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Quality Analysis of Extra Virgin Olive Oils – Part 7

Nutritive Benefits – Determination of Phenolic Compounds in Virgin Olive Oil Using the Agilent 1290 Infinity 2D-LC Solution

Application Note

Food Testing & Agriculture

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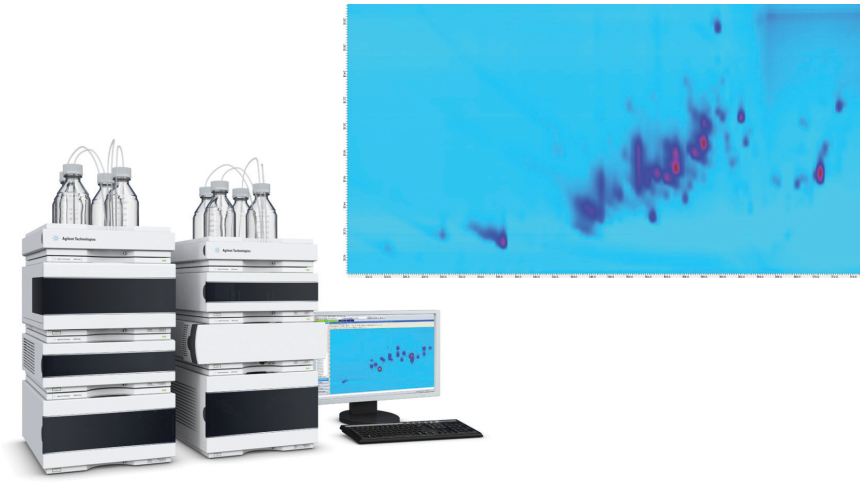
Abstract

Virgin olive oil contains a range of hydrophilic phenols that are associated with nutritional benefits, protect the oil against autoxidation, and are responsible for the characteristic bitter and pungent taste of the oil. This Application Note demonstrates that the Agilent 1290 Infinity 2D-LC Solution can be used to resolve the complex mixture of hydrophilic phenols contained in virgin olive oil by employing two reversed-phase separations. The combination of 2D-LC separation with detection of accurate *m/z* values by time-of-flight mass spectrometry (TOF-MS) enables the identification of hydrophilic phenols contained in virgin olive oils. Furthermore, differences between the compositions of hydrophilic phenols present in virgin olive oils can be investigated.



Introduction

For commercial and health-related reasons, the authenticity of vegetable oils is of great importance. In past years, chromatographic analysis of different compounds of vegetable oils, for example, fatty acids, triglycerides, waxes, and sterols has been used to authenticate vegetable oils[1]. Virgin olive oil is obtained from the fruits of the olive tree (*Olea europea L.*) by mechanical pressing without further refining processes[2,3], and without the use of thermal treatment. The analysis of thermally treated olive oils has been shown in previous Application Notes[4,5,6].

Virgin olive oil is associated with the health and nutritional benefits of a Mediterranean diet, and the presence of antioxidants plays an important role in this respect[7]. Antioxidants in virgin olive oil include tocopherols (vitamin E)[8], carotenoids, chlorophylls, and hydrophilic phenols. In addition to their nutritional benefits, hydrophilic phenols also protect the oil against autoxidation increasing shelf-life. They are also responsible for the characteristic bitter and pungent taste of virgin olive oil[7,9]. The concentrations of phenolic compounds in virgin olive oils depend on the olive cultivar, fruit ripening, geographic, and technological conditions[7].

Hydrophilic phenols present in virgin olive oil include phenolic acids, phenolic alcohols, flavonoids, secoiridoids, and lignans[2,7]. Among the phenolic acids, caffeic, vanillic, sinapic, ferulic acid, and many others, have been found in virgin olive oil[7,10-12]. Phenolic alcohols are mainly represented by hydroxytyrosol and tyrosol[7]. The intact olive fruit contains secoiridoids such as oleuropein and ligstroside. During mechanical pressing, hydrolysis reactions of the secoiridoids lead to the formation of the respective aglycons[7]. Oleuropein aglycon and ligstroside aglycon are present in virgin olive oil in the form of various isomers[3,13]. Flavonoids in virgin olive oil are represented by apigenin and luteolin, and lignans include pinoresinol and acetoxypinoresinol[7]. Figure 1 shows the structures of some hydrophilic phenols present in virgin olive oil.

The analysis of the total phenol content of virgin olive oil in accordance with the operation protocol of the International Olive Council (COI/T.20/Doc No 29, November 2009)[14] was shown in a previous Application Note[15]. In this Application Note, one dimensional analysis could not completely resolve the hydrophilic phenols present in virgin olive oil. Due to its high separation capability, comprehensive two-dimensional liquid chromatography can be used to improve the separation of hydrophilic phenols in virgin olive oil.

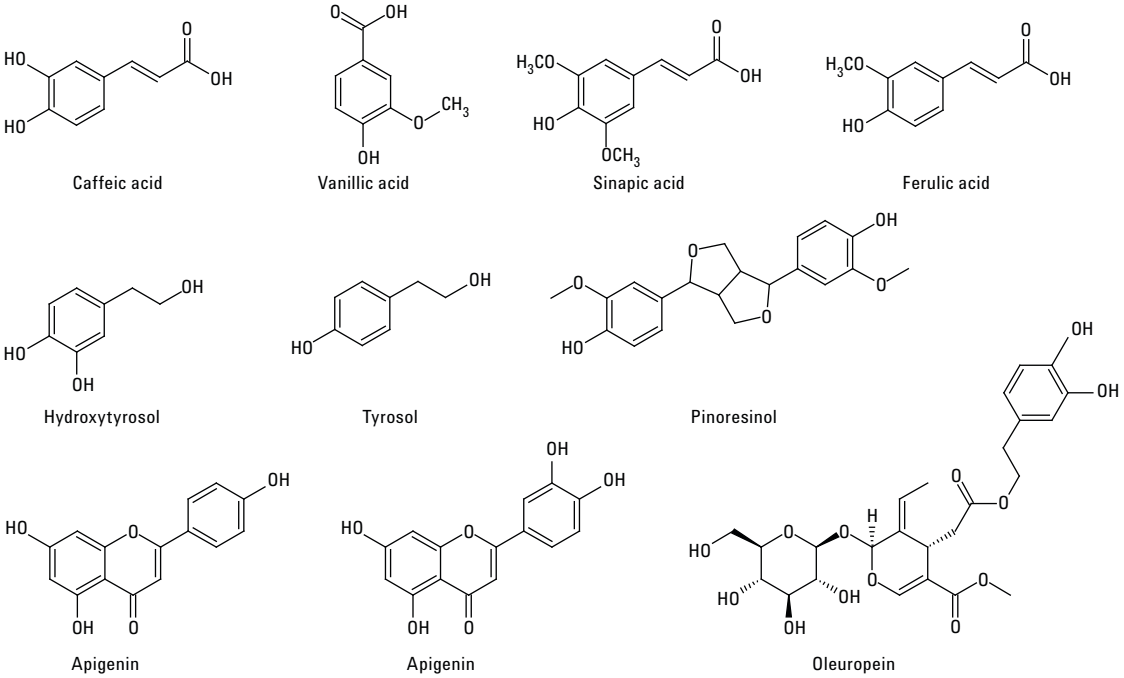


Figure 1. Examples of hydrophilic phenols present in virgin olive oil.

This Application Note shows the separation of hydrophilic phenols in virgin olive oils using an Agilent 1290 Infinity 2D-LC Solution. The method was developed using a mixture of 15 different hydrophilic phenol standards and a sample of virgin olive oil. Two reversed-phase separations were employed with different stationary phase chemistries and solvent conditions. Four virgin olive oils with high phenol content were analyzed, and the composition of the hydrophilic phenols present in those oils was compared. Identification of hydrophilic phenols in the olive oils was performed based on comparison of retention times (RT) with those of phenol standards as well as detection of accurate *m/z* values by TOF-MS in connection with literature data.

Experimental

Equipment

The Agilent 1290 Infinity 2D-LC Solution was comprised of the following modules:

- Two Agilent 1290 Infinity Binary Pumps (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A) with 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-Position/4-Port Duo valve (G4236A) equipped with two 60-µL loops
- Agilent 1290 Infinity Diode Array Detector (G4212A) with a 60-mm Max-Light Cartridge cell (G4212-60007)

Mass spectrometric detection was performed using an Agilent 6530 Accurate-Mass Q-TOF LC/MS System equipped with an Agilent Jet Stream ESI source (G1958-65538).

Columns

First dimension:
Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl, 2.1 × 150 mm, 1.8 µm (p/n 959759-912)

Second dimension:
Agilent ZORBAX RRHD Eclipse Plus C18, 3.0 × 50 mm, 1.8 µm (p/n 959757-302)

Software

- OpenLAB CDS A.02.01. (ChemStation Edition) with Agilent 1290 Infinity 2D-LC Solution add-on software.
- Agilent MassHunter Workstation Software, Data Acquisition Version B.05.01, Qualitative Analysis Version B.06.00.
- GC Image LCxLC Edition Software for 2D-LC data analysis from GC Image LLC., Lincoln, NE, USA.

Sample

Phenol standards were purchased from Sigma-Aldrich, St. Louis, MO, USA. Several olive oils were purchased directly from Italian olive oil farms.

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, EMD Millipore, Billerica, MA, USA).

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

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 (syringic acid 0.015 mg/mL in methanol/water 80/20 (v/v)) was transferred to the previously weighed sample. The sealed sample tube was vortexed for 30 seconds. After adding 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 rpm 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.

Thermostatted Column Compartment

- First dimension column on the right side at 25 °C
- Second dimension column on the left side at 60 °C

Valve

The valve was switched automatically after each second dimension modulation cycle of 30 seconds. The loops were used in a cocurrent manner (filled and eluted from the same side).

First dimension pump	
Solvent A	Water + 0.1 % formic acid
Solvent B	Methanol + 0.1 % formic acid
Flow rate	0.05 mL/min
Gradient	0 minutes, 5 % B 60 minutes, 95 % B 80 minutes, 95 % B
Stop time	80 minutes
Post time	30 minutes
Second dimension pump	
Solvent A	Water + 0.1 % formic acid
Solvent B	Acetonitrile + 0.1 % formic acid
Flow rate	3 mL/min
Gradient	0.00 minutes, 5 % B 0.35 minutes, 15 % B 0.36 minutes, 5 % B 0.50 minutes, 5 % B
Gradient modulation	0.00 minutes, 5 % B to 15 minutes, 5 % B to 60 minutes, 35 % B to 65 minutes, 65 % B 0.35 minutes, 15 % B to 15 minutes, 15 % B to 60 minutes, 60 % B to 65 minutes, 95 % B 0.36 minutes, 5 % B to 15 minutes, 5 % B to 60 minutes, 35 % B to 65 minutes, 65 % B 0.50 minutes, 5 % B to 15 minutes, 5 % B to 60 minutes, 35 % B to 65 minutes, 65 % B
Post time	30 minutes

Autosampler	
Injection volume	20 µL
Sample temperature	6 °C
Needle wash	6 s in methanol
Diode Array Detector	
Before detection, the effluent from the second dimension column was split approximately 1:1 between the DAD and the MS.	
Wavelength	260 nm/4 nm, Ref.: 360 nm/100 nm 280 nm/4 nm, Ref.: 360 nm/100 nm
Data rate	80 Hz
Mass Spectrometer	
Instrument	Agilent 6530 Accurate-Mass Q-TOF LC/MS System
Ionization mode	Negative
Acquisition rate	10 spectra/s
Jet Stream ESI source conditions	
Gas temperature	300 °C
Gas flow	9 L/min
Nebulizer	60 psi
Sheath gas temperature	350 °C
Sheath gas flow	12 L/min
Capillary	–4,500 V
Nozzle	–300 V

Method

The first and second dimension gradients visualized by the 1290 Infinity 2D-LC Solution add-on software are shown in Figure 2.

Results and Discussion

The 1290 Infinity 2D-LC Solution method for the separation of hydrophilic phenols in virgin olive oil was optimized using a mixture of 15 different hydrophilic phenol standards (Table 1) and a sample of virgin olive oil with high phenol content. Figure 3 shows the separation of the hydrophilic phenol standards with UV detection at 280 nm (Figure 3A) and MS detection (Figure 3B). The components of the standard mixture were automatically detected by the software peak detection algorithm.

The comparison of the 2D-LC chromatograms of the mixture of hydrophilic phenol standards with UV detection at 280 nm (Figure 3A) and MS detection (Figure 3B) shows that apigenin (15) could not be detected by UV detection at 280 nm, but by MS detection. The mass spectrometric detection also revealed a coelution of cinnamic acid (13) and pinoresinol (14). Homovanillyl alcohol (4), conversely, was only detected by UV detection. The broad signal, visible in the first-dimension RT range from 35 minutes to 45 minutes of the 2D-LC-MS chromatogram (Figure 3B), also appeared in a blank run, and could possibly originate from an impurity present in the solvents used.

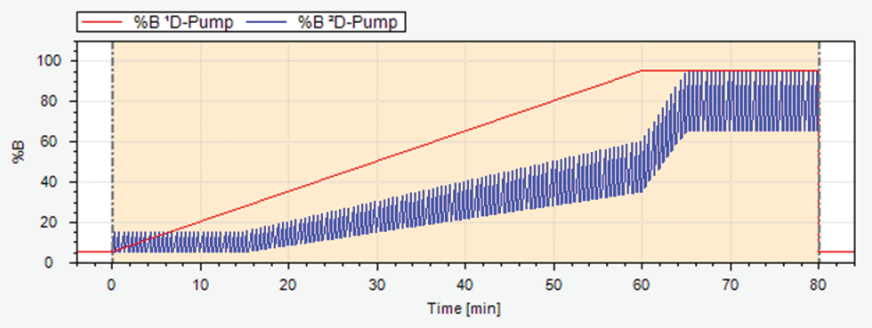


Figure 2. First and second dimension gradients.

Table 1. Compounds contained in the mixture of hydrophilic phenols and their RT in the first and second dimension.

Compound	Compound name	UV detection at 280 nm		MS detection	
		RT I (min)	RT II (sec)	RT I (min)	RT II (sec)
1	Gallic acid	16.50		16.53	
2	Hydroxytyrosol	24.50			
3	Tyrosol	30.00			
4	Homovanillyl alcohol	33.00	12.48	n.d.	n.d.
5	Caffeic acid	34.00			
6	Vanillic acid	34.50			
7	Syringic acid	36.00			
8	*p*-Coumaric acid	40.00			
9	Ferulic acid	41.50		41.53	
10	Benzoi acid	n.d.	n.d.	45.03	
11	*o*-Coumaric acid	45.50			
12	Oleurope	48.00			
13	Cinnami acid	52.00		52.03	
14	Pinoresin	n.d.	n.d.	52.03	
15	Apigen	n.d.	n.d.	56.03	

n.d. = not detected

The developed 2D-LC separation method was applied to the analysis of four different olive oil samples with high phenol content[15] purchased from Italian olive oil farms. Figure 4 exemplarily shows the 2D-LC chromatogram of an olive oil with UV detection at 260 nm. UV detection at 260 nm was more suitable for the analysis of the olive oils than UV detection at 280 nm, as more compounds could be detected at this wavelength. In the first-dimension RT range from 42 minutes to 65 minutes, compounds that would coelute in a one-dimensional separation could be resolved in the second-dimension separation.

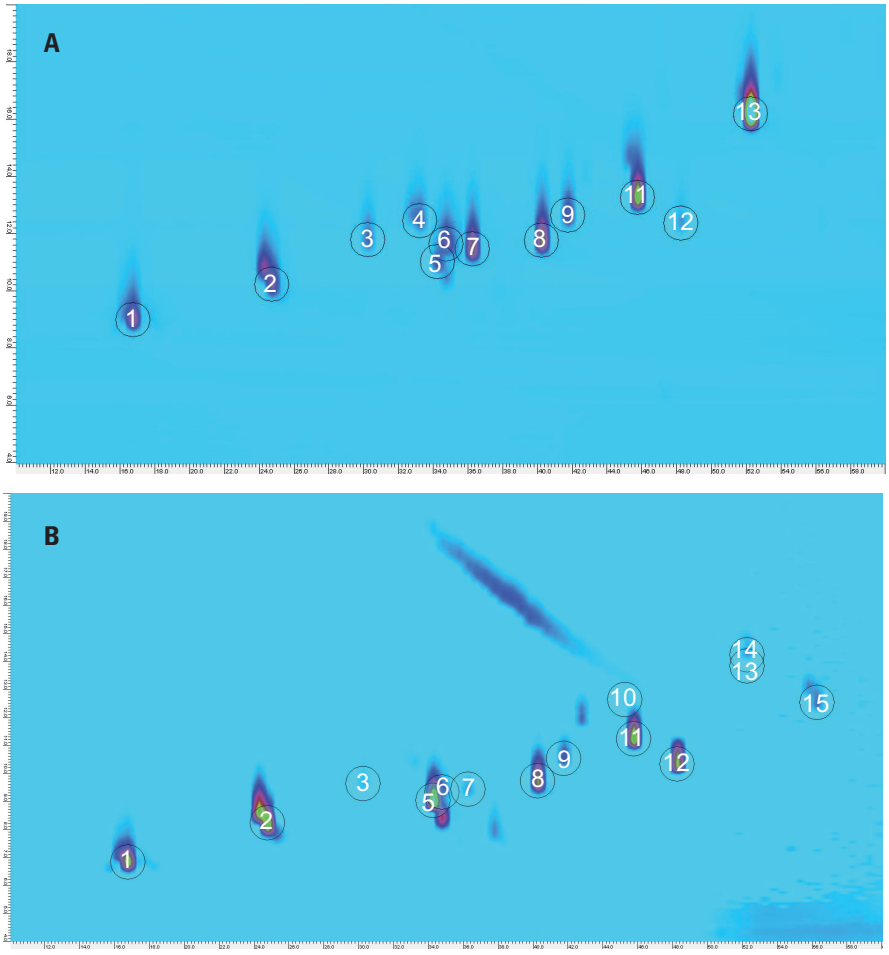


Figure 3. 2D-LC chromatogram of the mixture of hydrophilic phenol standards. (A) UV-detection at 280 nm; (B) MS-detection.

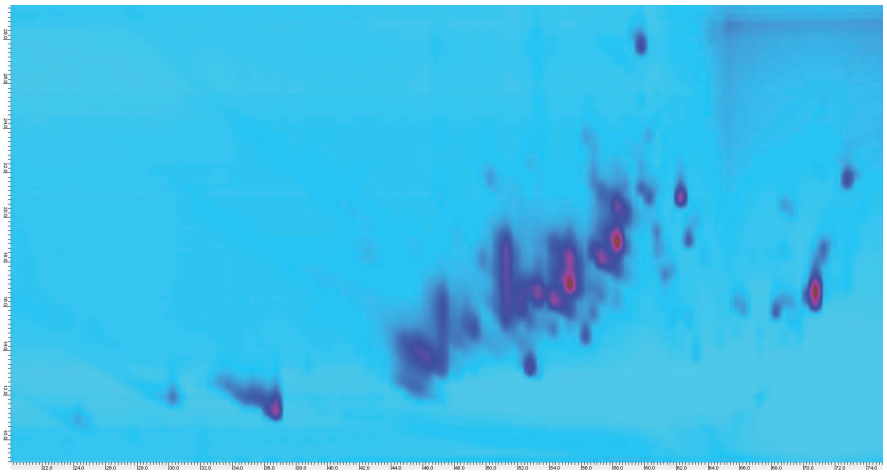


Figure 4. 2D-LC chromatogram of an olive oil with UV-detection at 260 nm.

To enable identification of compounds present in the olive oils but not contained in the mixture of hydrophilic phenol standards, detection of accurate *m/z* values by TOF-MS was used. Figure 5 shows the 2D-LC/MS chromatograms of the different olive oil samples analyzed. The detection of peaks was performed automatically using the software peak detection algorithm. Among the compounds contained in the mixture of hydrophilic phenol standards, only hydroxytyrosol and apigenin could be detected by 2D-LC/MS in all olive oils analyzed.

The detection of accurate *m/z* values in connection with literature data on hydrophilic phenols present in virgin olive oils[3,10-13] showed that the main hydrophilic phenols present in the olive oils analyzed are different isomers of oleuropein aglycon, ligstroside aglycon, and aglycons of decarboxymethyl oleuropein, decarboxymethyl ligstroside, and decarboxymethyl 10-hydroxy oleuropein. Furthermore, elenolic acid, luteolin, apigenin, hydroxytyrosol, and hydroxytyrosol acetate were identified in all olive oils analyzed. Table 2 lists the hydrophilic phenols detected in all olive oils together with their RTs in the first and second-dimension, and their theoretical *m/z* values.

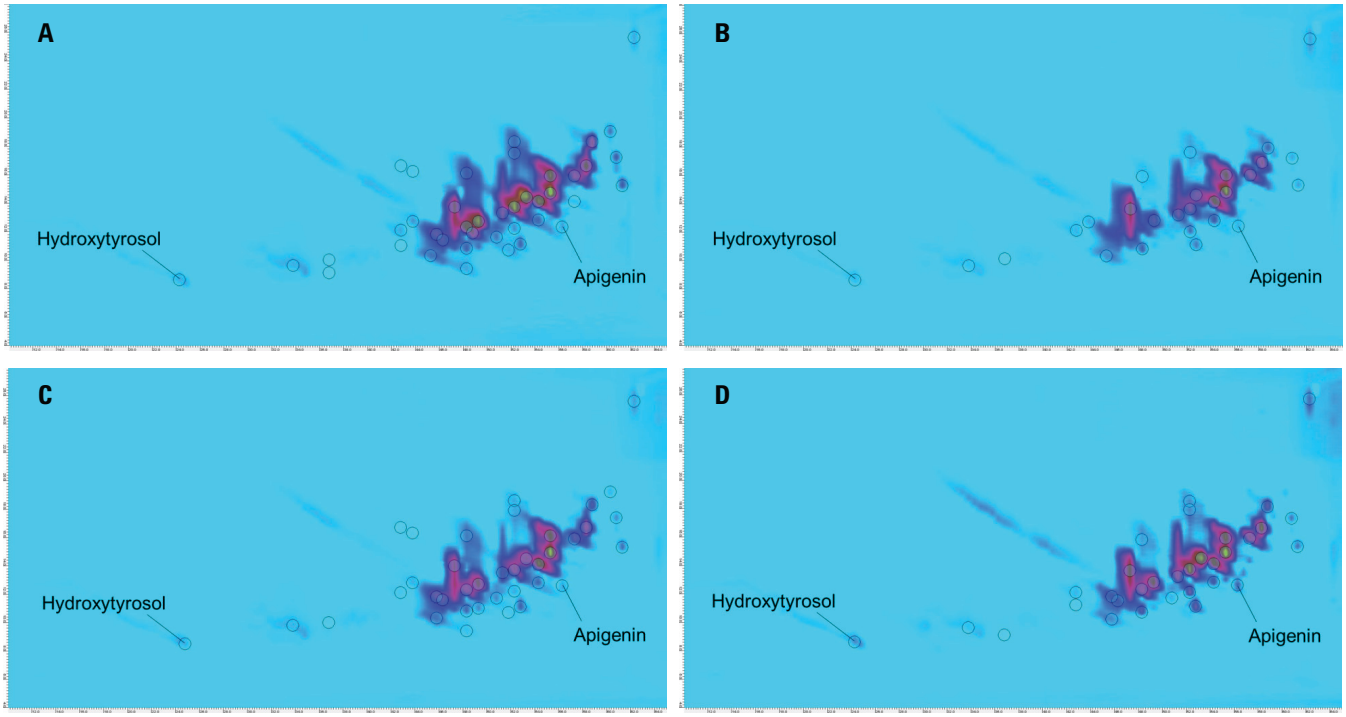


Figure 5. 2D-LC chromatograms of the olive oils A–D analyzed with MS detection.

Table 2. Hydrophilic phenols identified in all olive oil samples.

Compound name	Formula	Theoretical *m/z*	Mean RT I (min)	Mean RT II (sec)
Oleuropein aglycon	$C_{19}H_{22}O_8$	377.1242	48–55	13.6–16.0
Ligstroside aglycon	$C_{19}H_{22}O_7$	361.1293	52–61	14.5–17.7
Decarboxymethyl oleuropein aglycon	$C_{17}H_{20}O_6$	319.1187	47–52	12.1–13.7
Decarboxymethyl ligstroside aglycon	$C_{17}H_{20}O_5$	303.1238	51.03	13.29
Decarboxymethyl 10-hydroxy-oleuropein aglycon	$C_{17}H_{20}O_7$	335.1136	**48.03**	1
Elenolic acid	$C_{11}H_{14}O_6$	241.0718	**33–45**	9.6–
Luteolin	$C_{15}H_{10}O_6$	285.0405	52.53	11.09
Apigenin	$C_{15}H_{10}O_5$	269.0455	56.03	12.47
Hydroxytyrosol	$C_8H_{10}O_3$	153.0557	24.16	8.57
Hydroxytyrosol acetate	$C_{10}H_{12}O_4$	195.0663	42.53	12.09

To show the differences between the compositions of hydrophilic phenols present in the olive oils analyzed, the percent responses of the individual peaks detected in the olive oils were averaged to generate an average percent response for each substance detected. The percent responses calculated by the GC Image LCxLC Edition Software were exported. Using Excel, the differences between this average percent response and the percent responses of the individual peaks detected in the olive oils were calculated and are shown in Figure 6.

Olive oils A–C were produced at the same olive oil farm in Italy, and olive oil D originated from a second Italian olive oil farm. One difference between olive oils A–C and D that can be recognized from Figure 6, is that the flavonoids apigenin and luteolin show higher percent responses in olive oil D than in olive oils A–C. This could be related to differences in the cultivation of the olives or the production of the oils.

Conclusion

This Application Note demonstrates that the Agilent 1290 Infinity 2D-LC Solution can be used to significantly improve the separation of hydrophilic phenols contained in virgin olive oil compared to a one dimensional separation. The combination of 2D-LC separation with detection of accurate *m/z* values by time-of-flight MS enabled the identification of several hydrophilic phenols contained in the olive oils analyzed. Furthermore, differences between the compositions of hydrophilic phenols present in the olive oils can be easily investigated and shown using data generated by the GC Image LCxLC Edition software.

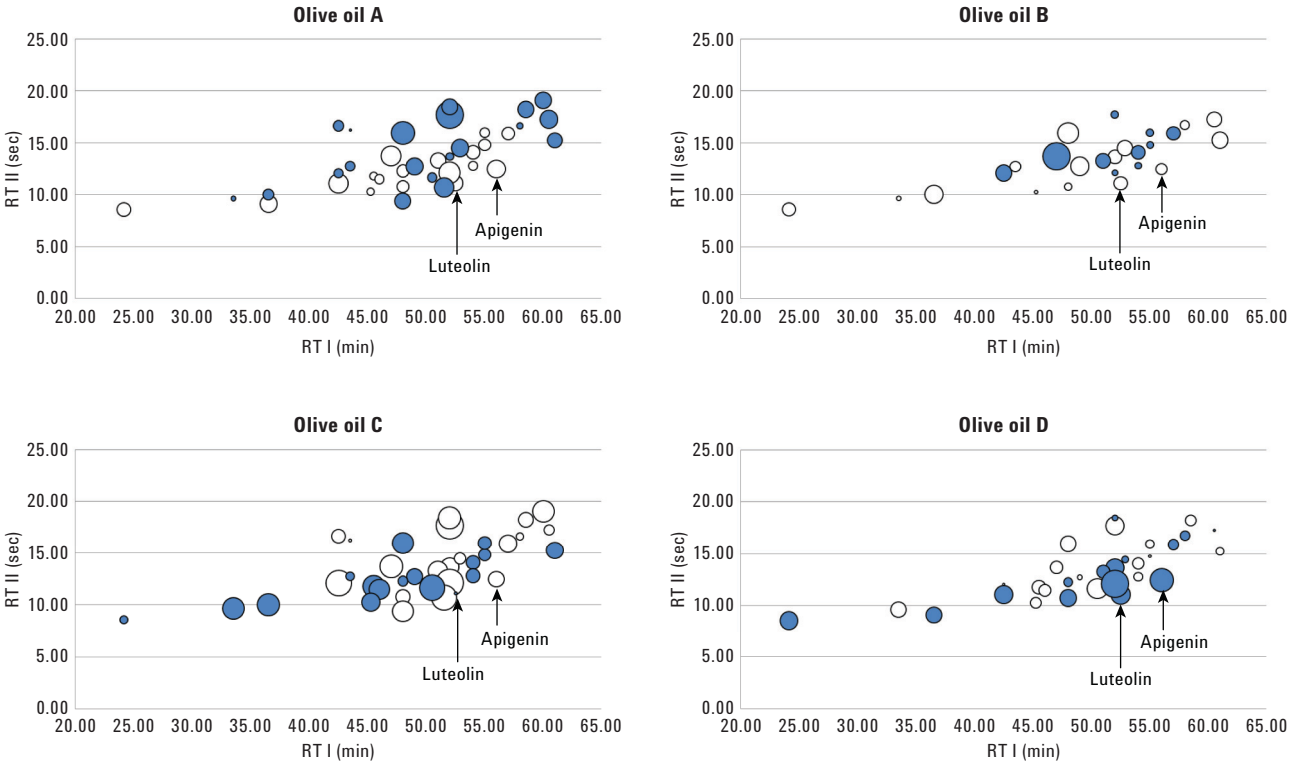


Figure 6. Differences between peak detection in each olive oil and an average of the olive oils analyzed (blue circles indicate peaks with a higher percent response than the average percent response of that substance; white circles indicate peaks with a lower percent response than the average; areas indicate differences).

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