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Determination of diacylglycerol isomers in vegetable oils by solid-phase extraction followed by gas chromatography on a polar phase

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Abstract

Diacylglycerol (DG) isomers in vegetable oils were determined by several chromatographic techniques. The use of reversed-phase high-performance liquid chromatography on LC-18 under isocratic conditions was inappropriate, since no complete resolution of the 1,2- and 1,3-isomers appearing at low retention time was achieved. In addition, overlapping of some peaks, absence of peaks of the minor components and interferences by sterols were observed. On the other hand, the isolation of the polar fraction by solid-phase extraction (SPE) using a bonded diol phase and further analysis of the silyl derivatives by capillary GC on 65%-phenyl-methylsilicone, was carried out successfully. No interferences by other components were found and isomerisation by passing the DGs through the SPE column was negligible. The procedure is easy, fast and reproducible, allowing the quantitation and separation of DGs according to their carbon number, their isomeric structure (1,2 and 1,3) and the degree of unsaturation. The effect of unsaturation on the DG retention times depends on the number and arrangement of the double bonds in the molecule. Applications to some vegetable oils are shown.

Keywords: Vegetable oils; Oils; Solid-phase extraction; Diacylglycerols

1. Introduction

Diacylglycerols (DGs) are found in edible vegetable oils in low amounts (between 1 and 10%). They are formed as intermediate products in the biosynthesis of triacylglycerols (TGs), as well as by acidic and enzymatic hydrolysis of TGs during oil extraction, refining and storage. Simultaneously, isomerisation processes produce changes in the composition of the DGs. Knowledge of the quantity and composition of DGs is, therefore, of great interest for the evaluation of the quality of the oil and of the treatments to which the oil is subjected.

Determination of DGs in fats can be performed using two different methodologies: (i) Isolation of the DGs and further analysis; (ii) direct analysis of the sample using instrumental techniques.

Among the procedures included in the first group, the oldest referred to in the literature is the separation of the DGs by preparative thin-

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layer chromatography (TLC), transformation of the ester groups into hydroxamic acid and colorimetric quantitation of the ferric complex formed after the reaction with iron perchlorate [1]. Subsequently, capillary gas chromatography on a low-polarity phase (5%-phenyl-methylsilicone) is applied to the silyl derivatives of the DGs isolated by TLC [2,3].

Recently, a method was proposed in which the polar compounds of the oil were isolated by solid-phase extraction (NH_2 phase) and the total DGs quantitated by high-performance size-exclusion chromatography (HPSEC) using PLGEL columns of 100 and 50 Å connected in series, with a refractive index (RI) or light scattering detector [4].

The direct method, however, has been more widely accepted. Thus, direct injection of the silylated oils into a gas chromatograph equipped with a low-polarity capillary column (SE 54 or similar) is a general method [5,6], which was standardised by the IUPAC Commission on Oils, Fats and Derivatives [7]. This procedure allows separation according to carbon number and molecular structure (1,2- and 1,3-isomers).

The introduction of capillary columns with greater polarity and high thermal stability (65%-phenyl-methylsilicone), allowed better resolution of the DGs, separation being achieved on the basis of carbon number, 1,2- and 1,3-isomeric structure and the number of double bonds in the molecule [8]. Application of direct gas chromatographic methods to vegetable oils implies the simultaneous injection of the TGs present in the sample. For this reason, they are recommended for the analysis of DG concentrates and fats and oils with a high DG content [7].

Other authors used high-performance liquid chromatographic techniques for direct analysis. Thus, Cortesi et al. [9] used reversed-phase chromatography over bonded C_{18} columns equipped with a refractive index detector. These authors followed the IUPAC standardised method for the determination of TGs [10]. The oil dissolved in acetone is injected directly onto the chromatographic system, the DGs appear as small peaks at the beginning of the chromatogram and are separated according to their equivalent carbon number (ECN). The use of HPLC with silica gel columns and a light scattering detector for the direct analysis of olive oil hydrolysis products gave chromatograms showing only two peaks, corresponding to the 1,2- and 1,3-DGs [11].

Recently, the use of ^{13}C NMR spectroscopy has been proposed for the analysis of the oil [12], and ^{1}H NMR spectroscopy for the analysis of the products arising from the reaction between the DGs and trichloroacetylisocyanate [13]. These NMR methods are used for the rapid quantitative determination of the 1,2- and 1,3-isomers, but no differentiation between the components of each series is achieved.

In spite of the wide variety of analytical methods described in the literature, none of them are completely adequate for the analysis of DGs in vegetable oils. In the present study, we describe the development of a rapid and simple method for the determination of these compounds in such matrices, verifying its repeatability and the absence of interferences.

2. Experimental[1]

2.1. Materials

All reagents were of analytical-reagent grade, except HPLC eluents that were HPLC grade.

The DGs 1,3-dimiristin 1,2-dimiristoyl-*rac*-glycerol, 1,3-dipalmitin, 1,2-dipalmitoyl-*rac*-glycerol, 1,3-distearin, 1,2-distearoyl-*rac*-glycerol, 1,3-

[1] The following abbreviations for fatty acids are used: L = linoleic acid, *cis,cis*-9,12-octadecadienoic acid, $C_{18:2}$; Ln = linolenic acid, *cis,cis,cis*-9,12,15-octadecatrienoic acid, $C_{18:3}$; M = miristic acid, tetradecanoic acid, $C_{14:0}$; O = oleic acid, *cis*-9-octadecenoic acid, $C_{18:1}$; P = palmitic acid, hexadecanoic acid, $C_{16:0}$; P_o = palmitoleic acid, *cis*-9-hexadecenoic acid, $C_{16:1}$; S = stearic acid, octadecanoic acid, $C_{18:0}$. The DGs are represented with a numeric prefix followed by the symbols of the two acyl groups. The order of appearance of the symbols has no relationship with the position of acyl groups in the glycerol molecule. The 1,2 prefix includes all *sn*-1,2- and *sn*-2,3- diastereoisomeric DGs. In the heterogeneous DGs, the 1,3 prefix depicts the enantiomeric pairs, whereas the homogeneous DGs comprise a unique compound with no chiral centre.

diolein and 1,2-dioleoyl-*rac*-glycerol were obtained from SIGMA (St. Louis, MO, USA).

The solid-phase extraction (SPE) columns (3 ml) packed with activated silica, and with the bonded phases diol and–NH_2 were from Supelco (Bellefonte, PA, USA). Silica gel for column chromatography was prepared by heating silica gel 60, particle size 0.063–0.200 mm (Merck, Darmstadt, Germany), at 160°C for 2 h and allowing it to cool in a desiccator. Then 5% (w/w) of water was added and shaken for 30 min. Fluorisil 100–200 mesh and aluminium type 5016 A (basic) activity stage I and aluminium oxide type 507 C (neutral) activity stage I were purchased from Fluka (Buchs, Switzerland).

2.2. Samples

A crude palm oil and various refined vegetable oils, such as olive, palm, peanut, rapeseed, soybean, sunflower and high-oleic sunflower, were purchased from refining plants. Two virgin olive oils were used, fresh and old, corresponding to an oil just obtained and one which has been stored for one month at 40°C, respectively.

2.3. HPLC analysis

The reversed-phase HPLC separations were carried out in an HPLC system consisting of an HP 1050 pumping unit (Hewlett-Packard, Avondale, PA, USA) equipped with a Rheodyne injection valve, an oven and an HP 1047A refractive index (RI) detector. Separations were performed on a Lichrospher 100 RP-18 (4 μm) column (25 cm × 4 mm I.D.) maintained at 40°C, using acetone–acetonitrile (1:1, v/v) as mobile phase, at a constant flow of 0.8 ml/min. Aliquots (5 μl) of solutions in acetone (50 mg/ml) of standard DGs and oil polar fractions isolated by SPE (see below) were injected onto the chromatographic system.

2.4. SPE–GC analytical method

Reagents

An internal-standard solution of 1,3-dipalmitin (1 mg/ml) was used for the analysis of oils with a low content of palmitic acid (olive, peanut, rapeseed, soybean, sunflower and high-oleic sunflower oils). For palm oil, an internal-standard solution of 1,3-dimiristin was used. Each solution was prepared by dissolving 25 mg of standard in a mixture of 2.5 ml of methylene chloride and 0.25 ml of diethyl ether. The solution was then diluted with hexane up to a volume of 25 ml.

Silylating reagent was prepared by adding 3 ml of hexamethyldisilazane and 1 ml of trimethylchlorosilane to 9 ml of anhydrous pyridine.

SPE isolation

A diol bonded-phase SPE column was placed in a vacuum elution apparatus and washed under vacuum with 4 ml of hexane. The vacuum was released immediately after the wash to prevent the column becoming dry. Standard solution (200 μl) and 500 μl of oil solution in *n*-hexane (0.2 mg/ml) were applied to the column and the solvent was pulled through, leaving the standard and the sample on the column. An amount of 6 ml of hexane–methylene chloride–ethyl ether (89:10:1, v/v) was applied to the column and a first fraction was collected. Subsequently, 4 ml of chloroform–methanol (2:1, v/v) was applied to the column and a second fraction collected. In order to check the separation of components, both fractions were analysed by TLC as described below. The second fraction was evaporated to dryness in a rotary evaporator under reduced pressure and the residue was treated with 200 μl of the silylating reagent and left at room temperature for a few minutes. Of the latter solution 1 μl was injected onto the gas chromatographic system.

The SPE column was washed with 3 ml of methanol and 3 ml of acetone consecutively and then dried by passing nitrogen through it. The columns can be used at least six times.

GC analysis

Chromatographic analysis of DGs was performed using a Chrompack (Middelburg, Netherlands) CP9000 gas chromatograph fitted with a flame ionisation detector and a split injection system (split ratio 1:30). Separations were carried out on a high-temperature fused-

silica capillary column (25 m × 0.25 mm I.D.) coated with 65%-methyl-phenylsilicone, with a thickness of 0.1 μm (Quadrex, New Haven, CT, USA). The operating conditions were: oven temperature 270°C for 4 min, then increased at 1°C/min to 295°C and held at 295°C for 1 min; injector temperature, 300°C; detector temperature, 325°C; and carrier gas hydrogen at 100 kPa.

Data acquisition and processing were carried out by a Chrom-Card Data System (Fisons, Altrincham, UK).

Recovery

Recovery data were calculated by comparing the results of 1,3-PP determinations on an olive oil matrix with those obtained from the GC analysis of silylated 1,3-PP. Six replicates were determined in each case.

2.5. TLC analysis

For the analysis of the two fractions separated by SPE, TLC on silica gel 60 (20 × 20 cm glass plates, 0.25 mm layer thickness; Merck) was applied. The plates were eluted with hexane–diethyl ether–acetic acid (79:20:1, v/v) and developed by spraying with H_2SO_4–water (1:1, v/v) and heating.

For the separation of 1,2- and 1,3-DGs, the second fraction eluted from the SPE was placed on the silica gel plate and eluted as above. Both ends of the plate were developed with iodine vapours, the two DG bands scraped off separately and the silica gel powder extracted with chloroform.

3. Results and discussion

3.1. HPLC analysis

Of all the methods described in the Introduction, reversed-phase HPLC analysis with a refractive index detector is the one that seems to offer the best prospects, because it is a direct method that performs simultaneous analysis of DGs and TGs [9]. To verify the chromatographic behaviour of the 1,2- and 1,3-isomers, standards and DG fractions isolated from sunflower, palm and fresh olive oils were analysed. The flow-rate used was lower than that described in the original method (0.8 ml/min instead of 1.2 ml/min) in order to achieve a better peak resolution. As reported by other authors [14], only a very slight separation between 1,3- and 1,2-isomers was achieved, the two LL isomers appearing as a broad single peak, and DGs with higher retention times yielding partially resolved peaks for each pair of isomers (Fig. 1). In the fresh olive oil, where the major components are in the 1,2-isomeric series, quantitation of the small amounts of 1,3-isomers was impossible. Finally, overlapping between DGs with different ECN and isomeric structure and interferences due to the sterols were observed. These facts, together with the interferences produced by the hydrocarbons reported in the original work, demonstrate the unsuitability of this HPLC technique for the analysis of DGs in vegetable oils.

3.2. SPE isolation

Another appropriate technique for the separation of DGs is gas chromatography using a capillary column. However, the direct injection of a vegetable oil is not advisable because a large amount of TGs would be injected onto the column, and these would have to be eluted using high oven temperatures over a long period. This would significantly increase the analysis time and reduce the life time of the column. For this reason, it was necessary to isolate the DGs prior to the chromatographic analysis.

Solid-phase extraction (SPE) in amino-phase columns has been described for the separation of fatty compounds in biological extracts [15]. However, the application of this method to the isolation of DGs from oils yielded DGs contaminated with TGs [4]. In addition, assays with 1,3-PP and 1,2-PP standards showed a significant isomerisation to 1,2-PP and 1,3-PP, respectively. Different columns with bonded phases and adsorbents were, therefore, tested. The results shown in Table 1 demonstrate that the amino-phase column produces a high rate of isomerisation for both isomers, an equilibrium ratio of

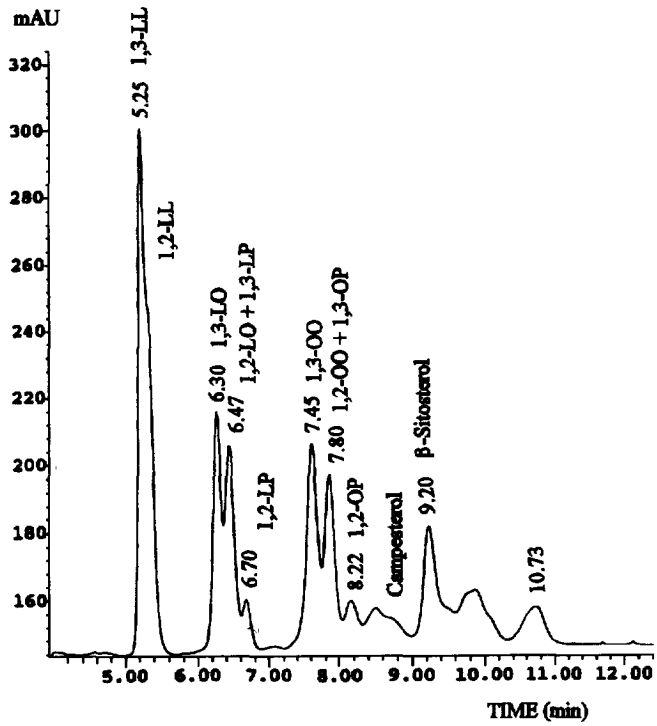

Fig. 1. Reversed-phase HPLC–RI chromatogram of the polar fraction isolated from a refined sunflower oil.

1,3/1,2 ≈ 2 being reached. On the other hand, the diol-phase and deactivated silica gel columns showed negligible isomerisation. The passage of an admixture of PP and oleic acid through diol-phase columns proved that the presence of free fatty acids does not produce isomerisation during the isolation of the corresponding fraction. Eluent and volume optimisation were established by checking olive oil fractions by TLC, so that hydrocarbons, waxes and TGs were eluted in the first fraction and aliphatic alcohols, triterpenic alcohols, methyl sterols, sterols and mono- and diacylglycerols in the second.

Table 1
Isomerisation rate (%) of PP isomers through SPE-bonded phases and adsorbents, using chloroform–methanol (2:1, v/v) as desorbent

Phase	Isomerisation (%)	
	1,3→1,2	1,2→1,3
Silica gel + 5% water	N	N
SPE diol	N	N
SPE activated silica	3.7	4.3
Florisil	11.4	21.5
Neutral alumina (grade 3)	25.3	61.4
Basic alumina (grade 3)	33.6	63.9
SPE amino	35.0	63.5

N = negligible.

3.3. GC analysis

For gas chromatographic analysis, silyl derivatives of DGs were prepared by treatment with a mixture of hexamethyldisilazane and trimethylchlorosilane in pyridine [16].

A column coated with 65%-methylphenylsilicone was considered the most appropriate for the gas chromatographic analysis, because it can be used at a high temperature (350°C) with very little bleeding and allows separation of the DGs on the basis of their

carbon number, the 1,2- and 1,3-isomeric structure and the number of double bonds [8].

Most of the vegetable oils (olive, rapeseed, sunflower, peanut, soybean) do not contain any dipalmitine; therefore, 1,3-PP was used as an internal standard. In the oils such as palm oil with high contents in P, a standard with a lower carbon number, like 1,3-MM, was used.

In the chromatograms of fresh olive and crude palm oil (Figs. 2 and 3), no interference from other compounds present in the injected fraction was found, since monoacylglycerols and alkanols appear in the solvent peak tail and the triterpenic alcohols, methyl sterols and sterols at low retention times. Furthermore, the β-sitosterol and the 24-methylencycloartanol peaks can be distinguished. If, as a consequence of a bad SPE separation, the TGs are eluted in the second fraction, they should appear at retention times of 30 min or higher.

The assignment of the chromatographic peaks was done by means of standards of 1,2- and 1,3-isomers of PP, OO and SS, which allowed the carbon number of the components associated with each peak group to be deduced, as well as the difference between the retention times of the 1,2- and 1,3-isomers. In a good-quality fresh olive oil, the 1,2-DGs are predominant [1,6] whereas in refined olive oil the 1,3-isomers are more abundant, the 1,3/1,2 ratio being approximately 2 [8]. Furthermore, the major peaks were assigned to DGs constituting the most probable combinations of the fatty acids present in the oil. The shifting of the retention times to higher values due to the unsaturation rate allowed the identification to be completed. Finally, the identifications were confirmed by separately analysing the 1,2- and 1,3-isomer fractions isolated from the oils by TLC on silica gel. Using these approaches, it was possible to identify most of the

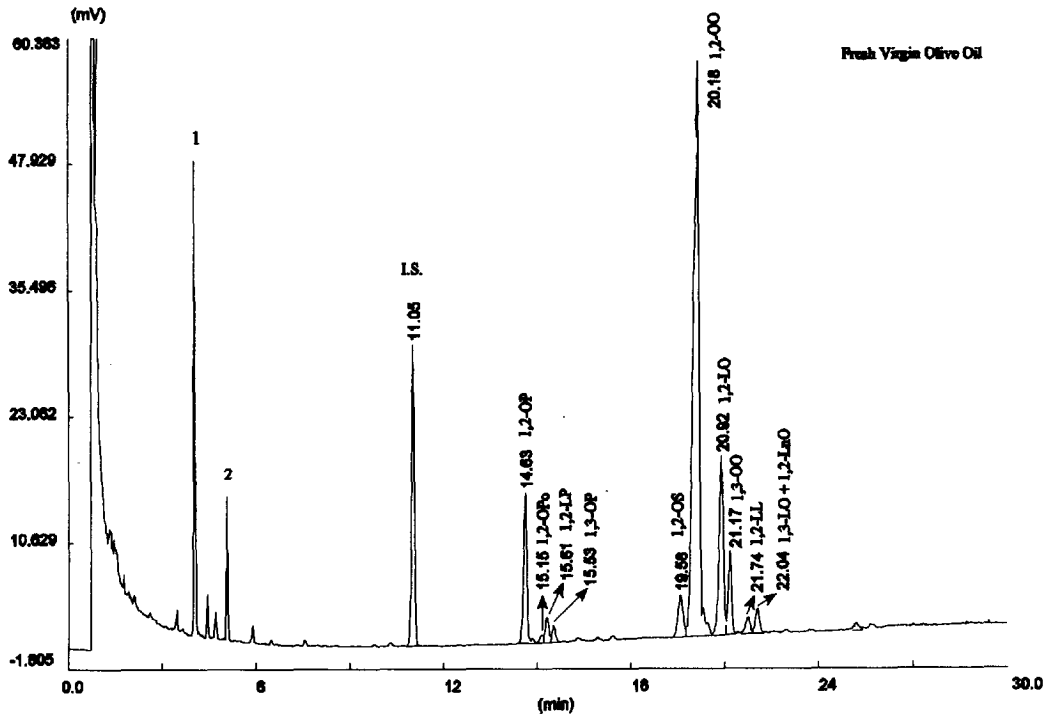

Fig. 2. Gas chromatogram of the polar fraction isolated from a fresh virgin olive oil. Peaks: 1 = β-sitosterol, 2 = 24-methylencycloartanol.

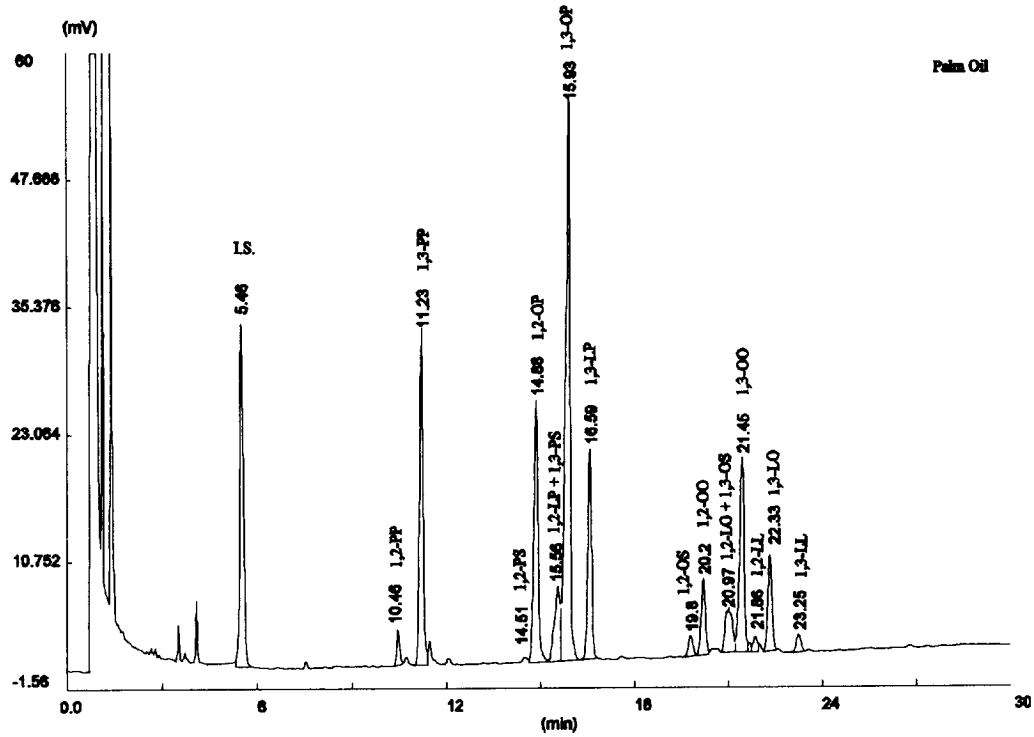

Fig. 3. Gas chromatogram of the polar fraction isolated from a crude palm oil.

peaks in the chromatograms of fresh olive oil (Fig. 2), crude palm oil (Fig. 3) and refined soybean oil (Fig. 4).

The equivalent carbon number (ECN) of the unsaturated DGs was calculated just as the equivalent carbon length of the fatty acids methyl esters [17]. The data shown in Table 2 indicate that one unsaturation produces an increased ECN, and this increases as the degree of unsaturation in the acyl group increases. Therefore, the retention time is related to the arrangement of double bonds in the molecule. Isomers having the same double-bond number show greater ECNs the higher the unsaturation that exists in one acyl radical (In Fig. 4, compare retention times of LnO and LL, and LS and OO.) Using the chromatographic conditions described in the Experimental section, the ECN of the elements of each isomeric series can be approximately calculated using the formula

$$ECN \approx CN + 0.15n_1 + 0.22n_2 + 0.29n_3$$

where CN is the number of carbons of the acyl groups, n_1 the number of double bonds in monounsaturated acyl groups, n_2 the number of double bonds in linoleoyl groups and n_3 the number of double bonds in linolenoyl groups, and the relationship between homologues of the 1,2- and 1,3-series being

$$(ECN)_{1,3} = (ECN)_{1,2} + 0.32.$$

3.4. Recovery and repeatability of SPE–GC

The mean area of the 1,3-PP peaks obtained with the six replicates for olive oil spiked with this compound and that corresponding to the direct analysis of the silyl derivative are shown in Table 3. It can be seen that the analytical technique has a good repeatability (R.S.D.$_r \approx$

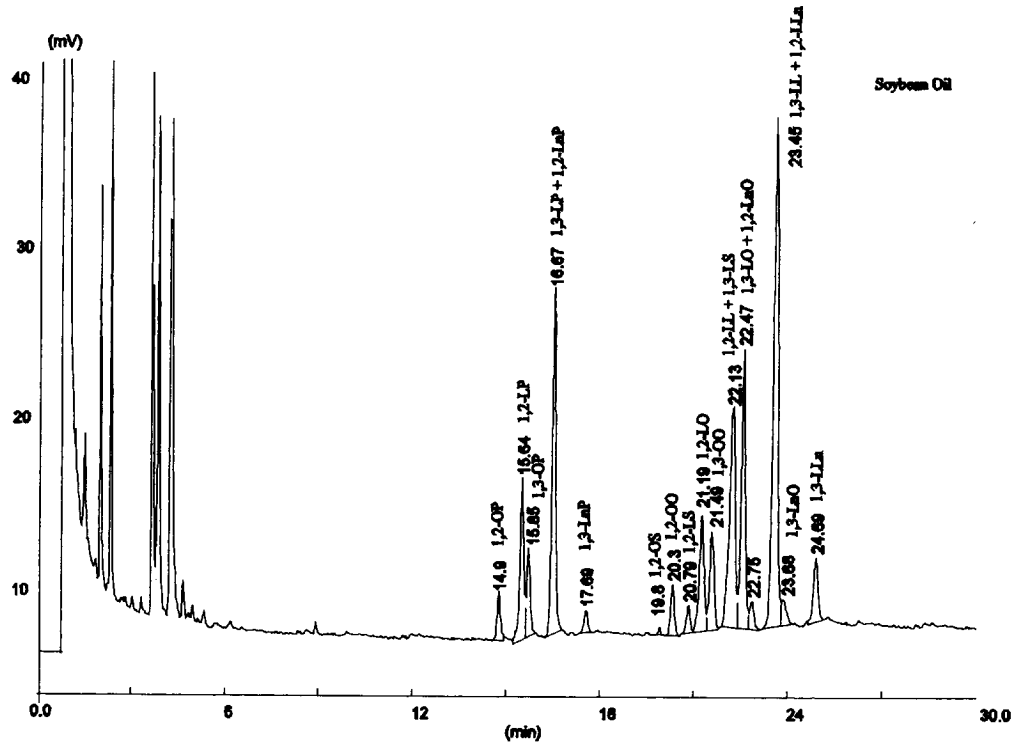

Fig. 4. Gas chromatogram of the polar fraction isolated from a refined soybean oil.

Table 2
Equivalent carbon number (ECN) shifts due to a double-bond gain in the acyl group

Number of double bonds	Diacylglycerol		ΔECN
	Initial	Final	
1	PS	OP	0.16
	SS	OS	0.16
2	PS	OP_o	0.29
	SS	OO	0.31
	PS	LP	0.49
	SS	LS	0.48
3	SS	LO	0.60
	PS	LnP	0.90
4	SS	LL	0.88
	SS	LnO	0.98
5	SS	LLn	1.25

Table 3
Evaluation of the recovery with the SPE–GC method. GC peak areas of 1,3-dipalmitin obtained by direct injection and by isolation from a mixture with olive oil

	1,3-PP + oil (SPE–GC analysis)	1,3-PP (direct analysis)
Mean area ($n = 6$)	436 076	419 447
$S.D._r$	13 161	11 874
$R.S.D._r$ (%)	3.02	2.83

3%) and the recovery is complete (104%). This demonstrates that the separation obtained with SPE diol columns is adequate.

The repeatability of the method was studied using a virgin olive oil sample and a new SPE column for each replicate and a crude palm oil sample using always the same column. Results are shown in Tables 4 and 5, respectively, and indicate a good repeatability for both assays.

Table 4
Repeatability data of diacylglycerol analysis by SPE–GC of a virgin olive oil using a new column each time

	Diacylglycerols (%)									Total (mg/g)
	OP		LP	OS	OO		LO		LL	
	1,2-	1,3-	1,2-	1,2-	1,2-	1,3-	1,2-	1,3- [a]	1,2-	
Mean ($n = 6$)	9.47	0.86	1.63	3.20	61.50	1.57	18.10	2.10	1.11	13.41
S.D.$_r$	0.075	0.053	0.016	0.025	0.449	0.051	0.174	0.054	0.015	0.208
R.S.D.$_r$ (%)	0.79	6.20	0.98	2.25	0.69	3.23	0.96	2.59	1.12	1.51

[a] There is a contribution of 1,2-LnO.

3.5. Applications

The analysis of a fresh virgin olive oil (Fig. 2) showed very high proportions of the 1,2-isomers (1,3/1,2 ratio, $R < 0.1$). This virgin olive oil stored at 40°C for a month showed a minor increase in total DGs (from 13.4 to 14.0 mg/g) and R near to 2 for each pair of DG isomers. Crude palm oil (Fig. 3) with an R-value higher than 2 ($R_{PP} = 16.6$) was heated at 110°C for 3 h and samples were taken every half hour. At the end of two hours, all R-values were near to 2, and remained constant thereafter. These facts suggest that the isomerisation equilibrium is reached with mild heating, the R-value being close to 2. This ratio should, therefore, pertain in recently refined oils since they are subjected to high temperatures (200°C approximately) during refining.

In the refined vegetable oils (olive, palm, peanut, rapeseed, soybean, sunflower and high oleic sunflower), R-values range from 1.2 to 2.7, but some exceptions such as PP in palm oil ($R_{PP} = 3.7$) and OP in soybean oil ($R_{OP} = 4.2$) (Fig. 4) were observed. This can be explained bearing in mind that the isomeric proportion of the hydrolysis products depends on the isomeric structure of the TGs. Thus, the palm oil that contains 1,3-dipalmitoyl-2-oleyl-glycerol but no 1,2-dipalmitoyl-3-oleyl-glycerol, gives very high concentrations of 1,3-PP. Therefore, if during storage the hydrolysis predominates over isomerisation, 1,3/1,2 ratios other than 2 can be expected.

In conclusion, the isolation of the polar fraction by SPE diol columns and the subsequent analysis of their silyl derivatives by GC with a capillary column coated with a polar phase, is a

Table 5
Repeatability data of diacylglycerol analysis by SPE–GC of a crude palm oil using the same column each time

	Diacylglycerols (%)										Total (mg/g)
	PP		OP		LP		OO		LO		
	1,2-	1,3-	1,2-	1,3-	1,2- [a]	1,3-	1,2-	1,3-	1,2- [b]	1,3-	
Mean ($n = 6$)	0.91	15.02	11.18	32.12	2.50	9.43	4.22	14.31	2.74	5.57	95.42
S.D.$_r$	0.083	0.178	0.604	0.199	0.219	0.205	0.207	0.351	0.269	0.086	1.389
R.S.D.$_r$ (%)	9.51	1.18	5.35	0.61	8.53	2.15	4.85	2.42	9.67	1.53	1.46

[a] There is a contribution of 1,3-PS.
[b] There is a contribution of 1,3-OS.

rapid, simple and reproducible analytical method for the identification and quantitation of DGs in vegetable oils.

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