Combination of adsorption and size-exclusion chromatography for the determination of fatty acid monomers, dimers and polymers

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ABSTRACT

A procedure based on adsorption and size-exclusion chromatography is proposed for evaluating fatty acid monomers, dimers and polymers in fats subjected to thermal oxidation. Starting from fatty acid methyl esters, two fractions of different polarity are obtained by silica column chromatography and submitted to a second separation by high-performance size-exclusion chromatography. The procedure allows the determination of unaltered fatty acids in addition to four groups of degradation compounds: non-polar fatty acid dimers, oxidized fatty acid monomers, polar fatty acid dimers and fatty acid polymers.

INTRODUCTION

The evaluation of minor components formed in heated or frying fats has always been difficult owing to the problems involved in their isolation and identification. However, advances in instrumental techniques have contributed to a better knowledge of the alterations that occur[1–3]. Several procedures for an objective evaluation of the alteration level in heated fats have been applied[4–8], including the determination of polar compounds proposed by IUPAC as a standard method for frying fats[4,9]. All these methods are based on the determination of the degradation compounds formed but give no idea about the structure or significance of such alteration compounds.

It is clear that a good definition of thermally oxidized fats must be based on the determination of alteration products, owing to their different chemical and nutritional significances. Some studies have contributed to this aim, but they are tedious and do not give quantitative results[10,11].

Previously, the possibilities of applying exclusion chromatography to the separation of alteration compounds produced during the heating of fats have been explored, and the direct analysis of fats[12–14] or their methyl ester derivatives[14–16] has been studied. The analysis is simple, as it is necessary only to dilute the fat or methyl esters in the appropriate solvent before the chromatographic determination, but the

resolution and detection of minor compounds are very poor owing to the presence of unaltered triglycerides or unaltered methyl esters as major components.

Application of the technique to concentrated fractions from fats has been reported[12,17,18] and the possibilities of the elucidation of the degradation mechanisms have been considered using a method based on the combination of column and high-performance size-exclusion chromatography[19]. The latter method allows the determination of the global alteration level of a fat sample and the contributions of the main groups of compounds coming from hydrolytic, oxidative and thermal degradations to the total alteration; it is also possible to apply the method to a better definition of non-heated fats.

In this paper, a similar combination of adsorption and high-performance size-exclusion chromatography (HPSEC) is proposed for the determination of fatty acid methyl ester derivatives, which allows the elucidation of oxidation and thermal alteration mechanisms. The procedure permits the differentiation and quantification of five groups of compounds: unaltered fatty acids, non-polar fatty acid dimers, oxidized fatty acid monomers, polar fatty acid dimers and fatty acid polymers.

EXPERIMENTAL

Standards

Methyl palmitate, stearate and oleate (Sigma, St. Louis, MO, U.S.A.) and two mixtures of pure fatty acid methyl esters obtained from edible fats and oils of different unsaturation levels were used as standards for the determination of unaltered fatty acids by HPSEC.

Non-polar dimeric methyl esters obtained from linoleic acid heated at 220°C under nitrogen, isolated by column chromatography and subsequently purified by thin-layer chromatography (TLC)[20] were used as standards for the determination of non-polar dimers by HPSEC.

Oil samples

The following oil samples were analysed: refined olive and sunflower oils, both without heating and after heating at 190 $\pm$ 2°C for 100 h; and pure olive oil, unheated and heated at 180 $\pm$ 2°C for 150 h, and a 1:1 mixture of the two.

Procedure

Analysis of the oils involved the following stages:

(1) Transesterification of the oil samples with sodium methoxide and hydrochloric acid–methanol and subsequent quantitative recovery of methyl esters[6].

(2) Separation of methyl esters by silica column chromatography, following the method proposed by IUPAC[9], with two modifications: (a) the use of hexane–diethyl ether (88:12) to elute the non-polar fraction, which permitted the recovery of both the unaltered fatty acids and non-polar dimers, and (b) final elution of the column with methanol to improve the recovery of the sample. Both fractions should be checked by TLC to confirm the efficiency of the separation. TLC was carried out on plates coated with silica gel 60 G (Merck, Darmstadt, F.R.G.). The plates were developed using hexane–diethyl ether–acetic acid (80:20:1, v/v/v) and the spots were revealed by exposure to iodine vapour.

(3) Separation of non-polar and polar fractions by HPSEC. The samples were analysed in a Konik (Barcelona, Spain) Model 500 A chromatograph with a 10-μl sample loop, a Hewlett-Packard 1037 A refractive index detector and two 100- and 500-Å Ultrastyragel columns (Water Assoc., Milford, MA, U.S.A.), connected in series and operated at 35°C. The columns were 25 cm × 0.77 cm I.D., packed with porous, highly cross-linked styrene–divinylbenzene copolymer (10 μm). HPLC-grade tetrahydrofuran served as the mobile phase with a flow-rate of 0.5 ml/min and the sample concentration was between 15 and 20 mg/ml in tetrahydrofuran.

RESULTS AND DISCUSSION

Fig. 1 shows the analytical scheme applied to a thermally oxidized fat, where the efficiency of the separation by TLC is also shown. The non-polar fraction consists of two groups of compounds: unaltered fatty acids, which can also be defined as non-polar monomers, and non-polar dimers. With the polar fraction, three peaks can be observed corresponding to oxidized monomers, oxidized or polar dimers and polymers.

The main difference from previous studies consists in the differentiation of five groups of compounds instead of three, owing to the first separation by column chromatography. It permits first the concentration of minor altered fatty acids and second the separation of monomers and dimers into two groups of different significance. Thus, monomers are separated into non-polar compounds and the monomers originating via oxidation, in the first and second fractions, respectively. For dimers, the non-polar compounds, eluted in the first fraction, are representative of thermal alteration as there is no oxygen involved in their formation[20]. On the another hand, oxidative dimers can be independently determined in the polar fraction.

The calibration graphs for the HPSEC determination of non-polar monomers and non-polar dimers are shown in Fig. 2. For non-polar monomers, three samples with different concentrations of the five standards indicated above were prepared. For non-polar dimers, 24 samples of different concentrations of standards were used, as representative of the non-polar dimers present in thermally oxidized oils.

The calibration graphs were plotted as peak area (y) against amount (x), according to the following equations: for non-polar monomers or unaltered fatty acids,

$$y = 0.104x - 0.252 \qquad (1)$$

and for non-polar dimers,

$$y = 0.115x - 0.121 \qquad (2)$$

For eqn. 1, $r = 0.9889$, $n = 15$ and concentration range = 1–18 mg/ml, and for eqn. 2, $r = 0.9905$, $n = 24$ and concentration range = 1–12 mg/ml.

Unaltered fatty acids and non-polar dimers were determined using these calibration graphs. As these two groups of compounds differ both in polarity and molecular weight, it is not difficult to prepare representative standards. However, quantitative results for oxidized monomers, oxidized dimers and polymers included in
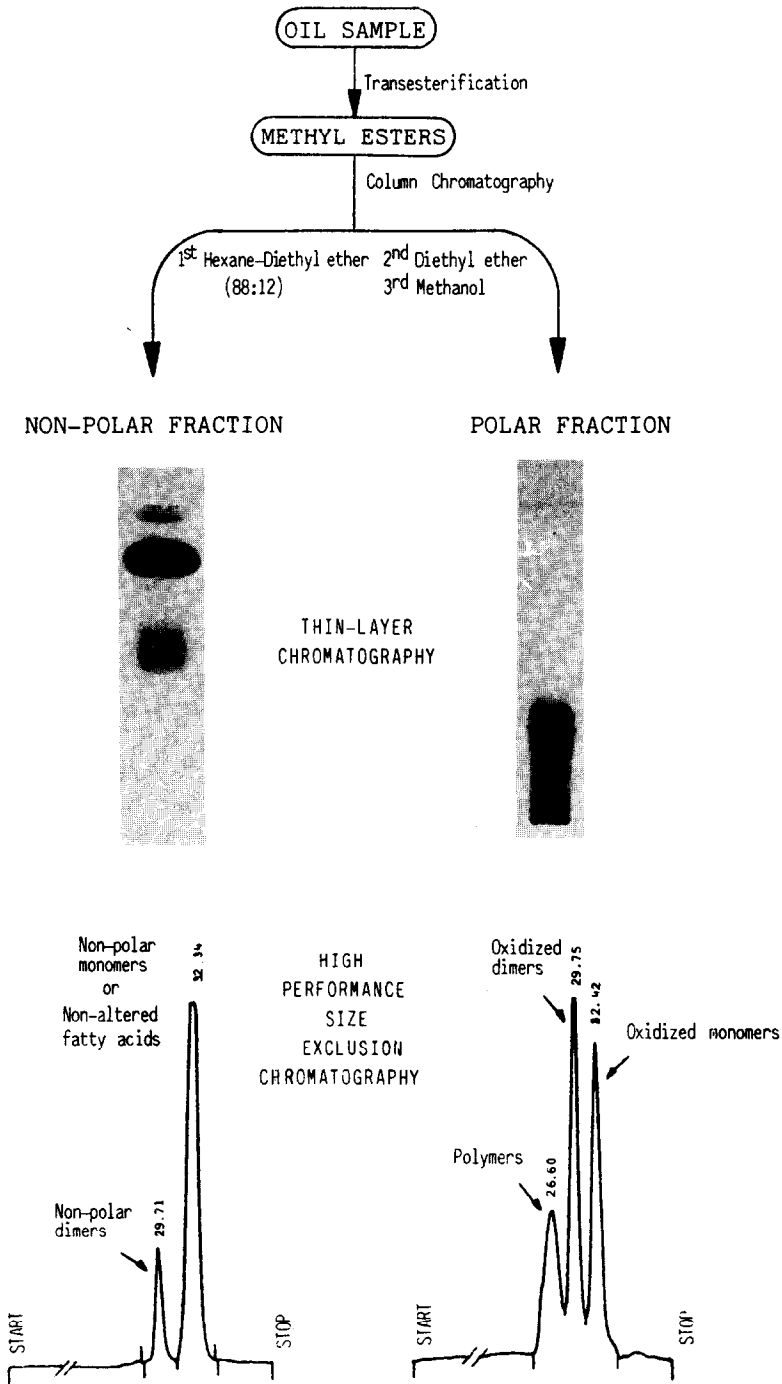
Fig. 1. Combination of column and size-exclusion chromatography for the determination of fatty acid methyl ester derivatives in heated fats.

the polar fraction were obtained by calculating their percentages relative to the total peak area. As it is known that compounds with very different chemical structures are involved in each peak[21,22], it is not possible to calculate precise response factors.

The determination of the five groups of compounds is shown in Table I. Each line lists the percentages of unaltered fatty acids or non-polar monomers, followed by those of the different groups of altered compounds. The last column shows the percentage of total altered fatty acids calculated by subtracting the percentage of unaltered fatty acid from 100. This is an interesting global measurement of acids undergoing thermal and oxidative alteration.

The results are means of four determinations in each instance. Limits of determination for the means ($\bar{x} \pm ts_{\bar{x}}$) can be easily deduced assuming a t distribution. The values of $ts_{\bar{x}}$ in Table I correspond to a 95% confidence level and three degrees of freedom ($t = 3.182$). As can be observed, reproducible results are obtained, in spite of the high value of t.

Polar compounds were not evaluated in unheated samples as their low proportion in comparison with the high content of unsaponifiable compounds eluting in the same fraction leads to difficulties in their determination.
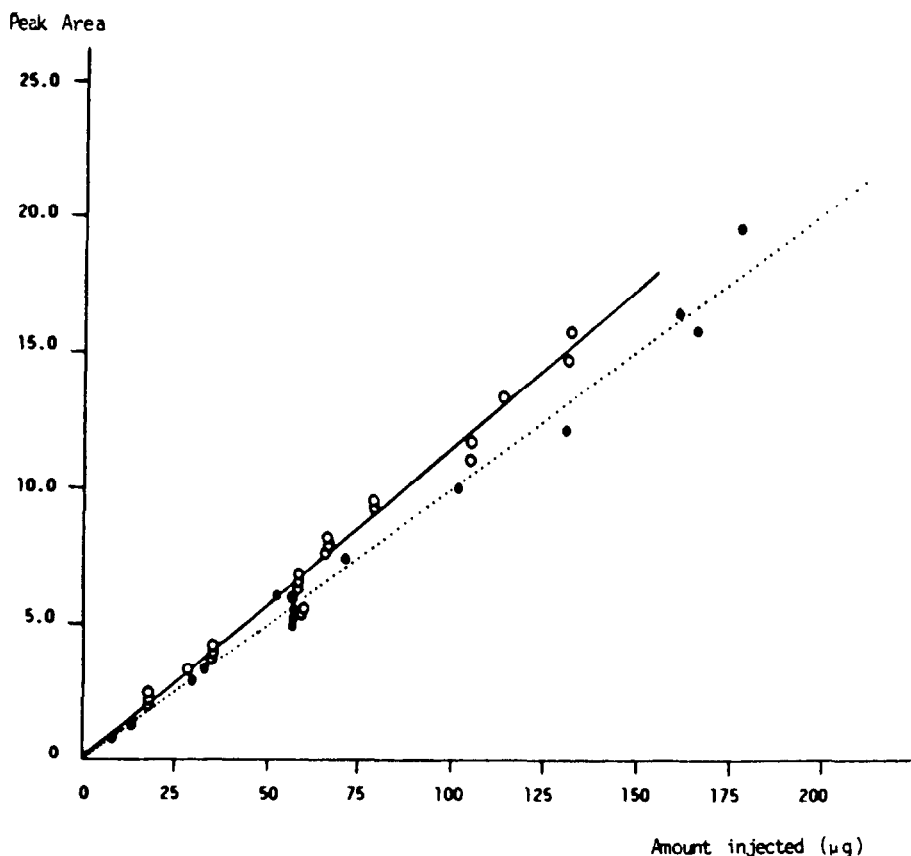

Fig. 2. Calibration graphs for (●) unaltered fatty acids and (○) non-polar dimers.

TABLE I

DETERMINATION OF NON-POLAR AND POLAR FATTY ACIDS IN THE INITIAL OILS AND AFTER HEATING (%, w/w ON FAT)

Sample	Treatment	Non-polar fraction				Polar fraction						Total altered fatty acids[c]
		Unaltered fatty acids		Non-polar dimers		Oxidized monomers		Oxidized dimers		Polymers		
		$\bar{x}^a$	$ts_{\bar{x}}^{b}$	$\bar{x}$	$ts_{\bar{x}}$	$\bar{x}$	$ts_{\bar{x}}$	$\bar{x}$	$ts_{\bar{x}}$	$\bar{x}$	$ts_{\bar{x}}$	
Refined olive oil	Unheated	98.7	0.5	—	—	—	—	—	—	—	—	2.3
	Heated	62.8	0.4	3.8	0.2	12.8	1.2	10.3	0.5	9.7	0.3	37.2
Refined sunflower oil	Unheated	97.5	0.3	—	—	—	—	—	—	—	—	2.5
	Heated	55.2	0.4	9.3	0.7	4.6	0.7	15.5	0.4	14.4	0.6	44.8
Pure olive oil	Unheated	98.3	0.5	—	—	—	—	—	—	—	—	1.7
	Heated	65.3	0.9	3.9	0.19	8.7	0.3	8.2	0.6	13.8	1.9	34.7
	Unheated–heated (1:1)	81.5	1.2	1.9	0.2	4.6	0.5	4.3	0.4	7.4	0.5	18.5

[a] Means of four determinations.

[b] $\bar{x} \pm ts_{\bar{x}}$: limits for the means (see text).

[c] Total altered fatty acids = 100 − unaltered fatty acids.

The results demonstrate that the proposed procedure permits comparisons to be made between samples with different unsaturation levels or subjected to different thermo-oxidative conditions. As can be observed, with similar heating conditions (100 h and 180°C) not only is the level of altered compounds higher in sunflower oil owing to its higher unsaturation, but also the distribution of such compounds is significantly different. Oxidized monomers reach higher percentages in olive oil whereas sunflower oil shows a greater concentration in of higher molecular weight compounds.

On the other hand, with similar unsaturation levels the greater percentage of polymers in the olive oil heated 150 h is notable, in spite of the similar altered fatty acid percentage. Hence the results clearly indicate that, indicating the alteration level of fats, the distribution of altered fatty acids provides useful information about the influence of oxygen and temperature on samples of unknown origin.

The main advantages of the proposed method are the following: a global measurement of thermo-oxidative alteration can be deduced from the contents of unaltered fatty acids; a substantial increase in the possibilities of the determination of polar compounds is obtained as oxidized and polymeric compounds can be determined without interference from less polar components, which are present in a majority; five groups of compounds (unaltered fatty acids, oxidized fatty acid monomers, non-polar fatty acid dimers, polar fatty acid dimers and fatty acid polymers) can be determined starting from the same sample; and it is possible to separate fatty acid dimers into two groups, non-polar and polar, and fatty acid monomers into unaltered and oxidized monomers. Non-polar dimers are specifically related to thermal degradation as there is no oxygen involved in their formation whereas oxidized monomers originate from the action of oxygen and their concentration in a sample is directly connected with oxidative alteration.

Finally, the proposed procedure can be combined with the similar evaluation proposed previously for glyceridic compounds[19] in order to obtain more information on fat alteration. This better chemical definition of thermally oxidized fats can be applied to nutritional studies involving edible frying fats, as it is well known that the oxidative and thermal degradations taking place in the unsaturated acyl groups of the triglyceride modify the physiological properties of the fat.

ACKNOWLEDGEMENTS

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