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Off-line coupling of non-aqueous reversed-phase and silver ion high-performance liquid chromatography–mass spectrometry for the characterization of rice oil triacylglycerol positional isomers

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Abstract

The determination of the triacylglycerol (TAG) profile in real world matrices is rather difficult as these compounds present a complex composition and are characterized by similar physico-chemical properties. This investigation is based on the high-performance liquid chromatography (HPLC) multidimensional determination of the TAG profile in terms of TAG species and positional isomers in a rice oil sample. The off-line bi-dimensional system was attained through the coupling of non-aqueous reversed-phase HPLC and silver ion (Ag)-HPLC. The primary column eluate was fractionated and the fractions of interest were then injected onto the secondary column, allowing the separation of several TAG positional isomers, unresolved in the first dimension. Peak assignment was carried out by combining retention data with atmospheric pressure chemical ionisation (APCI) MS spectra information. The fatty acid distribution along the glycerol backbone, determined by Ag-HPLC, was confirmed through diglyceride ion ratios derived from APCI-MS analysis. Method validation, where both precision and accuracy were measured, was carried out in preliminary applications on standard compounds. The analytical results obtained show that rice oil TAGs follow a distribution which can be considered typical for vegetable oils.

Keywords: Positional isomers; Non-aqueous reversed-phase liquid chromatography; Silver ion liquid chromatography; Triglycerides; Triacylglycerols; Glycerols; Fatty acids

1. Introduction

TAGs are the main constituents of lipidic foods. A triacylglycerol is formed almost invariably by long chain fatty acids linked to a glycerol molecule and is defined by the following basic attributes:

(a) total carbon number (CN), equal to the sum of the carbon atoms contained in the alkylic chains;
(b) fatty acid (FA) alkyl chain lengths;
(c) FA positions on the glycerol backbone;
(d) the degree of unsaturation;
(e) double bond position and configuration in each FA [1].

These features can be determined with well-known and widely used analytical techniques. The CN parameter and some (limited) information on the FA position can be attained through high-resolution gas chromatography (HRGC) or supercritical fluid chromatography (SFC) [2,3]. However, the most commonly employed technique in TAG research is HPLC.

Information concerning the degree of unsaturation and CN values can be obtained through non-aqueous reversed-phase (NARP) HPLC applications. This method foresees the employment of bonded-phase silica columns (especially the C_{18} type), which determine a separation on the basis of partition number (PN) values: PN = CN − 2DB, where DB is

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the total double bond number. The separation of TAGs with the same PN is critical and positional isomers cannot be resolved [4].

The silver ion (Ag) HPLC approach provides information on the double bond number/distribution and on FA double bond configuration (*cis/trans*). The separation mechanism, based on interactions between silver ions and FA double bonds [5,6], achieves positional isomer resolution: TAG retention behaviour is closely connected to the specific positions of unsaturated FAs on the glycerol molecule [7]. It must be noted, at this point, that TAG regioisomers are very important from a biochemical and nutritional viewpoint [8]. Furthermore, the knowledge of TAG stereo-specific structures is also gaining rapid importance for studies on structured triacylglycerols [9–11]. For most physical and nutritional purposes only the differentiation between the *sn*-2 and *sn*-1/*sn*-3 position is required [1,12–14], despite 1- versus 3-symmetry differences and consequently different biological properties [15]. Regio and stereo-specific determinations are extremely difficult to achieve, often requiring several operational steps and long sample treatments. Indirect methods such as enzymatic [16–19] and traditional chemical applications [20–23] find employment. Direct methods such as nuclear magnetic resonance spectrometry [24] and tandem MS [25–27] are characterized by relatively high costs and require complex calculations [8]. As such, Ag-HPLC represents a rapid alternative to traditional approaches, even though this method enables the separation of positional isomers only when specific experimental conditions are used [7].

Atmosphere pressure chemical ionisation (APCI) MS detection is widely employed in TAG analysis as it can provide useful information for the determination of a FA structure and position within a TAG molecule [13,28]. The differentiation of positional isomers is achieved through the evaluation of the relative intensities of the diacylglycerol ion fragments. Several techniques, based on the use of APCI-MS alone or combined with HPLC, have been developed for the determination of TAG composition and for the determination of positional isomers in natural matrices [12,13,28–33].

Electrospray ionisation (ESI) derived spectra contain only quasimolecular ions with no fragmentation. As such, the structural assignment of identical molecular mass TAGs can be unreliable. Nevertheless, the application of an Ag-HPLC–ESI-MS method achieved the thorough characterization of a mixture of TAGs [1].

The separation of all the TAGs present in a lipidic sample can be attained through the application of multidimensional techniques [8]. Several multi-modal chromatographic systems have been described: silver ion LC/reversed-phase LC [34], reversed-phase LC/silver ion LC [35], capillary SFC/capillary GC [36], Ag-pSFC/RP-CEC [37], comprehensive two-dimensional liquid chromatography (first dimension: Ag-; second dimension: RP-) [38], automated off-line pSFC (RP) × pSFC (Ag)/MS [39].

The present research is based on the determination of the regioisomeric TAG composition present in rice oil. This was achieved through the application of an approach characterized by the tandem use of NARP- and Ag-HPLC. The sample was initially fractionated in the NARP-mode; each TAG fraction was then subjected to secondary analysis on a silver ion column, in order to obtain regioisomer separation. Peak identification was carried out by APCI-MS detection. It must be emphasized that, although the economical and nutritional importance of rice oil is growing, the literature concerning this food product is quite scarce [40–44].

2. Experimental

2.1. Materials

Acetonitrile, acetone, n-hexane were HPLC-grade from Carlo Erba (Milan, Italy). The rice oil was purchased at a local supermarket. TAG standards were obtained from Larodan Pure Chemicals (Malmö, Sweden).

2.2. 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: palmitic acid (C16:0), S: stearic acid (C18:0), A: arachic acid (C20:0), O: oleic acid (C18:1, Δ^9), L: linoleic acid (C18:2, $\Delta^{9,12}$), Ln: linolenic acid (C18:3, $\Delta^{9,12,15}$).

An *sn*- (stereo-specifically numbered) prefix is used when the fatty acid distribution is known [45].

Preliminary studies on standard TAGs (*sn*-POO, *sn*-OPO, *sn*-POP, *sn*-PPO, *sn*-OLO, *sn*-OOL, *sn*-POS, *sn*-POL), and rice oil were carried out under NARP-conditions with both UV and APCI-MS detectors. This was achieved prior to multidimensional analysis in order to optimise separation conditions in the NARP mode and to obtain information concerning MS fragmentation.

2.3. NARP-HPLC preliminary analysis

Instrument: Shimadzu HPLC system equipped with two LC-10Advp pumps, a SCL-10Advp controller, a DGU-14A degasser, a SPD-10Advp UV detector, a 2010A mass spectrometer with an APCI interface. Conditions: column: Restek Ultra C_{18}, 250 mm × 4.6 mm i.d., 5 μm particle size. Solvents: acetone-acetonitrile (70:30), isocratic mode. Flow rate: 1 mL/min; Pressure:50 bar; injection volume: 20 μL. Acquisition wavelength: 210 nm. MS Conditions: nebulizer gas flow rate: 2.0 L/min; APCI mode: positive; APCI temperature: 400 °C; probe voltage: 3 kV; CDL (curved desolvation line) voltage: −34 V; Q Array (quadrupole array): scan; detector gain 1.7 kV; mass range: 450–1100 *m/z*. Rice oil was diluted in the mobile phase at 15 mg/mL. TAG standards were prepared at different concentrations (50, 25, 10 ppm).

2.4. Multidimensional analysis

2.4.1. First dimension: Micro-preparative NARP-HPLC-UV

Instrument: Shimadzu HPLC system equipped with two LC-10ADvp pumps, a SCL-10ADvp controller, a DGU-14A degasser, a SIL-10ADvp autosampler, a FRC-10ADvp fraction collector, a SPD-10ADvp UV detector, a CTO-10ADvp thermostatted oven. Except for the oven temperature (34 °C), the operative conditions were the same as in the RP-HPLC preliminary analysis. The rice oil sample was diluted in the mobile phase at 35 mg/mL; injection volume 20 μL. Three successive automatic collections were performed at a constant temperature (34 °C). All of the fractions collected were concentrated to dryness under nitrogen and were then solubilized in a volume ranging from 0.1 to 1 mL of hexane, depending on the relative abundance of every TAG in the original sample.

2.4.2. Second dimension: Ag-HPLC–APCI-MS

Instrument: Shimadzu HPLC system equipped with two LC-10ADvp pumps, a SCL-10ADvp controller, a DGU-14A degasser, a 2010A mass spectrometer with an APCI interface. Conditions: Column: Varian Chromspher 5 Lipids, 250 mm × 4.6 mm i.d., 5 μm particle size (Superchrom, Milan, Italy). Solvents: *n*-hexane–acetonitrile (99.5:0.5); isocratic mode. Flow rate: 1 mL/min, $P = 19$ bar; injection volume 20 μL. MS conditions were the same as in the RP-HPLC preliminary study.

3. Results

Twelve rice oil TAG fractions were obtained through the application of a micro-preparative NARP- HPLC technique. The chromatogram relative to this separation is illustrated in Fig. 1; also reported are the 12 fractions collected. Peak assignment was obtained through the combination of PN values and APCI-MS spectra information (Table 1). TAG relative abundances, also reported in Table 1, were attained through UV detection and the use of corrected area values [46].

Fig. 2 illustrates the chromatogram relative to a Ag-HPLC analysis performed on the rice oil sample. Peak identification was achieved through the use of DB values and MS information. As it can be observed, the determination of the positional isomer distribution for each TAG species would be impossible using this monodimensional approach, due to problems relative to peak overlapping between TAG isomers and other TAG species.

As aforementioned, standard compounds were analysed in preliminary applications in order to optimise and validate the method. It has been shown that the relative abundance of $[DG]^+$ in APCI-MS spectra of a specific TAG depends both on the type of instrumentation and on the experimental conditions employed [28]. Pure *sn*-OPO and *sn*-POO were analysed by RP-HPLC–APCI-MS in triplicate; an enlargement of the MS spectra obtained is shown in Fig. 3. As it can be seen, both spectra show fragments corresponding to the diglyceride ions $[OP]^+$ and $[OO]^+$. The ratio of the fragment intensities changes in relation to the position occupied by the three FAs, in accordance with data reported in literature [27–30]. In respect to the standard TAGs analysed in this investigation (*sn*-POO, *sn*-OPO, *sn*-POP, *sn*-PPO, *sn*-OLO, *sn*-OOL), it was found that the fragment intensity ratios for all TAGs were constant and independent from the FA nature. In the case of symmetric isomers (i.e. *sn*-OPO), the relative abundance of the diglyceride ions $[OP]^+$ and $[OO]^+$ were 100 and 28 respectively. In the case

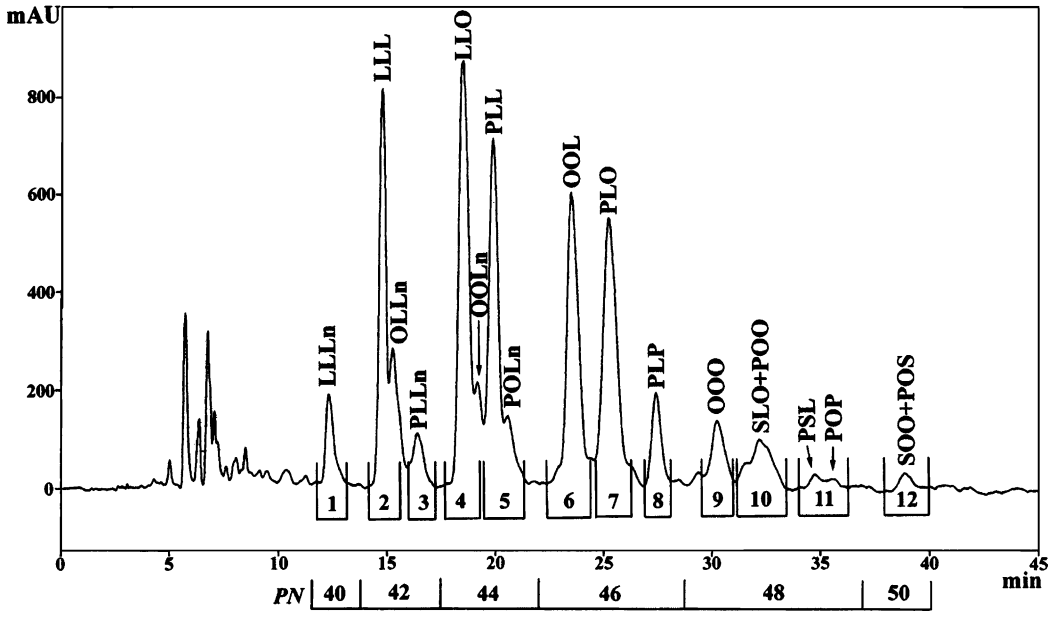

Fig. 1. NARP-HPLC–UV chromatogram of rice oil.

Table 1
Molecular mass, partition number, fragmentation ions obtained for TAGs identified in rice oil by RP-HPLC–APCI-MS

TAG	Relative abundance (%) ± S.D.	M_r	DB	CN	PN	$[M+H]^+$	$[DG]^+$	$[DG]^+$	$[DG]^+$
LLLn	0.92 ± 0.03	877.3	7	54	40	878	LnL 598	LL 600	
LLL	3.42 ± 0.10	879.4	6	54	42	880	LL 600		
OLLn	2.09 ± 0.39	879.4	6	54	42	880	OL 602	LLn 598	OLn 600
PLLn	1.41 ± 0.27	853.3	5	52	42	854	PL 576	LLn 598	PLn 574
LLO	7.93 ± 0.27	881.4	5	54	44	882	LL 600	LO 602	
OOLn	1.32 ± 0.06	881.4	5	54	44	882	OO 604	OLn 600	
LLP	6.28 ± 0.14	855.4	4	52	44	856	LL 600	LP 576	
POLn	1.66 ± 0.07	855.4	4	52	44	856	PO 578	OLn 600	PLn 574
OOL	11.98 ± 0.32	883.4	4	54	46	884	OO 604	OL 602	
PLO	14.80 ± 0.22	857.4	3	52	46	858	PL 576	LO 602	PO 578
PPL	4.13 ± 0.15	831.3	2	50	46	832	PP 552	PL 576	
OOO	14.68 ± 0.80	885.4	3	54	48	886	OO 604		
POO	14.22 ± 0.67	859.4	2	52	48	860	PO 578	OO 604	
SLO	1.98 ± 0.09	885.4	3	54	48	886	LO 602	SL 604	SO 606
PSL	1.51 ± 0.03	859.4	2	52	48	860	PL 576	SL 604	PS 580
POP	7.18 ± 0.71	833.4	1	50	48	834	PO 578	PP 552	
SOO[a]	4.59 ± 0.07	887.5	2	54	50	888	SO 606	OO 604	
POS	–	861.4	1	52	50	862	PO 578	SO 606	PS 580

TAG: triacylglyceride; M_r: molecular mass; DB: double bond; CN: carbon number; PN: partition number; $[M+H]^+$: pseudomolecolar ion; $[DG]^+$: diglyceryde ion.
[a] Coeluted with POS.

of asymmetric isomers (i.e. *sn*-POO), the relative abundance of the diglyceride ions $[OP]^+$ and $[OO]^+$ were 100 and 50 respectively. Precision was measured in five consecutive analysis performed on *sn*-POP and *sn*-PPO at a known concentration. R.S.D.s relative to area values were always lower than 2%. Mixtures with different ratios of the two standard TAGs were prepared and analysed five times each. The average relative abundance of each regioisomer in all mixtures was calculated without the support of correction factors and assuming that positional isomers give the same MS response. The experimental values attained differ within a 1 to 2% range in respect to the theoretical values. This result was important as many standard TAGs are not available.

Following this validated procedure, each TAG fraction derived from the primary column was re-injected onto the secondary column (triplicate analysis) for the determination of positional isomers. All fractions were analysed under the

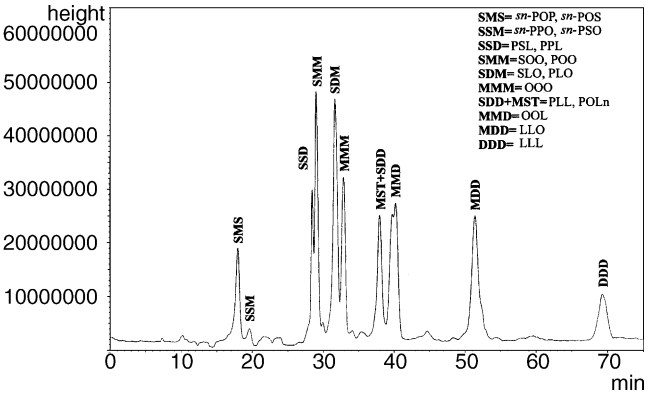

Fig. 2. Ag-HPLC–APCI-MS chromatogram of TAGs of rice oil. S: saturated fatty acid; M: monounsaturated fatty acid; D: diunsaturated fatty acid; T: triunsaturated fatty acid.

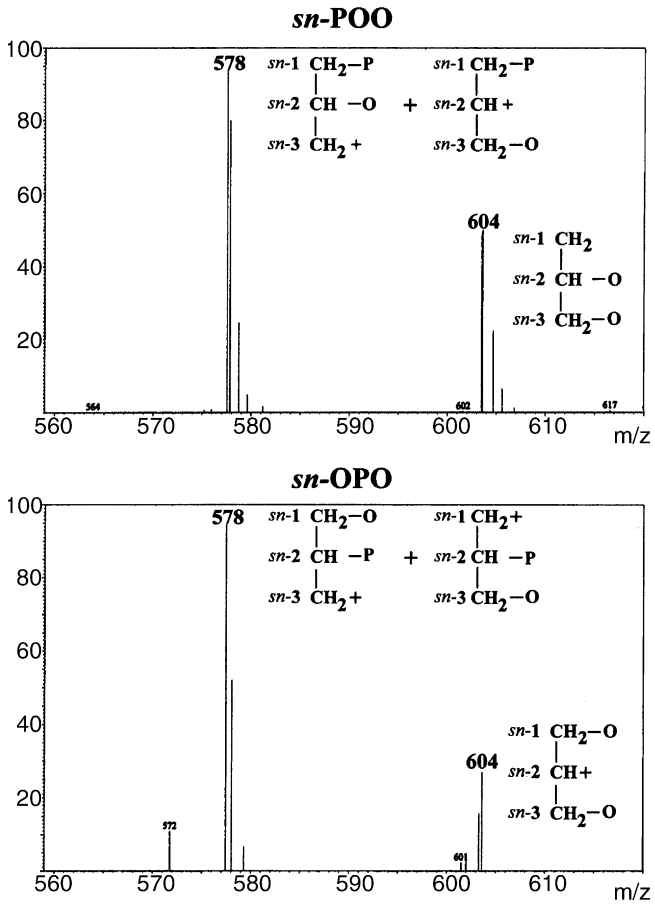

Fig. 3. APCI-MS spectra of *sn*-OPO and *sn*-POO standard isomers.

Table 2
Relative abundance (%) of TAG isomers in rice oil

TAG fraction	Isomers	Relative abundance (mol% of total)
POP	*sn*-POP	86
	sn-PPO	14
POS	*sn*-POS	83
	sn-SPO	17
POO	*sn*-POO	94
	sn-OPO	6
SOO	*sn*-SOO	98
	sn-OSO	2
PLP	*sn*-PLP	84
	sn-LPP	16
PLS	*sn*-PLS	85
	sn-LPS	15
PLO	*sn*-PLO	97
	sn-POL	
	sn-LPO	3
SLO	*sn*-SLO	70
	sn-LOS	27
	sn-LSO	3
PLL	*sn*-PLL	100
OOL	*sn*-OLO	40
	sn-OOL	60
LLO	*sn*-LLO	86
	sn-LOL	14

same analytical conditions (see Experimental section). As regards sample concentration, the fractions were appropriately diluted in relation to the amount of TAG contained in the relative fraction. In fact, sample concentration is a critical parameter in Ag-HPLC positional isomer separation [7]. Fractions, in relation to the TAG to be determined, were diluted in hexane to obtain an injected amount ranging from 0.5 to 3 μg. Regioisomer ratios, relative to rice oil TAGs containing FAs present in the matrix at a level higher than 1% (P, S, O and L) are listed in Table 2. Every fraction was analysed in triplicate and average values are reported. Coefficients of variation (CV%) ranged between 0.6 and 1.2%. A chromatogram relative to a multidimensional NARP-Ag application on TAG fraction 12 is illustrated in Fig. 4. As it can be seen, three TAG species were separated: POS, PLA and SOO. These rice oil components, characterized by the same *PN* value (50), undergo complete co-elution under our NARP-HPLC conditions. Also resolved are the positional isomers relative to the POS and SOO group: *sn*-POS, *sn*-SPO and *sn*-SOO, *sn*-OSO. The elution order is in agreement with that reported previously for Ag-HPLC [5,7,32]. In the case of PLA and SOO, both characterized by the same number of double bonds, the latter is more retained. The stronger interaction of SOO with the stationary phase is due to the presence of two double bonds on different FAs, thus interacting with different sites. Within the POS group, *sn*-POS was the first to elute as the unsaturated FA is located in the *sn*-2 position. The *sn*-POS MS spectrum presented three $[DG]^+$

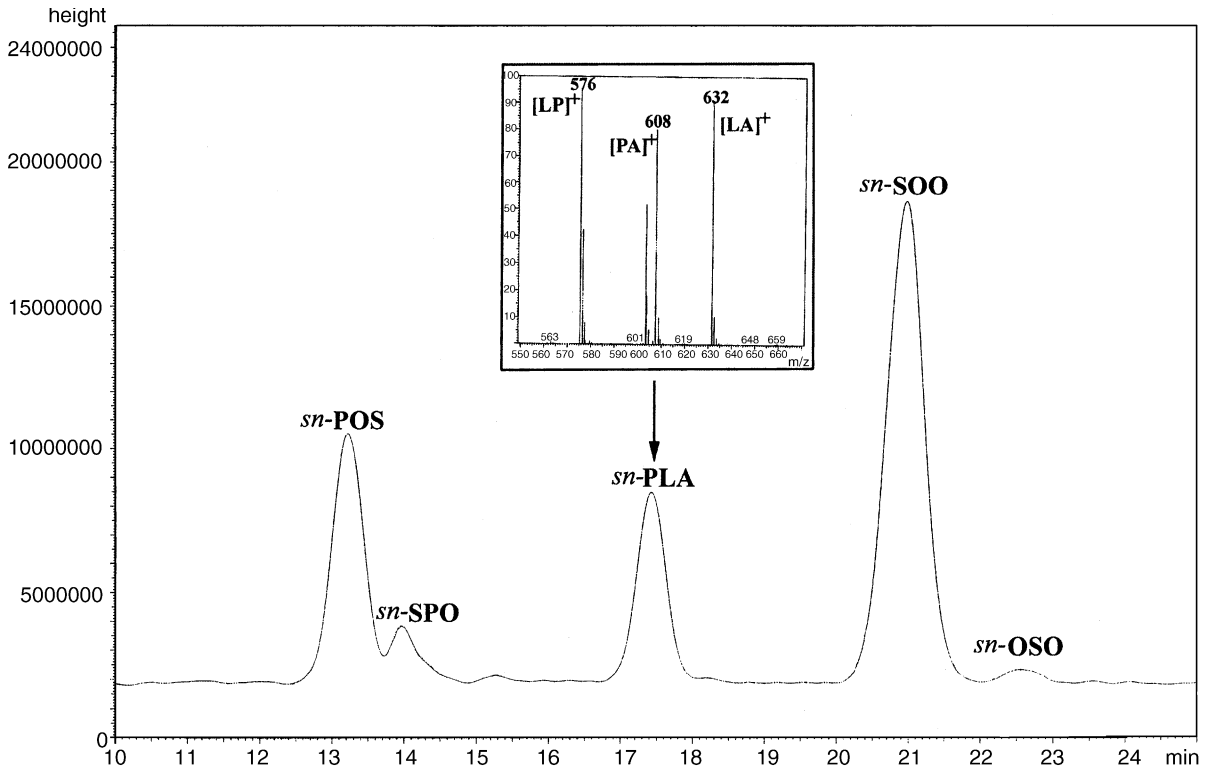

Fig. 4. Ag-HPLC–APCI-MS chromatogram of TAGs from fraction 12.

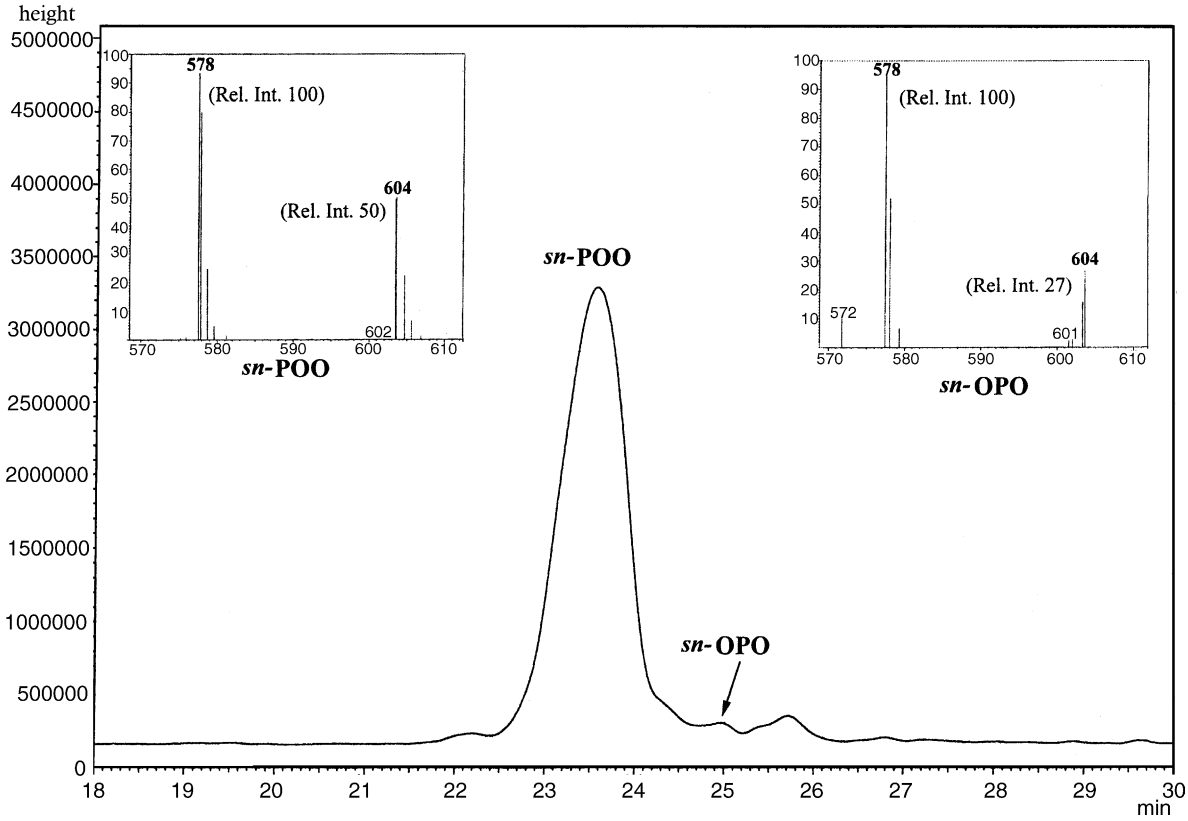

Fig. 5. Ag-HPLC–APCI-MS chromatogram of POO isomers (fraction 10).

ions, the less intense of which was the $[PS]^+$ ion. In fact, the loss of the FA in *sn*-2 position is the most hindered and was also observed for *sn*-SPO. The *sn*-PSO isomer was not detected; this could be connected to a low concentration or peak overlapping with *sn*-SPO.

The re-analysis of fraction 12 also revealed the presence of *sn*-PLA, initially undetected in NARP analysis on the entire sample; this was probably due to the low amount of this TAG in rice oil. No other regioisomers, relative to this TAG, were detected. Peak identification was achieved through mass spectra (the $[PA]^+$ ion was the less abundant) and retention behaviour.

The chromatographic results obtained for fraction 10 (POO/SLO) are shown in Figs. 5 and 6. These TAGs, characterized by the same PN value (48), are a critical pair in NARP-HPLC, while they show a very different elution behaviour in Ag-HPLC, as can be seen from the retention time values. Fig. 5, a 12 min expansion with corresponding MS spectra, illustrates the separation of *sn*-POO and *sn*-OPO in a 94:6 ratio. In fraction 11, the POP isomers (*sn*-OPP and *sn*-POP) are present in a 86:14 ratio. It must be concluded that even if there is a more favourable *sn*-2 position bonding for oleic acid in respect to palmitic acid, the different ratios are also dependent on the number of oleic and palmitic acid molecules in the TAG. A similar behaviour can be observed, for example, considering the results obtained for OOL and LLO.

Fig. 6 shows an expansion relative to the three SLO isomers: *sn*-SLO, *sn*-SOL and *sn*-OSL. In this group, TAG retainment is stronger as the degree of unsaturation in the external fatty acids increases. These regioisomers have, in fact, a more intense interaction with the stationary phase. *sn*-LSO, where the unsaturated FAs are present in the external positions *sn*-1 and *sn*-3, is more retained and completely resolved. The other two isomers are not baseline separated. However MS peak identification, considering the less intense $[DG]^+$ ion, is easily carried out, as shown in Fig. 7. Fig. 8 illustrates a partial separation relative to a critical

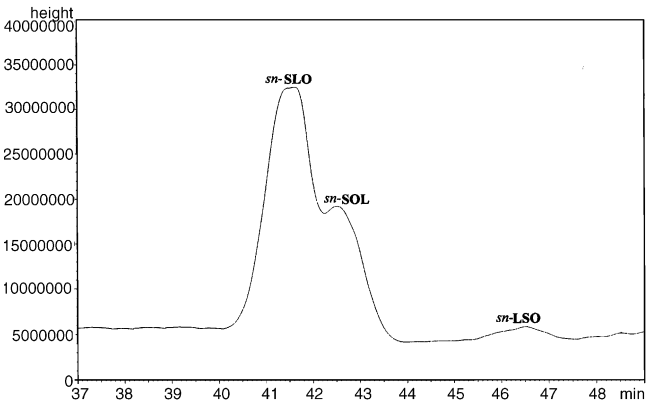

Fig. 6. Ag-HPLC–APCI-MS chromatogram of SLO isomers (fraction 10).

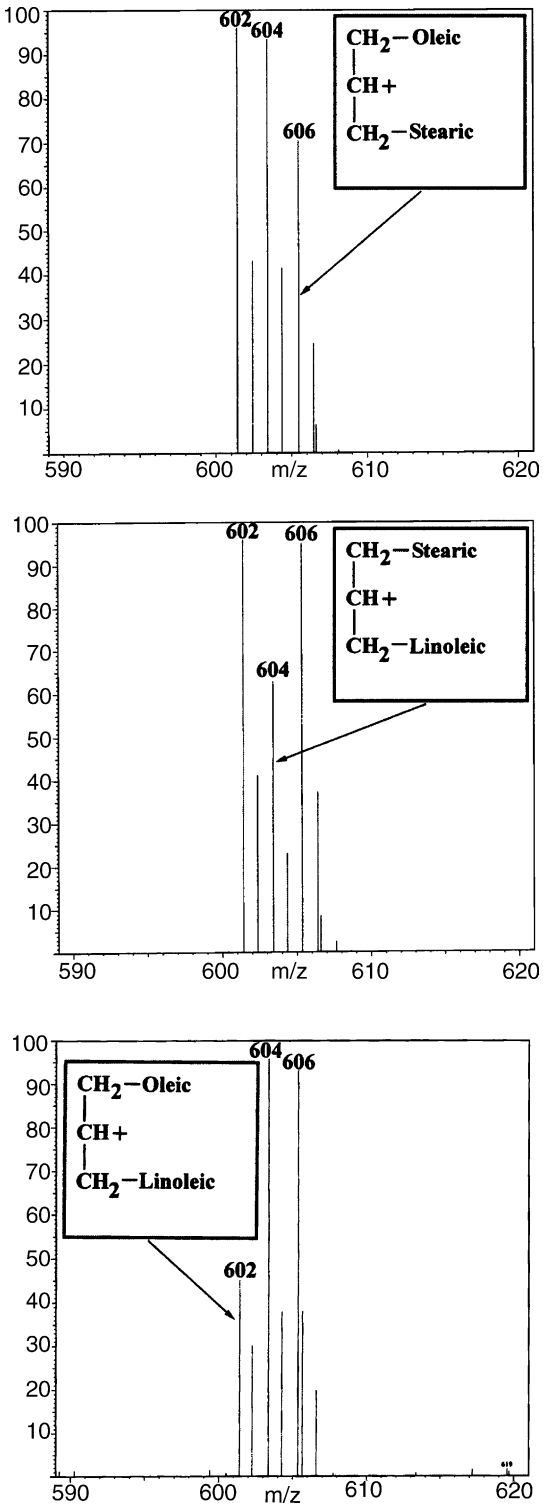

Fig. 7. APCI-MS spectra of SLO isomers.

pair of TAG isomers: *sn*-OLO and *sn*-OOL (fraction 6). Positional isomers, in fact, become more difficult to resolve when the DB value exceeds three and when the difference in the number of double bonds in the unsaturated FAs is only one [7]. Peak assignment was achieved following the above mentioned considerations. As was expected, from the

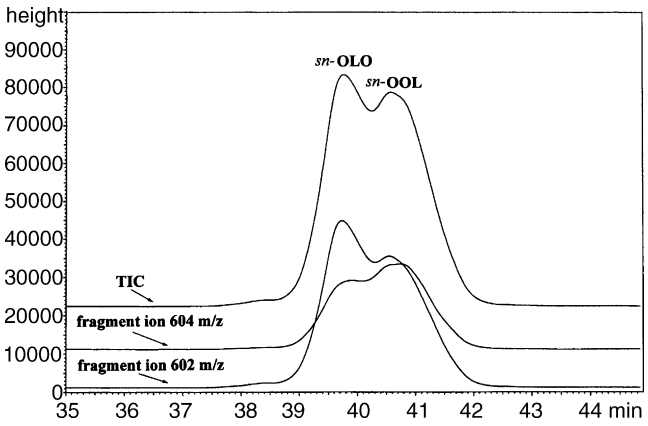

Fig. 8. Ag-HPLC–APCI-MS chromatogram of OLO/OOL isomers (TIC) and extracted chromatograms at *m*/*z* 602 and 604 (fraction 6).

MS extracted chromatograms at *m*/*z* 602 ($[OL]^+$) and 604 ($[OO]^+$), the $[OL]^+$ intensity is much higher for *sn*-OLO, while the $[OO]^+$ abundance shows a 2:1 ratio in favour of *sn*-OOL.

The results listed in Table 2 for the different rice oil TAGs are in accordance with the 1,3-random, 2-random distribution that is typical for vegetable oils [21]. In the case of TAGs containing both saturated and unsaturated fatty acids, the positional isomer with the unsaturated FA in the *sn*-2 position is always the most abundant. In particular, it is the only detected isomer when two diunsaturated fatty acids (DFAs) are present in the molecule, as was seen for PLL. If two saturated FAs (SFAs) and one DFA are combined, the ratio between the two isomers is about 85:15 as was observed for PLP and PLS. Approximately the same ratio is obtained when two SFAs are combined with a monounsaturated fatty acid (MFAs), as seen for POP and POS, where the isomer with the SFA in the *sn*-2 position ranged from 14 to 17%. This amount decreases to a 2–6% range when one SFA is associated with two MFAs, as can be observed for POO and SOO. For TAGs containing a SFA, a MFA and a DFA, such as PLO and SLO, the aforementioned isomer is present in a 3% quantity. In the case of OOL (one DFA, two MFAs), the positional isomer ratio *sn*-OLO/*sn*-OOL is 40:60. Whereas LLO is concerned, the relative isomer quantities differ greatly with a predominance of *sn*-LLO (86%) in respect to *sn*-LOL (14%). From the information reported in Tables 1 and 2 it has been calculated that the total amount of SFA in the *sn*-2 position is 3.5%.

In conclusion, the method here reported is easy to apply and can provide important information regarding TAG positional isomery in natural fats and oils. The combination of NARP and Ag-HPLC results very useful for the resolution of critical pairs, due to the different retention mechanism of TAGs under these two HPLC modes. The analysis of positional isomers under Ag-HPLC conditions was greatly improved by the use of a pre-separation step and also through APCI-MS detection. It must be noted, that the silver ion column analyses both on the initial sample and on the single

fractions presented problems regarding retention time reproducibilty. However, this had no negative effects on the analytical results obtained since MS detection was employed.

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