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Gas chromatographic determination of cholesterol and tocopherols in edible oils and fats with automatic removal of interfering triglycerides

E. Ballesteros, M. Gallego, M. Valcárcel*

Department of Analytical Chemistry, Faculty of Sciences, University of Córdoba, E-14004 Córdoba, Spain

Abstract

An automated gas chromatographic method for the simultaneous determination of cholesterol, α-tocopherol and α-tocopheryl acetate in edible oils and fats without derivatization is reported. Interferences from lipid material are avoided by using a continuous system to transesterify triglycerides with potassium methylate in methanol. The precision of the method is 1.9, 2.2 and 3.1% for cholesterol, α-tocopherol and α-tocopheryl acetate, respectively. The proposed method was validated by analysing a standard reference material of coconut oil (SRM 1563-2) with good results. The method features a high throughput, minimal sample handling and analyte specificity (lipid material does not interfere).

1. Introduction

Sterols and tocopherols belong to the group of nutritionally significant lipids that more and more often have to be routinely determined in foods. Cholesterol is the principal steroid in mammals; it occurs in lipids, either in free form or esterified with saturated and unsaturated fatty acids. This sterol is the precursor for the biosynthesis of other steroids such as sex hormones and bile acids in animal organisms. Tocopherol is an antioxidant that prevents or delays lipid oxidation and protects other biologically active compounds (e.g. vitamin A, ubiquinone, hormones and enzymes) from oxidation [1].

The above two molecular groups are commonly determined separately in foods by a variety of methods including spectrophotometry [2,3], high-performance liquid chromatography (HPLC) [3–10], supercritical fluid chromatography (SFC) [11] and gas chromatography (GC) [3,12–15]. The GC determination of cholesterol and tocopherols in complex matrices such as fats and oils includes several steps (e.g. saponification, extraction and purification) needed to remove the overwhelming amount of triglycerides (ca. 95% of all lipid), which may be deposited in the column and give rise to spurious results (broadened peaks or errors arising from adsorption and loss of polar solutes) [16].

Enzymatic methods have only been applied to cholesterol; in any case, the high cost and instability of enzymes have fostered the development of continuous methods where the enzyme is immobilized on a suitable support [17,18]. Our group has developed an automatic module that

* Corresponding author.

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permits the isolation, preconcentration, derivatization and GC determination of fatty acids in oil [19] and dairy products [20].

This paper reports on the use of a continuous system coupled to a gas chromatograph for the direct determination of cholesterol, α-tocopherol and α-tocopheryl acetate in oils and fats, and removal of interfering triglycerides. The triglycerides from food are transesterified to fatty acid methyl esters with potassium methylate in methanol, so they do not disturb the chromatographic separation and determination of cholesterol and α-tocopherols.

2. Experimental

2.1. Apparatus

A Hewlett-Packard 5890 A gas chromatograph equipped with a flame ionization detector and a 10 m × 0.53 mm I.D. dimethyl polysiloxane coated fused-silica column (film thickness 2.65 μm) (Hewlett-Packard, HP-1) was used. The chromatographic conditions were as follows: initial temperature, 210°C (10 min); 2.5°C/min ramp to 235°C; and 15-min hold at 235°C. The injector and flame ionization detector temperature were both 250°C and nitrogen was used as the carrier gas, at a flow-rate of 24 ml/min. Peak areas were measured by using a Hewlett-Packard 3392 A integrator.

The manifold used consisted of a Gilson Minipuls-2 peristaltic pump and a custom-made separator furnished with a Fluoropore membrane (1.0-μm pore size, FALP; Millipore) described elsewhere [21]. Poly(vinyl chloride) and Solvaflex pumping tubes for aqueous and organic solutions, respectively, and PTFE tubing for coils, were also used. A desiccating column (50 × 3 mm I.D.) packed with sodium aluminosilicate pellets was prepared. A Knauer 633200 6-port HPLC switching valve mounted over the injection port of the gas chromatograph (injected volume, 5 μl) [22], and a thermostated water bath were also used.

2.2. Standards and reagents

Cholesterol, α-tocopherol (vitamin E), α-tocopheryl acetate and 5α-cholestane were supplied by Sigma (Madrid, Spain). Acetyl chloride was purchased from Aldrich (Madrid, Spain). All other reagents (potassium methylate, ascorbic acid, sodium sulphate, methanol, *n*-hexane and petroleum ether) were supplied by Merck (Madrid, Spain). The oils and fats were purchased from local suppliers.

Standard solutions of cholesterol, α-tocopherol and α-tocopheryl acetate were prepared at a concentration of 4 g/l in *n*-hexane and stored in glass-stoppered bottles at 4°C. The optimal conditions for GC were established by using a mixture of 50 mg/l of each standard and the internal standard (5α-cholestane) in *n*-hexane. A 0.1 *M* potassium methylate solution in methanol also containing 20 mg/l of ascorbic acid (to avoid decomposition of α-tocopherols) was used as derivatizing reagent for triglycerides.

2.3. Procedure

The manifold used for the transesterification of triglycerides and determination of cholesterol, α-tocopherol and α-tocopheryl acetate in oils and fats is depicted in Fig. 1. It was oper-

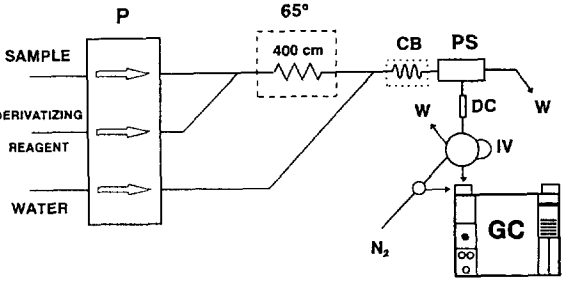

Fig. 1. Setup used for the continuous transesterification of triglycerides and determination of cholesterol, α-tocopherol and α-tocopheryl acetate. P = Pump; CB = cooling bath; PS = phase separator; W = waste; DC = desiccating column; IV = injection valve; GC = gas chromatograph.

ated as follows: an oil solution containing 10–250 mg of oil plus 250 μg of 5α-cholestane in 5 ml of *n*-hexane or a standard solution containing between 5 and 400 μg of cholesterol and α-tocopherols plus 250 μg of 5α-cholestane in 5 ml of *n*-hexane was continuously introduced onto the system at 0.4 ml/min and merged with a stream of 0.1 *M* potassium methylate and 20 mg/l ascorbic acid in methanol (flow-rate 0.4 ml/min) for the derivatization of the triglycerides, which were transesterified (i.e. converted into fatty acid methyl esters) in a 400 cm × 0.5 mm I.D. reaction tube that was heated at 65°C. The mixture was then cooled in a 100-cm coil immersed in a cooling bath (CB). Finally, a water stream flowing at 0.7 ml/min was introduced into the continuous system to remove excess derivatizing reagent and residual reaction products. The extract from the membrane separator (PS) was continuously circulated through the injection valve (IV) and the loop contents (5 μl) were injected into the nitrogen carrier gas and transferred to the chromatograph port. In order to prevent any water traces from reaching the column, the continuous system included a desiccating column (DC) prior to IV.

3. Results and discussion

3.1. Selection of chemical parameters

For this study, an automated system similar to that depicted in Fig. 1 and a spiked olive oil sample containing 100 mg of olive oil plus 150 μg of cholesterol, α-tocopherol and α-tocopheryl acetate, and 250 μg of 5α-cholestane (internal standard) in 5 ml of *n*-hexane were selected. Chemical variables were optimized by collecting the extracts from the membrane separator in 4-ml glass vials containing sodium sulphate and injecting 2-μl aliquots manually onto the chromatographic system by means of a syringe. First, two derivatizing reagents (potassium methylate and acetyl chloride) were used to overcome the problems posed by excess triglycerides in the oil samples. Both derivatizing reagents were prepared at a concentration of 0.2 *M* in methanol. Both allowed, cholesterol and α-tocopherols to be resolved; however, for the complete derivatization of triglycerides with acetyl chloride drastic conditions were necessary (viz. heating of the reactor at 75°C and stopping the flow for 10–15 min). Therefore, potassium methylate was chosen as the derivatizing reagent for triglycerides in the oils.

Two organic solvents, *n*-hexane and petroleum ether (boiling point range 50–70°C), were assayed as solvents for the spiked olive oil samples. *n*-Hexane was found to be more effective for the transesterification of the triglycerides and for the recovery of cholesterol and tocopherols from spiked olive oils than petroleum ether. For optimization of variables, an internal standard (5α-cholestane) frequently used in conventional procedures was selected.

The concentration of potassium methylate (the derivatizing reagent for triglycerides) in methanol was varied between 0 and 0.3 *M*. The detector signal for all triglycerides increased with potassium methylate concentration up to 0.05 *M*, above which it remained constant. A concentration of 0.1 *M* was found to be adequate for effective transesterification. The detector response for α-tocopherols was ca. 20% lower in the presence of the derivatizing reagent in methanol than that obtained when the α-tocopherol solution was only mixed with a stream of methanol containing no derivatizing reagent. Based on peak-area data, tocopherols were partially decomposed in the presence of potassium methylate. In order to avoid this shortcoming, ascorbic acid, usually employed in conventional methods [3,8], was added to the derivatizing reagent solution. As can be seen in Fig. 2, the decomposition of α-tocopherol and α-tocopheryl acetate was avoided above an ascorbic acid concentration of 10 mg/l. A derivatizing solution containing 0.1 *M* potassium methylate and 20 mg/l ascorbic acid was thus selected as optimal for the determination of α-tocopherols.

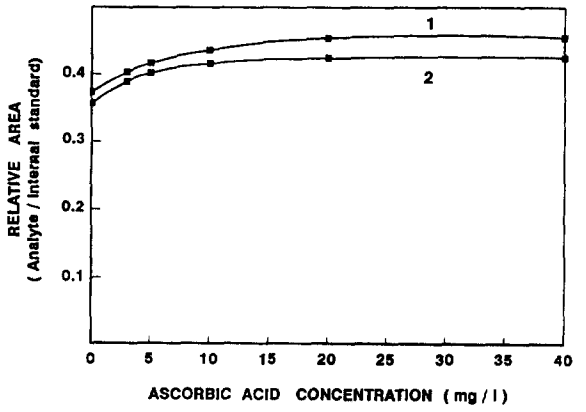

Fig. 2. Influence of the ascorbic acid concentration on the decomposition of α-tocopherols. 1 = α-tocopherol; 2 = α-tocopheryl acetate.

3.2. Optimization of the flow system and sample introduction device

Flow variables influencing the performance of the proposed system in the transesterification of triglycerides and direct determination of cholesterol, α-tocopherol and α-tocopheryl acetate were optimized by introducing a spiked olive oil sample containing 100 mg of oil, 150 μg of cholesterol and α-tocopherols, and 250 μg of 5α-cholestane in 5 ml of *n*-hexane into the system; the solution was then merged with a stream of 0.1 *M* potassium methylate and 20 mg/l ascorbic acid in methanol. The flow-rates of the sample and derivatizing solution influenced the transesterification of triglycerides. Thus, chromatographic signals for fatty acid methyl esters (transesterified triglycerides) increased with decreasing flow-rate of the sample and the reagents through increased derivatization. The best results were provided by a similar flow-rate for both solutions; thus, the same flow-rate of 0.4 ml/min was chosen for both the sample and the reagent flow. The water flow-rate had no effect over the tested range (0.2 to 1.0 ml/min) as the aqueous stream was only required to remove residual products, which may not reach the chromatographic column. A water stream flow-rate of 0.7 ml/min was thus chosen, which allowed effective separation of aqueous–organic phases. The influence of the length of the triglyceride derivatization coil was studied between 50 and 600 cm (0.5 mm I.D.) at 70°C. Above 300 cm, the peak areas for fatty acid methyl esters remained constant throughout the interval studied. A coil length of 400 cm, which resulted in a residence time of 1 min, was chosen.

Because some manual procedures require heating for the transesterification of triglycerides, we studied the effect of the derivatization coil temperature between 20 and 85°C by using a thermostated water bath. Increase of the temperature in the derivatization coil resulted in sharply increasing peak areas up to 60°C, while the peak areas remained virtually constant between 60 and 70°C, above which they decreased slightly. A temperature of 65°C was thus selected. However, this temperature decreased the aqueous–organic phase separation efficiency of the membrane separator and resulted in diminished repeatability of the analyte chromatographic peaks. This drawback was circumvented by inserting a cooling module consisting of a 100-cm coil immersed in a cooling bath (CB in Fig. 1).

The sample introduction device (the interface between the continuous system and the gas chromatograph) was an injection valve similar to that used elsewhere to couple an extraction unit with a gas chromatograph [22], with slight modifications for the present application. The loop of the injection valve (IV, 5 μl) was constructed from PTFE tubing and connected the valve to the instrument via a 10 cm × 0.3 mm I.D. stainless-steel tube with a needle soldered on one end that allowed direct fitting of the valve to the injection port of the gas chromatograph by inserting the needle into the septum of the instrument port. The carrier gas (nitrogen) inlet was split into two that were directly connected to one of the ports of valve IV and the chromatograph injection port, respectively. The flow-rate of the carrier gas was varied in order to reduce adsorption of cholesterol and α-tocopherols in the loop and/or connecting tube valve port, as well as to improve chromatographic resolution of the peaks. For this purpose, the total carrier gas flow-rate was varied between 10 and 45 ml/min. An overall gas flow-rate of 24 ml/min (flow-rate

through the valve and injection port, 17 and 7 ml/min, respectively) was selected as optimal.

3.3. Calibration of the system

The calibration graphs obtained under the optimum conditions shown in Fig. 1 and described under Procedure by plotting the analyte-to-internal standard peak-area ratio against the analyte concentration in *n*-hexane were linear throughout the tested range, viz. 1–80 μg/ml cholesterol or α-tocopherols. The equations for the calibration graphs ($n = 10$) are: for cholesterol, $y = 0.0005 + 0.0179$ ($r = 0.9991$); for α-tocopherol, $y = -0.0012 + 0.0152$ ($r = 0.9993$); and for α-tocopheryl acetate, $y = -0.0013 + 0.0142$ ($r = 0.9990$).

The detection limits, calculated as the concentrations yielding the minimum detectable signal in the chromatogram, were 0.5, 0.7 and 0.8 μg/ml for cholesterol, α-tocopherol and α-tocopheryl acetate, respectively. The precision of the method was checked on 11 samples containing 30 μg/ml cholesterol, α-tocopherol and α-tocopheryl acetate, and 50 μg/ml 5α-cholestane (internal standard). The relative standard deviations were 1.9, 2.2 and 3.1% for cholesterol, α-tocopherol and α-tocopheryl acetate, respectively.

3.4. Validation of the proposed method

The proposed method was validated by analysing a standard reference material of fortified coconut oil (SRM 1563-2) obtained from the National Institute of Standard Reference Materials (Gaithersburg, MD, USA) with a certified cholesterol and α-tocopheryl acetate content of 642 ± 6 μg/g and 158 ± 6 μg/g, respectively. Prior to analysis, the sample was melted by immersing each ampoule in warm water (25–30°C) and then sonicating for 10 min, according to the supplier's recommendations. A standard solution containing 200 mg of SRM 1563-2 coconut oil plus 250 μg of internal standard in 5 ml of *n*-hexane was introduced into the continuous system and merged with a stream of derivatizing reagent (0.1 *M* potassium methylate and 20 mg/l ascorbic acid in methanol). The sample was analyzed 5 times to obtain a mean value and standard deviation. The cholesterol and α-tocopheryl acetate concentrations obtained were 636.5 ± 7.7 and 160.5 ± 4.3 μg/g, respectively, which are consistent with the certified contents.

Finally, in order to assess the performance of the proposed method for the determination of α-tocopherol (not certified in the SRM 1563-2 material), the recovery of this α-tocopherol added to an olive oil sample (150 mg) was determined by performing three standard additions (350, 1400 and 2400 μg per g of oil) prior to addition of 250 μg of 5α-cholestane (internal standard) and dilution to 5 ml of *n*-hexane; the recoveries from five individual additions of α-tocopherol were close to 100% (viz. 97–99%) in all instances.

3.5. Analysis of edible oils and fats

The proposed method was applied to the determination of cholesterol, α-tocopherol and α-tocopheryl acetate in various types of vegetable oil (olive, sunflower-seed, corn, wheat-germ, grape-seed, castor-bean and sweet almond) and fats (margarine, butter and lard). For continuous analysis of oils and fats, three individual solutions containing 150 mg of each sample plus 250 μg of 5α-cholestane (internal standard) were diluted in 5 ml of *n*-hexane. The solutions were analyzed as described under Procedure. The results obtained are listed in Table 1. Only wheat-germ oil was found to contain the three analytes (cholesterol, α-tocopherol and α-tocopheryl acetate). The highest concentrations of α-tocopherol were found in sunflower-seed and wheat-germ oils; on the other hand, cholesterol was mainly found in animal fats. α-Tocopherol was only detected in the margarine sample, vegetable fat from corn oil. Fig. 3 shows typical chromatograms obtained in the analysis of sunflower-seed (A), wheat-germ oil (B) and butter (C). As can be seen in Fig. 3B all three analytes were detected in wheat-germ oil. The peaks preceding that for the internal standard in

Table 1
Concentrations (μg/g) of cholesterol, α-tocopherol and α-tocopheryl acetate in oils and fats as determined by the proposed method

Oil/fat	Cholesterol	α-Tocopherol	α-Tocopheryl acetate
Olive oil	–	175.8 ± 2.7	–
Sunflower-seed oil	–	473.0 ± 13.0	–
Corn oil	–	130.8 ± 4.3	–
Wheat-germ oil	82.4 ± 2.0	409.8 ± 10.1	2773.4 ± 70.8[a]
Grape-seed oil	45.6 ± 1.7	–	–
Castor-bean oil	91.6 ± 2.3	–	–
Sweet almond oil	58.1 ± 1.5	164.8 ± 5.3	–
Margarine	–	126.4 ± 3.1	–
Butter	1979.5 ± 62.8	–	–
Lard	953.7 ± 16.8	–	–

[a]Added to samples.

all the chromatograms of Fig. 3 correspond to fatty acid methyl esters and the solvent.

4. Conclusions

The determination of cholesterol and α-tocopherols in oil matrices entails removing the bulk lipid material by using an appropriate sample pretreatment (e.g. saponification, extraction of unsaponificable components, washing of the extracts), followed by normal-phase HPLC or GC. The proposed method overcomes the problems posed by the typically high triglyceride contents in oil by using a continuous derivatization system to transesterify the triglycerides in order to increase their volatility. However, because the derivatizing reagent decomposes α-tocopherols, it must include a reductant (ascorbic acid). The proposed method allows the determination of cholesterol and α-tocopherols in a wide variety of foods with high lipid contents such as oils and fats with minimal sample manipulation, and hence increased precision and sample throughput. If an even higher sensitivity is required, the organic extract from the phase separator can be evaporated with dry N_2 (cryogenic concentration) and subsequently injected manually.

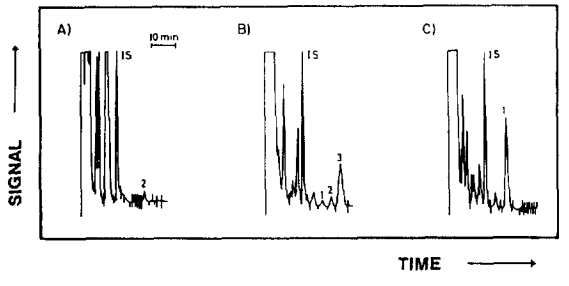

Fig. 3. Chromatograms obtained for a sample solution containing 150 mg of sunflower-seed oil (A), wheat-germ oil (B) and butter (C) in 5 ml of *n*-hexane. Peaks: 1 = cholesterol; 2 = α-tocopherol; 3 = α-tocopheryl acetate; IS = internal standard.

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