Quality Analysis of Extra Virgin Olive Oils – Part 6
Nutritive Benefits – Phenolic Compounds in Virgin Olive Oil

Suitable for Agilent
1260 Infinity III LC

Application Note

Food Testing & Agriculture

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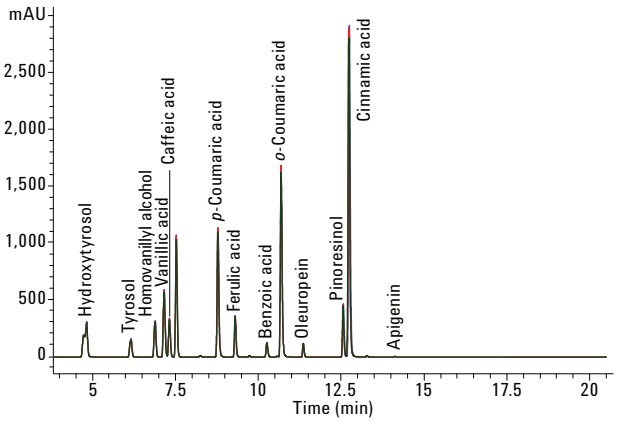
Abstract

Virgin olive oil contains a variety of hydrophilic phenols that are highly valuable to the quality of the oil with regard to sensory and health characteristics. This Application Note describes the analysis of hydrophilic phenolic compounds in olive oil samples, using both HPLC and UHPLC with ternary gradients on the Agilent 1260 Infinity Quaternary LC. The resolution, precision, linearity, and the calculated total phenol content of the analyzed olive oil samples was highly comparable after HPLC and UHPLC analyses. The run time was threefold reduced after UHPLC method transfer. In addition, over 80 % solvent was saved by also reducing the diameter of the column from 4.6 to 3 mm.



Verified for Agilent
1260 Infinity II LC

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Introduction

Virgin olive oil is obtained from the fruits of the olive tree (*Olea europea L.*) by mechanical procedures, without the use of any thermal or chemical treatment. Numerous methods have been developed for the evaluation of vegetable oils to ensure the authenticity of virgin olive oil with respect to adulteration, mislabeling, characterization, or misleading origin[1]. The analysis of thermally treated olive oils has been shown in previous application notes[2,3,4]. Due to nutritive benefits, virgin olive oil is a good source of several bioactive components related to highly chemoprotective effects on human health.

As well as the high amount of mono-unsaturated and polyunsaturated fatty acids, hydrophilic and lipophilic phenols are the most abundant antioxidants in virgin olive oil, affecting sensory and healthy characteristics. In addition, they protect the oil against autooxidation[5,6]. The levels of these bioactive components are dependent on genetic, agronomic, and environmental factors. Lipophilic phenols are represented as tocopherols (vitamin E) in virgin olive oil[7]. Other highly valuable antioxidants present in virgin olive oil, such as squalene[8], phytosterols, carotenoids, and hydrophilic phenols play a distinguished role in antioxidant activity, organoleptic characteristics, and shelf-life. Hydrophilic phenols include phenolic acids, phenolic alcohols, hydroxyisochromans, flavonoids, secoiridoids, and lignans. Figure 1 shows 14 examples of hydrophilic phenols found in virgin olive oil.

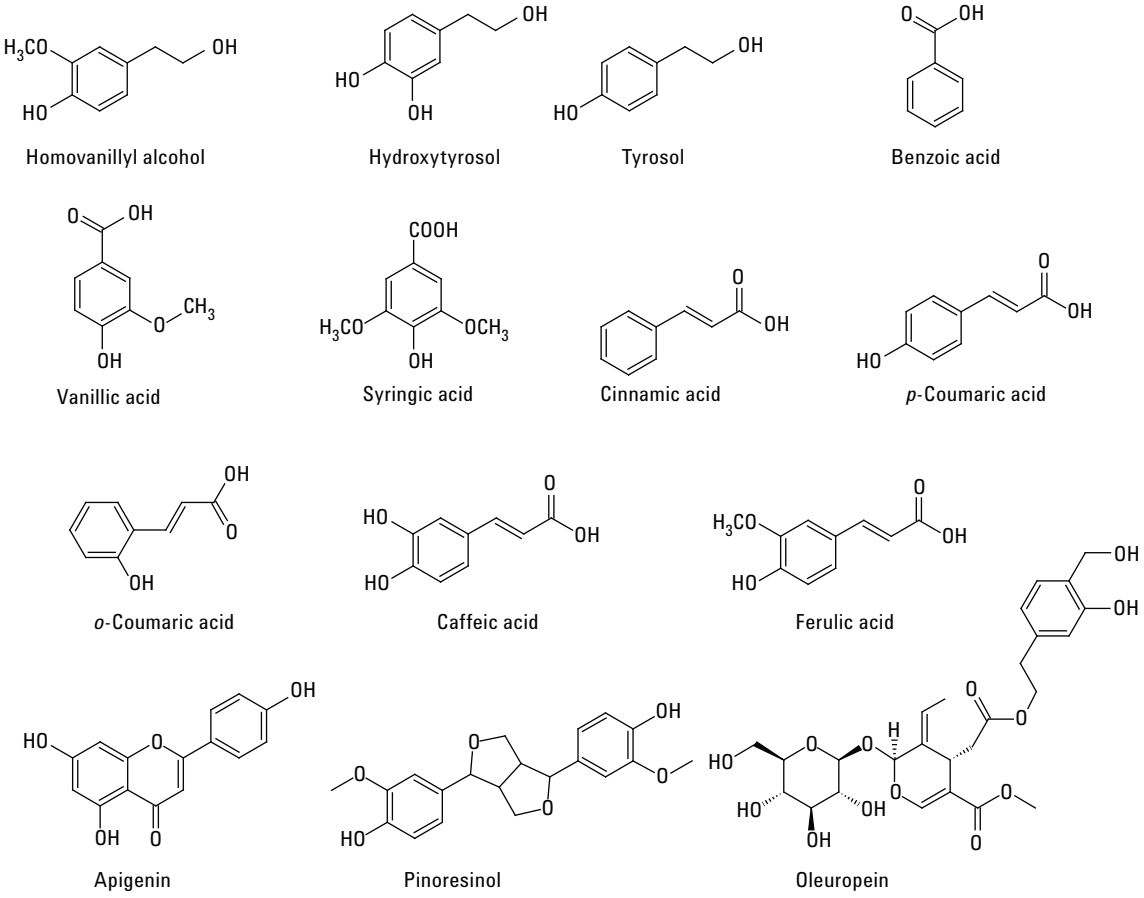


Figure 1. Structures of simple phenolic compounds usually found in virgin olive oil.

Phenolic acids, such as benzoic, vanillic, cinnamic, and many others are found in various plants, and play an important role in color and sensory quality. They can indicate geographical origin and olive cultivars. Phenolic alcohols such as hydroxytyrosol and tyrosol are present in fresh virgin olive oils at relatively low concentrations, but their amount is increased after storage, due to hydrolysis of secoiridoids. Apigenin and luteolin represent the class of flavonoids in virgin olive oil. Secoiridoids such as oleuropein, and lignans such as pinoresinol are the most abundant of these secondary metabolites. The concentration of secoiriroids and lignans is highly affected by agronomic conditions such as cultivar, ripening stage, or geographic origin[6].

Depending on the degree of information needed, different methods can be chosen for the determination of hydrophilic phenols in virgin olive oil. High performance liquid chromatography (HPLC) provides the combination of resolution, efficiency, versatility, and speed of analysis to enable both quantification of individual phenols and total phenol content.

This Application Note shows the analysis of the total phenol content in virgin olive oil in accordance with the operation protocol of the International Olive Council (COI/T.20/Doc no. 29, November 2009)[9]. This method enables a range of measurement from 30 mg/kg to 800 mg/kg. Depending on the agronomic conditions, the range of total phenols is typically between 100 and 500 mg/kg[10] for virgin olive oils. The amount of phenols in refined oils is considerably lower[11].

The analysis is carried out on an Agilent 1260 Infinity Quaternary LC Solution enabling method transfer to an UHPLC method, described in this Application Note. Linearity of two individual phenols, tyrosol and syringic acid, is determined together with precision of retention time and area for 14 different hydrophilic phenol standards. Additionally, the total amount of phenols is measured in 11 different olive oil samples. Linearity, precision, and the total content of the tested olive oil samples is compared between the HPLC and UHPLC methods.

Experimental

The Agilent 1260 Infinity Quaternary LC System consisted of the following modules:

- Agilent 1260 Infinity Quaternary Pump (G1311B)
- Agilent 1260 Infinity Autosampler (G1367E)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316A)
- Agilent 1260 Infinity Diode Array Detector (G4212B), equipped with 10 mm Agilent Max-Light Standard Cartridge Cell

Sample

Phenol standards were purchased from Sigma-Aldrich, St. Louis, MO, USA. Several olive oils were purchased directly from Italian olive oil farms or 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). Phosphoric acid was purchased from Merck, Darmstadt, Germany.

Sample preparation was carried out according the protocol from the International Olive Council (COI/T.20/Doc No 29, November 2009).

External standard solution:
Tyrosol 0.030 mg/mL and syringic acid 0.015 mg/mL in methanol/water 80/20 (v/v)

Internal standard solution:
Syringic acid 0.015 mg/mL methanol/water 80/20 (v/v)

A 2.0 g amount of olive oil was accurately weighed into a 15-mL tube. A 1-mL amount of the internal standard solution was transferred to the previously weighed sample. The sealed sample tube was vortexed for 30 seconds. After the addition of 5 mL of methanol/water 80/20 (V/V) extraction solution, it was again vortexed for exactly 1 minute before further extraction in the ultrasonic bath for 15 minutes at room temperature. Afterwards, the sample was centrifuged at 5,000 rev/min for 25 minutes. An aliquot of the supernatant phase was filtered through a 1-mL plastic syringe with Captiva Premium Syringe Filters Regenerated Cellulose, 4 mm, 0.45 µm (p/n 5190-5107) before injection into the HPLC system.

Columns

- Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm (p/n 959990-902) together with Agilent ZORBAX Eclipse Plus C18, Guard Columns, 4.6 × 12.5 mm, 5 µm (p/n 820950-936)
- Agilent ZORBAX Eclipse Plus-C18, 3 × 100 mm, 1.8 µm (p/n 959964-302), together with UHPLC Guard, Agilent ZORBAX Eclipse Plus C18, 3.0 mm, 1.8 µm (p/n 823750-901)

Chromatographic conditions

The separation is achieved using a ternary linear elution gradient with (A) water 0.2 % H_3PO_4 (v/v), (B) methanol and (C) acetonitrile. Table 1 shows the chromatographic conditions for the HPLC gradient as described in the protocol from the International Olive Council. Table 2 shows the chromatographic conditions after method transfer to UHPLC.

Software

Agilent OpenLAB CDS ChemStation Edition, Rev. C.01.05 [35].

Table 1. Chromatographic conditions, HPLC gradient.

Chromatographic conditions long gradient				
Ternary gradient				
	Time (min)	A) Water + 0.2 % H_3PO_4	B) Methanol	C) Acetonitrile
	0.00	96.00	2.00	2.00
	40.00	50.00	25.00	25.00
	45.00	40.00	30.00	30.00
	60.00	0.00	50.00	50.00
	70.00	0.00	50.00	50.00
	72.00	96.00	2.00	2.00
Stop time	72.00 minutes			
Post time	20.00 minutes			
Flow rate	1 mL/min			
Injection volume	20 µL			
Thermostat autosampler and FC	6 °C			
Temperature TCC	25 °C			
DAD	280 nm/4 nm, Ref.: 360 nm/100 nm			
Peak width	> 0.025 minutes (0.5 second response time) (10 Hz)			

Table 2. Chromatographic conditions, UHPLC gradient.

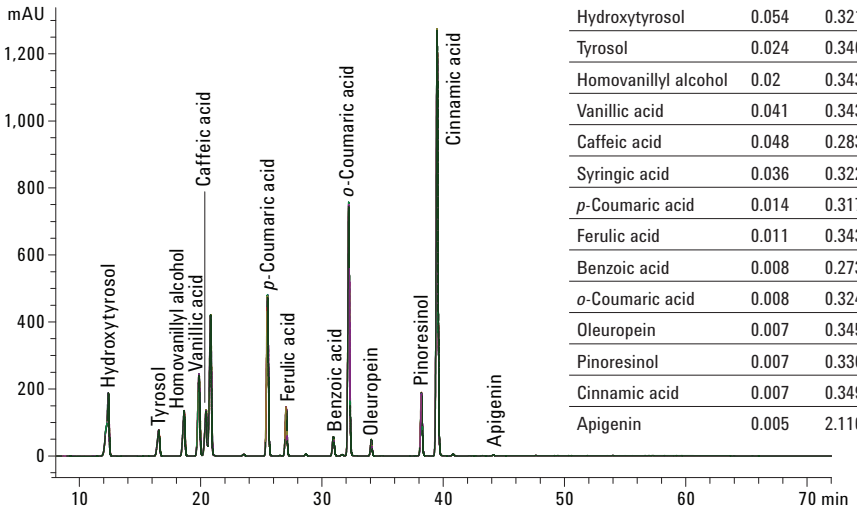
Chromatographic conditions short gradient				
Ternary gradient				
	Time (min)	A) Water + 0.2 % H_3PO_4	B) Methanol	C) Acetonitrile
	0.00	96.00	2.00	2.00
	11.00	50.00	25.00	25.00
	13.00	40.00	30.00	30.00
	17.00	0.00	50.00	50.00
	20.00	0.00	50.00	50.00
	20.50	96.00	2.00	2.00
Stop time	20.50 minutes			
Post time	7.50 minutes			
Flow rate	0.6 mL/min			
Injection volume	8.5 µL			
Thermostat autosampler and FC	6 °C			
Temperature TCC	25 °C			
DAD	280 nm/4 nm, Ref.: 360 nm/100 nm			
Peak width	> 0.013 minutes (0.25 second response time) (20 Hz)			

Results and Discussion

A mix of 14 phenol standards was separated using the HPLC ternary gradient from the International Olive Council protocol. The relative standard deviation (RSD) of retention time (RT) and area were evaluated over seven subsequent runs. Figure 2 shows the results of the separation. The analysis showed excellent precision of retention time and area. The RSD for RT was < 0.055 for all analyzed phenol standards. The area RSD was < 0.35 for all phenol standards except apigenin.

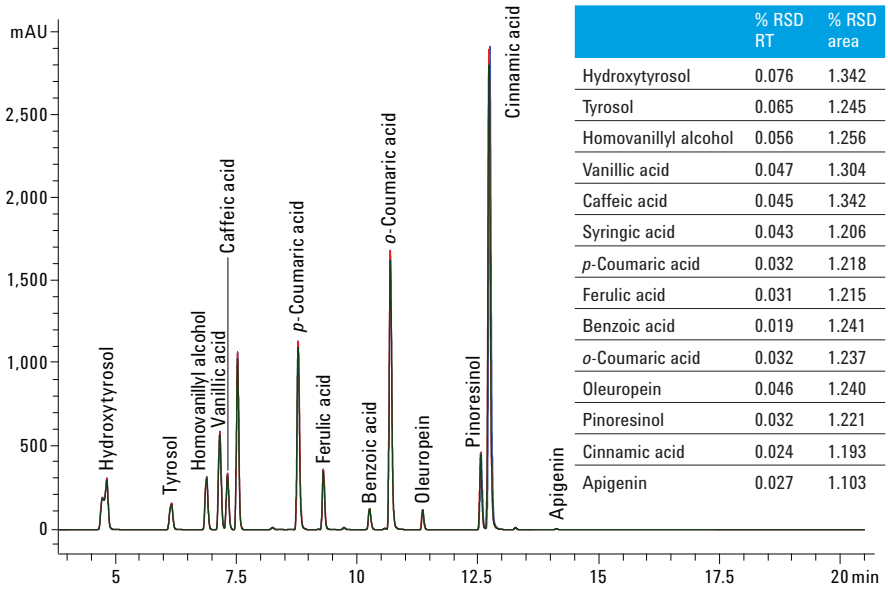
Because of a very long run time of over 70 minutes, the method was transferred to a UHPLC method using a sub-2 µm column (Agilent ZORBAX Eclipse Plus-C18, 3 × 100 mm, 1.8 µm). With this column, it was possible to reduce the gradient time down to approximately 20 minutes while maintaining excellent, even partly improved, resolution. By reducing the diameter of the column from 4.6 to 3 mm, it was also possible to save solvent (over 80 % per analysis). Figure 3 displays the UHPLC analysis together with the precision results for retention time and area. The precision of retention time and area were still excellent. The RSD for RT was < 0.08 for all analyzed phenol standards. The area RSD was < 1.35 for all phenol standards except apigenin.

The linearity of the HPLC and UHPLC methods were also compared. Seven different concentration levels for two different phenol standards were examined.



	% RSD RT	% RSD area
Hydroxytyrosol	0.054	0.321
Tyrosol	0.024	0.346
Homovanillyl alcohol	0.02	0.343
Vanillic acid	0.041	0.343
Caffeic acid	0.048	0.283
Syringic acid	0.036	0.322
p-Coumaric acid	0.014	0.317
Ferulic acid	0.011	0.343
Benzoic acid	0.008	0.273
o-Coumaric acid	0.008	0.324
Oleuropein	0.007	0.345
Pinoresinol	0.007	0.336
Cinnamic acid	0.007	0.349
Apigenin	0.005	2.110

Figure 2 - Overlay of seven runs of a mix of 14 phenol standards together with RSDs for retention time and area using a long HPLC gradient.



	% RSD RT	% RSD area
Hydroxytyrosol	0.076	1.342
Tyrosol	0.065	1.245
Homovanillyl alcohol	0.056	1.256
Vanillic acid	0.047	1.304
Caffeic acid	0.045	1.342
Syringic acid	0.043	1.206
p-Coumaric acid	0.032	1.218
Ferulic acid	0.031	1.215
Benzoic acid	0.019	1.241
o-Coumaric acid	0.032	1.237
Oleuropein	0.046	1.240
Pinoresinol	0.032	1.221
Cinnamic acid	0.024	1.193
Apigenin	0.027	1.103

Figure 3. Overlay of seven runs of a mix of 14 phenol standards together with RSDs for retention time and area using a short UHPLC gradient.

Tyrosol and syringic acid were diluted 1:3 from 0.33 mg/mL down to 0.46 µg/mL for tyrosol and from 0.156 mg/mL down to 0.21 µg/mL for syringic acid. Table 3 shows the results of the evaluation. Both analyses were highly linear with correlation coefficients of 1 for HPLC and 0.9997 for the UHPLC method.

Eleven different olive oil samples were prepared according to the protocol of the International Olive Council (COI/T.20/ Doc no. 29, November 2009). The analysis was carried out under HPLC and UHPLC conditions and the results were compared. The external standard, containing tyrosol and syringic acid as phenol standards, was analyzed first (results not shown). In accordance with the protocol, the value of the response factor ratio of syringic acid to tyrosol ($RRF_{syr/tyr}$) lay inside the required range of 5.1 ± 0.4. This enabled the final result to be expressed as tyrosol, using syringic acid as the internal standard.

The phenol content of the analyzed olive oil samples (natural and oxidized oleuropein and ligstroside derivatives, lignans, flavonoids and phenolic acids) is expressed in mg/kg. The result was calculated according to Formula 1.

$$\frac{mg}{kg} = \frac{\Sigma A \times 1000 \times RRF_{syr/tyr} \times W_{syr}}{A_{syr} \times W}$$

Formula 1

ΣA is the sum of the peak areas of the phenols (hydroxytyrosol, tyrosol, natural and oxidized oleuropein and ligstroside derivatives, lignans, flavonoids, and phenolic acids) recorded at 280 nm.

1,000 is the factor used to express the result in mg/kg.

$RRF_{syr/tyr}$ is the multiplication coefficient for expressing the final results as tyrosol.

W_{syr} is the weight of the syringic acid in mg used as internal standard in 1 mL of solution added to the sample.

A_{syr} is the area of the syringic acid internal standard recorded at 280 nm.

W is the weight of the oil used in grams.

Table 3. Linearity comparison between HPLC and UHPLC conditions.

Phenolic compound	Correlation coefficient HPLC	Correlation coefficient UHPLC
Tyrosol	1	0.99997
Syringic acid	1	0.99997

The phenols were identified by comparing the resulting chromatograms, both to the standards, and to the chromatogram and list in the protocol of the International Olive Council. For the HPLC method, all peak areas from 10 to 52 minutes retention time were summed. For the UHPLC method, all peaks areas from 3 to 16.5 minutes retention time were summed. Figure 4 shows a direct comparison of the chromatogram for the separation of a virgin olive oil with high phenol content after HPLC (4A) and UHPLC (4B) analysis. The phenols between 30 and 52 minutes (HPLC) and between 10 and 16.5 minutes (UHPLC) are not completely resolved. However, to determine the total phenol content, perfect resolution is not necessary in this retention time range. If higher resolution is desired, the Agilent 1290 Infinity 2D-LC Solution can be considered[12,13].

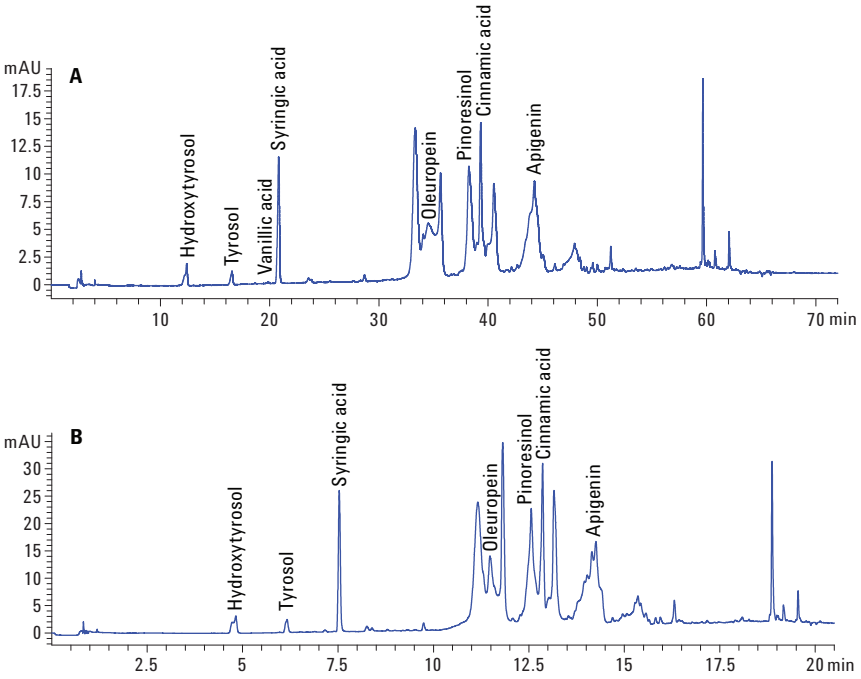


Figure 4. Chromatogram of a virgin olive oil with high phenol content (A) after HPLC analysis, and (B) after UHPLC analysis.

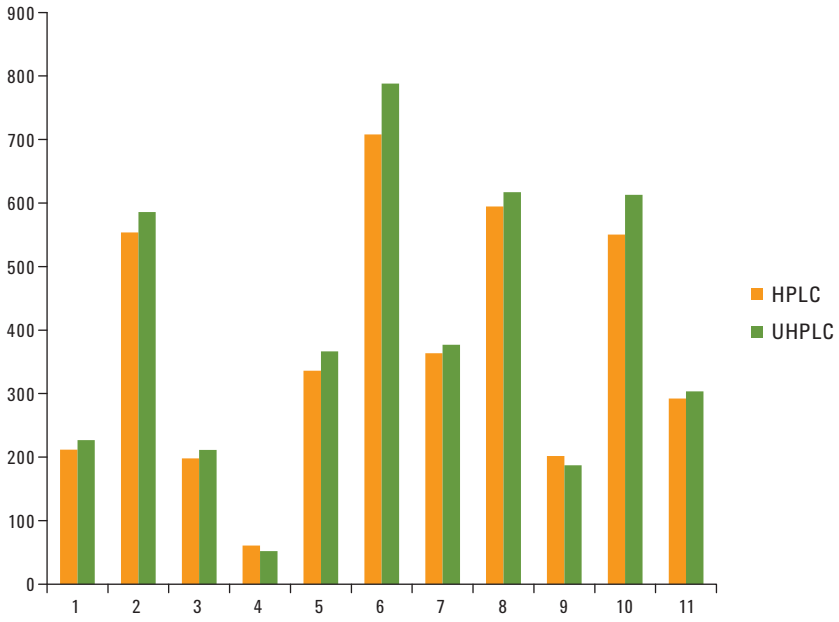
Figure 5 shows the results for the calculated phenol content in the analyzed olive oils. HPLC and UHPLC separation methods were directly compared with respect to their calculated total phenol content. The calculated total phenol content was comparable after HPLC and UHPLC separation. The total phenol content range found in virgin olive oils was from 198/211 up to 708/288 mg phenols per kg oil calculated after HPLC/UHPLC separation. Typically, the variability in the total phenol content originates from different agronomic conditions under which the oils were produced. Refined olive oil (sample 4) contained considerably fewer phenols due to loss during the refining processes.

Conclusions

The analysis of hydrophilic phenolic compounds in olive oil samples is shown with both HPLC and UHPLC methods using ternary gradients with an Agilent 1260 Infinity Quaternary LC. Both methods revealed excellent retention time and area precision as well as excellent linearity for the analysis of phenolic standard compounds.

Eleven olive oil samples were prepared according to the protocol of the International Olive Council by liquid–liquid extraction. All the samples were analyzed using the HPLC and UHPLC methods and the results were compared. The peak pattern plus resolution, and the calculated amount of total phenol content were comparable in the different oil samples. After HPLC separation, the calculated total phenol content ranged between 198 and 708 mg phenols per kg oil. After UHPLC separation, the calculated total phenol content ranged between 211 and 788 mg phenols per kg oil. In the refined olive oils sample, considerably fewer phenols were detected.

In summary, method transfer to the UHPLC method using sub-2 µm columns resulted in considerably shorter run times (threefold reduction in run time) while maintaining the same, or even improved, results. In addition, it was possible to save over 80 % of solvent by reducing the diameter of the column from 4.6 to 3 mm.



Oil	PP content in mg/kg - HPLC	PP content in mg/kg - UHPLC
1	212	227
2	554	586
3	198	211
4 (refined)	61	52
5	336	366
6	708	788
7	364	377
8	595	617
9	202	187
10	551	613
11	292	304

Figure 5. Calculation of phenol content in mg/kg oil. The calculations of the HPLC and UHPLC methods yielded similar results.

References

1. Aparicio and Ruiz, Authentication of vegetable oils by chromatographic techniques, *J. of Chromatography A*, 881, 93–104, **2000**.

2. S. Schneider, "Quality Analysis of Virgin Olive Oils – Part 1" *Agilent Application Note*, publication number 5991-1894EN, **2013**.

3. S. Schneider, "Quality Analysis of Virgin Olive Oils – Part 2" *Agilent Application Note*, publication number 5991-1985EN, **2013**.

4. S. Schneider, "Quality Analysis of Virgin Olive Oils – Part 3" *Agilent Application Note*, publication number 5991-1896EN, **2013**.

5. Beltrán *et al*., "Evaluation of virgin olive oil bitterness by total phenol content analysis" *Eur. J. Lipid Sci. Technol*. Volume 108, 193-197, **2007**.

6. El Riachy *et al.*, "Hydrophilic antioxidants in virgin olive oil. Part 1: Hydrophilic phenols: A key factor for virgin olive oil quality" *Eur. J. Lipid Sci. Technol.* Volume 113, 678-691, **2011**.

7. S. Schneider, "Quality Analysis of Virgin Olive Oils – Part 4" *Agilent Application Note*, publication number 5991-2180EN, **2013**.

8. S. Schneider, "Quality Analysis of Virgin Olive Oils – Part 5" *Agilent Application Note*, publication number 5991-2774EN, **2013**.

9. International Olive Council: COI/T.20/DOC. 29 - 2009 - DETERMINATION OF BIOPHENOLS IN OLIVE OILS BY HPLC, **2009**.

10. Owen *et al*., "Olive-oil consumption and health: the possible role of antioxidants" *The Lancet Oncology* Volume 1, 107-112, **2000**.

11. Garcia *et al.*, "Effect of refining on the phenolic composition of crude olive oils" *JAOCS* Volume 83, No. 2, 159-164, **2006**.

12. E. Naegele, "Qualitative and quantitative determination of phenolic antioxidant compounds in red wine and fruit juice with the Agilent 1290 Infinity 2D-LC Solution" Agilent *Application Note*, publication number 5991-0426EN, **2012**.

13. Reichenbach and Naegele, "2-Dimensional separation of polyphenols in beverages using the Agilent 1290 Infinity 2D-LC Solution and software-assisted 2D-LC data analysis for comparison of ingredients" *Agilent Application Note*, publication number 5991-1455EN, **2013**.

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