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Determination of aliphatic alcohols, squalene, α-tocopherol and sterols in olive oils: direct method involving gas chromatography of the unsaponifiable fraction following silylation

Jasminka Giacometti

Department of Chemistry and Biochemistry, Medical Faculty, University of Rijeka, Braće Branchetta 20, HR-51000 Rijeka, Croatia. E-mail: jasminka@mamed.medri.hr

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In general, analyses for aliphatic alcohols, sterols and tocopherols in vegetable oils are performed separately. A simple and reliable procedure is presented for the quantification of the alkanols, squalene, α-tocopherol and sterols in olive oils by a direct method involving gas chromatographic (GC) analysis of the unsaponifiable fraction after silylation. The method eliminates the need for a preliminary thin-layer chromatographic (TLC) fractionation prior to GC. External standard calibration with reference substances was used for the quantification of squalene, α-tocopherol and sterols and internal standard calibration for the quantification of aliphatic alcohols. The analyte recovery and the repeatability of the quantitative results were evaluated and were acceptable for routine use.

Introduction

The sterol and alkanol contents are very important for the investigation of the quality of vegetable oils, including olive oil. These compounds are present in the unsaponifiable fraction of olive oil and other vegetable oils and limitations on the contents of certain unsaponifiable components were established by EEC Directive 2568 (1991).[1] The main linear aliphatic alcohols present in olive oil are hexacosanol, octacosanol, tetracosanol and docosanol. Aliphatic alcohols are found in significantly larger amounts in olive-pomace oil than in other olive oils.[2] Mariani *et al.*[3] reported that the high content of total aliphatic alcohols in some olive oils is mainly due to the presence of the aliphatic alcohols in the free (non-esterified) form rather than as waxes. This phenomenon is attributed to adverse climatic conditions (prolonged drought).

Sterols are part of the large family of isoprenoids.[4] The conversion of farnesyl diphosphate into squalene marks the channelling of the isoprenoid pathway into the branch that produces sterols. Sterols are membrane components and as such regulate membrane fluidity and permeability. In plants, sterols are always present as a mixture, where they are substrates for the synthesis of a wide range of secondary metabolites, such as cardenolides, glycoalkanoids, pregnane derivatives and saponins.

For over 50 years the role of sterols has involved a substantial research effort in the analytical field.[5] Titration methods are unsuitable for the rapid qualitative or semiquantitative determination sterols in a wide range of products. Colorimetric titration of cholesterol after oxidation using various reagents has been described in many publications.[6] An enzymatic method using cholesterol oxidase has been marketed in the form of a reagent kit. Among the chromatographic methods, high-performance liquid chromatography (HPLC) is promising.[7] A fair number of the listed methods are used for specific applications and cannot be used for general foodstuff analysis.

Gas chromatography (GC) has been used for the determination of sterols and aliphatic alcohols, which were converted into their trimethylsilyl ether (TMS) derivatives. With the use of more selective stationary phases, low concentrations of sterols and aliphatic alcohols can be determined.[5] The methods that are proposed in the aforementioned UE Directive[1] are based on the isolation of the unsaponifiable fraction from the oil and separation of the sterol and aliphatic alcohol fractions by basic silica gel plate chromatography. These methods involve numerous manipulations. Several papers have described alternative methods to replace the official methods. Cortesi *et al.*[8] used HPLC to separate sterols, alkanols, erythrodiol and uvaol in the unsaponifiable fraction from olive oil and investigated the possibility of determining these analytes directly by HPLC. Several authors[9] proposed solid-phase extraction (SPE) for the clean-up of unsaponifiable solutions and separation of lipid classes.

The sterol fraction of virgin olive oil and the dehydration sterol products from refined oil have been successfully determined by the GC-MS analyses.[10,11]

In this paper, a GC method is proposed for the direct determination of the sterols, aliphatic alcohols, squalene and tocopherol contents without previous TLC fractionation. The direct analysis method uses programmed temperature conditions and silylation for derivatization of isolated unsaponifiables. The total run time is 75 min.

Experimental

Reference compounds and solvents

Potassium hydroxide in the form of pellets, chloroform, hexane and the reference substances, dihydrocholesterol (5α-cholestan-3β-ol; β-Cholestanol; 3β-hydroxy-5α-cholestane), cholesterol (5α-cholesten-3β-ol; 3β-hydroxy-5-cholestene), campesterol (24α-methyl-5-cholesten-3β-ol; (24*R*)-ergost-5-en-3β-ol), stigmasterol (3β-hydroxy-24-ethylcholesta-5,22-diene; 5,22-stigmastadien-3β-ol), β-sitosterol (5-stigmasten-3β-ol), arachidyl alcohol (eicosanol), behenyl alcohol (docasanol), tricosanol, tetracosanol, pentacosanol, hexacosanol, heptacosanol, octacosanol, squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) and (±)-α-tocopherol (vita-

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min E) were purchased from Sigma (St. Louis, MO, USA). Ethanol and a silylating mixture according to Sweeley *et al.*[12] (hexamethyldisilazane–trimethylchlorosilane–pyridine, 2 + 1 + 10 v/v/v) were purchased from Fluka (Buchs, Switzerland). Anhydrous sodium sulfate in the form of powder was purchased from J. T. Baker (Deventer, The Netherlands).

Instrumentation

GC analyses were carried out using an Autosystem XL (Perkin-Elmer, Norwalk, CT, USA) with a flame-ionization detector (FID). Chromatography software from Perkin-Elmer Nelson (Turbochrom 4, rev. 4.1.) was used for data acquisition from the FID. Hydrogen was obtained using a Claind hydrogen generator.

Samples

Ten olive oil samples were obtained from olive oil produced from a mixture various olive cultivars (Debela, Naska, Rosulja, Slatka, Plominka, Širnjača) and processed at an industrial level in an oil mill located in Punat, Island of Krk, Croatia, by using a continuous three-phase system with centrifugation. Four olive oil samples were obtained from olive oil produced from mixed olive cultivars (Buga, Carbonera, Bianchera and Leccino) and processed at an industrial level in an oil mill located in Vodnjan, Peninsula Istria, Croatia,[13] by using a continuous three-phase system with centrifugation. The olive oil samples obtained from these olive cultivars cover the total cultivated area in the region under investigation. All olive oil samples were collected from individual producers and stored at 4 °C and kept away from light until analysis. Table 1 and Fig. 1 shows the analytical results and a chromatogram of one of the analysed olive oils (acidity $< 1.0\%$).

Sample preparation

A mixture of 1 g of oil and 25 ml of saponification solution, ethanolic potassium hydroxide (2 mol l^{-1}), was transferred into a dark 50 ml flask and heated at 40 °C for 40 min. The heating was interrupted, 250 ml of water were added and the solution was transferred into a decanting funnel with the aid of 30 ml of hexane. The solution was shaken and left to separate. The hexane extract was washed repeatedly with 250 ml portions of water, passed through anhydrous sodium sulfate and evaporated to dryness in a rotary evaporator at 30 °C.

Preparation of standard solutions

Dihydrocholesterol was used as external standard (ESTD). Stock standard solutions of dihydrocholesterol in chloroform (0.1, 0.5, 1.0 and 2.0 mg ml^{-1}) for the determination of sterols were used for calibration. The relative standard deviations

Table 1 Retention times (RT), relative retention times (RRT) and relative standard deviations (RSD) for the absolute peak areas (mean of three replicates) for one of the test olive oils

Compound	RT/min	RRT[a]	RSD (%)
C_{20} (internal standard)	14.57	1.00	3.48
C_{22}	17.71	1.22	3.23
C_{23}	19.54	1.34	2.89
C_{24}	22.30	1.53	3.58
C_{25}	25.84	1.77	2.89
C_{26}	28.99	1.99	3.04
C_{27}	34.09	2.34	3.29
C_{28}	39.73	2.73	3.59
Squalene (external standard)	22.05	1.51	1.90
α-Tocopherol (external standard)	40.52	2.78	3.63
Dihydrocholesterol[b] (external standard)	39.73	2.73	3.56
Cholesterol	41.08	2.82	3.56
Campesterol	49.17	3.37	3.01
Stigmasterol	52.06	3.57	3.59
β-Sitosterol	57.30	3.93	2.97

[a] RRT, ratio of the retention time of each peak in relation to C_{20}. [b] Compound not contained in the olive oil.

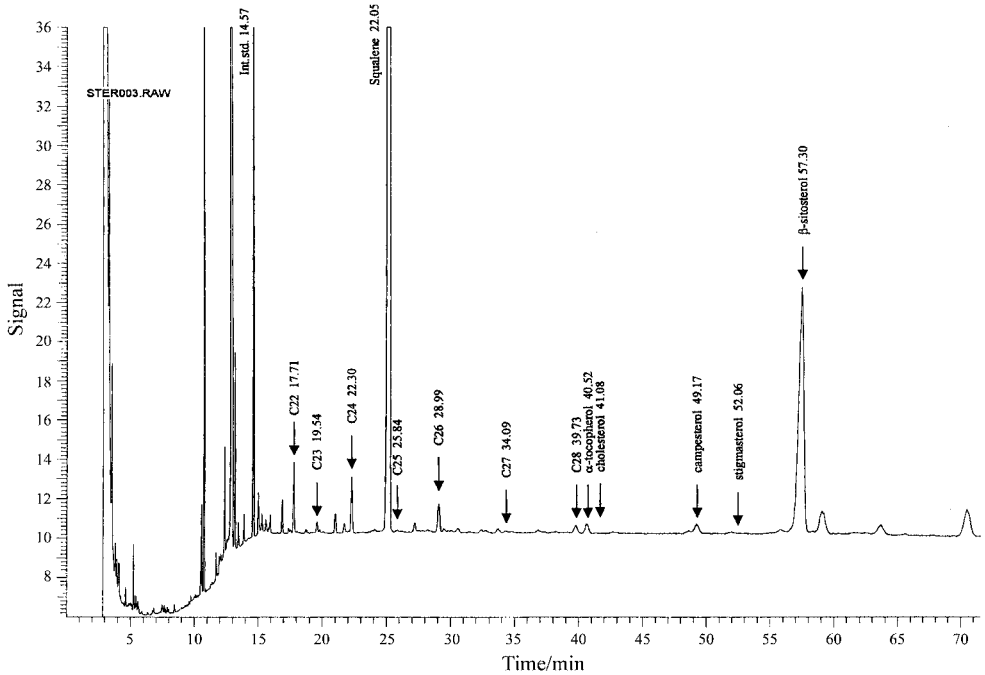

Fig. 1 Chromatogram of TMS derivatives of aliphatic alcohols, sterols, α-tocopherol and squalene on an SPB-5 capillary coloumn, 30 m × 0.53 mm id, coated with a 0.5 μm layer of poly(5% diphenyl–95% dimethylsiloxane). The GC oven was maintained at 180 °C for 1 min, than programmed at 8 °C min^{-1} to 270 °C and then held isothermal for 65 min. Splitting ratio, 100 : 1. Retention times (min) and compounds names are shown on the peaks. Peaks: Int. std. = eicosanol; C22 = docosanol; C23 = tricosanol; C24 = tetracosanol; C25 = pentacosanol; C26 = hexacosanol; C27 = heptacosanol; C28 = octacosanol.

(RSDs) of these concentrations of the standard solution obtained in three replicate analyses were between 2.88 and 4.35%. Stock standard solutions of cholesterol, campesterol, stigmasterol and β-sitosterol in chloroform (1.0 mg ml^{-1}) were used for calibration of the individual retention times (RTs) and appropriate mixed solution for the determination of relative retention times (RRTs) with respect to eicosanol.

Eicosanol was used as the internal standard (ISTD). Stock standard solutions of eicosanol in chloroform (0.1, 0.5, 1.0 and 2.0 mg ml^{-1}) for the determination of aliphatic alcohols were used for calibration. RSD values of these concentrations of the standard obtained in three replicate analyses were between 2.92 and 4.96%. Stock standard solutions of docosanol, tricosanol, tetracosanol, pentacosanol, hexacosanol, heptacosanol and octacosanol in chloroform (1.0 mg ml^{-1}) were used for determination of the individual RTs and an appropriate mixed solution was used for determination of RRTs with respect to eicosanol.

Stock standard solutions of squalene in chloroform (0.1, 0.5, 1.0 and 2.0 mg ml^{-1}) were used for the determination of squalene. RSD values for these stock standard solutions (three replicate analyses) were between 1.73 and 2.09%.

Stock standard solutions of vitamin E in chloroform (0.1, 0.5, 1.0 and 2.0 mg ml^{-1}) were used for the determination of α-tocopherol. RSD values for these stock standard solutions (three replicate analyses) were between 1.58 and 5%.

Preparation of derivatives

The unsaponifiable fraction was silylated according to Sweeley *et al.*[12] The reagents contained hexamethyldisilazane (HMDS) as the active ingredient, trimethylchlorosilane (TMCS) as catalyst and pyridine as solvent, all pre-mixed in appropriate proportions (2 : 1 : 10). The TMS derivatives were prepared by treating 2 mg of unsaponifiable fractions in chloroform. Silylating mixture (0.033 ml) and 0.1 ml of test solution dissolved in chloroform were added, shaken and allowed to stand for 15 min. After centrifugation, the supernatant (1 μl) was injected into the gas chromatograph.

GC determination

The test portions, in the form of TMS derivatives, were examined in triplicate and 1 μl of each test portion in chloroform was injected into the GC system. An SPB-5 capillary column (Supelco, Bellafonte, PA, USA), 30 m × 0.53 mm id, 0.5 μm film thickness, was used. Helium was used as the carrier gas with split injection (100 : 1). The analyses were carried out in the programmed temperature mode from 180 to 270 °C, with rate 8 °C min^{-1} and then isothermal for 65 min. The detector temperature was 300 °C and the injector temperature was 290 °C. The external standard for sterols was dihydrocholesterol and the internal standard for aliphatic alcohols was arachidyl alcohol. The results were expressed as both percentage and ppm of sterols and aliphatic alcohols in the unsaponifiable fraction.[13] α-Tocopherol and squalene were determined in the same fraction under identical analytical conditions.

Results and discussion

The determination of sterols and aliphatic alcohols in vegetable oils is complicated by the number of analytical steps involving numerous sample manipulations that can lead to loss of analyte and/or structural alterations. An analytical procedure was developed that eliminates the need for a preliminary TLC fractionation prior to GC.

Artho *et al.*[14] proposed an LC-GC method for the determination of minor components in edible oils and fats which involved GC of the oil following silylation. Direct analysis without previous saponification provided the most detailed information about the identity and the quality[15] of the oil. Villén *et al.*[16] described use of on-line reversed-phase liquid chromatography coupled to gas capillary chromatography (RPLC-GC) for the direct and simultaneous determination of free sterols, tocopherols and squalene in edible oils without the need for prior enrichment of the test sample.

Prior saponification and analysis of the unsaponifiables are preferred because of the easy determination of minor components in the oil.

Seven aliphatic alcohols (from C_{22} to C_{28}), four sterols, squalene and tocopherols were prepared individually and in an appropriate mixture and immediately assayed in triplicate by GC. Fig. 1 shows a typical gas chromatogram for one of the test olive oils with peaks of the aliphatic alcohol and sterol fraction, squalene and α-tocopherol. Peak identification was confirmed by comparison with the retention times of standards.

Table 1 shows the RTs and RRTs for one of the test olive oils and RSD values resulting from the absolute peak areas of the peaks obtained in three replicate analyses of an olive oil. The RSDs for identified sterols ranged between 2.97 and 3.59% and for aliphatic alcohols between 2.89 and 3.59%, and for squalene was 1.90% and α-tocopherol 3.63%. This means that it is possible to achieve results with high accuracy and precision using classical GC split injection.

Table 2 shows the concentrations of the analyte compounds in the test olive oil. The results obtained for all test olive oils by the proposed method were presented elsewhere.[13]

Analysis by official methods is lengthy but a faster method can be chosen only if it gives results that are not significantly different from those obtained using the official methods. The sensitivity of the GC method to the minor components was higher because only the unsaponifiable fraction was used (Table 2). An identical analytical procedure (including saponification) has been proposed to determine stigmasta-3,5-diene (STIG)[17,18] and sterols[19,20] of various vegetable oils and different processed olive oils.

The proposed method is preferred to conventional methods using TLC fractionation prior to GC since it requires little sample preparation for determination of the sterols, alkanols, α-tocopherol and squalene in olive oils.

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Table 2 Concentrations expressed in the form of percentage and ppm of analyte compounds in one of the test olive oils determined by GC analysis

Compound	Concentration (ppm)	Concentration (%)
C_{22}	35.836	0.621
C_{23}	1.641	0.028
C_{24}	39.646	0.687
C_{25}	0.000	0.000
C_{26}	32.671	0.566
C_{27}	0.000	0.000
C_{28}	2.011	0.035
Squalene	5450.610	86.705
α-Tocopherol	2.730	0.061
Cholesterol	0.000	0.000
Campesterol	0.580	0.003
Stigmasterol	0.237	0.005
β-Sitosterol	717.52	11.288

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