possible, the GC separations were made as fast as possible. Because the LC column groups the components based on polarity, all homologues will end-up in the same fraction. The separation of these homologues in GC is now very simple and can be performed on a very short column, albeit with a very wide temperature range generally being required.

For the transfer of the LC fractions to the GC, two interfaces were developed: a valve-based interface and a syringe-based interface. Splitting of the LC fraction was achieved by vaporising the LC column effluent into a hot injection chamber. By operating the system at a high split flow, only a small fraction of the effluent was actually transferred to the GC column. In this way overloading of the GC column (cf. above) was avoided and the volume of solvent reaching the detector reduced. The initial GC column temperature during transfer of the fractions to the GC is important. Ideally a temperature should be selected at which the solvent is not retained, while simultaneously the analytes are quantitatively trapped and refocused. The two interfaces developed are schematically depicted in **Figure 2**.

3.2.1 Valve-based interface

The interface is based on a six-port valve and uses the standard six-port switching valve incorporated in the Agilent LC 1100. The configuration of the valve is similar to that described by Verma et al. for on-line LC–GC [29]. A deactivated fused silica capillary (30 cm × 100 μm ID) is used to transfer the LC effluent into the heated GC injector. The transfer capillary is simply inserted into the heated injector through the septum to a depth identical to the normal needle penetration distance. In the flow position of the valve (solid lines), the LC effluent is transferred to the GC. In the stop-flow position (dotted lines), the LC flow is stopped and GC separation takes place. A small leak flow of carrier gas is created through a restriction capillary (2 m × 100 μm ID) installed in one of the ports of the valve. In this way the liquid remaining in the transfer capillary is discharged to waste and diffusion of solvent vapour into the injector is prevented. The volume of eluent discharged through the capillary during each GC run equals the volume of the transfer capillary (approx. 2 μL). This is a negligible volume, unless LC columns with a very low inner diameter are used. For such columns, on the other hand, flow rates are low and transfer capillaries with very narrow internal diameters can be used without causing excessive back pressure.

3.2.2 Syringe-based interface

The interface uses a commercially available 100-μL syringe with two side entrances/exits in the upper part of the barrel. The lower connection is used as the LC effluent entrance, while the upper line is connected to waste. The syringe plunger is used to select the direction of the solvent flow. With the plunger stamp positioned *between* the two connections (plunger up), the LC effluent flows down through the syringe barrel into the syringe needle. With the plunger stamp positioned *below* the entrance line (plunger down), the LC effluent is directed to waste. In the default position the syringe is in the up position with the needle outside the GC injector. When the GC returns to the initial ready status, the syringe moves into the GC injector (syringe down) and transfer of the LC fraction to the GC is commenced. After transfer the LC flow is stopped, the syringe withdrawn from the injector and the GC separation started. Because there is no permanent

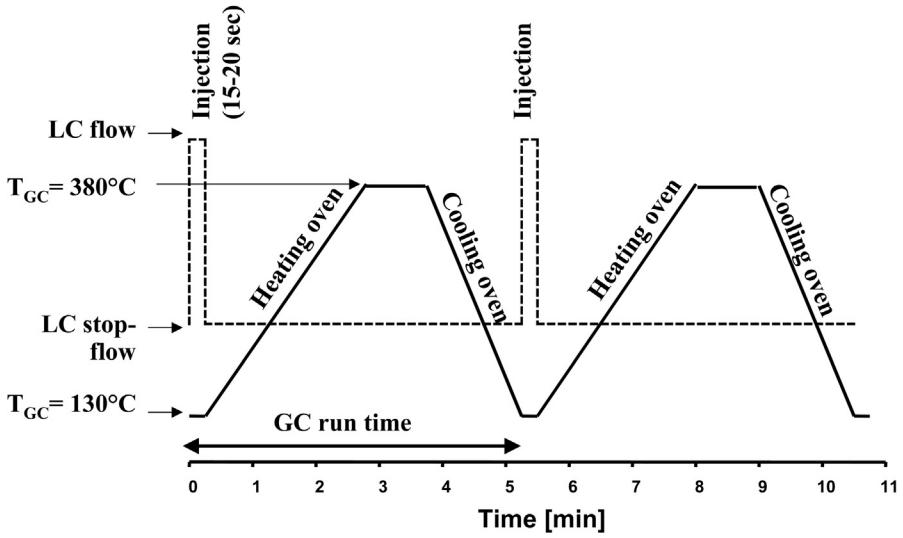


Figure 3. Sequence of events for automated LC × GC operation illustrated by using two consecutive injections.

connection between the LC and the GC, there is no need to create a leak flow to prevent solvent tailing: there is now no loss of sample due to transfer-line back flush as occurs in the valve-based interface.

3.2.3 Stop-flow operation

Figure 3 shows a typical sequence of events for the stop-flow operation used for both the valve-based and the syringe-based interface. As the GC returns to the initial ready status, the interface is switched to the transfer position and the LC flow is started. After the transfer of a fraction has been completed, typically after 15–20 s, the LC flow is stopped, the interface switched to the stop-flow position and the GC run started. After a GC cycle of heating and cooling, the GC returns to the ready status and the next fraction can be transferred. In the instrumental set-up the OPTIC injector is the 'master' unit controlling the operation of the various parts of the equipment that jointly make up the LC × GC instrument. It sends and receives the start/stop signals and controls the timing of the individual cycles. The OPTIC enters the ready state as soon as the GC(–ToF MS) sends a 'ready' signal to indicate that the GC system is in the initial state. Next, the OPTIC sends a start signal to the FOCUS to commence the transfer of a new fraction. After inserting the syringe into the injector, the FOCUS sends a start signal to the LC pump. After the selected LC fraction width has been eluted, the FOCUS transmits a stop signal to the LC pump, withdraws the syringe from the injector and sends a ready signal back to the OPTIC. The OPTIC logics control box now starts the heating of the injector (if necessary) as well as the pressure programme and, also, starts the GC(–ToF MS) run. It then waits for the ready signal of the GC before re-starting the entire protocol. **Table 2** shows an overview of the control protocol.

3.3 Applications and practical aspects

A typical example of a so-called bubble-plot AgLC × GC chromatogram of an edible oil is shown in **Figure 4**. The areas of the dots represent the areas of the GC peaks. Figure 4 was recorded with the AgLC column comprehensively interfaced to the high-temperature non-polar 'carbon-number' GC column using the valve-based interface and FID detection. The AgLC column separates the TAGs according to the number of double bonds (0 DB to ≧3 DB). A second separation based on carbon number (total fatty-acid chain-length) is afforded by the second-dimensional GC separation. In this experiment, a 4.6 mm ID LC column operated at a flow rate of 1 mL/min was used. To construct the comprehensive 2D chromatogram, 20-s time slices were transferred to the heated GC injector via the transfer capillary. This corresponds to fraction volumes of approx. 300 μL. The carrier gas system of the GC was operated in the constant-pressure mode at a pressure of 50 kPa.

From Figure 4 important information on the composition of the oil can be derived that would otherwise require several separate analyses. For example, the presence of un-even TAGs indicates the presence of animal fat. The series of smaller peaks between the TAGs with no (0 DB) and one (1 DB) double bond shows the presence of *trans* fatty acids (as indicated for the C52 TAGs), the origin of which can be derived from their carbon number distribution. From the analytical point of view it is interesting to see that TAGs with the same number of double bonds show up as slightly curved, banana-shaped, bands in the LC × GC chromatogram. The curvature indicates that retention in the two dimensions is correlated. Apparently TAGs with the same number of double bonds experience slightly different retention on the AgLC column depending on their

Table 2. Protocol for automated comprehensive LC × GC(–ToF MS).

	GC(–ToF MS)	OPTIC	FOCUS	Syringe	Syringe plunger	LC pump	Time [s]	Action/Status
Initialisation								
1	Ready	ready	waiting	up	down	running	0	Manual start to inject sample onto LC column
2	Ready	ready	waiting	up	down	running	60	LC void volume to waste
Repeated cycle								
3	ready	ready	waiting	up		hold		LC stopped. OPTIC sends start to FOCUS
4	ready	ready	running	down	up	hold		FOCUS starts running and inserts syringe into injector with plunger up
5	ready	ready	running	down	up	running	0	FOCUS re-starts LC pump and starts counting the fraction time
6	ready	ready	running	down	up	hold	15	LC pump is switched off after 15 s by FOCUS
7	ready	ready	running	up	up	hold		FOCUS withdraws syringe from injector and sends start to OPTIC
8	running	running	waiting	up	up	hold	0	OPTIC starts itself and GC(–ToF MS)
9	not ready	not ready	waiting	up	up	hold	300	GC(–ToF MS) run finished. GC cools down
10	ready	not ready	waiting	up	up	hold		GC(–ToF MS) sends ready to OPTIC to start next cycle
11								Repeat from line 3 until all LC fractions of interest are analysed
Finalisation								
12	ready	ready	waiting	up	down	running		System ready for next LC × GC(–ToF MS) analysis

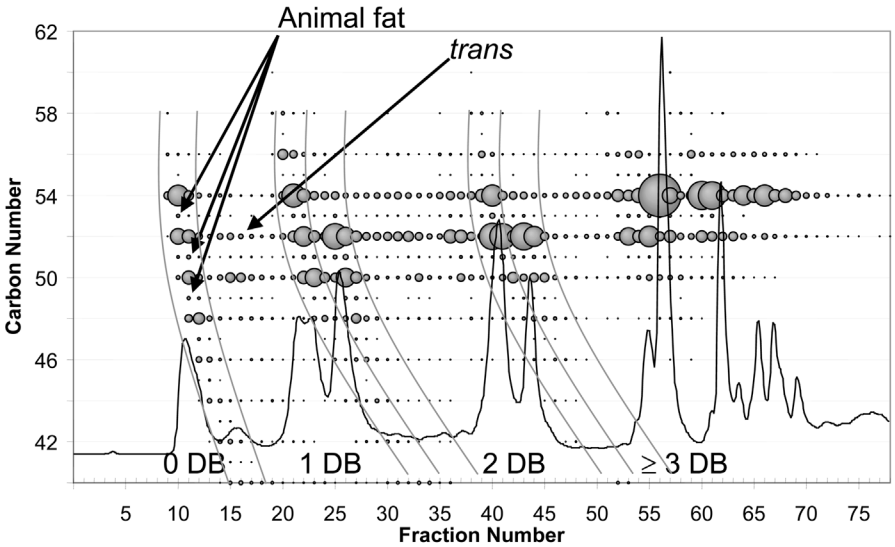


Figure 4. LC × GC–FID bubble plot of an edible oil. The *x* axis gives the LC fraction number, the *y* axis the carbon number of the TAG. The dot areas are a measure of the peak size and do NOT represent peak width. The reconstructed LC chromatogram indicates the saturated and the mono- and multiple-unsaturated (0 DB to >3 DB) TAGs. For GC conditions see text. LC conditions are given in Table 1 (10 cm × 4.6 mm ID, 3 μm Nucleosil, 10% $AgNO_3$-loaded silica).

carbon number. Longer TAGs elute somewhat earlier than the shorter TAGs.

The initial attempts at automated LC × GC were not without experimental difficulties. For example, a build-up of injector pressure was occasionally noticed upon transfer of an LC fraction. It turned out that the solvent that was evaporated in the hot injector recondensed in the split line and/or split valve of the PTV injector. This resulted in an increased flow resistance in the split line, build-up of pressure in the injector liner and, in the end, back-flow of solvent vapour into the carrier gas flow lines and contamination of the inlet lines. Such a situation should evidently be avoided, also because even a slight change in the flow resistance of the split line/valve will (temporarily) change the split ratio making quantification impossible. To avoid these problems two precautions were taken. First, the split valve was taken out of the control box of the OPTIC PTV unit and was positioned as close as possible to the injector. In this way the length of tubing between the injector body and the split exit was reduced to some 14 cm. In addition, to avoid recondensation of solvent in the split

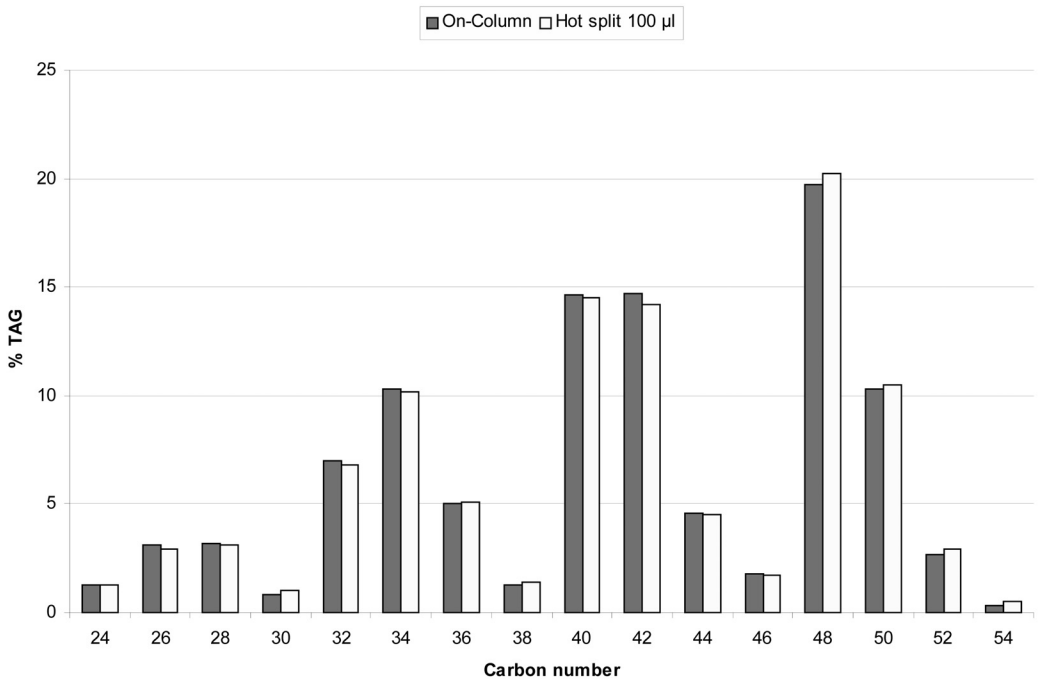


Figure 5. Comparison of two GC injection techniques of a standard TAG sample in dichloromethane. On-column injection: injection volume, 50 μL; column, 10 m × 530 μm ID × 0.17 μm. Hot split injection: injection volume, 100 μL; column, 5 m × 530 μm ID × 0.17 μm.

valve, this valve was put on top of an un-used heated injector block of the GC. When these precautions are taken, 4.6 mm ID columns operated at 1 mL/min can be used in automated comprehensive LC × GC. However, each time the large plug of solvent reached the detector, black soot was seen escaping from the FID. Although the FID 'recovered' remarkably fast, we decided to use a 2 mm ID LC column, in that way reducing the solvent load the injector and GC column have to cope with. The fraction volume was now reduced to 50 μL and the flow rate to 200 μL/min. Under these conditions, the system was used for several months without any problems.

To study analyte quantification in the automated LC × GC system, peak areas obtained for a TAG standard solution with (i) on-column injection and (ii) large-volume split transfer were compared. Results are shown in **Figure 5**. The two injection techniques gave closely similar results. The relative standard deviations (RSDs) of the sample composition were below 5% ($n = 6$) in all cases. Obviously, there was no discrimination of high- and/or low-boiling compounds; that is, this type of interface can be used for quantitative transfer of compounds from the LC to the GC system. The RSDs of the individual peak areas were about 7% ($n = 6$), and these of the retention times, around 1% ($n = 6$). The somewhat poorer repeatability of the retention times is most likely caused by the use of ballistic heating.

In the current set-up the column flow was approx. 13 mL/min. At a split flow of 150 mL/min this results in a split ratio of around 1:11 during sample introduction. Hence, approx. 9% of the solvent and the analytes were introduced onto the column, as was confirmed experimentally by comparing peak areas of a split injection and a normal-volume on-column injection. In conventional LC–GC performed to isolate and quantify trace compounds in a complex matrix, such a loss of sensitivity would clearly be unacceptable. In the present case, the aim of the LC step is not to isolate the triglycerides from the rest of the sample: the sample solely consists of triglycerides. The purpose of the LC step is to separate the TAG according to a certain property followed by a further separation according to a second, completely different, property.

One concern of the stop-flow mode of LC operation is band broadening in the LC column due to the frequent stopping/starting of the column flow. In addition, because the total run time of a LC × GC run can be as long as several hours, axial diffusional broadening can also start to occur. To study the effect of these parameters on the resolution in the LC dimension, an oil sample was analysed as 100 fractions of 12 s each. The LC column was the 2 mm ID Ag(I)-loaded ion-exchange column operated at 200 μL/min (for further details, see Table 1). The GC cycle time was 6 min. The total residence time of the compounds eluting last from the LC column hence was around 10 h! **Figure 6** shows the comprehensive chromatogram of this separation, and the reconstructed LC chromatogram. The last eluting LC peak is seen to be extremely narrow. This is due to the step gradient applied to elute this band. For the much more critical pair of the symmetrical (SOS) and asymmetrical (SSO) mono-unsaturated TAGs, very good peak shapes and a satisfactory resolution were also obtained: the reconstructed chromatogram shows base-

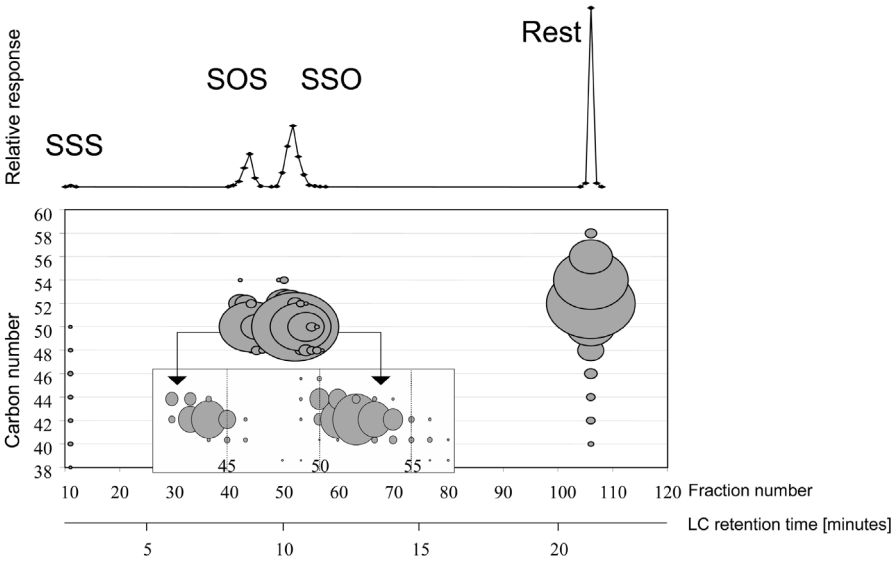


Figure 6. AgLC × carbon-number GC–FID bubble plot of an oil mixture (bottom) and reconstructed first-dimension LC chromatogram (top). LC column, 2 mm ID; LC flow, 0.2 mL/min; transferred fraction, 40 μL; total number of fractions, 100. The first 400 μL of the LC effluent were sent to waste. Bubble plot insert: enlargement/dot-rescale of SOS and SSO groups. SOS, all TAGs with one double bond in 2 position and saturated fatty acids in 1 and 3 positions (symmetrical mono-unsaturated TAGs). SSO, all TAGs with one double bond in 1 or 3 position (asymmetrical mono-unsaturated TAGs).

line separation. Not only is this good enough, it also is similar to what is usually obtained in a direct LC run of such a sample. The comprehensive stop-flow operation apparently does not adversely affect the first-dimension resolution. Whether this conclusion will also be true for lower-molecular-weight analytes that will have higher diffusion coefficients in the mobile phase still has to be investigated.

As already indicated above, an evident drawback of stop-flow LC × GC is the long total run time. Even if the GC run time is only 5 min, total LC × GC run times can easily be 5–10 h. Although the operation is fully automated, this still limits sample throughput to 2–5 samples/day. There are various options to reduce the total run time. Firstly, the sampling frequency can be reduced. The lower the number of LC fractions transferred, the shorter the total run time will be. However, if the number of fractions is too low, separation created during the LC run will be partly lost at the start of the GC analysis. That is, a compromise between run time and required resolution has to be found. A second option is to reduce the cycle time of the GC run [30]. By working with very short columns, high gas velocities and rapid temperature programming we were able to reduce the run time for TAG separations to less than 3 min. In that way we could reduce the total run time of a 50-fraction LC × GC run to less than 2.5 h. A last option would be adjustment of the fraction transfer frequency to the local complexity of the chromatogram, i.e. no fractions are transferred if no peaks elute from the LC column. For example, when recording the comprehensive chromatogram shown in **Figure 7**, no fractions were transferred during the LC void time. Ideally, transfer should be stopped at all larger baseline segments. In our case this offered an approx. 2-fold time gain.

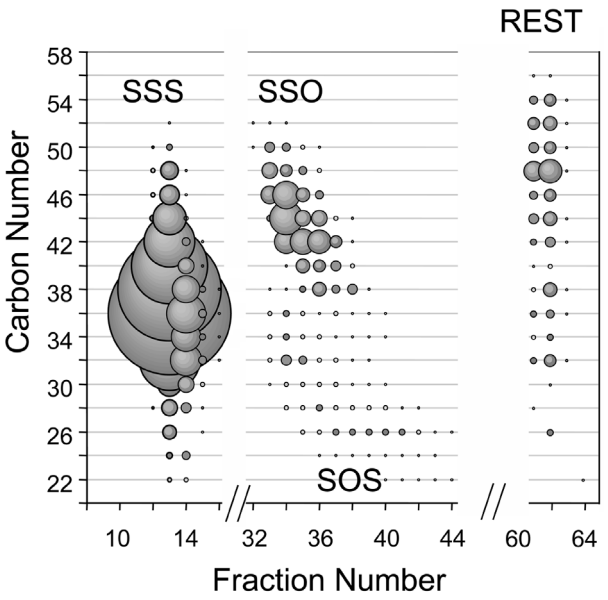


Figure 7. Local adjustment of the fraction transfer frequency. Only the fractions that contained the analytes of interest were analysed.

3.3.1 Enhanced resolution of LC × GC

As already mentioned in the Introduction, full separation of all TAGs in an oil/fat sample or of a complex FAME mixture is impossible using one-dimensional LC or GC. Even multiple AgLC column ensembles or very long capillary GC columns do not provide sufficient resolution. The enhanced resolution provided by LC × GC is demonstrated in **Figure 8** which shows the separation of butter FAMEs. The sample was analysed as 40 fractions of 15 s each using the syringe-based interface. Figure 8 shows a selection of second-dimension GC chromatograms. No band broadening due to elution into the barrel of the syr-

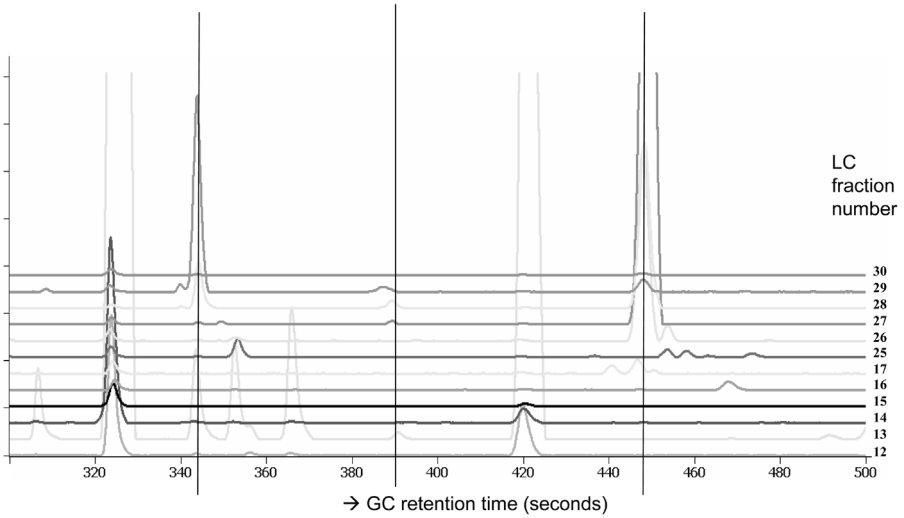


Figure 8. Enhanced resolution in comprehensive AgLC × GC FAME analysis. Vertical lines at retention times 342, 390 and 445 s indicate co-elution of compounds if only GC analysis is performed. GC traces of individual LC fractions, e.g. fraction 13, show co-elution problems if only LC analysis is performed.

inge was seen, presumably because of a plug-flow type flow pattern in the barrel. The superior resolution of the comprehensive system compared with LC alone can be nicely illustrated for fraction 13: GC analysis of this fraction indicates the presence of at least nine co-eluting compounds. Co-elution also occurs if only GC analysis is performed. Peaks are seen, for example, in several LC fractions at GC retention times of 345, 390, and 445 s. In other words, detailed separation requires the combined use of LC and GC in a comprehensive fashion unless selective MS detection is used, as will be discussed below. A parameter to consider in the LC × GC analysis of FAMEs is the initial temperature of the GC column. Because the compounds of interest are introduced as LC bands with a width of 15–20 s, refocusing on the column is required. For efficient refocusing of the lower-boiling C_4 FAMEs, the initial oven temperature had to be set at 40°C.

3.3.2 MS detection

Multidimensional combinations of LC and GC generate a wealth of chromatographic information. In the complex LC × GC chromatograms several hundreds of peaks can be seen. For their identification mass spectrometry is indispensable. For the MS system to be compatible with the LC × GC set-up, two important criteria have to be met: (i) a mass range wide enough to allow identification of the high-mass TAGs and (ii) the ability to maintain the high vacuum, even when a very large solvent cloud is introduced into the system. As regards the first criterion, it will be clear that neither sophisticated bench-top quadrupole systems with typical upper mass limits of 800 amu, nor modern ion-trap systems with upper mass limits of 650 amu meet this requirement. The MS system used here is a time-of-flight (ToF MS) instrument with an upper mass limit of 1000 amu. The system can maintain its vacuum at 10^{-7} torr even when large volumes of solvent are introduced. The merits of ToF MS detection for comprehensive chromatography including, e.g., its high data acquisition rate and deconvolution options have been described in detail by Dallüge et al. [31]. As regards the use of MS detection, with TAGs and FAMEs the main sample constituents show up as grouped bands in the 2D chromatograms. The identity of the compounds in these bands can often be derived from general knowledge of the sample, combined with a proper understanding of the factors that determine LC and GC elution orders. For final confirmation, model compounds can be injected. In other words, MS detection can be of help in such situations, but is not mandatory. In marked contrast, identification and identity confirmation of specific analytes or of minor constituents will generally require the use of MS. In addition, MS is a highly useful tool to introduce additional selectivity by plotting three-dimensional chromatograms for properly chosen extracted-ion traces.

Figure 9 shows the bubble plot of a diet butter TAG sample, based on LC × GC–ToF MS data. As an illustration, the inserts of this figure show the ToF MS spectra of three main sample constituents which were identified as C48:0, C50:1, and C52:2 TAGs. As has been convincingly demonstrated in earlier work [31, 32], such unequivocal information greatly facilitates the identification of other analytes and bands on the basis of double-bond (LC) and carbon-number (GC) information. For more complex samples as in, e.g., the LC × GC FAME analysis of a real butter, clustering can be less distinct and more individual peaks have to be identified. As an example, **Figure 10** shows a part of the LC × GC colour plot after conversion of the butter TAGs into FAMEs. Interpretation of the mass spectra in this region revealed the identity of most of the peaks. The linear FAMEs C14:0, C15:0, and C16:0 were identified, but some other peaks at GC retention times of 278 and 284 s were still unknown. The molecular ion *m/z*

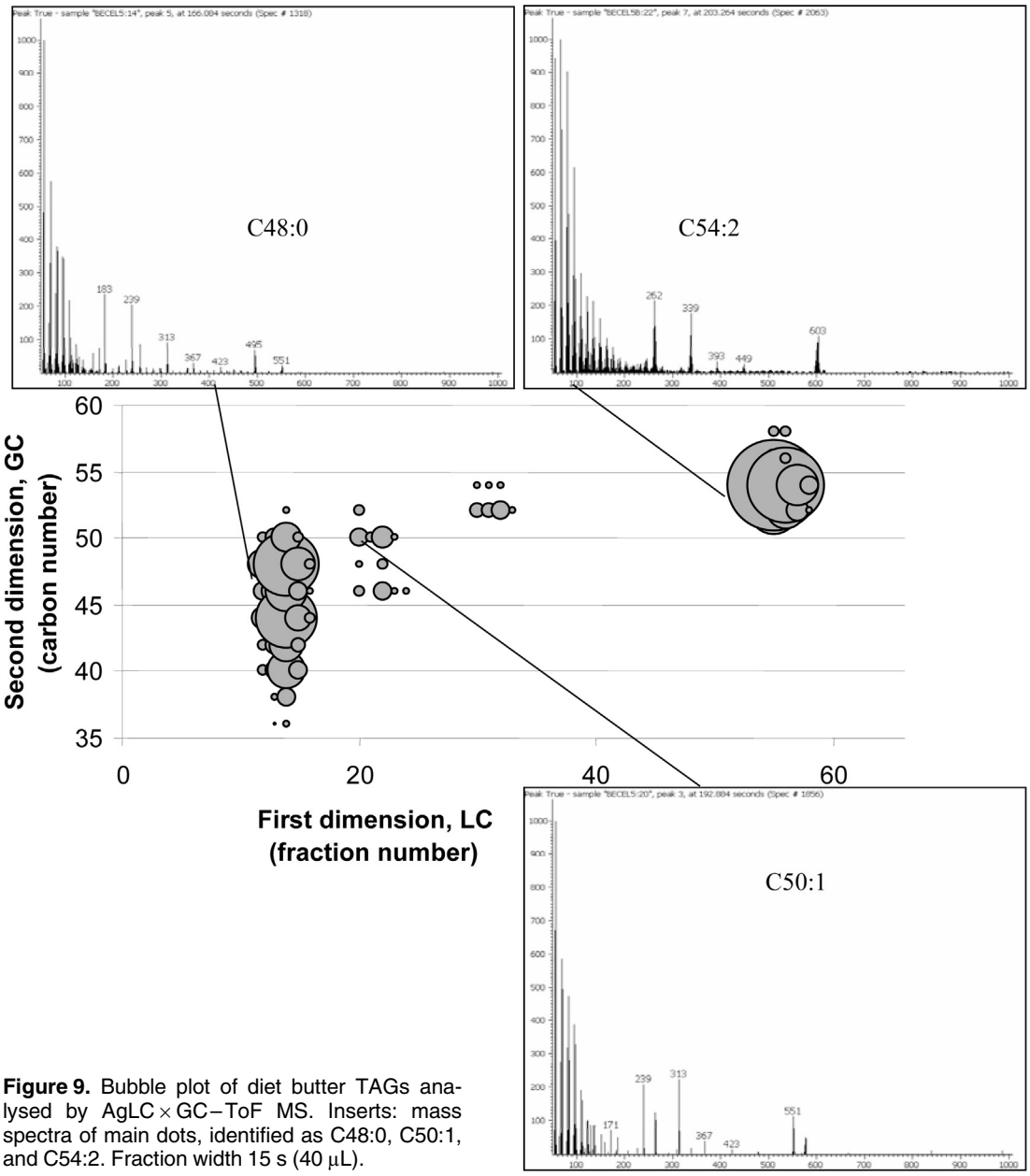


Figure 9. Bubble plot of diet butter TAGs analysed by AgLC × GC–ToF MS. Inserts: mass spectra of main dots, identified as C48:0, C50:1, and C54:2. Fraction width 15 s (40 μL).

256 in the mass spectra of these peaks clearly showed that the compounds were C15 FAMEs. Library search indicated the presence of a methyl branch in the fatty acid chain, but was not conclusive in terms of the position of the branch. For butter fatty acids branching can occur at the *iso* and *ante-iso* positions (positions 2 and 3 from the alkyl chain). The presence of the 199 amu fragment in the second unknown peak (GC retention time 284 s) indicates that this is the *ante-iso* isomer. Analogously, the peak at 306 s was identified as *iso*-C16:0. Clearly the very high peak capacity and resolving power of LC × GC complemented by the excellent selectivity of ToF MS provides and excellent tool for separation and identification of analytes in complex matrices.

4 Conclusions

The comprehensive two-dimensional combination of LC and GC provides an unsurpassed separation power for samples that are amenable to both modes of chromatography, but too complex to allow adequate resolution by a single GC or LC separation. Full automation of LC × GC is possible when using fairly simple interfaces based on commercially available hardware. Two interfaces, which utilize either a six-port switching valve or a dual side-port syringe, were developed. To match the LC sample load and the GC column loadability without resorting to extremely narrow LC columns, the LC effluent flow was split after evaporation in the hot split injector of the GC.

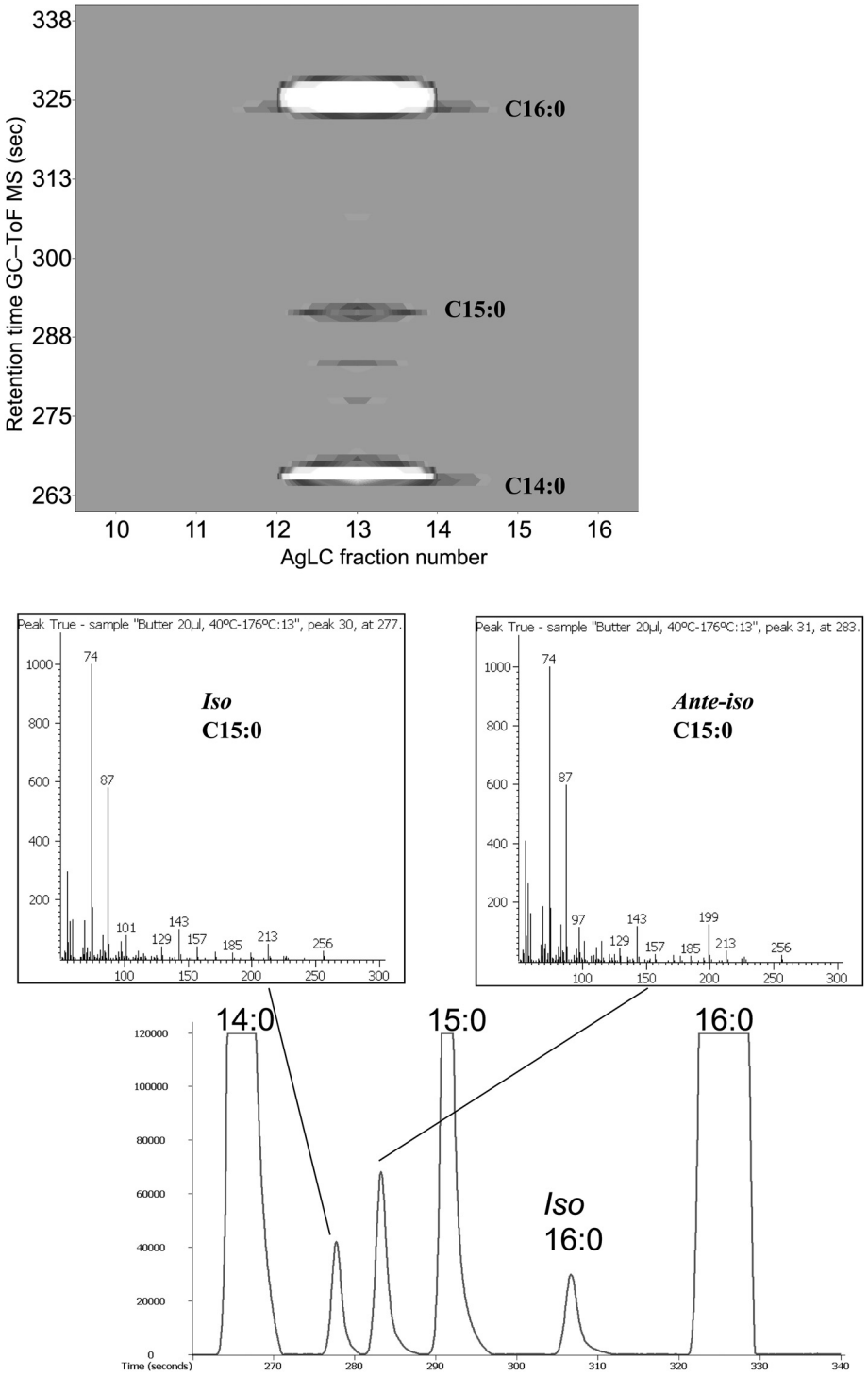


Figure 10. Zoomed LC × GC FAME colour plot (top) of real butter after conversion to FAMEs and part of GC–ToF MS chromatogram (trace *m/z* 74; bottom) of LC fraction 13 from the sample shown in Figure 9. The unknown compounds between FAME C14:0 and FAME C15:0 were identified by ToF MS as (left) *iso*-C15:0 and (right) *ante-iso*-C15:0. The peak between FAMEs C15:0 and C16:0 was identified as *iso*-C16:0 (no spectrum shown). For further discussion, see text. Fraction width 15 s (40 µL).

The usefulness of the automated LC × GC instrument was evaluated for triglyceride and FAME analysis. Both automated interfaces showed an excellent performance in terms of reliability, repeatability, and ease of use, and by now have been shown to allow unattended routine use of LC × GC in an industrial environment over a prolonged period of time. LC × GC yielded much more information on the samples than either of the individual separation modes. For example, the comprehensive coupling of silver-phase LC and carbon-number GC allowed separation of triglycerides according to two independent parameters: carbon number and number of double bonds. The information density of the data sets so obtained and the reliability of analyte identification, was further improved by

hyphenation of the system to a rapid-scanning time-of-flight mass spectrometer. When using LC × GC – ToF MS, several types of analysis in the edible oil and fat field, which were previously either impossible or extremely time-consuming, can now be performed rapidly and routinely.

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