Quality Analysis of Extra Virgin Olive Oils – Part 5
Nutritive Benefits – Determination of Squalene in Virgin Olive Oil

Application Note

Food Testing & Agriculture

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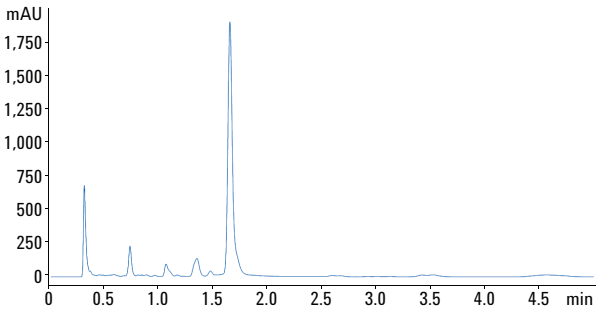
Abstract

Virgin olive oil is a good source of several bioactive components related to highly chemoprotective effects on human health. It is assumed that squalene is partially responsible for the beneficial effects of olive oil, including cancer protection. This Application Note presents the analysis of squalene in olive oil samples with both HPLC and UHPLC methods using the Agilent 1220 Infinity Mobile LC Solution. Olive oil samples were analyzed after fractional crystallization with high precision regarding retention time and area. In addition, high linearity was determined with low limits of detection and quantification.



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Introduction

Virgin olive oil is obtained from the fruits of the olive tree (*Olea europea* L.) only by mechanical procedures and without the use of any thermal or chemical treatment. To ensure authenticity of virgin olive oil regarding adulteration, mislabeling, characterization, or misleading origin, numerous methods have been developed for the evaluation of vegetable oils in the last two decades[1]. The analysis of thermally treated olive oils has been shown in previous application notes[2,3,4].

Regarding nutritive benefits, virgin olive oil is a good source of several bioactive components related to highly chemoprotective effects on human health. Besides the high amount of mono-unsaturated and polyunsaturated fatty acids, it contains highly valuable antioxidants such as vitamin E[5], phytosterols, carotenoids, phenols, and squalene, of which the analysis is highlighted in this Application Note. The levels of these bioactive components are dependent on genetic, agronomic, and environmental factors.

Squalene is a triterpene, containing six isoprene units ($C_{30}H_{50}$ hydrocarbon with six nonconjugated double bonds, Figure 1), primarily found in shark liver oil. Squalene represents a 0.2–0.7 % presence as phytosqualene in olive oil, but only 0.002–0.03 % in other common dietary fats and oils[6]. Squalene is an important intermediate in steroid biosynthesis pathways in both plants and animals. In the human body, it is essential in the biosynthesis of cholesterol, steroid hormones, and vitamin D. As a substantial part of the Mediterranean diet, it may be regarded as partially responsible for the beneficial effects of dietary olive oil, including cancer-protective effects[6,7].

This Application Note shows squalene analysis after sample preparation of different olive oils using fractional crystallization (after Nenadis and Tsimidou[8]). The squalene analysis was carried out on the Agilent 1220 Infinity Mobile LC Solution, which is a robust and rugged system for on-site measurement. Linearity, limit of detection (LOD), and limit of quantification (LOQ) were compared for HPLC and UHPLC analysis. Additionally, the amount of squalene was determined in nine olive oil samples.

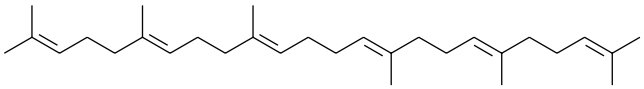

Figure 1. Squalene, a $C_{30}H_{50}$ hydrocarbon.

Experimental

The Agilent 1220 Infinity Gradient LC system with DAD (G4294B) was equipped with a dual gradient pump with integrated degasser, autosampler, column compartment, and the diode array detector. For transportation, the LC can be mounted on a transportation plate, Agilent 1220 Infinity Mobile Upgrade Kit (G4292A).

Sample

Squalene standard was purchased from Sigma-Aldrich, St. Louis, MO, USA. Several olive oils were purchased in local stores.

All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak).

Sample preparation was carried out according to Nenadis and Tsimidou[8].

The oil sample (0.5 g) was vortexed vigorously with 20 mL of methanol:acetone (7:3) in a 25-mL ground-glass stoppered test tube for 1.5 minutes and stored at –20 °C for 24 hours. The sample was rapidly filtered through a coarse filter paper. The solvent was evaporated in a rotary evaporator at 40 °C and the residue dissolved in 5 mL of acetone, which was then subsequently injected to the chromatographic system.

Columns

- Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm (p/n 959990-902) together with guard column (p/n 820950-920)
- Agilent ZORBAX RRHD Eclipse Plus C18, 3 × 50 mm, 1.8 µm (p/n 959757-302) together with guard column (p/n 823750-901)

Software

- OpenLAB CDS ChemStation Edition for LC & LC MS Systems, Rev. C.01.05 [35]
- OpenLAB CDS 3D UV Add-On software

Table 1. Chromatographic conditions.

	4.6 × 250 mm, 5 µm	3 × 50 mm, 1.8 µm
Solvent	A: acetonitrile:acetone (60:40)	
Flow rate	1 mL/min	0.8 mL/min
Isocratic	Stop time – 20 minutes	Stop time – 2.5 minutes
Injection volume	1–10 µL	1 µL
Temperature TCC	30 °C	35 °C
DAD	208 nm / 4 nm, Ref.: off	
Peak width	> 0.10 minutes (2.0 seconds response time) (2.5 Hz)	0.0063 minutes (0.13 seconds response time) (40 Hz)

Results and Discussion

The squalene standard and the prepared olive oil samples were analyzed under HPLC (4.6 × 250 mm, 5 µm column) and UHPLC (3 × 50 mm, 1.8 µm column) conditions regarding linearity, LOD, and LOQ. Eight different concentration levels (from 5,556 µg/g oil down to 3 µg/g, 1:3 dilution) were prepared from the stock solution (50 mg/g) and the linear relationship was determined between the peak area and the corresponding concentrations. LOD and LOQ were defined as the signal-to-noise ratio of 3:1 and 10:1, respectively. Table 2 shows the results of the evaluation. Both methods showed very high linearity with coefficients of determination (R^2) of 0.9997 for UHPLC and even 1.0 for HPLC. LOD and LOQ were improved approximately three times using the UHPLC conditions.

Nine different olive oil samples were prepared according to Nenadis and Tsimidou[8] using fractional crystallization at –20 °C. Figure 2 shows the analysis of the prepared oil HPLC conditions according to Nenadis and Tsimidou[8]. Six consecutive runs were analyzed for precision regarding retention time and area. The relative standards deviation (RSD) of retention time and area was found to be excellent – below 0.05 % respectively 0.28 %.

To shorten the analysis time of the squalene in olive oil, the method was transferred to a UHPLC method using an Agilent ZORBAX RRHD Eclipse Plus C18, 3 × 50 mm, 1.8 µm column. In comparison to a 4.6-mm id column, it was also possible to save solvent (approximately 80 % per analysis) using lower flow rates with the 3-mm column, but gaining similar resolution. Figure 3 displays the UHPLC analysis together with the precision results for retention time and area.

The RSD of retention time and area was found to be below 0.01 % and 0.45 %, respectively, for six consecutive runs, proving excellent precision for the short analysis.

Table 2. Linearity and LOD/LOQ - comparison between HPLC and UHPLC conditions.

	R^2	LOD (ng)	LOQ (ng)
HPLC	1	1.72	5.73
UHPLC	0.9997	0.53	1.77

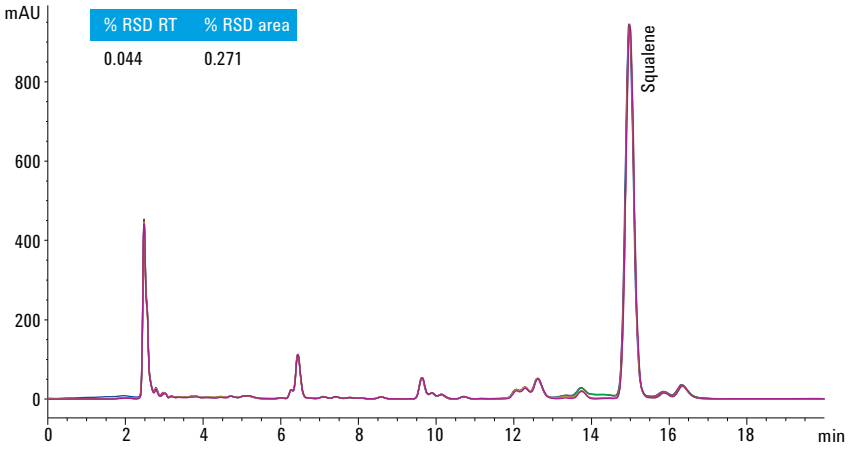

Figure 2. Six consecutive runs of olive oil after fractional crystallization using an Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm.

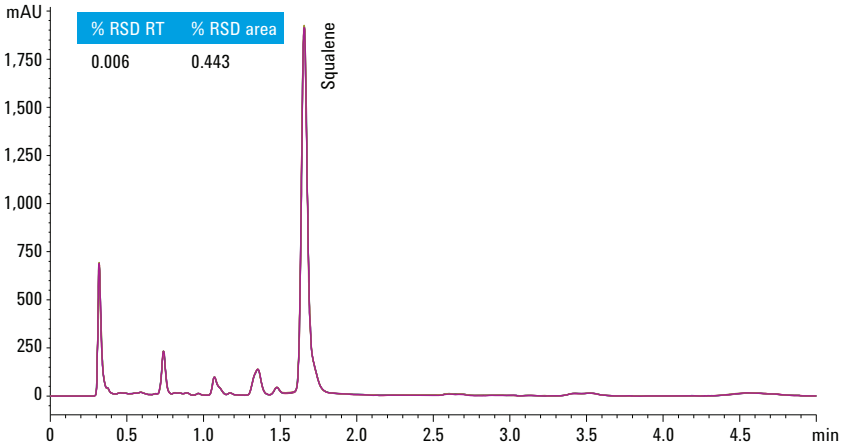

Figure 3. Six consecutive runs of olive oil after fractional crystallization using an Agilent ZORBAX RRHD Eclipse Plus C18, 3 × 50 mm, 1.8 µm.

The squalene content of the different olive oils was determined with UHPLC after sample preparation using fractional crystallization. The content ranged from 1,333 to 2,790 mg squalene per kg oil for virgin olive oils (Table 3), which was consistent with the amount described in literature. Depending on the olive cultivar, a squalene content from 800 to 12,000 mg/kg oil can be present[8]. In refined oil, the squalene content was substantially decreased (700 mg/kg, Table 3), as already shown by Nergiz and Celikkale[9], but still essentially higher compared to seed oils with an mean content of up to 260 mg/kg[9].

Table 3. Determined amount of squalene in different olive oils.

Oil	Squalene in mg/kg oil
Olive 1 (virgin)	2790
Olive 2 (virgin)	2553
Olive 3 (virgin)	2513
Olive 4 (virgin)	2350
Olive 5 (virgin)	2090
Olive 6 (virgin)	1560
Olive 7 (virgin)	1313
Olive 8 (virgin)	1333
Olive 9 (refined)	700

Conclusions

The analysis of squalene in olive oil samples was shown with both HPLC and UHPLC methods using the Agilent 1220 Infinity Gradient LC with DAD. Both methods revealed excellent retention time and area precision as well as excellent linearity. LOD and LOQ were in the one-digit ng range using the HPLC conditions and approximately three times better for the UHPLC conditions using the short, sub-2 µm particle column.

Nine olive oil samples were prepared using fractional crystallization to obtain squalene in the liquid fraction of the olive oil prior to LC analysis. The amount of squalene found was as expected.

The squalene analysis can be carried out on-site in a mobile laboratory, for example, directly on olive farms, using the Agilent 1220 Infinity Mobile LC Solution due to its resistance against shocks or vibrations during transportation.

In summary, the Agilent 1220 Infinity Gradient LC with DAD is perfectly suited for the quantification of squalene in olive oil.

References

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