A Rapid Method To Determine Sterol, Erythrodiol, and Uvaol Concentrations in Olive Oil

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ABSTRACT: A rapid, accurate, and efficient method for determining the sterol, uvaol, and erythrodiol concentrations was developed to meet International Olive Council (IOC) certification criteria for extra virgin olive oil (EVOO). The unsaponifiable fraction of the sample (0.2 g) was separated with a diatomaceous earth column, and the sterol and triterpenic dialcohols were isolated with a novel base-activated silica solid-phase extraction (SPE) cartridge cleanup protocol. The improved method and the IOC method provided identical pass/fail results ($n = 34$) for each of the six sterol and erythrodiol/uvaol IOC criteria used to assess olive oil. This method was validated, and recoveries of stigmasterol (88%) and β-sitosterol (84%) were greater than previously published values obtained using the IOC method. This method requires approximately one-third the time required to complete the IOC method and has great utility for the rapid screening of EVOO to detect adulteration, false labeling, and an inferior product.

KEYWORDS: Sterols, phytosterols, olive oil, extra virgin olive oil, erythrodiol, uvaol, solid-phase extraction

■ INTRODUCTION

Olive oil has historically been sought for its culinary value and suspected medicinal qualities. Many of the purported health benefits of olive oil, such as lowering cholesterol levels, decreasing the risk of heart disease, and benefiting insulin levels and blood sugar control are attributed to sterols, which have also been credited with anti-inflammatory, antibacterial, antioxidant, antifungal, and anticancer properties.[1−3]

Extra virgin olive oil (EVOO) is the grade of olive oil most highly valued for its superior taste and reported health benefits; it is also the most expensive olive oil to produce. The high cost has created an illegal market for false labeling and adulteration of EVOO to increase profit margins.[4] For example, some reports estimate that adulteration of EVOO with hazelnut oil in the European Union (EU) is a multimillion euro operation annually.[5] Opportunists have reportedly also adulterated olive oil with canola (rapeseed), soy, mustard, other seed oils, and refined olive oil.[4,6] The sterol composition of olive oil can be used to detect adulteration of virgin olive oil with adulterant oils and is one of the criteria used internationally to assess the quality of olive oil.[4] Elevated levels of certain compounds in olive oil are indicative of adulteration by seed or refined oil. For example, cholesterol occurs only in very small amounts in plant oils, and olive oil adulterated with soybean, grapeseed, or sunflower oil can be detected on the basis of the concentrations of campesterol and stigmasterol.[7] Brassicasterol is present in rapeseed and mustard oils but absent or present only in trace amounts in olive oils, and thus is a good identifier of potential adulteration of olive oil.[7] As such, the ability to accurately and efficiently determine the sterol composition of olive oil is useful in detecting a variety of potential adulterants.

The sterol fraction has also been used to gauge maturation, quality, type, and geographic region of cultivar used to produce olive oil.[8] Stigmasterol and Δ-5-avenasterol have been shown to increase as the fruit matures "up to a point which coincides with the maximum oil content of the olive fruit, and thus provide a potential gauge for determining optimal harvest time.[7]

Erythrodiol and uvaol are triterpenic dialcohols that are most abundant in the epicarp (the outermost portion) of olives. Thus, while solvent extraction leads to high amounts of these compounds in refined olive oil, they are absent or present in low levels in EVOO.[9]

According to the International Olive Council (IOC), the total sterol content for edible virgin olive oil must be at least 1000 mg/kg (0.1%, m/m), and the erythrodiol and uvaol contents must not be greater than 4.5% of the total sterol content.[10] The desmethylsterol composition as a percentage of total sterols must be as follows: cholesterol ≤ 0.5%, brassicasterol ≤ 0.1%, campesterol ≤ 4.0%, stigmasterol ≤ campesterol, Δ-7-stigmastenol ≤ 0.5%, and apparent β-sitosterol (β-sitosterol + Δ-5-avenasterol + Δ-5,23-stigmastadienol + clerosterol + sitostanol + Δ-5,24-stigmastadienol) ≥ 93.0.[10]

The sterol content of olive oil is typically determined by saponification, isolation of the unsaponifiable fraction, separation of the desmethylsterols, and gas chromatography−flame ionization detector (GC−FID) analysis. Conducting these tasks in accordance with official methods is time-consuming and labor-intensive.[11−13]

The official methods specify saponification of the oil sample for 20 min after the sample solution becomes clear. However, saponification of as little as 100 mg of sample has been carried out with maximum recovery (99.6%) in as little as 20 min.[14−16] The liquid/liquid extraction (LLE) procedure in the official methods is prone to emulsions and requires lengthy washing

steps, and the thin-layer chromatography (TLC) cleanup is time-consuming and has low throughput and low recoveries.[17]

Several published efforts have been made to improve the efficiency of the analysis of the sterol composition of oils. Some of these methods reported the composition of the major sterol components but were unable to identify the composition of the minor sterol components and/or the triterpenic dialcohols and, therefore, were not suitable for the analysis or certification of olive oil according to IOC criteria.[14] Other methods have been successful in determining the sterol composition of olive oil, yet these methods have either required additional equipment, such as high-performance liquid chromatography—mass spectrometry (HPLC—MS), and/or time-consuming methods, such as TLC cleanup, and therefore, did not substantially improve upon the current IOC method.[18−22]

Solid-phase extraction (SPE) has been used to isolate the sterol component from the unsaponifiable fraction. However, thus far, the published use of SPE has either failed to sufficiently separate all of the sterols and triterpenic diols required for IOC certification criteria or required complicated HPLC fractionation following SPE cleanup.[16,23,24]

The objective of this study was to develop an efficient and effective method that uses the same chemical principles as the current IOC method to determine the quality of olive oil based on IOC sterol composition criteria. The method described uses a small sample size, replaces LLE in separatory funnels with a diatomaceous earth column, replaces the TLC cleanup with base-treated silica SPE cleanup, and requires no additional instrumentation than that required by the IOC method. Quantitation is performed using GC−FID analysis. This method incorporates more efficient extraction and cleanup practices that are based on the same separation mechanisms employed in the IOC method, and therefore, the results produced from this method can be used to assess olive oil quality in accordance with IOC sterol content and composition criteria.

■ MATERIALS AND METHODS

Samples. Samples were obtained from commercial stores in California through collaboration with the University of California, Davis Olive Center. All samples were analyzed without knowledge of their origin or identity.

Reagents. Cholestanol (99.6%), campesterol (99%), stigmasterol (96.5%), β-sitosterol (95%), uvaol (97%), erythrodiol (99.5%), cholesterol (99%), 2,7-dichlorofluorescein diacetate (99.8%), chlorotrimethylsilane (99.4%), hexamethyldisilazane (99.7%), and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, derivatization grade) were obtained from Sigma-Aldrich.

Ethyl ether (laboratory grade), hexanes (Optima grade), anhydrous ethanol (histological grade), methanol (Optima grade), toluene (HPLC grade), acetone (certified ACS grade), ethyle acetate (pesticide grade), pyridine (99.8% anhydrous), potassium hydroxide (certified ACS grade), and anhydrous sodium sulfate (granular, 10−60 mesh, certified ACS grade) were obtained from Fisher Scientific.

Preparation of Standard Solutions. Standard stock solutions of cholestanol (1 mg/mL), stigmasterol (1 mg/mL), and β-sitosterol (0.1 mg/mL) were prepared in methanol and stored at 4 °C. 2,7-dichlorofluorescein diacetate was prepared in 0.2% ethanolic solution and made slightly basic by adding 2 drops of 2 N ethanolic potassium hydroxide.

IOC Method. Preparation and analysis were carried out in accordance with the IOC method of analysis.[11] The fatty (olive oil) substance, with added cholestanol as an internal standard (IS), was saponified with potassium hydroxide in ethanolic solution, and the unsaponifiables were then extracted with diethyl ether. The sterol

fraction was separated from the unsaponifiable extract by chromatography on a basic silica gel plate. The sterols recovered from the silica gel were transformed into trimethyl-silyl ethers and were analyzed by capillary-column gas chromatography.

Saponification. Cholestanol (100 μg, IS) was added [approximately 0.2% solution (m/V sample)] to a 16 × 150 mm screw-capped test tube (Fisherbrand). The solvent was completely removed under a stream of nitrogen (Organomation Associates, Inc., N-EVAP 112 with OASYS heating system) at 40 °C. The oil sample (550 μL, approximately 0.500 g) was added to the sample tube, and the mass recorded was to 0.1 mg. Ethanolic KOH (5 mL, 2 N) was added to each sample tube, which was capped [polytetrafluoroethylene (PTFE)-lined screw cap] and placed in an oven at 80 °C for 40 min.

Separation of the Unsaponifiable Fraction. Distilled water (5 mL) was added to each digest. The unsaponifiable fraction was extracted from each sample 3 times with 6 mL fractions of ethyl ether. The extracts were pooled into a 125 mL separatory funnel and washed with distilled water until the wash water was neutral. Diethyl ether was removed under a stream of nitrogen (Zymark TurboVap LV evaporator), and the extracts were reconstituted in 250 μL of ethyl acetate and stored overnight at 4 °C.

Separation of the 4-Desmethylsterol and Triterpenic Diol Fraction. Basic silica gel plates (DC-Fertigfolien precoated aluminum sheets, Alugram Nano-SIL G, 20 × 20 cm, Machery-Nagel GmbH, Düren, Germany) were immersed in 0.2 N ethanolic potassium hydroxide for 10 s, dried in a fume hood for 2 h, placed in an oven at 100 °C for 1 h, and stored in a desiccator for up to 7 days. Extract (100 μL) was plated (40% of sample; approximately 0.2 g), and plates were developed in a chamber containing a 95:5 toluene/acetone mixture (v/v). Plates were removed, dried in a fume hood, and lightly sprayed with a slightly basic 2,7-dichlorofluorescein ethanolic solution. Sterol bands were identified under ultraviolet light (using cholesterol as a reference solution) and cut from the sheet. Sterols were extracted with 5 mL of warm ethyl acetate.

Preparation of Sterols and Triterpenic Diols for GC−FID Analysis. Ethyl acetate extracts were evaporated to dryness under nitrogen, reconstituted in ∼1 mL of acetone, re-evaporated to dryness under nitrogen, and placed in an oven at 100 °C for 10 min to complete drying. Samples were derivatized by the addition of 250 μL of 9:3:1 pyridine/hexamethyldisilazine/chlorotrimethylsilane (v/v/v) and immediately analyzed.

GC−FID Analysis. GC analysis was conducted using an Agilent 7890A GC−FID equipped with a split/splitless injector. A 30 m × 250 μm × 0.25 μm Agilent Technologies HP-5MS phenyl methyl siloxane 1909IS-433 column was used. The inlet and detector temperatures were 280 and 300 °C, respectively. The oven temperature was isothermal at 260 °C for a run time of 55 min. Sterol derivatives (1 μL) were injected in a 15:1 split mode. The hydrogen flow rate was 30 mL/min; the air flow rate was 400 mL/min; and the helium (makeup gas) flow rate was 25 mL/min. The retention time of the IS was 22.1 min, and the retention time of β-sitosterol was 33.5 min. Identification of all other sterols and diols was determined on the basis of the retention time relative to that of β-sitosterol and by comparison to retention times of known standards.

Quantitation. The peak areas were calculated with Agilent ChemStation OpenLAB, and quantitation was performed relative to the peak area of the known concentration of IS in accordance with the IOC method.

Improved Method. Preparation and analysis were carried out according to the principles established in the IOC method of analysis.[11] The fatty (olive oil) substance, with added cholestanol as an IS, was saponified with potassium hydroxide in ethanolic solution, and the unsaponifiables were then extracted with diethyl ether. The sterol fraction was separated from the unsaponifiable extract by SPE on a base-activated silica cartridge. The sterols recovered from the silica cartridge were transformed into trimethyl-silyl ethers and analyzed by capillary-column gas chromatography.

Sample Preparation. The IS (40 μg) was added to each screw-capped test tube, and the solvent was completely removed under a stream of nitrogen. The oil sample (220 μL, approximately 0.200 g)

Table 1. Intraday Repeatability Average, Standard Deviation, and Percent RSD $(n = 7)^a$

sample	cholesterol (%)	brassicasterol (%)	campesterol (%)	stigmasterol (%)	Δ-7 stigmastenol (%)	uvaol + erythrodiol (%)	apparent β-sitosterol (%)	total sterol (mg/kg)
average	0.205	<0.05	3.51	0.455	0.190	1.40	94.7	1500
standard deviation	0.0246		0.0617	0.0129	0.0234	0.387	0.0914	27.0
percent RSD	12.0		1.76	2.84	12.3	27.6	0.0965	1.80

^{a}Concentrations listed under individual sterols are a percentage of the total sterol content.

Table 2. Interday Repeatability Average, Standard Deviation, and Percent RSD $(n = 5)^a$

sample	cholesterol (%)	brassicasterol (%)	campesterol (%)	stigmasterol (%)	Δ-7-stigmastenol (%)	uvaol + erythrodiol (%)	apparent β-sitosterol (%)	total sterol (mg/kg)
average	0.201	<0.05	3.53	0.467	0.208	1.22	94.6	1510
standard deviation	0.0159		0.0385	0.0295	0.0370	0.186	0.206	64.8
percent RSD	7.91		1.09	6.32	17.8	15.2	0.218	4.29

^{a}Concentrations listed under individual sterols are a percentage of the total sterol content.
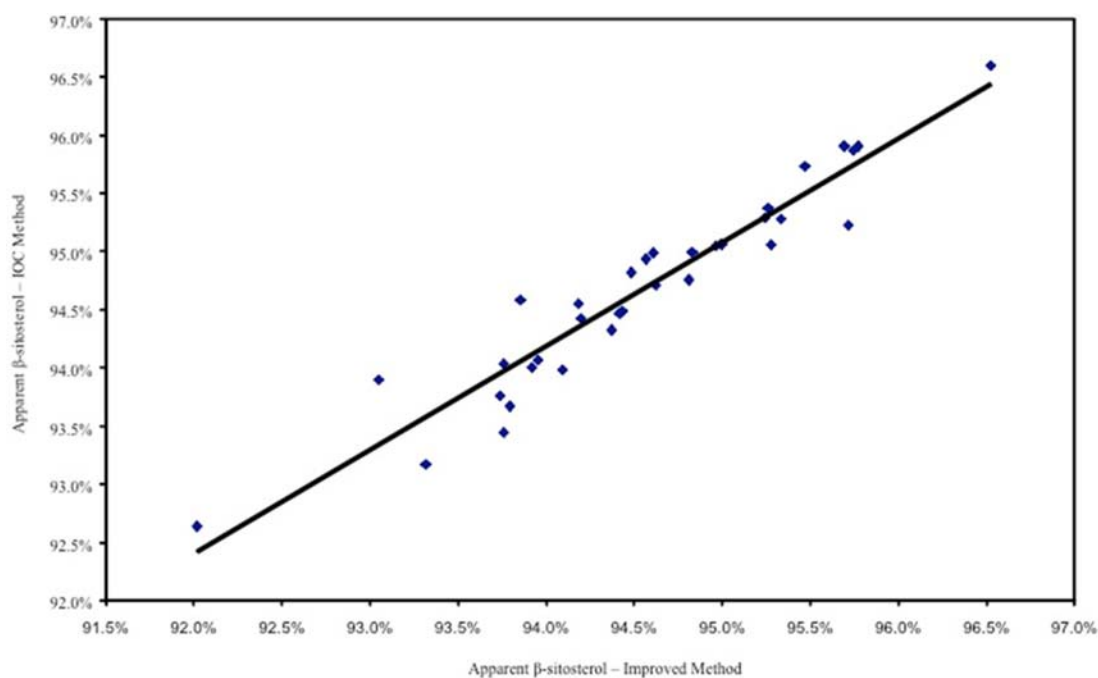

Figure 1. Comparison of apparent β-sitosterol concentrations obtained using the IOC method and the improved method for 34 samples. $r^2 = 0.9112$, and the regression equation is $y = 0.8924x + 0.103$.

was added to each sample tube, and the mass was recorded to 0.1 mg. Ethanolic KOH (2 mL, 2 N) was added, and tubes were capped and placed in an oven at 80 °C for 40 min. Following saponification, 18 mL of water was added to the test tubes, which were mixed by inversion.

Separation of the Unsaponifiable Fraction. Saponified fractions were loaded onto diatomaceous earth cartridges (Chem Elut, 20 mL, unbuffered, Agilent Technologies, Santa Clara, CA). At 15 min after loading, the unsaponifiable fraction was eluted with 60 mL of diethyl ether, and the eluate was collected after passing through approximately 5 g of anhydrous sodium sulfate. The extracts were evaporated to dryness under a stream of nitrogen and reconstituted in 5 mL of hexane for immediate isolation of fractions on silica cartridges.

Separation of the 4-Desmethylsterol and Diol Fraction. Silica cartridges (Agilent Mega BE-1, 1 g, 6 mL, Agilent Technologies, Santa Clara, CA) were conditioned with 10 mL of hexane. Ethanolic KOH solution (1 mL, 0.2 N) was loaded onto each cartridge and then immediately washed with 5 mL of hexane. The extract was loaded, and each cartridge was washed with 100 mL of 98:2 hexane/diethyl ether

(v/v). The column was eluted with 10 mL of 60:40 hexane/diethyl ether (v/v).

Preparation of Sterols and Diols and GC–FID Analysis. The eluate immediately was evaporated to dryness under nitrogen, reconstituted in ~1 mL of acetone, re-evaporated to dryness under nitrogen, and placed in an oven at 100 °C for 10 min to complete drying. Sterols and diols were derivatized with 250 μL of 3:1 pyridine/BSTFA (v/v) and heated at 80 °C for 15 min prior to GC–FID analysis.

GC–FID analysis and quantitation were performed in the same manner as the previously described IOC method.

Method Validation. Peak areas were calculated with Agilent ChemStation OpenLAB, and quantitation was performed relative to the peak area of the known concentration of IS in accordance with the IOC method. Method accuracy was assessed by fortification of EVOO (n = 5) with stigmasterol (25 mg/kg) and β-sitosterol (400 mg/kg). Method precision was assessed measuring the reproducibility of results of one sample on the same day (n = 7) and on 5 different days. The standard deviation (s) of seven replicate injections of 1 mg/mL cholestanol (1 mg/mL) and the slope of the standard concentration
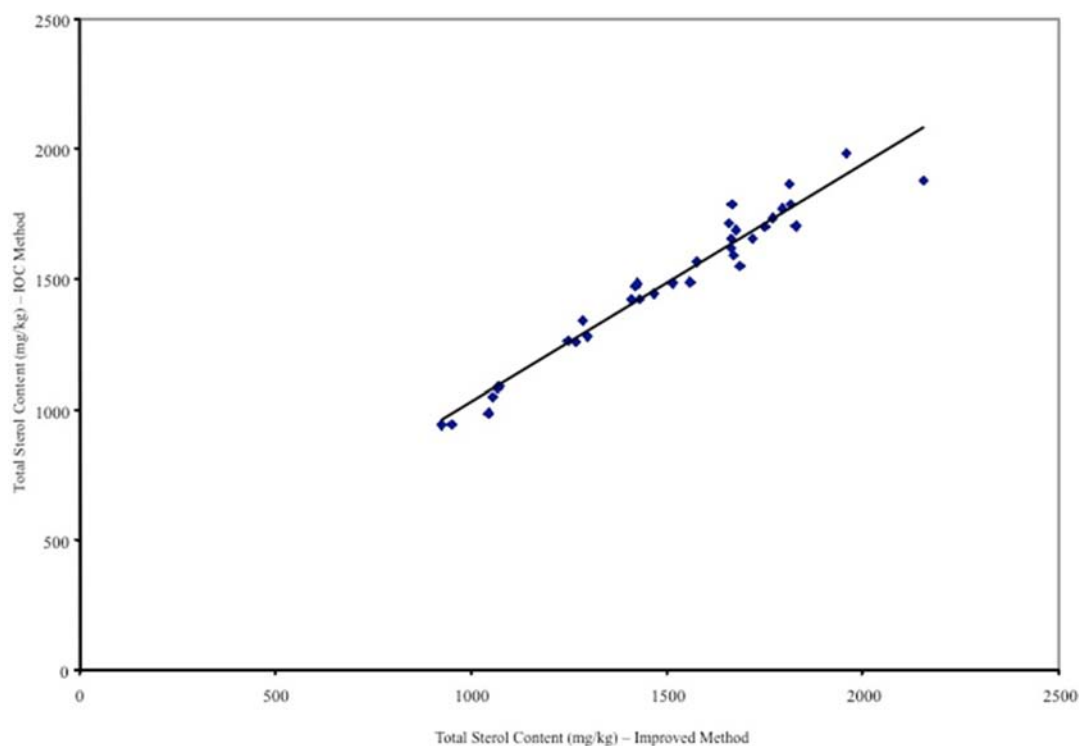
Figure 2. Comparison of total sterol (milligrams of sterols per kilogram of oil sample) concentrations obtained using the IOC method and the improved method for 34 samples. $r^2 = 0.9495$, and the regression equation is $y = 0.9123x + 115.66$.
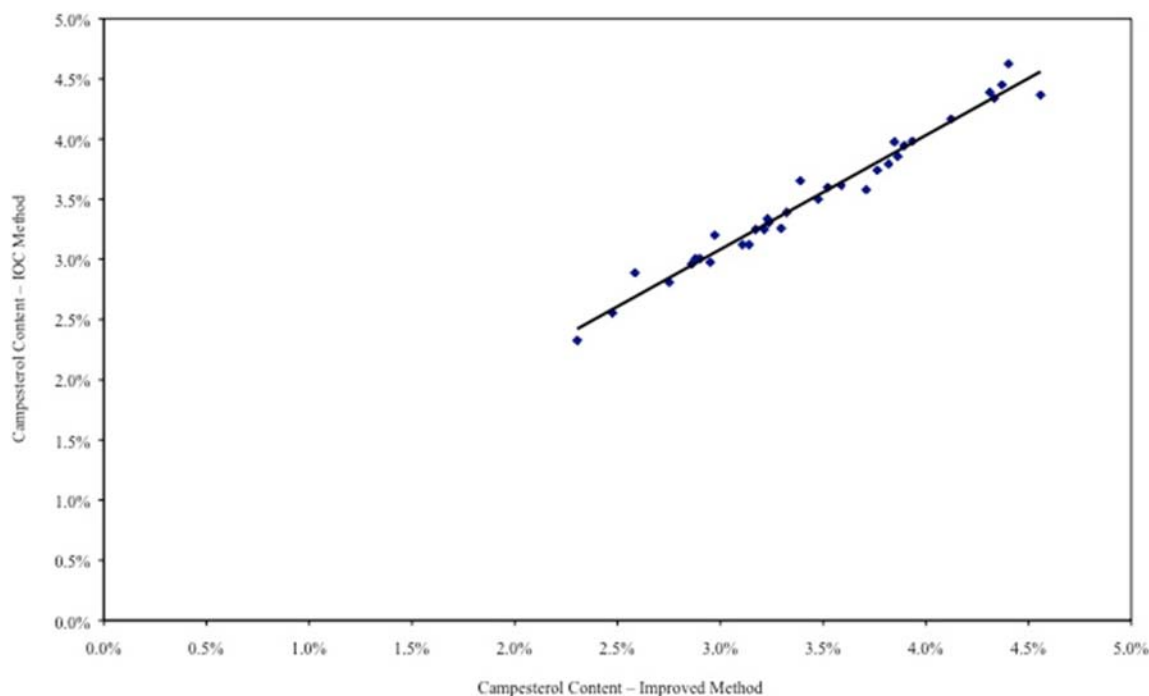

Figure 3. Comparison of campesterol concentrations obtained using the IOC method and the improved method for 34 samples. $r^2 = 0.9729$, and the regression equation is $y = 0.9507x + 0.0023$.

curve (m) were determined. The limit of quantitation (LOQ, $10s/m$) and the limit of detection (LOD, $3s/m$) were calculated. Instrument response linearity was assessed for cholestanol (1−3000 mg/kg), stigmasterol (1−40 mg/kg), and β-sitosterol (1000−3000 mg/kg).

■ **RESULTS AND DISCUSSION**

The improved method was accurate, precise, and yielded results comparable to the IOC method. Fortification recoveries of stigmasterol and β-sitosterol using this method were 88 and 84%, respectively ($n = 5$). Recovery of stigmasterol and β-sitosterol using the IOC-specified TLC method has been reported to be 61 and 65%, respectively.[17] Intraday ($n = 7$)
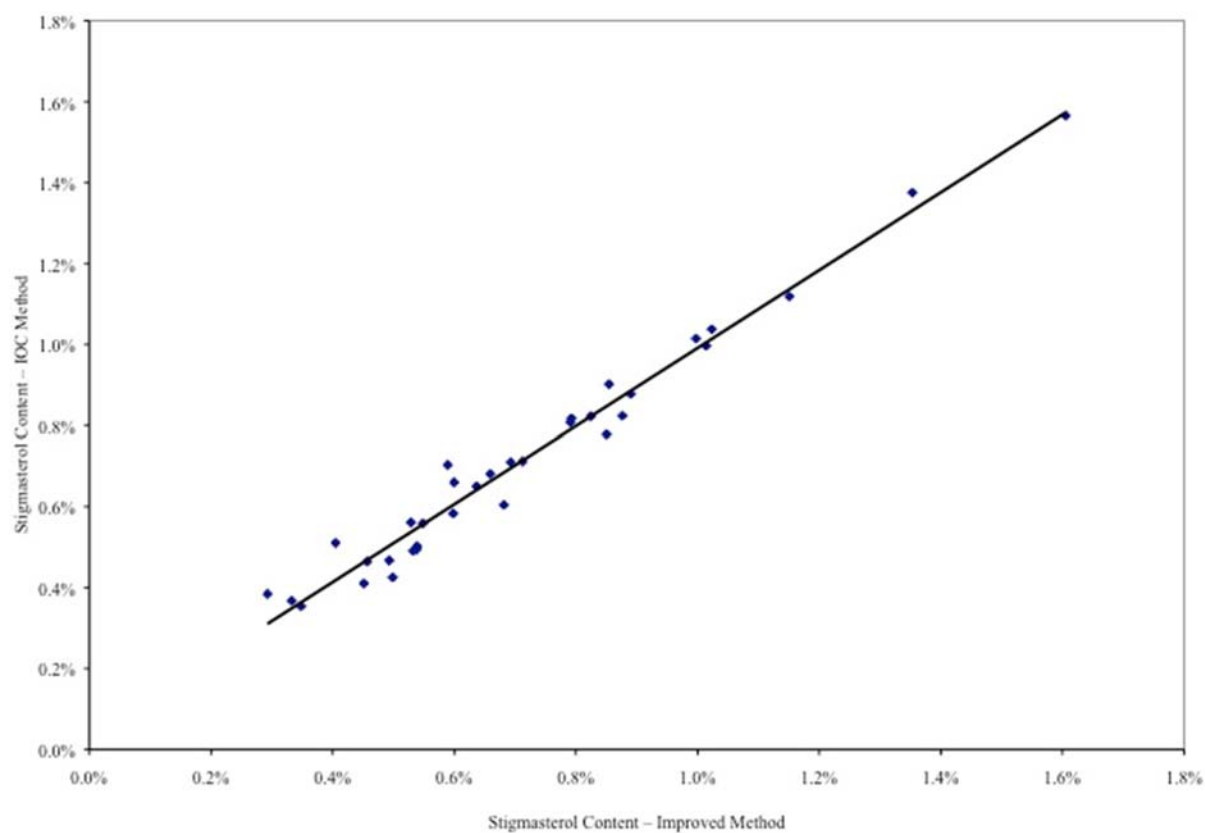
Figure 4. Comparison of stigmasterol concentrations obtained using the IOC method and the improved method for 34 samples. $r^2 = 0.9735$, and the regression equation is $y = 0.9618x + 0.003$.
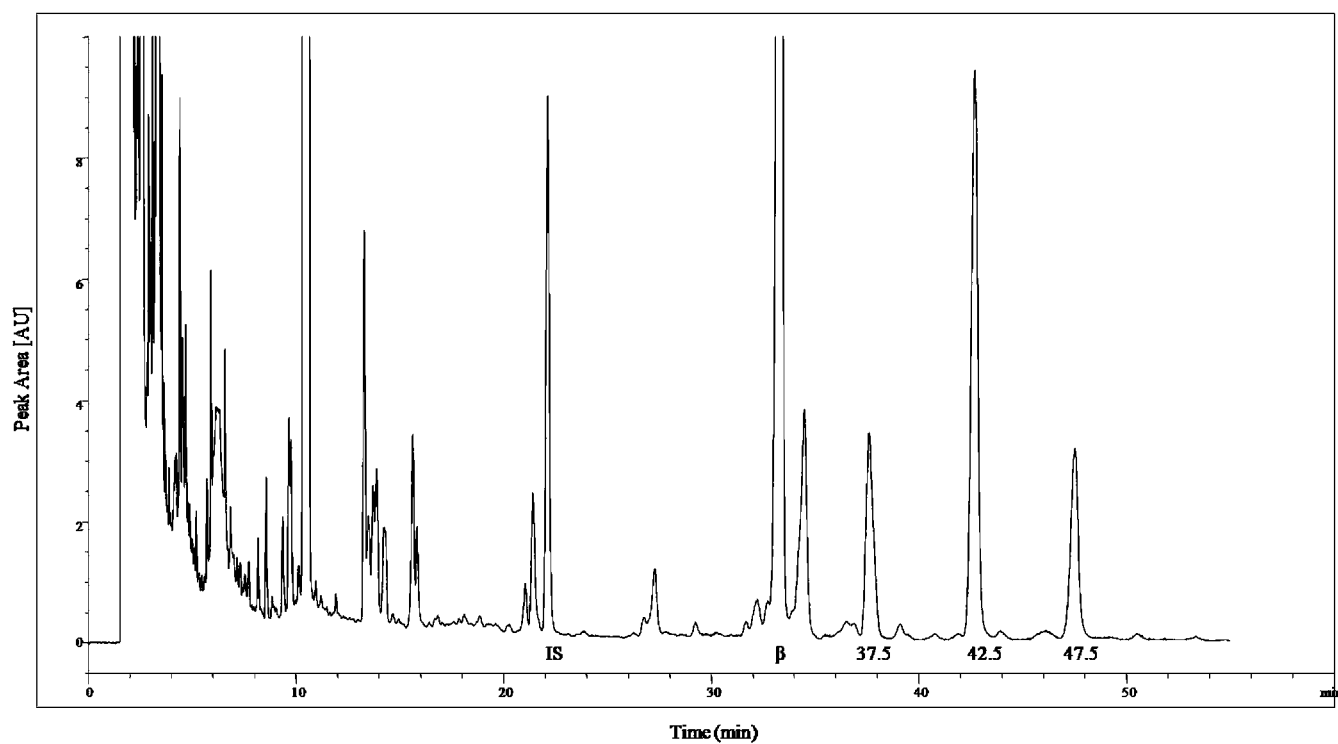

Figure 5. GC−FID chromatogram of the unsaponifiable fraction (without silica gel cleanup) of the olive oil sample obtained by SLE using diatomaceous earth. Peaks identified are the internal standard (IS), β-sitosterol (β), and the three primary interfering peaks at 37.5, 42.5, and 47.5 min. Desmethylsterols elute between 21 and 55 min.
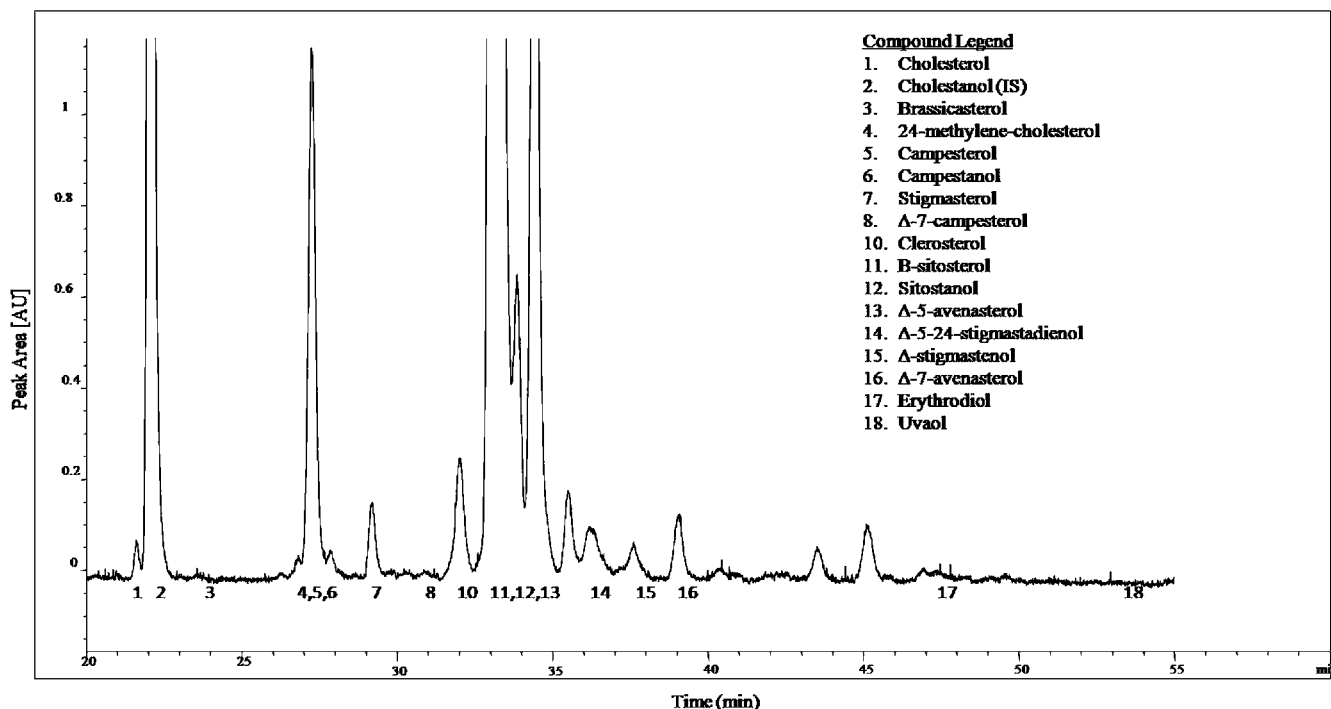
Figure 6. GC–FID chromatogram of the desmethylsterol fraction of the olive oil sample obtained according to the IOC LLE/TLC method.
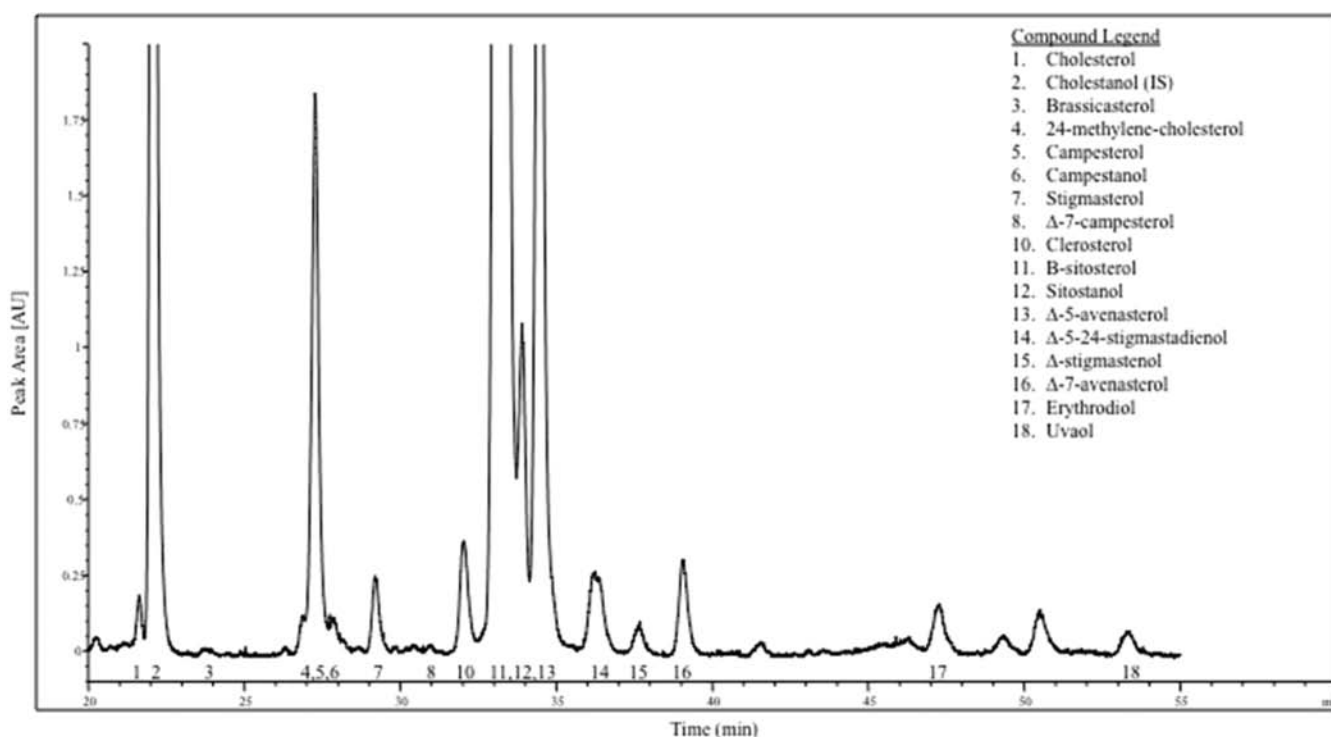

Figure 7. GC–FID chromatogram of the desmethylsterol fraction of the olive oil sample obtained according to the improved method.

relative standard deviation (RSD) values were less than 3% for total sterol, apparent β-sitosterol, campesterol, and stigmasterol contents (Table 1). Uvaol plus erythrodiol yielded the highest RSD values but were present in concentrations well below the IOC limits for EVOO. Analysis of the same sample over a 5 day period yielded less than 7% RSD for these analytes, with the exception of uvaol plus erythrodiol (Table 2). The inter- and intraday RSD values for the method are very comparable to the IOC repeatability values for the official method.[25] Samples (n = 34) were analyzed using the modified IOC method and the improved method. Method correlation coefficients (r^2 values) exceeded 0.90 for apparent β-sitosterol (r^2 = 0.9112; y = 0.0.8924x + 0.103), total sterol (r^2 = 0.9495; y = 0.9123x + 115.66), campesterol (r^2 = 0.9729; y = 0.9507x + 0.0023), and stigmasterol (r^2 = 0.9735; y = 0.9681x + 0.003) compositions (Figures 1–4). An important criterion for the improved

method was that it provided the same assessment of olive oil as the IOC method. Both methods provided identical pass/fail results for each of the six sterol and erythrodiol/uvaol IOC criteria used to assess olive oil grade, thus demonstrating that the improved method produces results consistent with the IOC method. The instrument LOD and LOQ were 0.051 and 0.17 μg/mL, respectively, corresponding to method LOD and LOQ of 0.06 and 0.2 mg/kg, respectively. Importantly, the LOQ was below the IOC brassicasterol limit of 1 mg/kg for a sample with a minimum sterol content of 1000 mg/kg of oil sample (the threshold level for virgin olive oil). The standard concentration curves for cholestanol (1−3000 μg/mL), stigmasterol (1−40 μg/mL), and β-sitosterol (1000−3000 μg/mL) were linear, with r^2 values greater that 0.9997.

The novel procedure improves upon the official IOC method for sterol analysis in several respects. Saponification of a large mass of oil sample requires bulky and expensive reflux and condenser equipment and a substantial amount of solvent. The improved method uses minimal sample mass, thus enabling simultaneous saponification of large numbers of samples in an oven. Extraction of the unsaponifiable fraction using LLE is time-consuming and manually intensive. Heavy emulsions are typical, requiring lengthy waiting periods for the emulsion to settle and multiple washing steps to achieve a neutral pH. The large volumes commonly used in the IOC method require time-consuming rotary evaporations. Alternatively, extraction of a small sample mass using diatomaceous earth enables one person to easily prepare 30 or more samples in 1 working day. Solid supported liquid extraction (SLE) using diatomaceous earth employs a separation mechanism similar to LLE performed with separatory funnels. The aqueous layer is adsorbed and distributed over the solid phase in a thin film, and the unsaponifiable fraction is eluted with diethyl ether. However, extraction using SLE is much faster and prevents the formation of emulsions.

The unsaponifiable fraction contains triterpenic dialcohols, 4-desmethylsterols, 4-monomethyl sterols, 4,4′-dimethylsterols, aliphatic alcohols, triterpenic alcohols, prenols, α-tocopherol, and hydrocarbons.[26] Desmethylsterols and triterpenic dialcohols elute from the column between 21 and 55 min. Without TLC or base-activated silica cartridge cleanup, several compounds in the unsaponifiable fraction coelute with the compounds of interest during GC−FID analysis (Figure 5). These interfering compounds were found in the two prominent bands that migrated immediately ahead of the desmethylsterol band on the TLC plates (data not shown). Unmodified silica SPE did not provide sufficient resolution to separate the sterols and triterpenic dialcohols from these interferences. The use of base-activated silica SPE cartridges enabled sufficient isolation of the desmethylsterol class and triterpenic dialcohols for olive oil analysis. The IOC method and the improved method isolated the desmethylsterol fraction from the unsaponifiable mass of the same oil sample with similar results (Figures 6 and 7). The absence of erythrodiol and uvaol in the chromatogram produced from the IOC method was possibly a result of not cutting the desmethylsterol band broadly enough to include the triterpenic dialcohols, which do not migrate as far as the desmethylsterols on TLC.[26] The potential for this error is avoided using base-activated silica SPE in the improved method, because the triterpenic dialcohols are eluted with the desmethyl sterols after the remaining unwanted fractions of the unsaponifiable mass have been washed from the cartridge with 100 mL of 98:2 hexane/diethyl ether (v/v) (the 98:2 wash

fraction was determined in preliminary tests to be the optimal solvent to remove the unwanted fractions without eluting the fraction of interest). Preliminary studies further demonstrated that 10 mL of 60:40 hexane/diethyl ether (v/v) was a sufficient volume to remove all of the fraction of interest from the silica cartridge. The increased instrument response in Figure 7 compared to that in Figure 6 is likely due to the higher recovery of compounds when using the improved method compared to that of the IOC method (as previously discussed herein); the same sample mass was injected in both cases.

This improved method is reliable and repeatable and provides sterol composition results more quickly and efficiently than the IOC method. The data produced enable assessment of the olive oil quality in accordance with IOC sterol criteria. This method also requires less solvent, less laboratory equipment, and less manipulation to complete than the IOC method. It is particularly noteworthy that this method is a modification of the IOC method in that it extracts the unsaponifiable fraction and isolates the sterol fraction using the same principles established in the IOC method of analysis. Moreover, this research demonstrates the most rapid method to date to determine the olive oil quality in accordance with IOC sterol composition and content criteria. Sterol and triterpenic dialcohol data necessary to determine the sterol composition of olive oil in accordance with IOC standards can be obtained for more than 30 samples by one technician in 1 working day.

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Notes
The authors declare no competing financial interest.

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