Determination of Squalene in Olive Oil Using Fractional Crystallization for Sample Preparation

Nikolaos Nenadis and Maria Tsimidou*

Laboratory of Food Chemistry and Technology, School of Chemistry, Aristotle University of Thessaloniki, 54006 Thessaloniki, Greece

ABSTRACT: A simple, rapid method for the determination of squalene in virgin olive oil was developed using RP-HPLC with detection at 208 nm. Fractional crystallization from methanol/acetone (7:3, vol/vol) was applied to obtain squalene in the liquid fraction of the oil prior to HPLC. Elution of squalene was then carried out isocratically with acetone/acetonitrile (40:60 vol/vol) within 11 min. The detection limit was 23 mg/kg, and the limit of quantification 79 mg/kg. The precision of the crystallization procedure (CV% = 3.76, $n = 7$) and the mean recovery (92.5 and 81.5% for the 7,000 and 700 mg/kg levels of addition, respectively) were satisfactory. The method is easily applicable to fulfill future needs for nutrition labeling.

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KEY WORDS: Fractional crystallization, nutrition labeling, RP-HPLC, squalene, virgin olive oil.

The interest in the health benefits of squalene is steadily increasing and the potential effects of its daily intake are being evaluated. Squalene is mainly found in nonedible shark liver oil. Virgin olive oil is a major source of phytosqualene, with a content ranging from 800 to 12,000 mg/kg depending on the olive cultivar (1–3). If virgin olive oil were the sole source of dietary fat, squalene intake would be more than 200 mg/d, whereas the daily intake in the United States is estimated to be only 30 mg/d (4). On the basis of these considerations, the interest in developing analytical methods for its determination in virgin olive oil and other edible fats to meet future needs for nutrition labeling is reasonable.

Squalene, which is a $C_{30}H_{50}$ hydrocarbon with six nonconjugated double bonds, is determined by titrimetric (5) or chromatographic procedures (1–3,6–13). In all reported methods, tedious, multistep sample preparation procedures seem to be inevitable. For example, sample preparation according to the official AOAC method (5) involves saponification, extraction of the unsaponifiable matter with large quantities of solvents, fractionation through column chromatography, and other treatments just before titration. The same applies for both GC and LC methods. In our opinion, the most interesting aspect in the existing methodologies is that the majority of them have been developed for the determination of other components such as FFA (2), tocopherols (3,12,13), sterols (7,12,13), and waxes (8), and not for squalene, which was simply codetermined. Thus, GC analysis of squalene presupposes removal of TAG, fractionation of the unsaponifiable matter into several classes of compounds through column chromatography(12) or TLC (6,9), and in some cases derivatization (2). In the existing HPLC methods, both normal- (3) and reversed-phase (RP) (10), sample treatment using alkaline digestion, extraction, and even distillation is employed. By using LC coupled with GC, direct analysis of squalene can be accomplished if the capital cost, technical details, and long elution time (22 min) are not taken into account (7,12).

The aim of this study was the development of a rapid, simple, and low-cost method for the determination of squalene in virgin olive oil. Such a method would be useful for routine analysis of a large number of samples in the industry or in official analytical laboratories with respect to future dietary and food labeling demands (14). Fractional crystallization replaces the alkaline treatment of the sample, thus avoiding the use of large quantities of toxic solvents. A short analysis time was a prerequisite during this endeavor. RP-HPLC at 208 nm was performed for the determination of squalene in the liquid fraction of the oil after crystallization at −22°C.

MATERIALS AND METHODS

Standards and samples. Squalene (98–100% purity) was purchased from Sigma Chemical Co. (St. Louis, MO). Elais SA (Piraeus, Greece) donated the virgin olive oil samples. The purified olive oil TAG fraction, used in the recovery studies, was prepared according to Lampi *et al.* (15).

Solvents. HPLC-grade solvents were used without further purification. Acetone (analytical grade) and distilled methanol were used for oil fractionation. All solvents were purchased from Riedel de Häen (Seelze, Germany).

Apparatus. The solvent delivery system consisted of an SSI liquid chromatography pump (model 300; Scientific Systems Inc., State College, PA) equipped with an SSI pulse damper (model LP-21 LO pulse) and a UV-Vis detector (SPD-10AV; Shimadzu Co., Kyoto, Japan). A Hewlett-Packard, Model HP 3396 Series II electronic integrator (Avondale, PA) was used for recording and quantifying the chromatographic peaks. An RID-6A refractive index detector (Shimadzu Co.) was also employed in certain experiments. Injection was by means of a Rheodyne 7125 injector (Cotati, CA) with a 20 μL loop. Column temperature was controlled using an SSI model 207 column oven.

Fractional crystallization. Olive oil (0.5 g) was shaken vigorously by means of a mechanical shaker (vortex) with 20

*To whom correspondence should be addressed.
E-mail: tsimidou@chem.auth.gr

mL of a mixture of methanol/acetone (7:3, vol/vol) in a 25-mL ground-glass stoppered test tube for 1.5 min and stored at –22 ± 1°C for 24 h (15). The supernatant was then rapidly filtered through a coarse filter paper. The solvent was evaporated under vacuum at 40°C and the residue was dissolved in acetone (5 mL).

Chromatographic analysis. The mobile phase was acetone/acetonitrile (40:60, vol/vol), and the elution was performed at a flow rate of 1 mL/min on a Nucleosil C_{18}, 5 µm (250 × 4 mm i.d.) column (Macherey-Nagel, Düren, Germany). The injection volume was 10 µL. The analytical column was kept at 30°C. Two kinds of detectors were tested: a refractive index detector set at a full-scale sensitivity $16 \cdot 10^{-6}$ RIUFS (refractive index units full scale) and a UV-Vis detector set at 208 nm [attenuation $10 \cdot 10^{-3}$ AUFS (absorbance units full scale)]. Identification was through spiking with a squalene standard solution and spectral data of chromatographic peaks. Quantification was based on the use of a calibration curve.

RESULTS AND DISCUSSION

As mentioned by Cortesi *et al.* (17), squalene in olive oil could possibly be determined simultaneously with TAG by using RP-HPLC and refractive index detection. If this is true, then squalene determination in olive oil should be a simple and direct procedure, as no sample preparation would be required. Starting with a mixture of acetone/acetonitrile (50:50, vol/vol) at a flow rate of 1.5 mL/min, as proposed by Cortesi *et al.* (17), squalene was eluted within 6 min, although the separation from other lipids (e.g., DAG) that eluted close to squalene was not satisfactory and often misleading (Fig. 1A). In an attempt to improve the separation, the content of acetonitrile was increased. A mixture of acetone/acetonitrile, (40:60, vol/vol) at 1 mL/min was found to be the most suitable to elute squalene within 11 min (Fig. 1B). This change in solvent composition lengthened the overall elution time and reduced resolution of certain TAG species. Thus, we decided to reduce the presence of the TAG that also cause a rapid deterioration of the analytical column and to optimize the method solely for the squalene determination.

Fractional crystallization is a mild process often employed in industry for the modification of fats. Less attention has been paid to using this process in the analysis of lipids, although it is considered one of the earliest tools to use in carrying out lipid speciation (18). To remove the majority of TAG, in particular, those having an equivalent carbon number greater than 44, fractional crystallization of the oil sample was achieved by using a procedure developed for the detection of adulteration of olive oil with lipids rich in PUFA (16). Crystallization was carried out in a laboratory freezer at –22°C for 24 h. Trials were also made for longer (60 h) and shorter (15 h) storage periods. The preferred 24-h crystallization period should not be considered as a long preparation step because it does not require constant supervision by the analyst. Another advantage is the use of nonworking hours for the completion of crystallization. Such a procedure is suitable and convenient in routine analysis, permitting

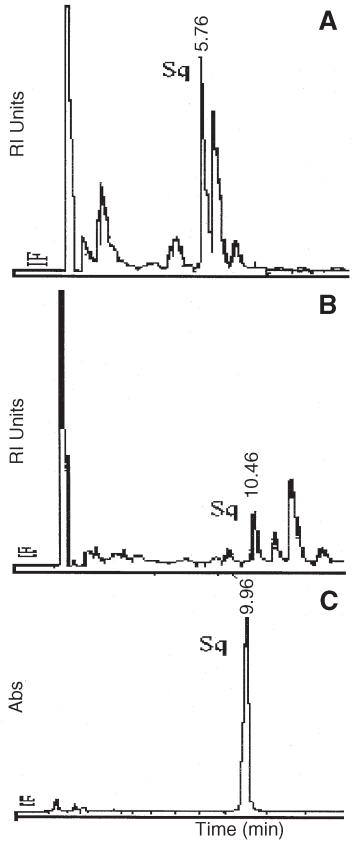

FIG.1. HPLC analysis of squalene. (A) Using acetone/acetonitrile (50:50, vol/vol), 1.5 mL/min, refractive index detection; (B) using acetone/acetonitrile (40:60, vol/vol), 1 mL/min, refractive index detection; (C) using acetone/acetonitrile (40:60 vol/vol), 1 mL/min, detection at 208 nm. RIU, refractive index units; abs, absorbance.

the analyst to handle large numbers of samples with little effort. The chromatographic analysis time, including column conditioning between runs, also is reduced to one-third because the analyte is found in the nonsolid fraction of the oil. An extra benefit is the increase in the column life as it is not burdened with the more saturated species expected at the end of the chromatographic run.

Detection of TAG is usually made with the use of a refractive index detector, a nonselective and nonsensitive system for analytical work. With this system, the detection limit (a signal-to-noise ratio of 3) was 50 mg/kg. Detection at 208 nm was considered more suitable, as illustrated in Figure 1C. At this wavelength the other compounds eluting near squalene are not detected (Fig. 1B). The detection limit value was 23 mg/kg, and the limit of quantification (a signal-to-noise ratio of 10) was 79 mg/kg. For this reason, a UV detector was used in all subsequent experiments. To our knowledge, no literature data are available for detection limits so that no comparison could be made. Under the assay conditions, a linear relationship between the concentration of squalene and the UV absorbance at 208 nm was obtained over the range 20–400 mg/kg. The nine-point linear curve [$y = b(\pm SD)x + a(\pm SD)$] was $y = 2 \cdot 10^7 (\pm 6.86 \cdot 10^5)x + 2 \cdot 10^6 (\pm 1.66 \cdot 10^6)$, the coefficient of determination $R^2 = 0.98$, and the standard error SE = $4.29 \cdot 10^5$.

TABLE 1
Squalene Content of Commercial Virgin Olive Oils[a]

Sample	Squalene content (mg/kg oil)	Sample	Squalene content (mg/kg)
1	4,478	8	5,017
2	6,520	9	5,213
3	3,752	10	2,497
4	5,735	11	2,078
5	6,476	12	3,009
6	3,833	13	2,993
7	5,545	14	4,984

[a]Measurements in duplicate.

Seven replicates of the same virgin olive sample were prepared by the above method and analyzed by RP-HPLC. Each sample was measured twice, and after every two samples a standard solution (250 mg/kg) was injected. The precision of the method was satisfactory (CV% = 3.76). Precision data of similar size (CV% = 1.37–1.97) were given by Lanzón *et al.* (19) after cold alkaline methylation using squalane as internal standard. To determine measurement precision, a squalene standard solution was injected five times; the calculated CV% was 4.05.

To examine the recovery of the procedure, a purified olive oil TAG fraction, prepared by a method repeatedly applied in our laboratory (15), was used as the lipid substrate. The experiment was made after checking the squalene content of the substrate (803 ± 38 mg/kg, $n = 5$). The recovery studies were carried out for two levels of standard squalene addition (7,000 and 700 mg/kg) and five replications ($n = 5$) of the crystallization procedure at each level. The recovery (%) at both levels was satisfactory (92.5 ± 6.7 and 81.5 ± 4.0, respectively). From the proposed methods, recovery data are given only in one case (3), where saponification was used for the sample preparation, although the reported values were not properly substantiated with statistical data.

The applicability of the method was then tested for a number of commercial virgin olive oil samples. Squalene levels for these samples are given in Table 1. In all samples squalene levels were significant, verifying that virgin olive oil is an important source of dietary squalene.

Consequently, the chromatographic determination of squalene by the proposed method is a simple, rapid, and straightforward procedure requiring low quantities of consumables. The detection limit by using selective detection at 208 nm was 10-fold lower than that achieved through refractive index detection. Fractional crystallization as a method for sample preparation prior to HPLC analysis was effective and simple; it could be adopted easily as part of a routine to meet analytical needs for nutrition labeling.

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