APPLICATIONS

Determination of Sterols in Olive Oil using Supported Liquid Extraction (SLE), Solid Phase Extraction (SPE) and GC-FID

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Introduction

Olive oil has been prized since ancient times for its nutritional, medicinal, cosmetic, and even ceremonial value. In the modern era, scientific research on the health benefits of extra virgin olive oil (EVOO) has been a major driving force behind an increase in global interest. Unfortunately, as a high-value food product, EVOO is a frequent target of fraud, often in the form of adulteration. A 2011 study by the UC Davis Olive Center reported that 70 % of samples from the five top-selling imported EVOO brands taken from California supermarkets failed official chemical tests for purity.[1] The report concluded that adulteration with refined olive oil was one of the main causes of failure. However, adulteration with other vegetable oils such as canola, sunflower, soybean and hazelnut oil is also thought to be widespread.

Several official methods for detecting adulterants and determining olive oil quality have been established by the International Olive Council (IOC) to guarantee authenticity and safety. One such method is the determination of sterol concentrations.[2] A sterol profile provides an important criterion for distinguishing virgin olive oil from refined oil as well as a fingerprint for the identification of several seed oil adulterants and even geographical origin.[3] The current IOC method involves saponification of an oil sample with potassium hydroxide, followed by liquid-liquid extraction (LLE). The unsaponifiable fraction, which represents 1-2 % of the total mass of olive oil, is isolated from the dried organic layer, then cleaned up by thin-layer chromatography (TLC). The sterol and triterpene diol fractions are scraped off the TLC plate and reconstituted, then derivatized and analyzed by GC-FID. The LLE step is labor-intensive, consumes large amounts of solvent, is prone to emulsions and includes repeated time-consuming washing steps. The TLC step is also time-consuming and has a low throughput and modest recoveries.[4]

Recently, an improvement to the official sterol method has been reported by Mathison and Holstege and implemented at the UC Davis Olive Center. The LLE step is replaced by supported-liquid extraction (SLE) on a diatomaceous earth (DE) cartridge, and the TLC step is replaced by solid phase extraction (SPE) on a silica gel column.[5] The improved method eliminates several problems and allows multiple samples to be prepared in parallel. The goal of the present work is to show the utility of the improved method for determining sterols, erythrodiol and uvaol in olive oil, using a Strata® DE SLE cartridge and a Strata Si-1 SPE cartridge for sample preparation and a Zebron™ ZB-5*PLUS*™ column for GC-FID analysis.

Materials and Methods

Reference standards were purchased from Sigma-Aldrich®. A Strata DE SLE 60 cc cartridge with a loading capacity of 20 mL was used for the SLE. Drying tubes were prepared from 6 mL disposable syringes hand-packed with anhydrous sodium sulfate (ca. 6 g) over a plug of glass wool. Strata Si-1 was chosen for the SPE cartridges and a Zebron ZB-5*PLUS* column was used for GC analysis. EVOO (certified by the California Olive Oil Council) and canola oil were purchased from a supermarket in Torrance, CA. A 50:50 mixture (v/v) of EVOO and canola oil was used as an example of adulterated olive oil.

Internal Standard Preparation

Add 40 µL of 1 mg/mL cholestanol in chloroform to a clean, dry 20 mL screw-top test tube and evaporate to dryness under a nitrogen flow.

Saponification

1. Add 200 mg of olive oil sample to the test tube containing the internal standard.
2. Add 1.5 mL of 2M Potassium hydroxide in 95 % Ethanol.
3. Cap the tube and heat in an 80 °C oven for 25 minutes.
4. Mix sample gently to ensure homogeneity (sample should appear as a clear solution) and continue heating for an additional 25 minutes.
5. After heating, add 13.5 mL of deionized water and mix. The entire diluted volume is now ready to load onto the SLE cartridge.

SLE (Supported Liquid Extraction) Protocol

Cartridge:	Strata®-DE SLE cartridge, 20 mL loading capacity, 60 cc Tube
Part No.:	8B-S325-VFF
Load:	Diluted sample (from saponification step 5) plus 2 x 1 mL DI water rinse (17 mL total volume, gravity flow)
Wait:	15 minutes
Extract:	3 x 15 mL Diethyl Ether (gravity flow)
Evaporate:	Dry under N_2 at 40 °C (greenish-yellow, oily residue)
Reconstitute:	5 mL of Hexane

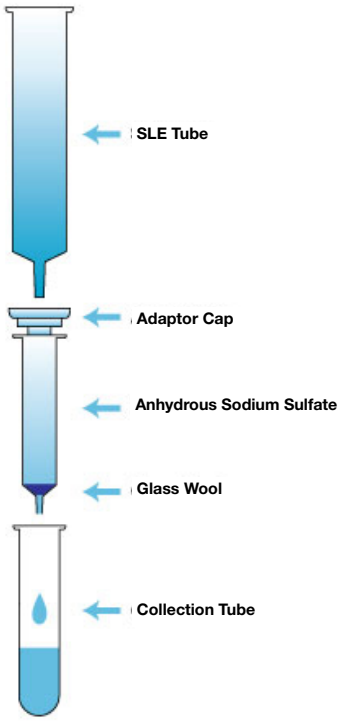
SPE (Solid Phase Extraction) Protocol and Derivatization

Cartridge:	Strata Si-1 (1 g/6 mL) tube
Part No.:	8B-S012-JCH
Condition:	1. 2x 6 mL Hexane 2. 1 mL 0.2M Potassium hydroxide in 95 % ethanol
Equilibrate:	5 mL Hexane immediately after potassium hydroxide elution
Load:	Reconstituted SLE extract (5 mL) followed by 2x 1 mL Hexane rinses
Wash:	85 mL Hexane/Diethyl ether (98:2) under 3" Hg vacuum, flow rate of 2 mL/min.*
Elute:	10 mL Hexane/Diethyl ether (60:40)
Dry:	Dry under N_2 at 50 °C. After evaporating to dryness, add 3-4 drops of acetone and then re-evaporate under N_2 to remove any occluded water. Place in 100 °C oven for 10 minutes.
Derivatization:	250 µL Pyridine/BSTFA (3:1) at 80 °C for 30 minutes

* To handle the large volume of eluant, a 60 mL empty reservoir tube was attached to the 6 mL SPE tube.

The sample is then analyzed by GC-FID.

Figure 1.
SLE setup with sodium sulfate drying tube attached to an SLE column. (Gravity Flow)



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GC Conditions

Column:	Zebron™ ZB-5*PLUS*™
Part No.:	7HG-G032-11
Dimensions:	30 m x 0.25 mm x 0.25 µm
Injection:	Split 5:1 @ 280 °C, 1 µL
Recommended Liner:	Zebron PLUS Single Taper Z-Liner™ (for Agilent® systems)
Liner Part No.:	AG2-0A13-05
Carrier Gas:	Helium @ 0.9 mL/min (constant flow)
Oven Program:	260 °C for 70 min
Detector:	FID @ 300 °C
Samples:	Analytes were derivatized with BSTFA / pyridine (1:3)

Figure 2.
Standards

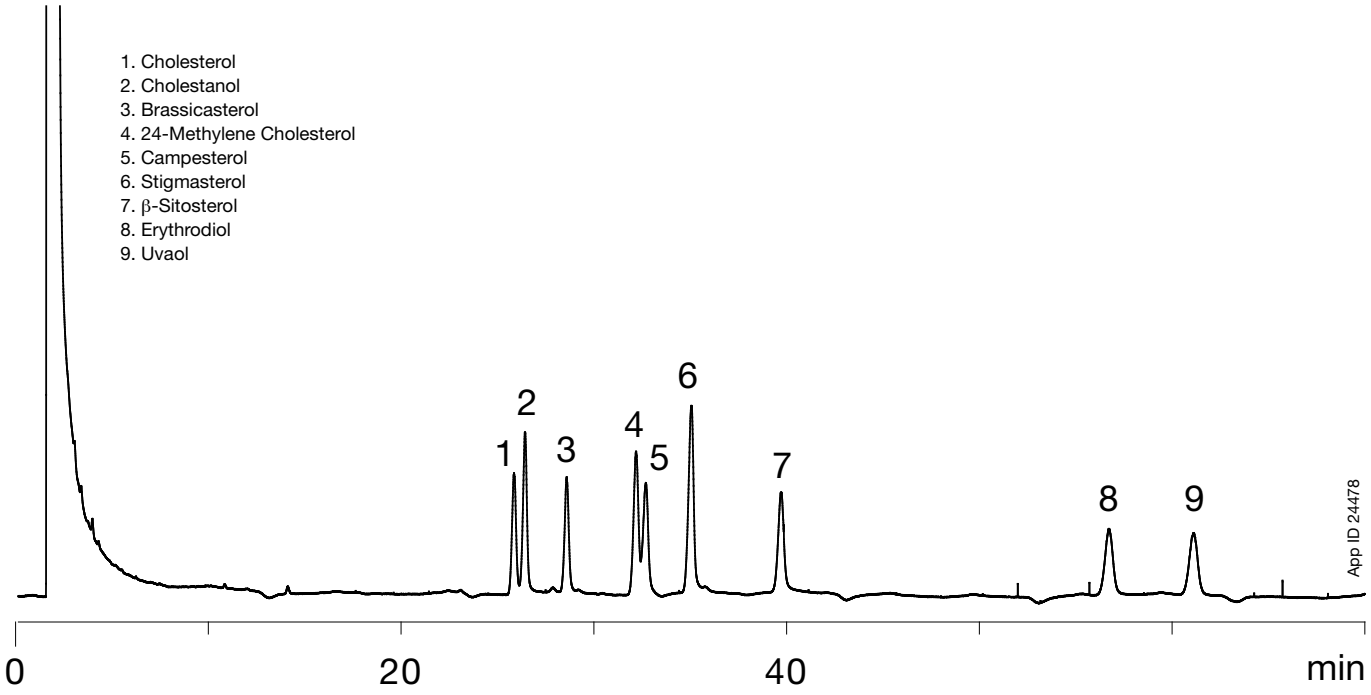

Figure 3.
Extra Virgin Olive Oil Sterols

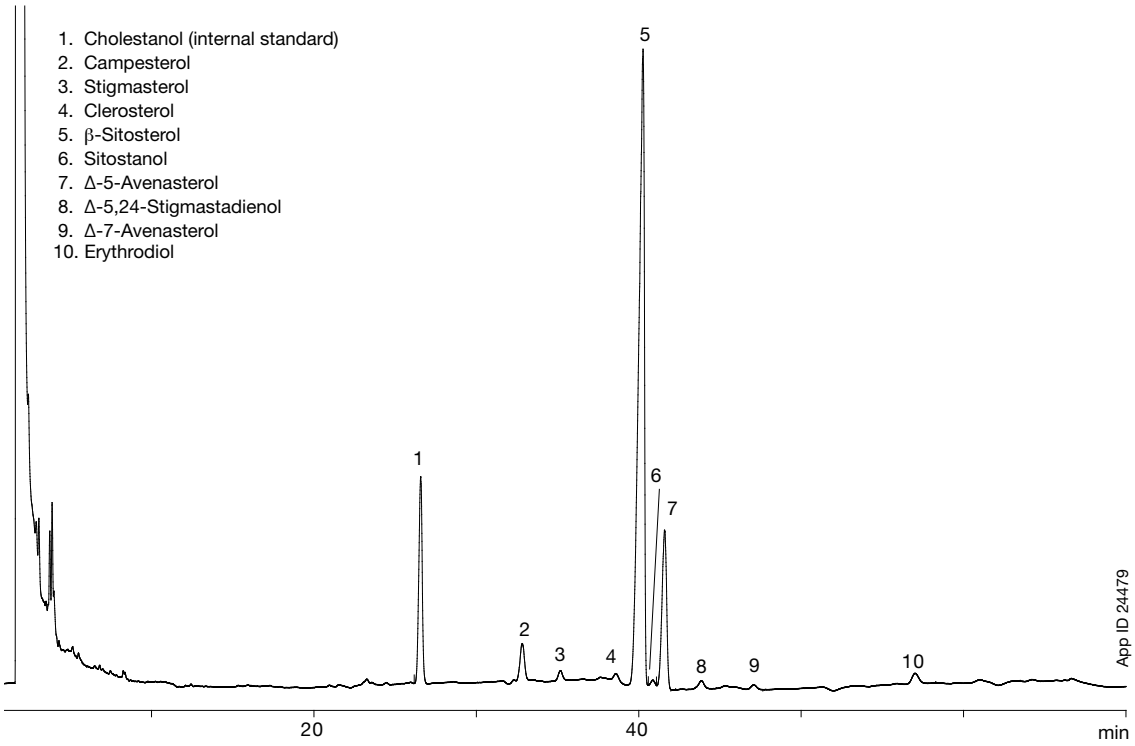

Figure 4.
Adulterated Olive Oil:Extra Virgin Olive Oil/Canola Oil (50:50)

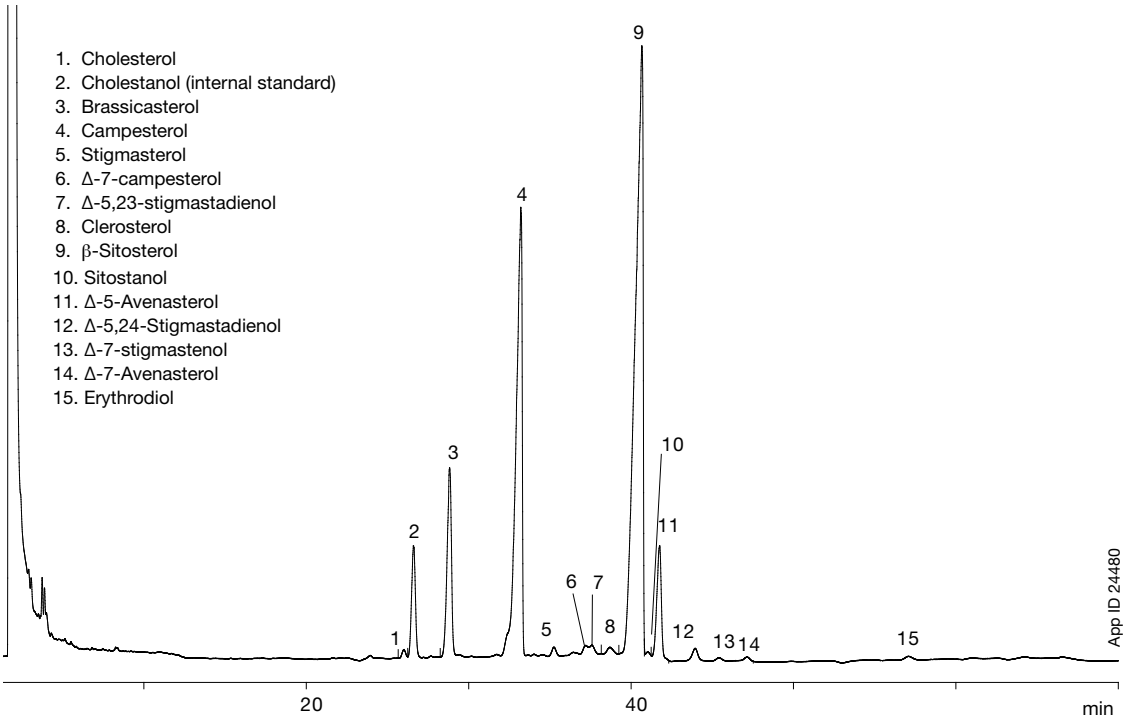

Table 1.
IOC Sterol Criteria for Virgin Olive Oil Classification

Standard Name	IOC Standard Criteria for Virgin Olive Oil	Extra Virgin Olive Oil		Adulterated Olive Oil	
		% Recovery	% RSD (n=3)	% Recovery	% RSD (n=2)
Apparent β-Sitosterol *	≥ 93.0 % of total sterols	94.6 %	0.3	60.3 %	1.2
Cholesterol	≤ 0.5 % of total sterols	not detected	-	0.3 %	13.3
Brassicasterol	≤ 0.1 % of total sterols	not detected	-	8.7 %	0.7
Campesterol	≤ 4.0 % of total sterols	3.8 %	6.8	29.1 %	1.3
Stigmasterol	≤ Campesterol (≤ 4.0 % of total sterols)	1.0 %	9.0	0.6 %	45.0
Δ-7-Stigmastenol	≤ 0.5 % of total sterols	not detected	-	0.7 %	2.9
Uvaol + Erythrodiol	≤ 4.5 % of total sterols	1.8 %	31	0.3 %	1.0
Total Sterols	**≥ 1000 mg/kg**	**1324 mg/kg**	**6**	**4221 mg/kg**	**1.0**

*Apparent β-sitosterol = β-sitosterol + Δ-5-avenasterol + Δ-5,23-stigmastadienol + clerosterol + sitostanol + Δ-5,24-stigmastadienol. Total sterols = cholesterol + 24-methylene cholesterol + brassicasterol + campesterol + campestanol + stigmasterol + Δ-7-campesterol + Δ-5,23-stigmastadienol + apparent β-sitosterol + Δ-7-avenasterol.

Results and Discussion

A chromatogram of nine commercially available analyte standards is shown in **Figure 2**. **Figure 3** shows a chromatogram of an EVOO sample obtained using the Zebron™ ZB-5*PLUS*™ GC column. All peaks were well resolved. A 70 minute run time and isothermal conditions were necessary to obtain good separation. Analytes were identified through a combination of GC-MS, chromatograms of analytical standards and a comparison of the relative retention times (analyte/β-sitosterol) to literature values.[2] It should be noted that the chromatogram in **Figure 3** is very similar in appearance to chromatograms of virgin olive oil found in the literature.[2,5] Importantly, this chromatogram is clearly distinguishable from that of an EVOO sample adulterated with 50 % canola oil (**Figure 4**), in which the brassicasterol and larger campesterol peaks are especially prominent. In contrast, it was difficult to visually distinguish a physical sample of the 50:50 mixture from a sample of pure extra virgin olive oil.

More precise classification of oil quality according to the IOC standards for purity was afforded by quantitation of the analyte peaks. The IOC sterol-related criteria for classification of virgin olive oil are summarized in **Table 1**, along with the relevant sample results. The EVOO sample passed all eight standards, while the adulterated sample failed to meet the standards for total apparent β-sitosterol,

brassicasterol, campesterol and Δ-7-stigmastenol.

The accuracy of the analysis method was assessed by analyzing EVOO samples (n=2) fortified with six reference standards. Recoveries for the analytes brassicasterol (82 %), campesterol (75 %), stigmasterol (77 %), β-sitosterol (100 %), erythrodiol (134 %) and uvaol (118 %) were obtained. These results compare favorably with reported recoveries using the IOC method.[4] The method results were found to be reproducible in terms of chromatogram appearance and quantification.

The improved method allowed an analyst to extract 16 samples in parallel, with the entire analysis completed in two days, and the SLE step required relatively little time, effort and attention compared to a conventional LLE procedure. Additionally, lab cleanup was easy, with no separatory funnels or collection flasks to wash. Further observations and tips on carrying out the full procedure are described below.

In the saponification step, it was found that using a Pasteur pipette was a cleaner, more reproducible way to transfer an oil sample to a test tube rather than using a standard pipettor with polypropylene tips. Thirteen to fourteen drops were approximately equivalent to 200 mg of sample. Once the saponification reaction neared completion, the samples took on a primarily light gold appearance, although some samples from different sources were a deeper orange to orange-red. Differences in color did not affect subsequent analysis.

When performing the SLE step, it was necessary to have a margin between the total aqueous sample volume and the nominal loading capacity of the DE sorbent, to account for potential lot-to-lot variability in water absorption capacity and to avoid accidentally exceeding this limit. Overloading the cartridge by as little as 10 % beyond the nominal capacity led to significant water breakthrough and a failed extraction. In some attempts, water breakthrough overwhelmed the capacity of the sodium sulfate and caused complete blockage of flow. It is speculated that the creation of soaps inherent to the procedure exacerbated the problem. A margin of 12.5 % less than the nominal capacity gave consistently good results. The amount of potassium hydroxide used was adjusted to maintain a concentration of 0.2 M in the diluted loading solution.

The extracts were dried under continuous flow by using an attached drying tube made from a disposable 6 mL syringe and 6 g of anhydrous sodium sulfate packed over a plug of glass wool. However, for a large batch of samples, prepacked 5 g/20 mL Strata® Sodium Sulfate Giga tubes (Part No.: 8B-S124-LEG) may be more convenient to use. In order to ensure an even flow under gravity, these must be vented with a pre-drilled hole near the top of the tube. In the SPE cleanup step, it was important to first activate the silica with 0.2 M ethanolic potassium hydroxide prior to sample loading. Without activation, interferences appeared in the chromatogram and the resolution was poorer.

Conclusion

The use of large volume Strata DE SLE tubes followed by SPE on Strata Si-1 was found to be an effective and relatively rapid method for determining sterol, erythrodiol and uvaol concentrations in olive oil. Acceptable method accuracy was demonstrated by recoveries of six selected analytes ranging from 75 % to 134 %, which are comparable to or better than those reported using the IOC method. Importantly, the SLE and SPE method allowed clear, quantitative distinction between extra virgin olive oil and adulterated olive oil. The EVOO sample passed all eight IOC sterol criteria and the adulterated sample failed four. Equally important, the improved method eliminates the need for large cumbersome glassware which must be cleaned between analyses, and the method was able to process up to 16 samples in parallel over two days, including the GC-FID analysis. The possibility of more improvements to minimize analysis time and increase throughput also exists.

References

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5. Mathison, B. and Holstege, D. A Rapid Method to Determine Sterol, Erythrodiol, and Uvaol Concentrations in Olive Oil. *J. Agric. Food Chem.*, 2013, 61, 4506-4513.

Ordering Information

Strata® DE SLE

Part No.	Description	Unit
8B-S325-VFF	Strata DE SLE, 60cc Tube	16/pk

Strata Si-1 (Silica) SPE Tubes

Format	Sorbent Mass	Part Number	Unit
Tube			
	100 mg	8B-S012-EAK	1 mL (100/box)
	200 mg	8B-S012-FBJ	3 mL (50/box)
	500 mg	8B-S012-HBJ**	3 mL (50/box)
	500 mg	8B-S012-HCH**	6 mL (30/box)
	1 g	8B-S012-JCH**	6 mL (30/box)
Giga™ Tube			
	500 mg	8B-S012-HDG	12 mL (20/box)
	1 g	8B-S012-JDG	12 mL (20/box)
	2 g	8B-S012-KDG	12 mL (20/box)
	5 g	8B-S012-LEG	20 mL (20/box)
	10 g	8B-S012-MFF	60 mL (16/box)
	20 g	8B-S012-VFF	60 mL (16/box)
	50 g	8B-S012-YSN	150 mL (8/box)
	70 g	8B-S012-ZSN	150 mL (8/box)

Zebron™ ZB-5*PLUS*™ GC Columns

ID (mm)	df (µm)	Temperature Limits (°C)	Part No.	with 5 m Guardian
30 Meter				
0.25	0.25	-60 to 360/370	7HG-G032-11	7HG-G032-11-GGA
0.25	0.50	-60 to 360/370	7HG-G032-17	—
0.25	1.00	-60 to 360/370	7HG-G032-22	—
0.32	0.25	-60 to 360/370	7HM-G032-11	—
0.32	1.00	-60 to 360/370	7HM-G032-22	—

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