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Rapid, quantitative determination of polar compounds in fats and oils by solid-phase extraction and size-exclusion chromatography using monostearin as internal standard.

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Abstract

A rapid and simple method was developed for quantitation of polar compounds in fats and oils using monostearin as internal standard. Starting from 50 mg of oil sample, polar compounds were obtained by solid-phase extraction (silica cartridges) and subsequently separated by high-performance size-exclusion chromatography into triglyceride polymers, triglyceride dimers, oxidized triglyceride monomers, diglycerides, internal standard and fatty acids. Quantitation of total polar compounds was achieved through the internal standard method and then amounts of each group of compounds could be calculated. A pool of polar compounds was used to check linearity, precision and accuracy of the method, as well as the solid-phase extraction recovery. The procedure was applied to samples with different content of polar compounds and good quantitative results were obtained, especially for samples of low alteration level.

Keywords: Oils; Fats; Polar compounds; Solid-phase extraction; Monostearin; Triglycerides; Diglycerides; Fatty acids

1. Introduction

Quantitation of polar compounds has been the most useful method for quality evaluation of frying fats since its development in the late seventies [1,2]. The basis of the method is the separation of 1 g of fat in a silica column to obtain two fractions of different polarity which can be determined gravimetrically. The polar fraction includes all the alteration products and, in consequence, the higher the polar fraction, the lower the quality of the frying fat. Collaborative tests conducted by the International Union of Pure and Applied Chemists (IUPAC) demonstrated that the method is simple, exact and reproducible and, moreover, the efficacy of the separation can be easily checked by thin-layer chromatography (TLC) [3]. Nevertheless, it is a silica, solvent and time-consuming technique.

Separation of polar compounds by exclusion chromatography was later proposed [4] as a complementary evaluation of the main groups of compounds differing in molecular mass, i.e., polymers, oxidized monomers and diglycerides, representative of thermal, oxidative and hydrolytic alteration, respectively. This methodology has not only been applied to the area of frying fats [5–8] but it has also contributed to the development of an extended number of applications in oxidation studies [9–11], quality control of

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refined oils [12,13] and characterization of virgin oils [14–17].

Solid-phase extraction (SPE) has been proven of utility for the separation of polar compounds either by using silica [18–20] or amino-bound cartridges [21]. Nevertheless, in order to obtain a good efficiency of the separation, it is necessary to start from milligram quantities of sample. Under these circumstances, gravimetric determination would not provide similar levels of accuracy and precision, especially in samples with low levels of polar compounds.

The objective achieved in the present study was the development of a rapid, simple method for the determination of polar compounds by using monostearin as internal standard (I.S.). In a first step, polar compounds were obtained by SPE starting from 50 mg of oil and quantitative results were subsequently attained through exclusion chromatography analysis. Thus, the overall analysis enables quantitation of total polar compounds and their distribution.

2. Experimental

2.1. Chemicals

Silica cartridges for SPE were Sep-Pak columns supplied by Waters (Milford, MA, USA). TLC plates (Silica Gel 60) and silica for column chromatography (Silica Gel 60, particle size 0.063–0.200 mm) were purchased from Merck (Darmstadt, Germany). Monostearin (purity >99%), used as I.S., was obtained from Nu-Chek-Prep (Elysian, MN, USA). Tetrahydrofuran was HPLC grade and other solvents, namely, light petroleum (b.p. 40–60°C), diethyl ether, diisopropyl ether and acetone, were of analytical grade.

2.2. Samples

A pool of polar compounds obtained from refined and used frying oils by adsorption chromatography [3] was used to check linearity, accuracy, precision and SPE recovery. Ten samples of fats and oils of different origins and variable levels of polar compounds, ranging from 3 to 35%, were selected to apply the proposed methodology.

2.3. Analytical procedure

2.3.1. Internal standard solution

A stock solution of the I.S. (5 mg/ml) was prepared by dissolving 500 mg of monostearin in 100 ml of diisopropyl ether.

2.3.2. Sample preparation

A 250-mg mass of oil was weighed into a 10 ml volumetric flask. A 1-ml volume of the I.S. solution was added and the flask was filled up to the mark with light petroleum.

2.3.3. Solid-phase extraction

A vacuum manifold (Supelco, Bellefonte, PA, USA) was used for all the experiments. Sep-Pak silica columns (1 g) were conditioned before use by rinsing with 10 ml of initial mobile phase (light petroleum–diethyl ether, 90:10) taking care to prevent the columns from drying out. Following the conditioning step, 2 ml of the oil solution, thus containing 50 mg of oil and 1 mg of I.S., were placed on the column and the solvent was passed through while the sample was retained on the column. Next, the non-polar fraction was eluted with 15 ml of light petroleum–diethyl ether (90:10). A second fraction containing polar compounds and the I.S. was eluted with 15 ml of diethyl ether. Non-polar and polar fractions were evaporated under reduced pressure and redissolved in 1 ml of tetrahydrofuran for further analyses by TLC and high-performance size-exclusion chromatography (HPSEC). The samples were analyzed in triplicate by reusing the same Sep-Pak column. Before reuse, the column was rinsed with 5 ml of acetone and then dried by passing nitrogen through it.

2.3.4. Thin layer-chromatography

Efficiency of the separation of non-polar and polar fractions by SPE has to be checked necessarily as it is essential to achieve accurate quantitative results. Thus, separation of fractions was routinely monitored by TLC, using small plates of Silica Gel 60 (5×10 cm aluminum plates, 0.25 mm thickness). Plates were deliberately overloaded, eluted with light petroleum–diethyl ether–acetic acid (80:20:1) and visualized with iodine vapours. A neat separation between the two fractions is normally found.

2.3.5. High-performance size-exclusion chromatography

Fractions of polar compounds were analyzed by HPSEC in a Konik 500A chromatograph (Konik, Barcelona, Spain) with a 10-μl sample loop. A refractive index detector (Hewlett-Packard, Pittsburgh, PA, USA), one 100 Å and one 500 Å Ultrastyragel column (Waters Associates, Milford, MA, USA) connected in series operated at 35°C. The columns were 25 cm×0.77 cm I.D., packed with a porous, highly cross-linked styrene–divinylbenzene copolymer (<10 μm). High-performance liquid chromatography grade tetrahydrofuran served as the mobile phase with a flow of 1 ml/min.

Quantitation of total polar compounds (PC% on sample) was achieved through the I.S. method, as follows:

$$PC\% = \frac{(\Sigma A - A_{I.S.}) \times c_{I.S.}}{A_{I.S.} \times c_{oil}} \times 100$$

where, ΣA is the sum of areas of all peaks, $A_{I.S.}$ is the peak area of the I.S., $c_{I.S.}$ is the concentration of I.S. in the starting sample solution (mg/ml) and c_{oil} is the concentration of oil in the starting sample solution (mg/ml)

Amounts of each group of compounds, i.e., triglyceride polymers, triglyceride dimers, oxidized triglyceride monomers, diglycerides and fatty acids, were calculated from the individual peak areas and percentage of total polar compounds, assuming equal response factors [4].

2.4. Linearity, precision and accuracy

The pool of polar compounds previously defined in Section 2.2. was used. Solutions containing increasing concentrations of polar compounds in tetrahydrofuran, ranging from 2 to 20 mg/ml, and a fixed concentration of I.S. (1 mg/ml) were prepared and analyzed by HPSEC. Linearity was checked by performing linear regression analyses of peak-area ratio polar compounds/I.S. vs. polar compound concentrations.

Precision was determined by replicate analysis ($n=3$) and was expressed as the relative standard deviation (R.S.D.%). Accuracy was calculated based on the differences between the mean calculated and the concentrations added, and expressed as percentage found.

2.5. Solid-phase extraction recovery

To determine the extraction efficiency, samples of polar compounds containing 2 and 20 mg/ml of polar compounds and 1 mg/ml of I.S. in light petroleum–diethyl ether (90:10) were analyzed as outlined in Section 2.3. by SPE and HPSEC. Recovery was calculated by comparing the results obtained with those from direct HPSEC injection.

2.6. Determination of total polar compounds by IUPAC Standard Method

Total polar compounds were also determined gravimetrically according to the IUPAC Standard Method [3]. Briefly, starting from 1 g of fat, nonpolar and polar fractions were separated by silica gel column chromatography using 150 ml of light petroleum–diethyl ether (87:13) and 150 ml diethyl ether as elution systems, respectively.

3. Results and discussion

A representative chromatogram of the separation obtained by exclusion chromatography is shown in Fig. 1, wherein monostearin was added as I.S. (filled peak). This chromatogram corresponds to the pooled sample of polar compounds used to check linearity, accuracy and precision. Monostearin was used as I.S. given that monoglycerides are normally in negligible, not detectable amounts in fats and oils. As can be observed, the peaks resolved, eluting by inverse order of molecular mass, were triglyceride polymers, triglyceride dimers, oxidized triglyceride monomers, diglycerides, monoglycerides and fatty acids, this last peak also including polar unsaponifiable fraction. The analysis requires a total run time of just 15 min, after which quantitation of the overall groups of compounds can be attained. In cases where a better resolution is required, additional columns can be

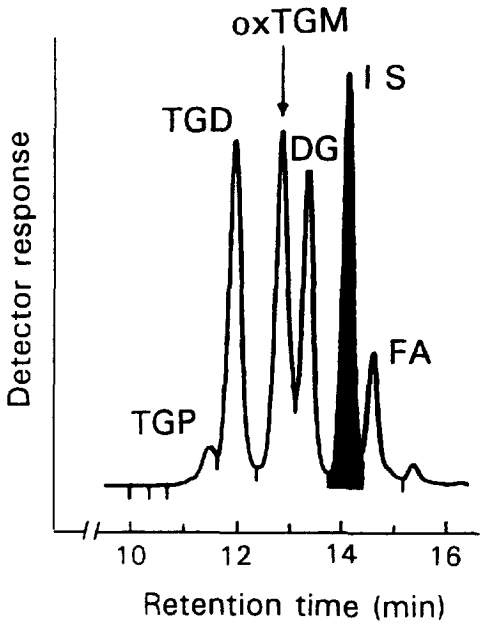

Fig. 1. High-performance size-exclusion chromatogram of polar compounds, with monostearin added as internal standard (I.S.). Retention times: 11.5 min, triglyceride polymers (TGP); 12.0 min, triglyceride dimers (TGD); 12.9 min, oxidized triglyceride monomers (oxTGM); 13.4 min, diglycerides (DG); 14.1 min, I.S. and 14.6 min, fatty acids (FA).

used. One more 100 Å column would improve separation in the low-molecular-mass range while one more 500 Å column would result in better efficiency of separation in the range of high-molecular-mass. Nevertheless, increasing the number of columns would also bring about considerable drawbacks, such as higher solvent consumption, longer total run times and higher pressure. On the other hand, it is important to check the efficacy of the SPE separation prior to the HPSEC analysis by TLC, as indicated in Section 2, since it is the only proof of an efficient elimination of nonpolar triglycerides which otherwise would elute at the same retention time as that of oxidized triglyceride monomers.

Polar compounds in fats and oils are very variable both in total amount and composition depending on the origin of the samples. Normally, expected polar compound percentages range from 3 to 30%, lower values corresponding to virgin and refined oils while higher being typical of used frying fats and oils. Thus, the concentrations selected for checking linearity, accuracy and precision were 20, 15, 10, 7.5, 5, 3 and 2 mg/ml of polar compounds (in all cases also containing 1 mg/ml of I.S.), corresponding to oils with polar compound levels up to 40%, according to the proposed procedure starting from 50 mg of oil.

The response of total polar compounds was checked in the range of application for this analysis. As expected for refractive index detection, the response was linear in the range of polar compounds injected (from 2 to 20 mg/ml). The linear regression equation for polar compounds was $y=1.03x-0.08$ ($n=21$). The regression coefficient was 0.998 and the standard errors for regression, intercept and slope were 0.433, 0.167 and 0.015, respectively. It is interesting to note that 95% confidence limits for the slope (0.99–1.06) included the value 1 which indicate that response factors for polar compounds and I.S. were similar.

Accuracy and precision results are given in Table 1, where found values (%) have been obtained by applying the same response factors for polar compounds and the I.S.. Precision, expressed as the R.S.D.%, ranged from 3.0 to 5.7% while accuracy, expressed as found amount (%) ranged from 100 to 103.3%. As can be observed, R.S.D. were greater for higher concentrations. This is due to the low proportion of the I.S. in the samples with high concentrations of polar compounds since a small variation in the percentage of I.S. in replicated chromatograms would originate a considerable variation in the polar compounds quantitated. The opposite would occur when the percentage of I.S. is high.

Given that a maximum number of 5 peaks corre-

Table 1
Accuracy and precision of polar compounds determination

Added (mg/ml)	Found (%)	R.S.D.[a] (%)
20.0	103.0	4.7
15.0	101.3	4.5
10.0	102.0	3.2
7.5	100.0	3.5
5.0	102.0	5.7
3.0	103.3	4.8
2.0	100.0	3.0

[a]R.S.D.% = (Standard deviation/Mean found) × 100
$n=3$

sponding to the different groups of polar compounds are expected, a minimum value of 10% of the total area for the peak corresponding to the I.S. is recommended. Thus, for samples containing very large amounts of polar compounds, a higher dilution of the sample and a higher amount of I.S. added would give rise to more reproducible results.

Excellent results were found for SPE recovery. The general procedure was applied to the samples of polar compounds at the highest and lowest concentration (2 and 20 mg/ml). Mean values for triplicates were 100 and 102%. In both cases, 1 ml of sample solution containing 1 mg of I.S. was applied to the cartridge. The entire sample was recovered in the polar fraction and no differences were found between results obtained by direct injection and after SPE. This means that recovery was quantitative for both polar compounds and I.S. In both cases, 10 ml of diethyl ether were sufficient to elute the polar fraction, although an excess of 5 ml was considered convenient.

Table 2 shows results of the application of the procedure to ten samples with different contents of polar compounds, ranging from 3 to 35%. Polar compounds were also determined in triplicate by the classical methodology starting from 1 g of fat [3]. Results obtained are presented in Table 3. There were no significant differences between the mean values obtained by the two different procedures, although, as previously commented for Table 2, the lower the polar compound concentration, the lower the standard deviation. In view of these results, it seems that quantitation using I.S. would be especially adequate for samples with low level of polar compounds, otherwise giving higher R.S.D.% by gravimetric determination.

Table 2
Quantitative determination of polar compounds (%, w/w) in fats and oils of different origin

Sample	Internal standard method		Gravimetric determination	
	Mean	R.S.D.(%)[a]	Mean	R.S.D.(%)
1	3.7	12.1	3.5	21.1
2	4.8	7.3	5.1	12.0
3	6.7	7.3	7.1	9.0
4	7.5	4.0	7.8	10.0
5	13.0	4.2	13.6	5.1
6	18.5	3.5	18.0	4.8
7	20.1	4.6	20.7	4.0
8	24.3	3.1	25.2	1.9
9	25.4	2.4	25.1	1.8
10	34.9	3.2	34.6	2.3

[a] R.S.D.% = (Standard deviation/Mean found) × 100
$n = 3$

Table 3
Distribution of polar compounds by HPSEC in fats and oils of different origin (mg/g of oil)

Sample	TGP	TGD	oxTGM	DG	FA
1	–	–	5	23	10
2	–	9	19	15	5
3	–	8	39	14	6
4	2	19	25	21	8
5	–	14	30	78	9
6	23	62	63	26	11
7	25	72	80	17	7
8	35	107	81	12	8
9	64	84	57	42	6
10	113	114	68	42	13

Abbreviations: TGP, triglyceride polymers; TGD, triglyceride dimers; oxTGM, oxidized triglyceride monomers; DG, diglycerides; FA, fatty acids.
Results are means of three determinations.

Besides determination of total polar compounds, the method proposed here enables quantitation of the main groups of alteration compounds. Table 3 shows distribution of polar compounds in the ten samples analyzed (data for total polar compound contents in Table 2). Results are expressed as mg/g oil and were calculated from the percentages of each group of compounds in the chromatogram and the polar compound concentrations. R.S.D. for the different groups of compounds ranged between the following values: 0.9–8.5% for triglyceride polymers; 1.2–9.8% for triglyceride dimers; 2.0–17.4% for oxidized triglyceride monomers; 1.8–15.6% for diglycerides and 2.7–15.4% for fatty acids.

Samples 1, 2 and 3 correspond to a virgin olive oil, refined sunflower oil and soybean oil, respectively, the first one characterized by the absence of polymeric compounds. The other samples are used frying oils with different levels of alteration. Among them, sample 5 is a palm oil, of low alteration level (13% polar compounds), predominantly comprised of diglycerides. Samples 8 and 9, both around the limit established in different European countries for fat rejection (25% polar compounds), presented very

close levels of polar compounds but further analysis of polar compounds by HPSEC showed that distribution of alteration products differed clearly between them. For instance, the amount of triglyceride polymers in sample 9 was double that in sample 8. Finally, sample 10 was far beyond the discarding limit and presented very high amounts of dimers and polymers, accounting for approximately 20% of the total sample.

In conclusion, by using SPE, good quantitative results were obtained, particularly for samples of low alteration level. Among the advantages over the classical methodology, it is worthy to note that silica and solvents are drastically reduced. Also, less than one hour is required to carry out the complete analytical procedure, including quantitation of total level and distribution of polar compounds. The methodology proposed is being currently applied to oxidation studies, in order to follow-up the oxidative process during early and advanced stages, and to determine the action of antioxidants.

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