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Review

Comprehensive two-dimensional chromatography in food analysis

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Abstract

Comprehensive two-dimensional (2D) chromatographic techniques can be considered innovative methods, only quite recently developed. Since their introduction to the chromatographic community, these techniques have been used in several fields and have gained an excellent reputation as valuable and powerful analytical tools. The revolutionary aspect of comprehensive multidimensional (MD) techniques, in respect to classical MD chromatography, is that the entire sample is subjected to the 2D advantage. The resulting unprecedented separating capacity makes these approaches prime choices when analysts are challenged with highly complex mixtures. Furthermore, in the case of automated systems, instrumental analysis times are roughly the same as in monodimensional applications. The present review reports various comprehensive chromatographic applications on different food matrices. The GC × GC section highlights two fundamental aspects for component separation/identification: the exceptional peak capacity and the formation of group types on the 2D space plane. The LC × LC section reports the employment in food analysis of a recently developed multidimensional normal-phase (NP)-reversed-phase (RP) high performance liquid chromatography (HPLC) system. Also reported are comprehensive LC × GC and packed column supercritical fluid chromatography (pSFC × pSFC) applications in this field.

Keywords: Food analysis; Comprehensive two-dimensional chromatography

Contents

1. Introduction

Food, either naturally occurring, processed or cooked, is consumed both for the maintenance and enjoyment of human

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life. Food products are very complex mixtures that contain many nutrients of organic (lipids, carbohydrates, proteins, vitamins) and inorganic (water, minerals, oxygen) nature. In addition to natural constituents, they may contain xenobiotic substances that come mainly from technological processes, agrochemical treatments or packaging materials. Analytical methods allow the qualitative and quantitative determination of the main components of food samples but can also be selective and sensitive enough to permit the determination of minor components. The analysis of food products may be directed to the assessment of food quality and authenticity, the control of a technological process, the determination of nutritional values and the detection of molecules with a possible beneficial or a toxic effect on human health. Consequently, a major objective in food chemistry concerns the continuous improvement and development of analytical techniques [1].

Monodimensional chromatographic processes are widely applied in the analysis of food products. Although such methods often provide rewarding analytical results, the complexity of many naturally occurring matrices exceeds the capacity of any single separation system. As a consequence, in the past years considerable research has been dedicated to the combination of independent techniques with the aim of strengthening resolving power.

The concept of comprehensive chromatography has its origin in thin-layer chromatography (TLC); the first 2D TLC separation, which consisted in a chromatographic migration in one direction followed by a second migration at a right-angle to the first, was first reported in 1944 [2]. It is unquestionable, that, although this approach may be useful in some analytical applications, it would be impractical in the separation of highly complex samples [3,4].

The development and employment of automated twin-column comprehensive chromatographic methods in all fields is gradually increasing. This is due to the fact that these techniques are characterized by a greatly enhanced resolving power as compared with single column and classical MD approaches. It is well-known, that even in low complexity samples there is generally random peak distribution which requires high plate number values for total peak resolution. Davis and Giddings affirmed that in a single column application, when the number of components exceeds 37% of the peak capacity, statistically peak resolution is compromised. Furthermore, several peaks are the result of two or more co-eluting components [5]. Real world samples are usually characterized by a variety of chemical groups and, consequentially, by random peak distribution, therefore requiring a high separating power.

A comprehensive two-dimensional system should possess the following features:

- All components in a sample mixture are subjected to two separations in which their displacement depends on different factors.
- Any two components separated in the first dimension must remain separated in the second dimension.
- The elution profiles from both dimensions are preserved [6,7].

A typical comprehensive separation is achieved, generally, on two distinct columns connected in series with a special transfer system located between them. The type of interface used is linked to the specific methodology. The function of the interface is to cut and then release continuous fractions of the primary column effluent onto a fast separation column. In order to achieve comprehensive analysis and to preserve the 1D separation, the bands injected onto the secondary column must undergo elution before the following re-injection. Secondary retention times must be, at the most, equal or less than the duration of a single modulation period. During the development of the 2D chromatograms, the interface is engaged in the following heart-cutting process.

Effective comprehensive analysis is achieved with at least three or four modulations per peak [8]. The effect of this process can be seen in Fig. 1a and b, relative to an unmodulated and modulated peak. The latter, in fact, is sliced into five distinct fractions all detected at specific intervals which correspond to the modulation period. In this case, the peak in Fig. 1a is representative of a single compound, and so further component separation does not arise. The sum of the five peak areas is equal to that of the single unmodulated peak. The unravelling of a triple component peak (Fig. 2a) through modulation can be observed in the raw bidimensional chromatogram reported in Fig. 2b. The latter can be considered the sum of three sequential secondary analyses, in which 1D eluate fractions are resolved into three components (α, β and γ). The peaks present in a single 2D chromatogram are characterized by the same monodimensional retention time, corresponding to the beginning of the secondary analysis, and by

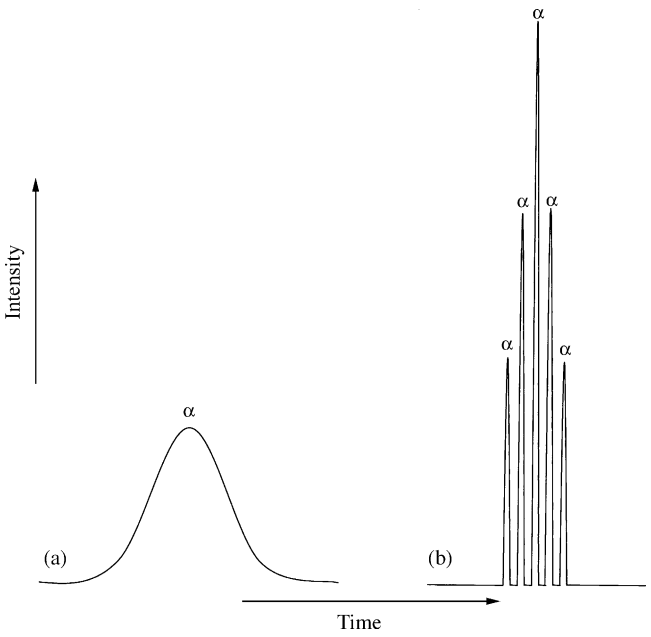

Fig. 1. (a) Unmodulated peak relative to a single component (α). (b) Peak after modulation.

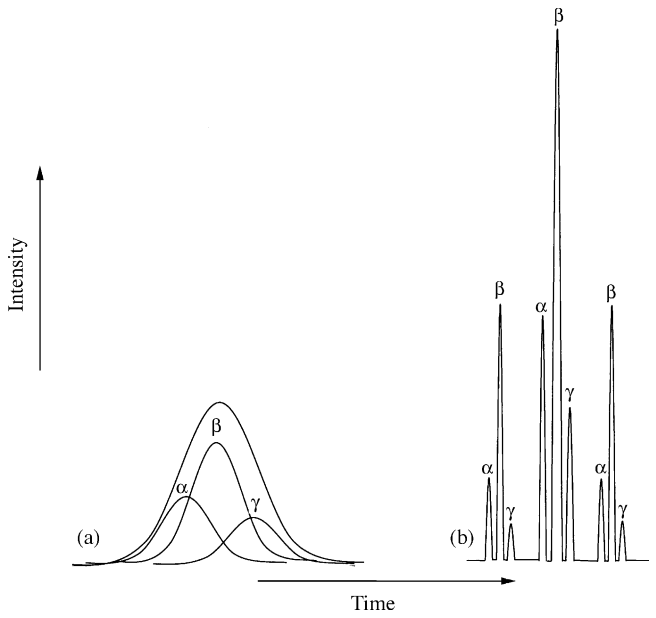

Fig. 2. (a) Unmodulated multi-component peak (α, β and γ). (b) Multi-component peak after modulation.

different second column elution times. If we consider the series of 3 peak areas relative to component α, they correspond, obviously, to their relative concentration in each heart-cut.

Comprehensive chromatographic data can be better appreciated when transformed in a 2D contour plot. In the latter, each component is revealed as an oval-shaped peak and is defined by two retention time coordinates. Contour peak colours and dimensions are related to the total quantity of each compound present in the sample. The 2D plane can accommodate many more peaks, and thus has a much higher peak capacity than a single axis 1D chromatogram. The possible overlapping of two differently structured molecules is statistically improbable, as this would require equal elution times on two columns characterized by distinct separating mechanisms.

The benefits in terms of separation power in comprehensive two-dimensional chromatography are enormous if compared to single dimension chromatography. Giddings has shown theoretically that the peak capacity can be greatly enhanced by coupling columns that separate according to different (orthogonal) retention mechanisms [7,9,10]. Ideally, for fully orthogonal methods, the peak capacity becomes the product of the peak capacity in each dimension. Resolution is equal to the square root of the sum of the squares of the resolution for each dimension.

2. Comprehensive two-dimensional gas chromatography

Over the past decades, monodimensional GC has been the most common approach used for the unravelling of food volatile and semi-volatile composition. This well-established method has a proven effectiveness for the separation of simple samples and provides, usually, a great deal of valuable information. Whenever confronted, though, with more intricate mixtures many peaks are the result of two or more co-eluting compounds. This problem can be overcome through the linking of a quadrupole mass spectrometer to a gas chromatograph (GC–qMS), which is a valid approach not only for single peak assignment and quantitation [11] but also for the correct identification of multi-component peaks through the use of deconvolution techniques [11,12]. It is obvious, though, that the library assignment of unknowns is more reliable (especially for the more complex matrices) when high quality mass spectra relative to resolved compounds are obtained. Total component separation is, therefore, always desirable, but often complicated to achieve. The modification of a column parameter such as increase in length, while extending analysis times, has a limited effect on resolving power because of the square-root dependence of R on N. For example, the use of a 400 m column (1.3×10^6 N — peak capacity 1000) in an 11 h analysis on a gasoline sample was far from sufficient for total matrix separation [13]. Another more effective way of increasing the maximum number of compounds separable within a given time in a chromatographic system is the linkage, through an interface (usually a switching valve), of two or more independent columns [3]. In gas chromatography, the first MDGC system with two capillary columns was developed 40 years ago [14]. Conventional MDGC is a heart-cutting method which involves the transfer of selected groups of unresolved compounds from a primary column onto a secondary column which are connected by an interface. The two columns employed have approximately equal peak capacities and may be operated in either a single or two distinct GC ovens. The 1D zones, subjected to re-analysis, are selected through monodimensional applications prior to MDGC analysis. Several food sample applications, especially chiral separations, have been reported in literature [15–17]. Although MDGC is a valuable analytical tool, it is also characterized by a few drawbacks such as high time costs, the complex instrumentation and by the fact that the whole procedure requires considerable technical expertise. Continuous heart-cutting, applied to the entire sample, is cause of loss of 1D resolution [18]. Furthermore, although the implementation of multiple intermediate traps or parallel secondary columns may allow the bidimensional separation of a substantial part of the 1D eluate, the resulting GC equipment is grossly elaborate [3].

The step from heart-cutting to comprehensive two-dimensional GC was achieved by Liu and Phillips in 1991 [19]. The GC × GC methodology has been reviewed recently [20]. The interest towards this method has grown rapidly, especially in the last couple of years as can be seen in Fig. 3 (approx. number of comprehensive GC publications).

The heart of the GC × GC process is the presence of a modulator between the two dimensions. The function of the modulator is to trap, refocus and then release continuous fractions of the primary column effluent onto a shorter "fast" column. The different approaches through which

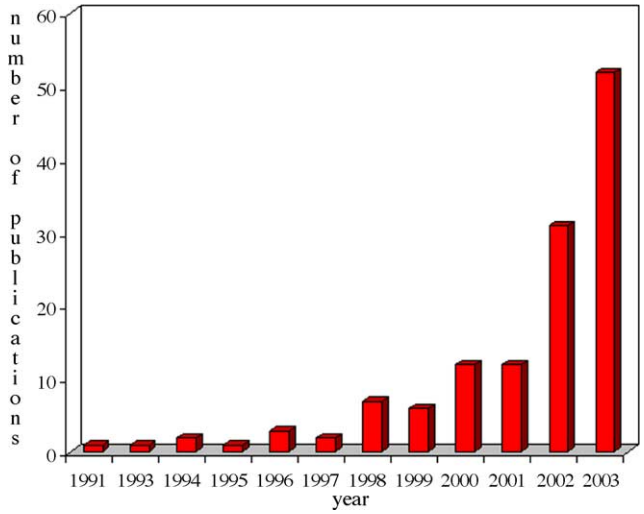

Fig. 3. Approximate number of GC × GC publications in the years from 1991 to 2003.

modulation has been achieved are thermal [21,22], valve [23,24] and cryogenic [25–27]. The longitudinally modulated cryogenic system (LMCS), developed by Kinghorn and Marriott [27] and the thermal sweeper of Phillips et al. [22] have been the most widely used interfaces.

The LMCS, positioned at the head of the second column, exploits the freezing effect of an internal CO_2 stream to initially entrap and refocus analytes that enter this region. The following longitudinal movement of the trap along the column exposes the previously cooled zone to the heat of the GC oven, thus ejecting a rapid pulse of entrapped components onto the secondary column (Fig. 4).

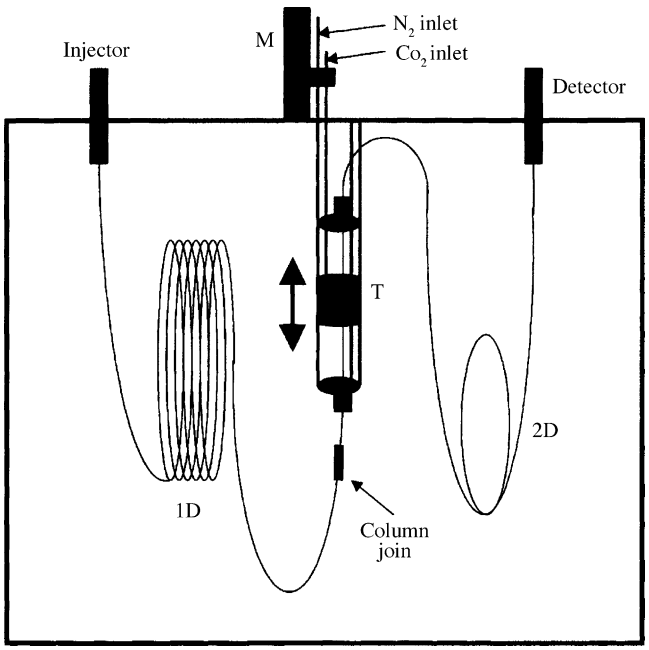

Fig. 4. The LMCS system: the modulator (M) moves the cryogenic trap (T) up and down as required, according to the modulation period (reprinted from [3], reproduced with permission from © Wiley).

The thermal sweeper is formed essentially by a slotted heater connected perpendicularly to a rotating shaft. Analyte entrapment is achieved, at ambient GC oven temperature, on a length of thickly coated column positioned between the first and second dimension (phase-ratio focusing). The successive rotation (downstream direction) of the heater over the aforementioned column section enables at first the heart-cut, then zone compression and finally rapid band injection onto the secondary column (Fig. 5).

Modulation cycles, in both thermal and cryogenic interfaces, are performed continuously on successive fractions of the entire 1D eluate. Short 2D narrow bore columns (typically 50–100 cm × 0.1 mm i.d.) perform rapid, high resolving power separations on the compressed 1D samples. A modulation period of 5 s will enable the re-analysis of 1200 slices of a 60 min monodimensional eluate. Furthermore, a 1D peak with a 20 s band width, will be divided approximately in four cuts, all subjected to consecutive secondary separations.

It must be noted that GC × GC separations are characterized by a substantial increase in the signal-to-noise ratio. One major effect of modulation is, in fact, enhanced sensitivity through the formation of compressed solute bands which makes the technique particularly suitable for trace-level component detection.

In order to achieve GC × GC analysis it is necessary to employ columns with nearly independent separating mechanisms. The parameters upon which analytes are separated in GC are basically two: boiling point and polarity. Most

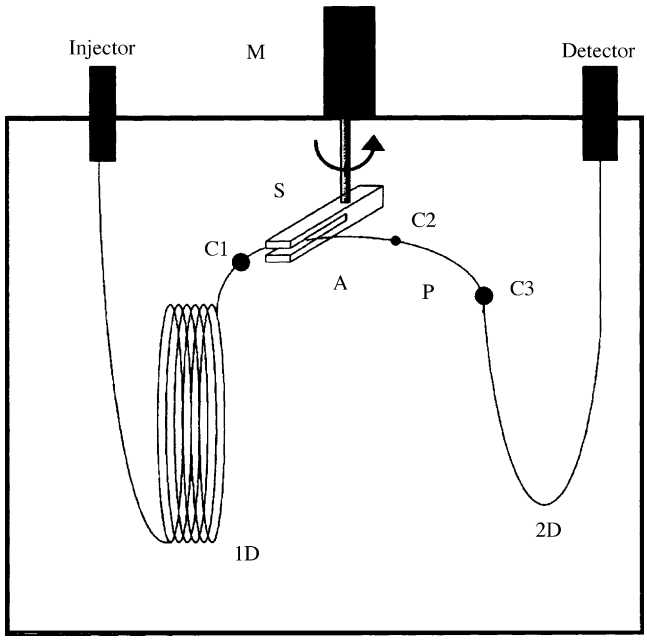

Fig. 5. The thermal sweeper system: column A accumulates primary dimension solutes until the slotted heater (S), rotated by the modulator (M) focuses and then expels them onto the uncoated column (P). C1, C2 and C3 are column connections (reprinted from [3], reproduced with permission from ©Wiley).

investigations reported in literature are based on the use of a primary apolar and a secondary polar column. This type of combination, in fact, provides a true orthogonal separation. In this case, 1D solutes are differentiated solely on their relative vapor pressures while specific interactions on the 2D stationary phase enable the separation of analytes characterized by the same or similar boiling-points. It must be noted that total orthogonality is not always the most suitable choice and other column combinations must be considered during method optimization. Whilst the best possible separation is always the ultimate goal in any GC method, also of fundamental importance in comprehensive GC, is the formation, when possible, of characteristic patterns on the 2D plane and the avoidance of solute wrap-around. This last aspect can be caused by excessive second dimension retention especially when using a polar column. In general, the choice of the most appropriate column set depends, mainly, on the physicochemical properties of the sample components.

As aforementioned, GC × GC peak widths are typically very narrow and, as such, altogether comparable to those observed in fast GC [28]. Compatible detection methods, as a consequence, necessitate rapid acquisition capacities. Flame ionization detector (FID) systems are characterized by rapid data acquisition rates (250 Hz max) and are the most commonly used. Time-of-flight mass spectrometers (TOF MS) have a demonstrated effectiveness for the positive identification of comprehensive GC analytes [29,30]. This type of MS possesses a higher scan speed in respect to traditional quadrupole systems [11] and is capable of supplying sufficient spectra per peak (at least 10) for reliable component assignment.

Although a series of peak quantification methods have been reported in the literature, solute relative quantitation can be considered, at present, the achille's heel relative to 2D comprehensive GC ([20] and references reported therein). This is due to the lack of truly suitable software and, as a consequence, quantitative determinations are scarcely reported in literature. It must be noted, though, that progress is being made in this field, which also concerns the combination of 2D GC separation with chemometric data analysis. A chemometric technique, the generalized rank annihilation method (GRAM), has been used to quantify overlapping peaks in a valve-modulated GC × GC study [31]. An independent column temperature control (isothermal in the second dimension), applied in this research, although guaranting the bilinear structure of the data was, in itself, a severe restriction. In a later work, the percent weight quantification of target naphthalenes in fuel samples was achieved through a 5-min temperature-programmed comprehensive GC separation in conjunction with trilinear partial least squares (Tri-PLS) [32]. Tri-PLS calibration models were constructed from 2D chromatograms that were retention time corrected through rank minimization retention time alignment. In general, it can be affirmed that the development and availability of user-friendly software will make this powerful separative technique even more widely accepted.

2.1. GC × GC fat and oil analysis

Fatty acids (FA), the building blocks of fats and oils, play a fundamental role in the maintenance of good health. As such, the GC determination of the FA profile in fats and oils is very important. It is well known that in order to achieve this type of separation, it is necessary to carry out methylic transesterification on the sample prior to gas chromatographic analysis. The procedure allows the formation of the more volatile (in respect to free fatty acids) fatty acid methyl esters (FAMEs). Lipidic foods are considered to be moderately complex matrices (especially fish oils) and, as such, a single capillary column is sufficient for their separation. To the authors knowledge, three researches on this type of food sample have been reported [33–35]. All refer to the formation of ordered patterns of structurally related FAMEs: esters characterized by the same number of double bonds tend to align themselves along distinct bands. This aspect is particularly interesting as it can be exploited for the identification of compounds when no commercial standards are available. An excellent example of the aforementioned predictive GC × GC potential is shown in Fig. 6 (an expansion derived from a 2D chromatogram reported in [35]) which illustrates a 2D chromatogram expansion relative to the menhaden oil (one of the most complex lipidic foods) C_{18} family. Comprehensive GC was achieved with an apolar–polar column set. Within the C_{18} class, five groups of homologs (C:0 to C:4) elute at regular intervals forming an orderly group-type pattern: 1D analyte retention times (min. axis) gradually increase as the number of double bonds within the ester decrease. The opposite is true for the 2D analyte retention times (sec. axis), which increase with the number of double bonds. In the case of fatty acid methyl esters with the same number of double bonds, retention will be stronger for the FAME with the lowest ω value (i.e. $C_{18:1\omega9}$ and $C_{18:1\omega7}$) on both columns.

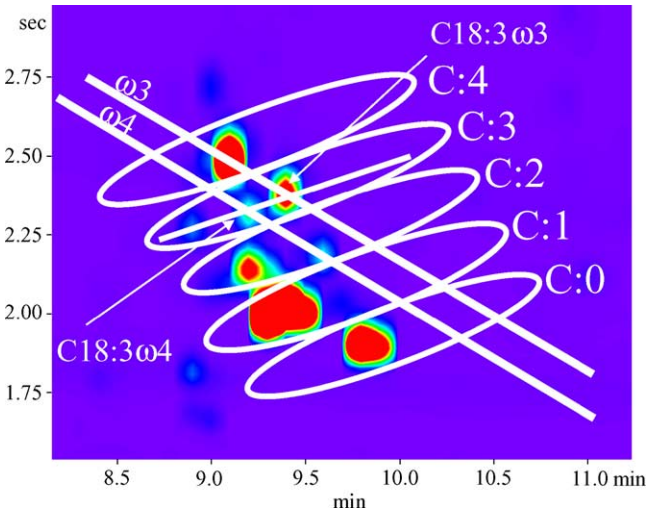

Fig. 6. A 3.5 min 2D chromatographic expansion containing the C_{18} FAMEs family relative to a menhaden oil application (reprinted from [35] with permission from Elsevier, © 2003).

These apolar–polar elution sequences determine an orderly peak distribution on the 2D space plane: diagonals can be drawn through components characterized by the same number of double bonds (i.e. $C_{18:3\omega3}$ and $C_{18:3\omega4)}$) and through those with the same ω number (i.e. $C_{18:3\omega3}$ and $C_{18:4\omega3}$). The point of intersection relative to the $C_{18:3}$ and the $C_{18\omega3}$ diagonal coincides with the $C_{18:3\omega3}$ peak and the point of intersection relative to the $C_{18:3}$ and the $C_{18\omega4}$ diagonal coincides with the $C_{18:3\omega4}$ peak. For these and other FAMEs, reliable peak assignment could have been achieved with this type of deduction. The formation, in this application, of highly ordered 2D chromatographic patterns was linked to the type of sample components that not only presented the same ester group but also linear hydrocarbon structures.

2.2. GC × GC essential oil analysis

Essential oils are mainly used in food and perfume industries, while several isolated components are also employed for their pharmacological and anti-microbial properties. They can be classified as moderately to highly complex samples and, as such, a single capillary GC column often proves to be insufficient for complete separation.

The employment of comprehensive GC in essential oil separation has been recently reviewed [36]. More 2D research on this type of matrix has been reported than for any other food sample. Dimandja et al. determined the fingerprints of the volatile fraction of peppermint and spearmint essential oils, predicting the future use of GC × GC for the detection of fraud and the identification of sources of poisoning [37]. A 2D separation carried out on vetiver oil, one of the more complex essential oils (more than 150 components), has been described [38]. In this research, many 1D peaks were discovered to be the result of six to eight overlapping compounds showing a much greater complexity than what can be suspected from single column analysis. Headspace solid-phase microextraction (HS-SPME) coupled to GC × GC, applied to the analysis of eucalyptus oil, achieved the resolution of about 580 volatiles compared to the 60 components resolved in a single column application [39]. A comparison of tea tree and lavender oil 2D fingerprints was carried out by Marriott and coworkers [40]. The investigation confirmed the potential utility of GC × GC for essential oil differentiation. The superior resolving power of comprehensive GC in respect to monodimensional GC [41] and the use of GC × GC–TOF MS for peak assignment [30] has been described in further research on lavender oil. Comprehensive GC–qMS applications have also been described: the reliable identification of 65 Egyptian geranium oil components was achieved through the employment of a rapid scanning qMS used at a reduced mass scan range. Bidimensional analysis, in this case, was carried at low pressure outlet conditions allowing an increase in the secondary column efficiency [42].

The characterization of the chiral profile in essential oils can provide useful information on authenticity, quality and geographic origin. Moreover, enantiomers can possess different biological activities. The gas chromatographic separation of enantiomers is achieved through the use of cyclodextrin derivatives as chiral selectors [43]. This type of stationary phase is obviously very selective towards optically active components, but provides a much less rewarding analytical result for other essential oil components.

A 1D enantio-separation followed by a 2D polarity based separation was successful in resolving key chiral and achiral components in Australian tea tree oil providing an alternative to MDGC methods [44]. A vapor pressure primary separation and a fast chiral analysis in the second dimension has also been reported in the literature [45]. This separation, performed in vacuum outlet conditions (through qMS hyphenation), was carried out on a bergamot oil sample, the most valuable citrus essential oil [46]. The use of a 0.25 mm i.d. column in the second dimension enabled rapid and efficient enantioseparations. The 2D space plane relative to a GC × enantio-GC–qMS application on the above mentioned matrix (the enantiomeric monoterpene region) is shown in Fig. 7. The separation of the compounds of interest was achieved in 8.5 min.

A comparison between a monodimensional GC–MS analysis and a 2D application on a lemon oil sample has been carried out [47]. The single column analysis was carried out on a polyethylene glycol stationary phase column and was characterised by substantial interferences between the oxygenated monoterpene and sesquiterpene hydrocarbon fraction. As a consequence, several peaks underwent partial or complete coelution hindering reliable MS identification. The GC × GC separation, carried out on a 1D polar and 2D apolar column set, achieved the thorough separation of the sample components.

In general, it can be said that the distribution of essential oil volatiles does not follow a regular pattern as can be observed

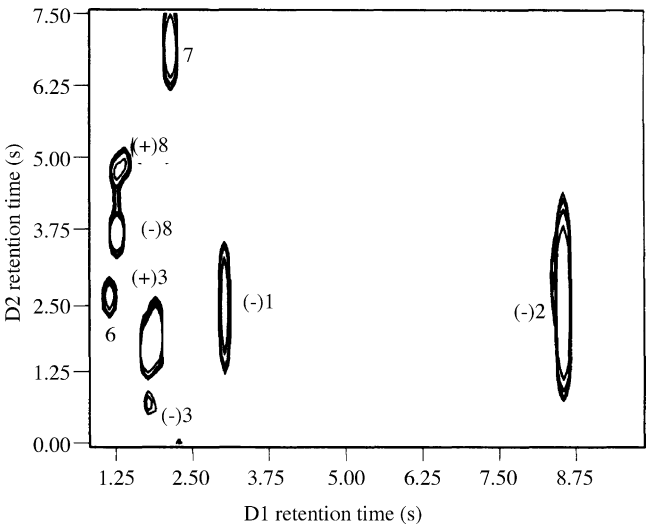

Fig. 7. 2D space plane relative to the bergamot oil monoterpene region. Identity of peaks: (1) linalool, (2) limonene, (3) 1,8-cineole, (6) R,S-α-pinene, (7) γ-terpinene, (8) sabinene (reprinted from [45] with permission from American Chemical Society, © 2002).

in FAMEs analysis. The reason for this is that, although essential oil analyte groups derive from the same number of terpene molecules, they have different hydrocarbon structures and/or functional groups. The distribution of terpene molecules with the same functional group (i.e. esters, alcohols and aldehydes), though, is generally more related.

In the above applications, the total number of separated components is certainly higher than what can be obtained from a single, or even two distinct monodimensional GC runs. Furthermore, the methods presented are certainly capable of supplying the same information without the drawbacks connected to classical multidimensional approaches [3,10].

2.3. GC × GC food contaminant analysis

The single column GC analysis of food contaminants is considered a cumbersome (sometimes impossible) challenge, as the presence of numerous target trace-level compounds must be identified within complex sample extracts. As such, several 1D peaks are often the result of two or more co-eluting components hindering MS peak assignment. In this field, the use of comprehensive GC has a demonstrated effectiveness. GC × GC with micro-electron-capture detection (μECD) has been applied to the analysis of polychlorinated biphenyls (PCBs) contained in a cod liver extract [48]. In this study, peak identification was achieved through the use of standard compounds and the comparison of group-type patterns. Further GC × GC–μECD applications, for the trace analysis of polychlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls in food extracts (fish oil, vegetable and spiked milk) have been described [49]. The reliable identification of pesticides in spiked and non-spiked vegetable extracts by GC × GC–TOF MS has been investigated [50]. A carrot extract 2D chromatogram, containing several hundred resolved compounds, relative to this research is shown in Fig. 8. The highly concentrated peaks, A16 and A18, were found to be respectively hexadecanoic and octadecanoic acid. Trace level amounts of chlorfenvinphos (approx. 10 pg/μl) were separated from interfering solutes as can be seen in the expansion reported in Fig. 9a. As a consequence, pure mass spectra were obtained for this pesticide (Fig. 9c). The same was not achievable through 1D GC–MS as can be seen in the same figure (Fig. 9b, d and e). Further studies concerning the improved separation/identification of pesticides in fruit products from matrix co-extracts have also been reported [29]. Very low levels of pesticide residues, equal to those required for the analysis of baby food, were confirmed.

2.4. Other food applications

Only few other GC × GC food applications have been described. Headspace solid-phase microextraction comprehensive GC methods for the analysis of garlic and ginger volatiles have been reported [51,52]. The use of SPME, a highly sensitive and selective extractive procedure, in combination with a GC method capable of resolving the extracted analytes gave here and in other investigations some very rewarding results. The semi-volatile profiles of three ginseng species were determined by Marriott et al., through a comprehensive GC–qMS approach [53]. In this research, a reduced mass scanning range was used in order to operate the MS at the highest possible acquisition rate. The instrumental set-up proved to be useful for peak attribution but did not provide solid quantitative data. GC × GC and TOF MS detection, on the other hand, was successfully employed in a qualitative/quantitative study on trace-level key flavour components in dairy products [54]. Alcoholic beverages have also been the object of two-dimensional analysis: the *trans*-resveratrol content of red wine samples, extracted through SPME with on-fibre derivatization, was determined [55]. Further studies on wine have concerned the use of coupled first dimension shape-selective stationary phase columns for the separation of positional isomers [56]. This approach proved to be effective in the separation of a number of small, volatile molecules (based on the butyl hydrocarbon group) which are found in wines. A further HS-SPME–GC × GC investigation was applied to the separation of one of the most complex food matrices: the roasted coffee bean volatile fraction [57]. The latter is characterized by the presence of thousands of mainly volatile components, belonging to several classes of compounds and in a large range of concentrations. In this research, the full resolving power of comprehensive GC was necessary for volatile and semi-volatile separation. The 2D application, carried out on a polar–apolar column set, achieved the separation of approximately 1000 Arabica coffee bean volatiles (Fig. 10).

In preliminary GC × GC applications on this matrix, the minimum modulation temperatures supplied by the pressurized CO_2 in the LMCS system proved to be insufficient for the entrapment of early-eluting components, which ended either unseparated or grossely broadened through the lack of zone compression. It is well known that a modulation temperature of a 100 °C below the analyte elution temperature is required for effective entrapment [58]. This problem was overcome with a substantial decrease of the minimum modulator temperature obtained through an increase in the CO_2 cylinder pressure. A lighter gas (nitrogen), introduced into the tank, enabled an increase in the carbon dioxide pressure of about 100 atm. In this case, the highly volatile compounds were effectively compressed leading to an outstanding improvement in peak quality that was maintained for all the coffee bean early-eluting peaks.

3. Comprehensive two-dimensional liquid chromatography

In gas chromatography, although several stationary phases with differing selectivities are available, retention is more or less dependent on solute vapor pressures. LC techniques, on the contrary, are characterized by a wider variety of separation mechanisms with truly different selectivities. As such,

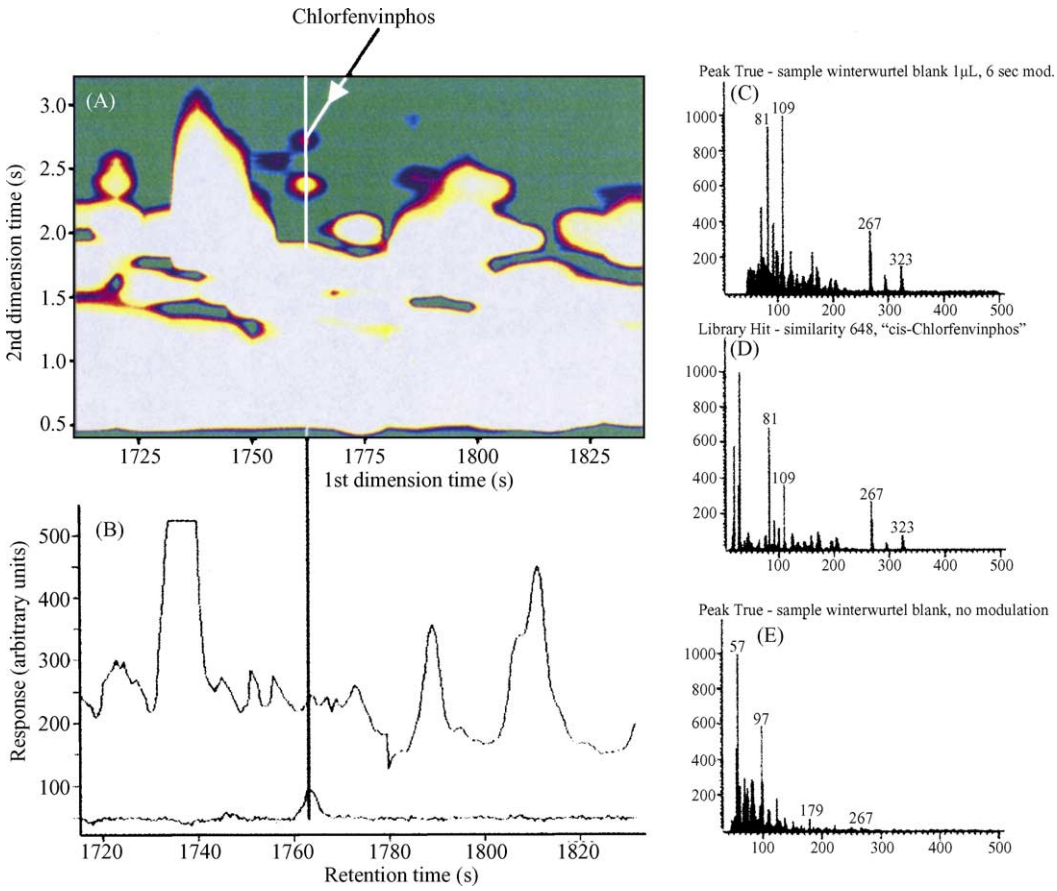

Fig. 9. GC × GC–TOFMS vs. 1D-GC–TOFMS for the analysis of a carrot extract. (a) GC × GC–TOFMS contour plot blow-up. (b) 1D-GC–TOFMS chromatogram of the same region; upper trace, TIC scaled to 1%; lower trace, *m*/*z* 323 ion trace. (c) Mass spectrum obtained after bidimensional separation showing characteristic chlorfenvinphos *m*/*z* values. (d) Chlorfenvinphos library spectrum. (e) Mass spectrum obtained after 1D GC–MS analysis (reprinted from [50] with permission from Elsevier, © 2002).

the number of theoretically achievable orthogonal MDLC combinations is higher. It must be noted, though, that the combination of certain LC mode-types can present a series of difficulties if not impossibilities, such as mobile phase immiscibility, precipitation of buffer salts, 1D mobile phase-2D stationary phase incompatibility [3,10].

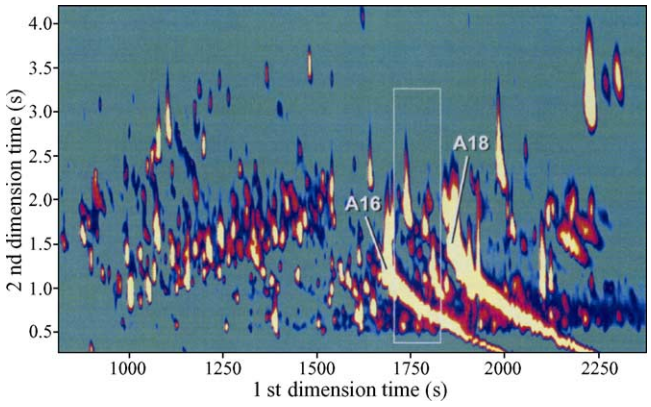

Fig. 8. 2D space plane relative to a carrot extract analysis (reprinted from [50] with permission from Elsevier, © 2002).

Off-line multidimensional techniques are frequently exploited in LC in the pre-treatment of complex matrices. This is achieved through the collection of primary column fractions and their successive re-injection onto a secondary column at a later time. Although the off-line approach is very simple it presents a series of disadvantages: it is time-consuming, difficult to automate and the possibility of sample contamination and artefact formation is high. Furthermore, analytical reproducibility is low.

Classical on-line MDLC is attained through the connection of two columns by means of a high pressure switching valve which entraps a specific quantity of 1D eluate, usually in a loop, and directs it onto the 2D column. On-line techniques offer the advantage of ease of automation and greater reproducibility in a shorter analytical time. These systems, on the other hand, are more difficult to operate and need specific interfaces. Furthermore, the method is typically heart-cutting and, thus, does not achieve a complete bidimensional separation on the entire initial sample.

The first comprehensive two-dimensional LC systems were introduced by Erni and Frei [59], followed by Bushey and Jorgenson [60] in the 1980s.

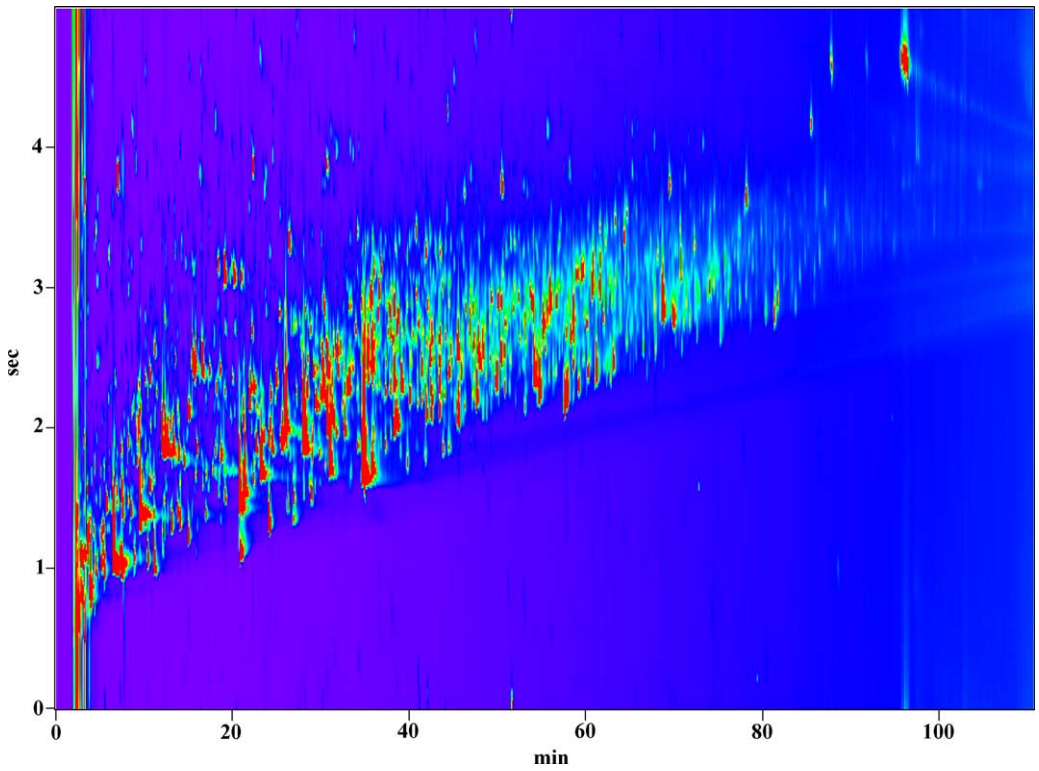

Fig. 10. 2D space plane relative to an HS-SPME–GC × GC application on Arabica roasted coffee beans (reprinted from [57] with permission from © Wiley-VCH Verlag GmbH & Co.).

The comprehensive LC systems, that have been developed, are based mainly on two methods: the use of an 8- or 10-port valve equipped with two sample loops that allow continuous transfers from a primary micro-bore LC column to a second fast column, or the use of a valve that allows transfer from a conventional column to two parallel fast secondary columns, without the use of storage loops.

The use of a 1D micro-bore LC column is a suitable approach for different reasons:

- the small column i.d. ensures a minimization of dilution and provides flow-rates that are compatible with secondary dimension sample injection volumes;
- a pre-concentration step at the head of the secondary column is not necessary and solvent incompatibility between different separation modes is avoided.

Although the lower sample capacity of these columns can be considered a handicap, it must be noted that they are employed successfully in MDLC pre-separation steps. The limited decrease in efficiency observed, which is linked to large injection volumes, is tolerable.Bushey and Jorgenson have developed a comprehensive multidimensional HPLC system for the analysis of bovine serum protein digests [60] and bacterial cell lysate (as well as standard biomolecules) [61]. They used a micro-bore cation exchange column in the first dimension, and a computer controlled eight-port valve as interface. The valve was equipped with two storage loops filled alternately by the effluent from the first column. A second pump forced the loop material onto a second column where the analysis was run quickly, so that the effluent from the first column was sampled by the second column continuously, without the use of stopped flow methods. The second column may be a size exclusion (SEC) column [60] or a reversed-phase C_{18} column [61], and detection was carried out by UV in the first case, by UV and MS in the second.

The combination of a micro-bore column in the first dimension and a conventional column in the second dimension, connected by a multiport switching valve has been used for the comprehensive two-dimensional analysis of synthetic polymers [62]. In this case, a LC × SEC system was developed, using an improved (symmetrical) configuration based on a single 10-port switching valve equipped with two equal volume injection loops as interface.

The employment of a 1D conventional column was reported by Opiteck et al. [63] for the 2D separation of peptides derived from the enzymatic digestion of standard ovalbumin and bovine serum albumin. The LC × LC interface, in this case, was characterized by two parallel fast secondary columns rather than storage loops. The storage loop system works well when the first dimension flow rate is much lower than that of the second dimension. In this kind of system, the primary mobile phase should have a very low strength so that analytes can be trapped at the head of the secondary columns during the loading step. The columns used were a SEC

followed by RP with 1.5 μm size non-porous C_{18} particles, that produced fast and efficient analysis.

A similar approach in protein analysis was followed by Wagner et al. [64] and by Unger et al. [65]. A conventional ion exchange column operated at 1 ml/min was used in the first dimension and two parallel C_{18} bonded 1.5 μm particle size non-porous columns in the second dimension, operated at a flow rate of 2.5 ml/min in gradient mode. The analytes were transferred alternately to one of the two C_{18} columns using a 10-port valve and deposited in an on-column focusing mode at the head of one column while the analytes on the other column were eluted.

Recently, a simple, automated 2D LC system equipped with an electronically controlled, 12-port valve has been used for the analysis of aromatic amines and non amines, operating both dimensions under comparable reversed-phase conditions [66]. In this case, orthogonal separation was achieved by tuning the operating parameters, such as mobile phase strength, temperature, buffer strength, in conjunction with column selectivity.

3.1. LC × LC food analysis

In comparison to GC × GC, the number of LC × LC food applications reported in the literature are much less. Nakashima et al. reported a comprehensive LC application for the analysis of triacylglycerols (TAGs) in vegetable oils [67]. The bidimensional analysis, achieved through a stopped flow method, was based on the employment of a 1D micro-bore Ag-column and a 2D octadecylsilyl (ODS) column. The columns were connected by a 10-port switching valve with a single sample loop.

Recently, a system based on the use of a micro-bore silica column operated in normal-phase mode in the first dimension, and a monolithic type column operated in reversed-phase mode in the second dimension has been developed [68]. This automated approach was applied to the separation of the oxygen heterocyclic components (coumarins and psoralens) of a lemon essential oil. The use of normal and reversed-phase mode in the two dimensions can be helpful in the separation of complex mixtures of a natural origin, containing uncharged molecules of comparable dimension, different in polarity and hydrophobicity. The primary micro-bore column (isocratic elution) was connected to a 2.5 cm monolithic column (gradient elution) through a 10-port valve equipped with two 20 μl sample loops. The presence of two loops in the switching interface enabled the simultaneous accumulation of a 20 μl eluate fraction (60 s) from the first dimension and the re-injection of the previous 20 μl cut onto the secondary column. The application of a 20 μl/min flow rate through the first column enabled one injection per minute onto the secondary column. In this way, all the effluent from the first column was subjected to the 2D separation.

The bidimensional analysis relative to the above mentioned sample is shown in Fig. 11. Peak assignment was

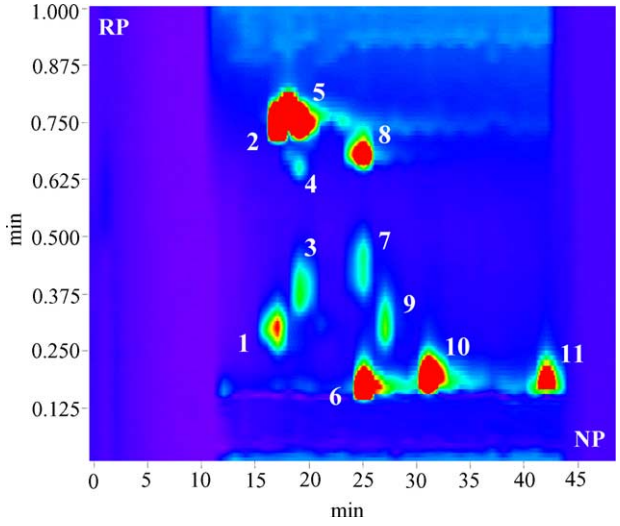

Fig. 11. 2D space plane relative to a LC × LC application on a lemon essential oil. Peak identification: (1) unknown, (2) 5-geranyloxypsoralen, (3) 5-isopentenyloxypsoralen, (4) 5-geranyloxy-8-methoxypsoralen, (5) 5-geranyloxy-7-methoxycoumarin, (6) 5,7-dimethoxycoumarin, (7) 5-methoxy-8-isopentenyloxypsoralen, (8) 8-geranyloxypsoralen, (9) 5-isopentenyloxy-8-epoxyisopentyloxypsoralen, (10) 5-epoxyisopentyloxypsoralen, (11) 5-methoxy-8-(2,3-epoxyisopentyloxy)psoralen. NP: normal phase; RP: reversed-phase (reprinted from [68] with permission from American Chemical Society, © 2002).

achieved through the comparison of retention times in the single dimensions and the relative bidimensional location, with those of standard compounds (when available) and UV spectra obtained with a photo diode array detector.

The main limitation in the development of a MDLC system, where the two dimensions are operated in NP- and RP-mode, is mobile phase incompatibility as the introduction of large volumes of an incompatible solvent yields broadened and distorted peaks [69]. The use of a micro-bore column in the first dimension enables the injection of a small sample volume onto the secondary column, making the transfer of incompatible solvents from the first to the second dimension possible without peak shape deterioration or losses in resolution. In fact, no problems arose from the transfer of the small volumes of 1D eluate (75% *n*-hexane/25% ethyl acetate), which were immiscible with the aqueous mobile phase (50% acetonitrile/50% water), present in the secondary monolithic column. Another fundamental aspect to be considered was that the eluent strength of the initial 50% acetonitrile/50% water mobile phase at the head of the 2D column was lower than that of the primary 75% *n*-hexane/25% ethyl acetate eluent. This enabled the effective refocusing of the 1D solute bands at the head of the secondary column. A repetitive gradient was then necessary to elute the fraction within 48 s (plus 12 s equilibration time). The use of a monolithic column in the second dimension allowed both rapid equilibration times and high flow rates (4 ml/min) with no loss in terms of resolving power [70].

4. Comprehensive two-dimensional liquid–gas chromatography in food analysis

The analysis of very complex mixtures is often complicated by the fact that sample components belong to a variety of chemical classes and are present in a wide range of concentrations. As such, several compounds cannot be resolved by monodimensional GC. In this respect, less complex and more homogenous mixtures can be attained by LC fractionation of the matrix prior to GC separation. The multidimensional LC–GC approach combines the selectivity of the LC separation with the high efficiency and sensitivity of GC separation, enabling the separation of compounds with similar physico-chemical properties in samples characterized by a great number of chemical classes.

The employment of off-line LC–GC methods is frequent and although the collection and handling of liquids is simple, the method is long, laborious and involves a series of steps that can be the cause of contamination, loss of sample, etc. These disadvantages can be avoided by the application of classical multidimensional on-line LC–GC [71]. Furthermore, sample pre- or post-treatment are not necessary as the set-up is fully automated. These systems, on the other hand, are expensive, difficult to operate and are characterized by complicated interfaces. The major problem, relative to this technique, is the transfer of large quantities of liquid from the first to the second gaseous dimension. Several methods have been developed to enable the introduction of 1D liquid heart-cuts onto the secondary column [72–75]. This is achieved through the selective removement of the solvent, thus allowing the reinjection of a sharp analyte band onto the 2D column. Various multidimensional on-line LC–GC food applications have been reported in the literature with many concerning the unsaponifiable fraction of edible oils and fats [76–78].

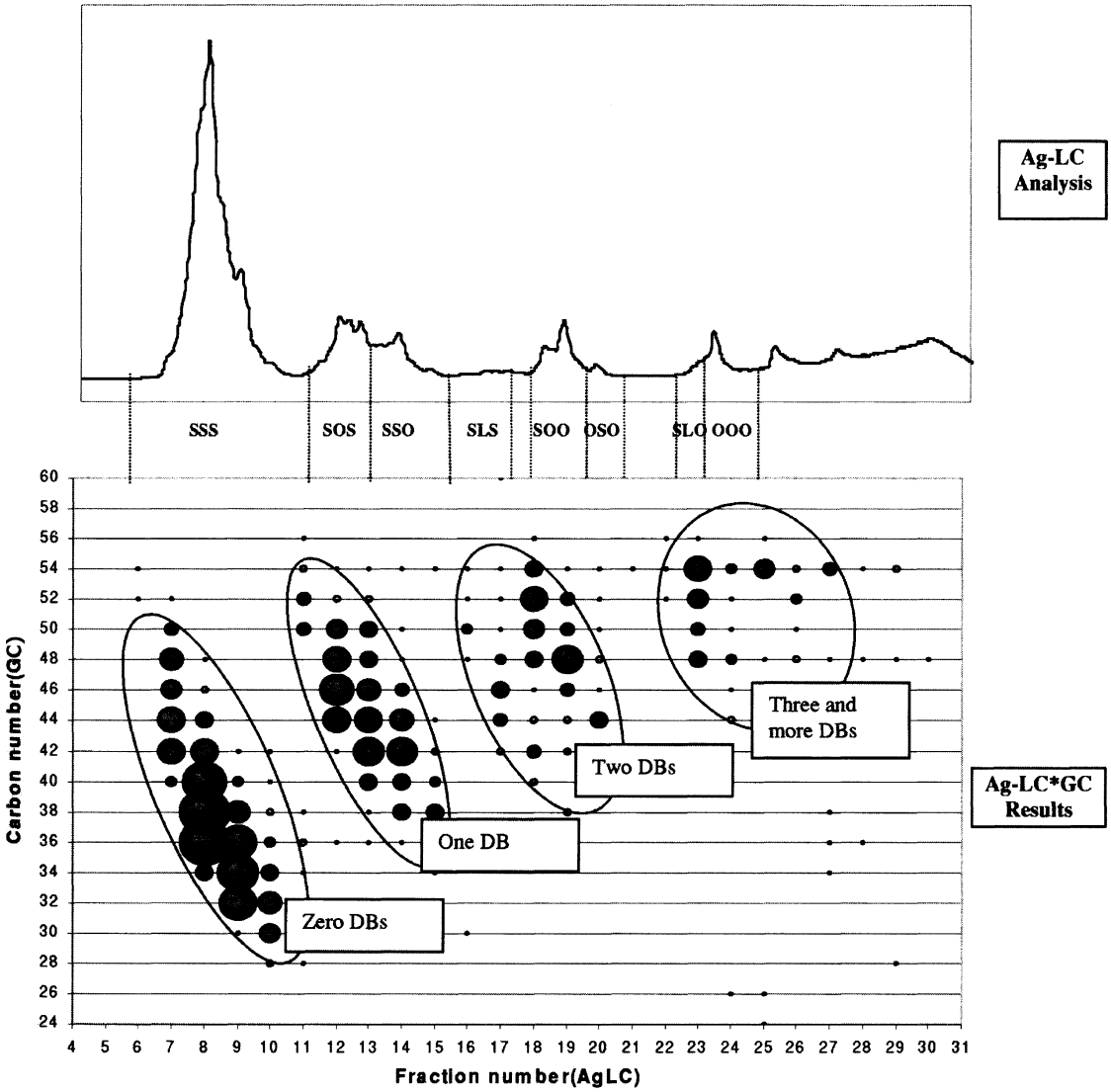

Fig. 12. Off-line comprehensive AgLC × GC separation of a palm kernel oil. Symbols: S refers to a saturated FA. O refers to a mono-unsaturated FA and L to the diunsaturated counterpart. DB: double bond (reprinted from [81] with permission from Elsevier, © 2002).

Comprehensive LC–GC can be considered, at present, a largely unexplored field of research. To the authors knowledge, only two automated on-line LC × GC systems have been developed. The first, in order of time, was applied to the headspace analysis of volatile organic compounds in water [79]. The instrumental set-up was based on the use of a short primary LC column (water mobile phase) and the employment of an innovative interface that allowed the transfer, with the support of a stream of helium, of the solutes onto the secondary GC column. This type of approach is only applicable to the analysis of highly volatile analytes. The second LC × GC system, applied to TAG analysis, was based on the use of a primary silver-loaded column and a secondary short wide-bore GC column [80]. The fast vaporization and transfer of the LC fractions was obtained through the use of a programmed temperature vaporizor in the split mode as an interface.

A single comprehensive off-line LC–GC food application has been described in the literature and, as such, will be reported in this section. The method, applied to edible lipids, was developed by Janssen et al. [81] in order to study the feasibility of comprehensive LC × GC in TAG classification. In the research, fractions of primary column eluate were collected and then later injected onto the secondary column. Fig. 12 shows the comprehensive AgLC × GC "dot-plot" relative to a palm kernal oil. In this case, TAGs were separated in the first dimension on a degree of unsaturation/symmetry effect basis as can be seen in the top part of the figure (monodimensional Ag-LC application) while the GC secondary analysis was achieved on a CN (carbon number: acyl carbon number of the fatty acids bonded to the glycerol molecule) basis. A series of different comprehensive applications, based on the combination of different column sets, were described: silver ion LC/carbon number GC, silver ion LC/FAME GC and normal-phase LC/carbon number GC.

5. Comprehensive two-dimensional pSFC chromatography in food analysis

To the authors knowledge only two fully automated pSFC × pSFC systems have been developed. The earlier method was based on the use of a primary column operated under subcritical conditions and a secondary column under supercritical conditions (both had the same ODS packing material) connected with a 10-port valve. Applications on natural fats and oils were carried out [82]. The subsequent study (pSFC × pSFC-MS), also applied to the analysis of triglycerides (vegetable oils), foresaw a primary SN (separation number: the carbon number minus the number of double bonds) separation and a secondary separation based on the degree of unsaturation [83]. The comprehensive result for four main fractions relative to a peanut oil is illustrated in Fig. 13: the *x*-axis illustrates the primary analysis by RP-pSFC while the *y*-axis shows the secondary silver ion-pSFC separation. The *z*-axis shows additional third dimension atmospheric pressure chemical ionization (APCI)–MS information for the SN 51 fraction.

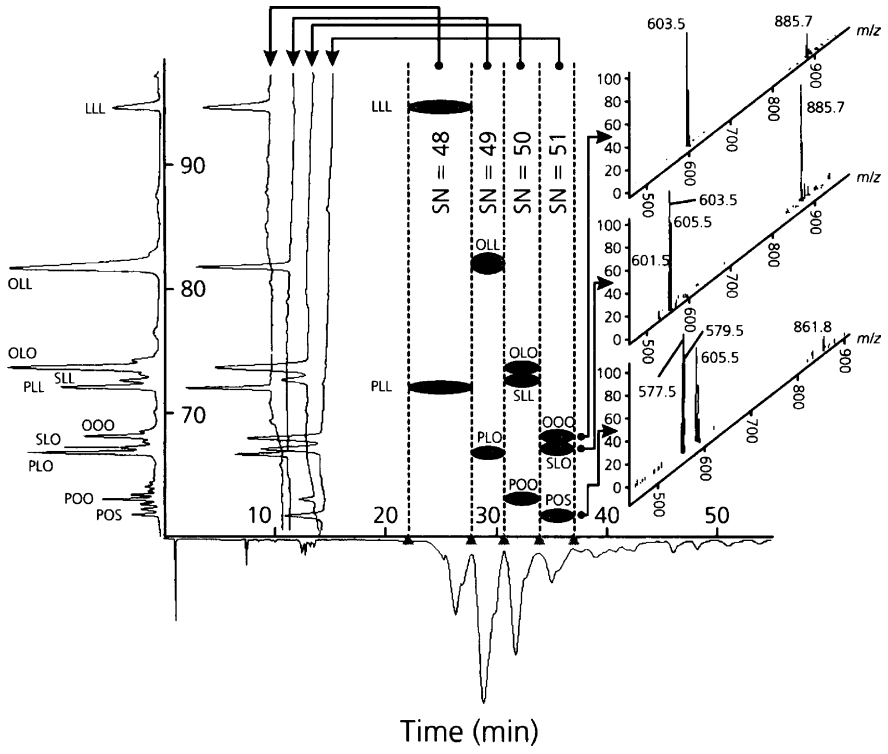

Fig. 13. Comprehensive pSFC × pSFC result for peanut oil. Symbols: palmitic acid (P), stearic acid (S), oleic acid (O), linoleic acid (L) (reprinted from [83] with permission from Prof. Pat Sandra (editor)).

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