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Silver-ion reversed-phase comprehensive two-dimensional liquid chromatography combined with mass spectrometric detection in lipidic food analysis

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Abstract

The triacylglycerol (TAG) profiles present in real world lipidic samples are usually quite complex and, as such, monodimensional high-performance liquid chromatographic (HPLC) techniques are inadequate when challenged with such matrices. In this respect, the complementary use of silver-ion (Ag) and non-aqueous (NA) reversed-phase (RP) HPLC can be exploited if thorough TAG separations are required. The present investigation reports the employment of a newly developed comprehensive LC (LC × LC) system, based on the different separation mechanisms of the aforementioned techniques, and applied to a rice oil sample. The approach was successful in the separation of a high number of solutes, otherwise unachievable through monodimensional LC. Furthermore, the use of atmospheric pressure chemical ionization mass spectrometry (APCI-MS), as detection system, provided a third analytical dimension boosting the identification power of the comprehensive chromatographic method.

Keywords: Comprehensive two-dimensional liquid chromatography; Triacylglycerols; TAG; APCI-MS

1. Introduction

Triacylglyceroles, the most abundant compounds contained in fats and oils, are esters formed mainly by three long-chain fatty acids (FAs) bonded to a glycerol molecule. TAGs are defined by a variety of specific features: total carbon number (CN), FA chain lengths and positions, double bond number (DB), position and configuration relative to each FA [1]. The possible number of FA combinations along the glycerol backbone is high and thus the determination of TAG profiles is usually a cumbersome task.

Non-aqueous reversed-phase high-performance liquid chromatography is commonly employed in TAG analysis. The elution order, in this type of approach, is dependent on increasing partition numbers (PN = CN − 2DB). Although the separation of TAGs (in simple samples and under optimized experimental conditions) with the same PN is possible, this is considered a very difficult task especially in the case of more complex samples [2].

Silver-ion HPLC is also widely used in TAG separations. In this case, the elution sequence is connected to an increasing degree of unsaturation and to the position/configuration of the double bonds within each FA. The separation mechanism is based on specific silver-ion/double bond interactions [3–7].

As far as detection is concerned, APCI-MS has gained quite a firm foothold in HPLC triacylglycerol analysis as it can provide valuable information on FA structures and positions [8–11]. Other detection systems employed in TAG analysis and worthy of note are evaporative light-scattering [12,13] and electrospray ionization MS [1,14,15]. It is obvious that reliable peak assignment, for any type

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of detector, is dependent on the degree of chromatographic separation.

The need for better separations has enhanced, in the last two decades, the growth of multidimensional (MD) chromatography. In LC (as in any other chromatographic technique), a substantial increase in resolving power can be attained through the combination of two separation steps with different selectivities. Off and on-line MD LC separations have been employed in the analysis of complex samples [16,17]. Off-line multidimensional Ag-HPLC/NARP-HPLC has been applied to vegetable [18] and fish oils [19]. A later approach (also off-line) was based on a primary RP fractionation and a secondary silver-ion separation [20]. It must be emphasized that these types of applications, although relatively easy to carry out and to optimize, present a series of disadvantages such as the time costs, the high possibility of sample contamination and the low analytical reproducibility [7,17,21]. On-line methods, which may be either "heart-cutting" or "comprehensive", offer a greater reproducibility in a shorter analytical time but they are more difficult to operate and require specific interfaces. Generally, on-lines systems consist of two columns (ideally with orthogonal separation mechanisms) connected in series through a switching valve which diverts specific volumes of 1D effluent onto the secondary column. The recent progresses achieved in comprehensive LC have obscured, in terms of importance, conventional "heart-cutting" MD methods. The latter, as it is well known, can achieve 2D analysis only on specific 1D eluate fractions, while LC × LC methods can extend the bidimensional advantage to the entire initial sample. The gains, in terms of resolving and identification (formation of group-type patterns on the 2D plane) power, are enormous. It must be added though, that while the first LC × LC system was introduced more than 25 years ago [22], comprehensive LC is not widespreadly employed within the chromatographic community especially if compared to other comprehensive chromatographic methodologies (i.e. comprehensive GC). This is due mainly to the fact 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 [23].

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 [24–28], 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 [29–31]. In terms of TAG analysis, Nakashima et al. reported a comprehensive LC application on vegetable oils [32]. The separation, achieved through a stopped flow method, was based on the employment of a 1D micro bore Ag column and a secondary octadecylsilyl column. Recently, comprehensive LC × GC [33] and supercritical fluid chromatography (SFC × SFC) [34] have been applied to lipid analysis.

A recent research reported an innovative LC × LC system based on the combination of a 1D micro-column, operated in the normal-phase mode (adsorption) and a 2D monolithic, operated in the reversed-phase mode which was successfully applied to the analysis of a lemon essential oil [28]. The comprehensive LC system herein described, which was developed on the basis of this previous experience, differed basically in the presence of a primary Ag micro-column and an APCI-MS (instead of a photo diode array detector) as detection system. These modifications enabled the complete separation of rice oil TAGs, a group of components which are of fundamental importance in food chemistry. The use of APCI-MS as detection system added a third analytical dimension and provided reliable component identification.

2. Experimental

2.1. Abbreviations

Triacylglycerols are defined by means of three letters corresponding to the fatty acid linked to the glycerol backbone. The abbreviations used in this paper are P for palmitic acid (C16:0), S for stearic acid (C18:0), A for arachic acid (C20:0), O for oleic acid (C18:1, Δ^9), L for linoleic acid (C18:2, $\Delta^{9,12}$).

2.2. Samples

HPLC grade acetonitrile, *n*-hexane and isopropanol were purchased from WWR International (Poole, UK). All solvents were used as received. The rice oil was purchased at a local supermarket. Pure standard compounds, obtained from Larodan Pure Chemicals (Malmö, Sweden), were as follows: OOP, POP, OOL, POL, POS, PLS, PPL, OSO, LOL, OOO and LLL.

2.3. Instrumentation and chromatographic conditions

Preliminary studies on the standard components and rice oil were performed on a Shimadzu HPLC system (Kyoto, Japan) consisting of a LC-10AD vp solvent delivery unit, a SCL 10A vp controller and a SPD-10A vp UV detector, equipped with a U-Z view micro-cell (LC Packings, Amsterdam, The Netherlands). Samples were injected by means of a Rheodyne 7725i two-position six-port valve (Rheodyne, Rohnert Park, CA, USA) with a 1 μL injection loop. The HotSep micro-bore column packed with Nucleosil 100–5 SA (150 mm × 1 mm I.D. stainless steel; 5 μm particle size) was purchased from G & T Septech (Kolbotn, Norway). The Ag column was prepared by flushing the column with a 1 M silver nitrate aqueous solution [3]. Acetonitrile and *n*-hexane [0.5–0.8% (v/v) ACN in *n*-hexane] were used as the mobile phase in isocratic mode. Standard and sample solutions were diluted in *n*-hexane at concentrations that yielded an adequate detector response. The mobile phase flow-rate was main-

tained at 13 μL min^{-1} using a ProteCol flow-splitter (SGE International, Ringwood, VI, Australia). The detection wavelength was set at 210 nm.

LC × LC instrumentation: the primary dimension isocratic composition was 0.7% (v/v) ACN in *n*-hexane. The second dimension gradient system consisted of two Shimadzu LC-10AD vp solvent delivery units connected in parallel to a gradient mixer, a Shimadzu DGU-14A degasser, a Shimadzu SCL-10A vp controller and a Shimadzu LCMS-2010A mass spectrometer with an APCI interface. The secondary monolithic column was a Chromolith Performance RP-18 (100 mm × 4.6 mm I.D.), purchased from Merck (Darmstadt, Germany). Isopropanol (A) and acetonitrile (B) were used as mobile phase in the following gradient mode: 0.00–0.20 min 80% B; 0.20–0.40 min 25% B; 0.40–1.00 min 0% B; 1.00–1.40 min 0% B; 1.40–1.41 min 80% B; 1.41–1.50 min 80% B. The mobile phase flow-rate was maintained at 4 mL min^{-1} (the second dimension was also used independently during the optimization procedure). The following APCI-MS parameters were applied: mass spectral range: 500–1000 *m*/*z*; interval: 0.1 s; scan speed: 6000; APCI mode: positive; drying gas flow: 2.5 L min^{-1}; APCI temperature: 400 °C; curved desolvation line (CDL) temperature: 230 °C; CDL voltage −34 V; probe voltage +3 kV; quadrupole array (Q array): scan; detection gain +1.5 kV. MS data acquisition was performed by Shimadzu LabSolutions V2.04 software. The eluate flow-rate was diminished to 1 mL min^{-1} prior to MS analysis through a T-piece union.

A schematic of the developed comprehensive LC system is illustrated in Fig. 1. The columns were connected with an electronically controlled two-position 10-port Supelpro switching valve equipped with two 20 μL sample loops (Supelco, Milan, Italy), controlled by a method editor software (Shimadzu Class vp. 5.0). The two loops work simultaneously and alternately as follows: while one (loading position) accumulates a specific volume of primary silver-phase column eluate (19.5 μL every 90 s, working at a flow-rate of 13 μL min^{-1}), the other (injection position) releases the previously collected "cut" onto the secondary monolithic column. At the end of each rapid second dimension analysis the loop positions are inverted through the switching valve. This alternate process is applied continuously throughout the entire LC × LC run. When the system is not in use the two liquid chromatographs can be used separately with minimal modifications in terms of plumbing. The valve was switched every 90 s by the Class vp programmed external events. The ASCII data, created using the export function of the LabSolutions software, were converted into a matrix with rows corresponding to a 90 s duration and data columns covering all successive second dimension chromatograms using the Comprehensive Chromatography Converter 1.0 software (Avantech, Angri, Salerno, Italy). Contour representation of the 2D chromatograms was achieved through the same software.

3. Results and discussion

The initial part of this investigation was dedicated to the optimization of both HPLC dimensions to be employed in the system. In this respect, it was the monolithic secondary column that was primarily tested as it was important to define the elution window in this dimension (re-injection plus second column analysis plus re-equilibration time) which is also equal to the transfer process period (the time necessary to fill one of the 20 μL sample loops). Furthermore, the transfer period is obviously dependent on the primary flow rate. It must be added that in terms of elution mode, the isocratic approach was immediately excluded for two reasons: fast (1–2 min) secondary TAG elution would have been impossible and the necessity of a low (initial) strength solvent mixture

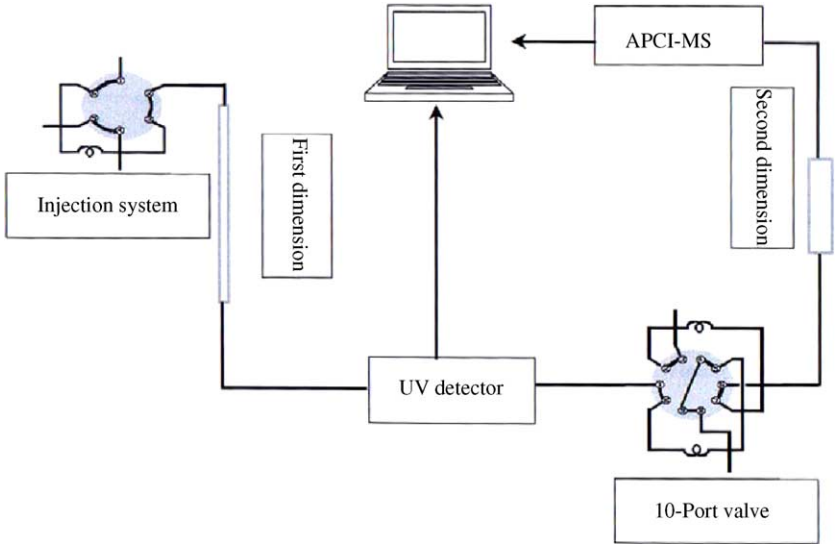

Fig. 1. Schematic of the LC × LC system.

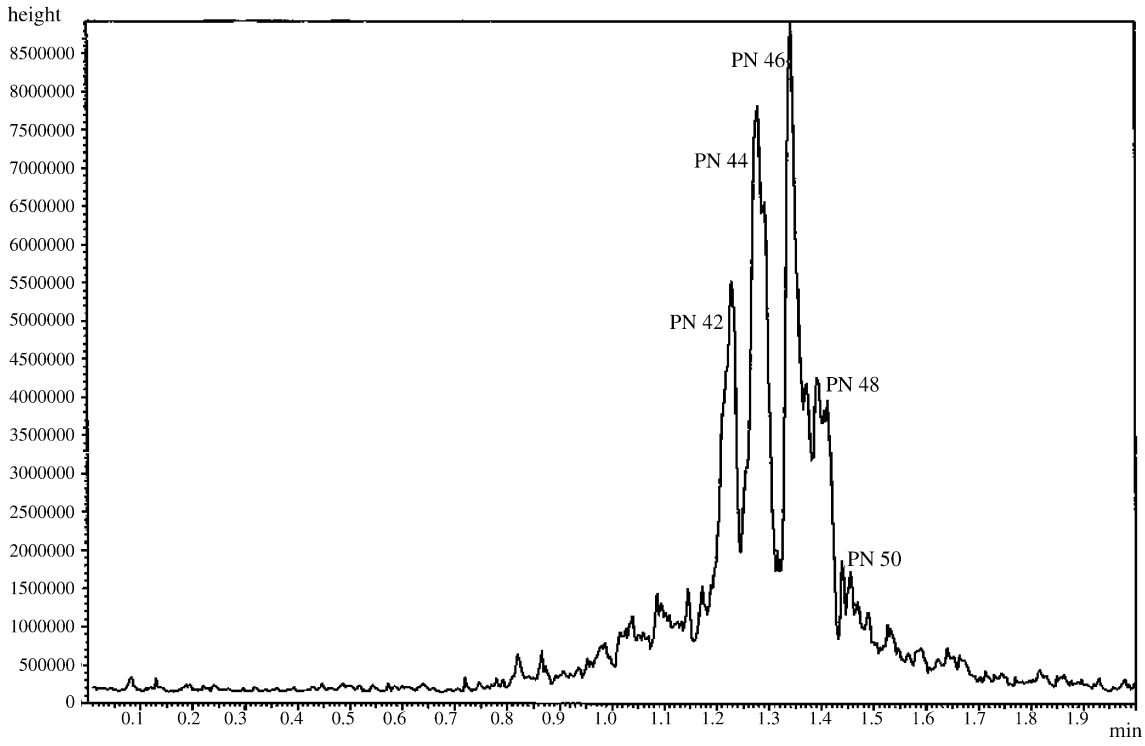

Fig. 2. Monodimensional RP-HPLC result for rice oil TAGs. For abbreviations refer to text. PN 42, LLL; PN 44, OLL; PN 46, OOL, POL, PPL; PN 48, OOO, OOP, PSL, POP; PN 50, SOO, POS.

for effective solute focalization (this last aspect will be later explained). The most satisfactory gradient elution separation was obtained in 90 s. This can be observed in Fig. 2, which illustrates the fast monodimensional RP-HPLC–APCI-MS chromatogram relative to rice oil TAGs that elute according to increasing PN values. Standard components were also used for peak assignment (in terms of PN values). The quality of chromatographic separation is quite good in consideration of the high applied flow rate (4 mL min^{-1}), which was necessary for the elution of all analytes within 90 s, and also in consideration of the amount of sample injected (20 μL), diluted in a solvent (*n*-hexane) which is stronger than the mobile phase (acetonitrile:isopropanol). It must be added that although the five peaks eluted within a narrow retention time window of about 18 s (from 1.15 to 1.45 min), the degree of resolution obtained is quite sufficient for LC × LC requirements as will be later shown. The excellent characteristics of monoliths in terms of brief re-equilibration times (0.09 min in this specific case), high resolving power and low pressure drop (due to their high permeability) are well-known [35]. These features makes these columns particularly suitable as secondary fast columns.

Considerable attention was, at this point, dedicated to the research of the most adequate first column isocratic mobile phase composition. It must be added that many first (as well as second) column separations in several comprehensive chromatographic applications are a compromise (this can be considered one of the main disadvantages relative to these approaches), as they must enable both a decent separation and sufficient sampling of each peak leaving the first column. It is well known, that in order to achieve effective comprehensive chromatography, each peak must be sampled at least three or four times [36]. In first instance, the same mobile phase composition (0.5% ACN in *n*-hexane) as in a previous research was tested [21]. This solvent mixture has been successfully used in a secondary conventional silver-ion column separation in an off-line MD application for the analysis of rice oil TAGs. Under these analytical conditions the retention times of the later eluting rice oil TAGs were unacceptably long. This, as a consequence of the highly reduced flow rate. An increase in the mobile phase content in acetonitrile (0.7%, v/v) provided the best outcome in terms of the compromise aforementioned. The chromatogram relative to the optimized Ag-HPLC analysis of rice oil TAGs is reported in Fig. 3. As it can be seen, the application was achieved in under 125 min while the TAG elution order was that expected (increasing DB numbers, 1–6). Peak assignment was carried out through the employment of standard components and UV detection. The addition of higher quantities of the more polar solvent in the mobile phase (0.8% ACN) was unsuitable in terms of comprehensive LC: while on one hand this led to lower analytical times, on the other narrower solute bands and less peak resolution was observed. If analytical times on both columns are considered, the primary effluent will be divided into about 83 fractions, all of which will be subjected to further separation.

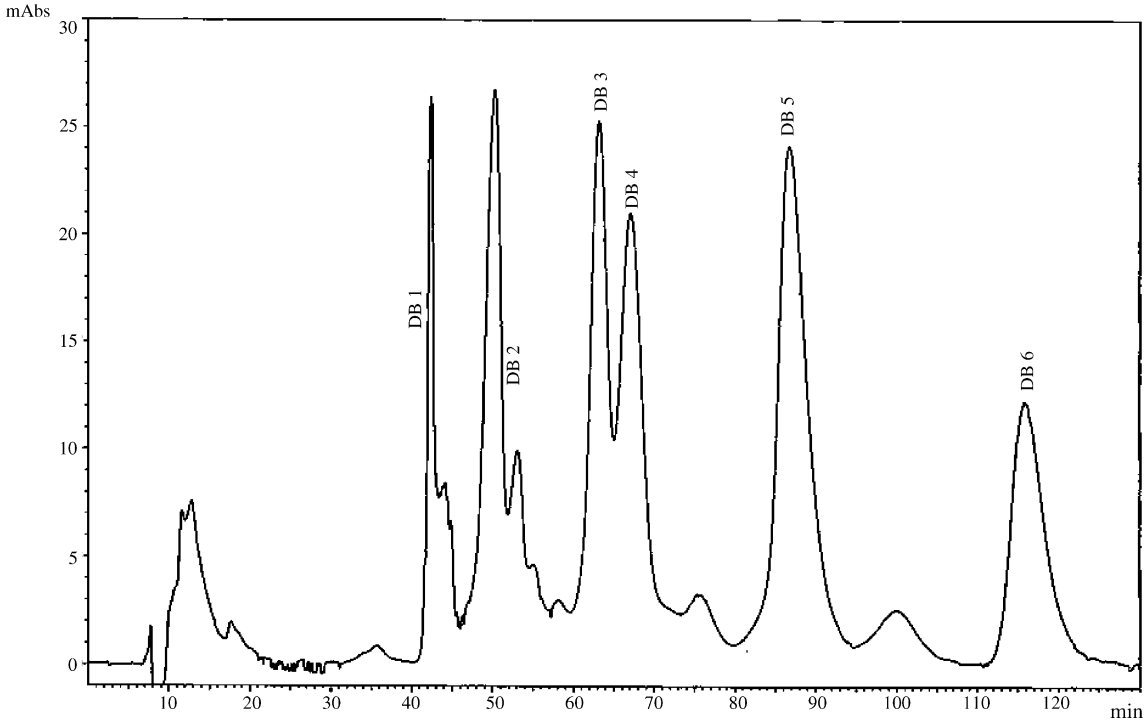

Fig. 3. Monodimensional Ag-HPLC result for rice oil TAGs. For abbreviations refer to text. DB 1, POS, POP; DB 2, PPL, OOP, PSL, SOO; DB 3, POL, OOO; DB 4, OOL; DB 5, OLL; DB 6, LLL.

The next step in this research, after the optimization of both dimensions, was the implementation and testing of the LC × LC system. In order to measure the effectiveness of the set-up, a simple standard TAG mixture was analysed. The sample consisted of 4 TAGs, three with a PN of 48 (POP, OOP, OOO) and one with a PN of 50 (POS), while DB values were 1 (POP/POS), 2 (OOP) and 3 (OOO). As aforementioned, the separation of TAGs with the same PN is a difficult task under RP conditions while the same can be affirmed for TAGs with the same DB value under silver-ion conditions. A contour plot expansion, relative to the standard TAG application, is illustrated in Fig. 4. As it can be seen, the separation of the four components is complete and they are located in characteristic positions of the 2D plane in relation to their PN and DB values.

A further aspect to be briefly mentioned is that one major limitation in the development of this type of MDLC system is mobile phase incompatibility as the introduction of large volumes of an incompatible solvent yields broadened and distorted peaks [37]. 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.

An important issue to be considered in any comprehensive technique is solute focalization: band zone compression is essential for rapid and effective secondary column separations. The band compression mechanism of this LC × LC system is as follows: the mobile phase sampled onto the secondary column operated in RP-LC mode, is stronger than

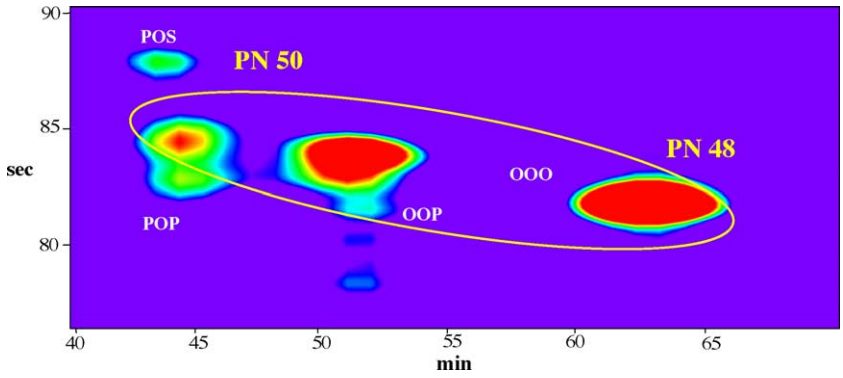

Fig. 4. LC × LC contour plot expansion for four standard TAGs. For abbreviations refer to text.

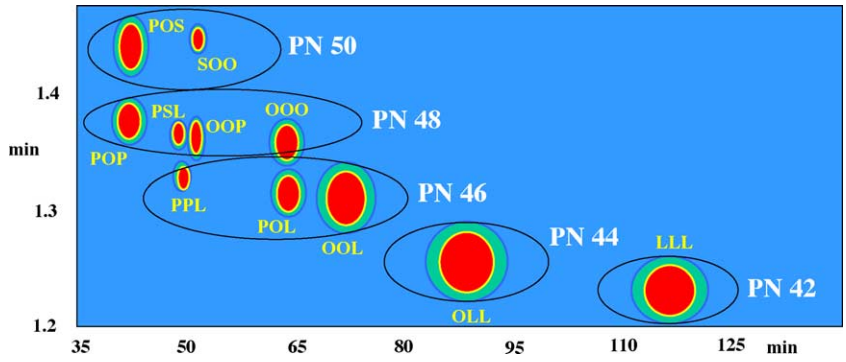

Fig. 5. LC × LC re-constructed chromatographic expansion for rice oil TAGs. For abbreviations refer to text.

the mobile phase at the head of the secondary column. In this case, a narrow eluate plug was attained both through the very fast transfer of the loop content from the first to the second dimension and the initial low eluent strength (80% ACN). It must be noted that the focalization of a cut of approx. 20 μL (a relatively large amount) required an unaltered mobile phase strength for 0.2 min. A repetitive gradient in the second dimension was then necessary to elute all the components of the fraction within the 90 s secondary analysis. As can be observed in Fig. 4, the maximum TAG peak base width (OOP) is approximately 2.5 s (retention along the *y*-axis) while the same compound presented an over 5 min peak base width in the first dimension (retention along the *x*-axis). Considering a 90 s cut relative to OOP, it can be affirmed that band broadening was reduced by at least 36 times (more if secondary column broadening is considered) through the zone compression mechanism. However, the solute focusing ability of the system was not measured.

The re-constructed contour plot expansion relative to a rice oil TAG analysis is illustrated in Fig. 5. The application was successful in resolving 11 different TAGs, characterized with DB and PN values ranging from 1 to 6 and 42 to 50, respectively. As it can be seen, four double-component peaks (POP + POS, PPL + PSL, OOP + SOO, POL + OOO)

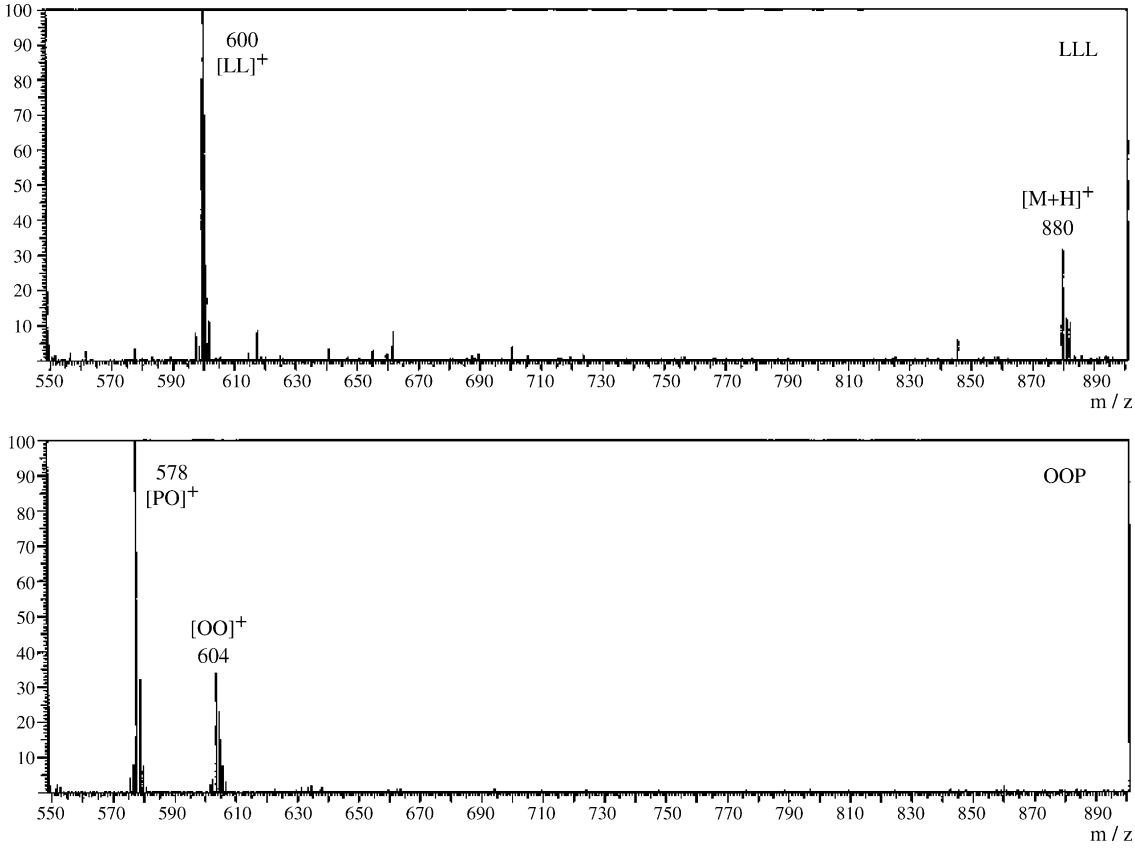

Fig. 6. Mass spectra relative to LLL and OOP. For abbreviations refer to text.

are fully resolved after their passage on the monolithic column. A further consideration regards a well-known and valuable (in terms of identicative potential) characteristic relative to comprehensive chromatography: the formation of group-type patterns on the 2D space plane. It can be observed, in fact, that TAGs with the same PN tend to align themselves along distinct bands (circled areas in the figure), while those with the same degree of unsaturation fall within vertical contour plot zones (not illustrated in the figure). As regards APCI mass spectrometric detection, it provided valuable TAG structural information. As previously reported [8], the APCI-MS spectra presented a pseudomolecular ($[M + H]^+$) ion, the intensity of which is related to the degree of unsaturation of the TAG molecule: the higher the degree of unsaturation, the more intense the pseudomolecular ion. In addition, the formation of diglyceride ($[DG]^+$) ions was observed, due to the loss of a fatty acid linked to the glycerol backbone. Fig. 6 reports MS spectra obtained for two TAGs separated by the comprehensive LC analysis. As can be seen, the MS spectra are in agreement with literature data: in the upper spectrum relative to LLL, the presence of the pseudomolecular ion ($[M + H]^+ = 880$) and the diglyceride ion (base peak $[LL]^+ = 600$) are evident. The fragmentation of OOP (lower spectrum in Fig. 6), on the other hand, is characterized by the formation of two abundant diglyceride ions: $[PO]^+$ (base peak = 578) and $[OO]^+$ (604). Bidimensional peak assignment was confirmed through the injection of standard TAG components. If the experimental conditions applied are considered, the MS supplied a data acquisition rate of 10 Hz and a mass scan range of 400–1000 U, which is wide enough to include all the signals relative to the TAGs present in rice oil. Under these conditions, the attainment of about 20 spectra for a 2D peak of about 2 s, proved to be more than sufficient for reliable peak assignment.

In conclusion, it must be noted that while the two separation mechanisms are truly different, this LC × LC approach is characterized by a lower peak capacity than what would be expected from a totally orthogonal system (ideally the product of the peak capacity relative to each column). In fact, as seen in Fig. 5, the first TAG starts eluting after about 70 s (PN = 42) and, as such, only the upper part (about 22–23%) of the 2D space plane is exploited. Nevertheless, the degree of solute resolution can be considered completely satisfactory. The authors retain that a more rapid elution of such a TAG molecule (maintaining the same peak separation) is extremely difficult. Obviously, this aspect is linked to the specific characteristics of these analytes and in the case of lipids containing TAGs with lower PN values, RP column retention times would decrease and, thus, a larger 2D chromatographic space would be available.

4. Conclusions

The aforedescribed Ag-LC × RP-LC–APCI-MS approach can be considered an additional contribution to the field of comprehensive LC methods. It can be affirmed that it has demonstrated to be an effective tool for the thorough determination of TAG profiles in fundamentally important food matrices. Furthermore, the system is easily assembled and not difficult to use. At present, the gradient mode is being tested in the first dimension in order to extend the applications of this system to fats and oils containing TAGs with a wider PN and DB range. In general, this type of approach (1D micro-column and 2D monolithic) is very versatile as it can be employed for the analysis of a variety of compounds over a wide range of polarities. Future research in this field will be dedicated to both improvement of the existing comprehensive LC methods and development of other LC × LC systems.

Acknowledgments

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