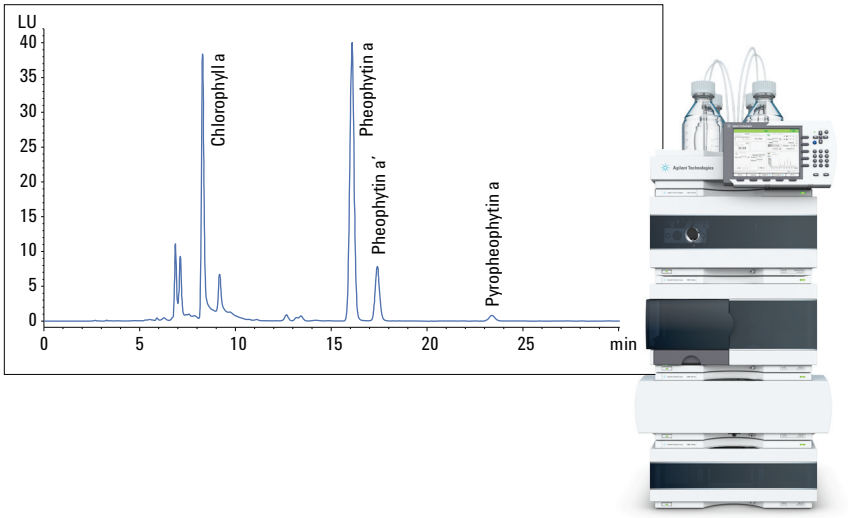
Quality analysis of virgin olive oils – Part 3

Thermal treatment analysis – determination of the chlorophyll a and a' degradation products pheophytins a, a' and pyropheophytin a using the Agilent 1260 Infinity LC System with Fluorescence Detector

Application Note

Food Analysis & Agriculture



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Abstract

This Application Note shows the analysis of the chlorophyll degradation products pheophytin a, a' and pyropheophytin a after sample preparation according to ISO 29841:2009(E). Pyropheophytin a was found in longer stored virgin olive oils as well as in partly refined olive oil. Fresh olive oil (about three months old) revealed only minor amounts of pyropheophytin a. In addition to a longer analysis time of 30 minutes, the establishment of a short run was possible with a 50-mm column, still obtaining great resolution using sub-2 µm particles. Using the Agilent 1260 Infinity LC with fluorescence detection, virgin olive oil can be analyzed for proper storage conditions or potential refining processes.

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Introduction

There are different important quality characteristics for virgin olive oils. In addition to the determination of stigmastadienes[1], and the analysis of the concentration of polymerized triacylglycerides[2] in olive oil, the analysis of chlorophyll degradation products is a further important factor.

Chlorophyll content reduces up to 80% during the oil extraction process, using only mechanical and physical processes for extraction[3]. This results in a considerably reduced chlorophyll level in the oil compared to the olive fruit itself. The pigment composition depends quantitatively and qualitatively on the genetic basics of the olive plant. The chlorophyll a content is approximately three times higher than the chlorophyll b content[4].

During the extraction processes, the chlorophyll a and b pigments (initially found in the fruit) are converted into the more stable pheophytins due to the release of acids after the mechanical breakdown of the plant tissue[5]. Pheophytins are chlorophyll molecules where the central Mg^{2+} is replaced by two hydrogen ions, (Figure 1).

In addition, a slowly developing decarbomethoxylation takes place on C-13 of the pheophytin molecule (R_2), which originates pyropheophytin[6]. Freshly extracted virgin olive oils do not contain pyropheophytins. However, it is generated during storage in different amounts depending on the conditions to which the oil has been exposed. This reaction is boosted by heat treatments during refining processes, especially during deodorizing[6].

That leads to the possibility of using the degradation products of chlorophyll as a marker for quality and traceability of virgin olive oils. With the obtained values, it can be determined if an olive oil, labeled as virgin, was properly stored or, in contrast, if the virgin olive oil was cut with refined oils. For all investigations, it is essential to consider the exact harvesting date.

Experimental

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

- Agilent 1260 Infinity Quaternary Pump (G1311B)
- Agilent 1260 Infinity Autosampler (G1367E)
- Agilent 1290 Thermostatted Column Compartment (G1316A)
- Agilent 1260 Infinity Fluorescence Detector (G1321B), equipped with standard FLD flow cell

Sample

The chlorophyll a standard was purchased from Sigma-Aldrich, St. Louis, MO, USA and was dissolved in acetone. Pheophytin was generated by acidification of the chlorophyll standard using formic acid. Additional heating over 100 °C further produced pyropheophytin, which was, together with the pheophytins, used as retention time reference standards. Several olive oils (virgin and partly refined olive oils) were purchased in local stores. The SPE extraction was carried out using Bond Elut SI cartridges 1 g, 6 mL (p/n 12256008). Sample preparation was carried out according to ISO 29841:2009(E).

Solvents

All solvents used 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).

Chlorophyll species	Mg	R_1	R_2
Chlorophyll a	+	$C_{20}H_{39}$	CH_3COOH
Pheophytin a	–	$C_{20}H_{39}$	CH_3COOH
Pyropheophytin a	–	$C_{20}H_{39}$	H

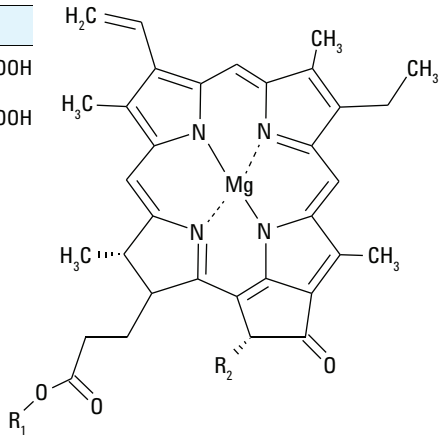

Figure 1
Structure of chlorophyll and its degradation products.

Columns

- Agilent ZORBAX SB-C18, 4.6 × 250 mm, 3.5 µm (p/n 884950-567)
- Agilent ZORBAX RRHT SB-C18, 4.6 × 50 mm, 1.8 µm (p/n 822975-902)

Software

OpenLAB CDS ChemStation Edition for LC & LC MS Systems, Rev. C.01.04 [35]

	Long run	Short run
Mobile phase:	Water:methanol:acetone (4:36:60)	
Flow rate:	1 mL/min	1.2 mL/min
Isocratic run:	Stop time – 30 minutes	Stop time – 8 minutes
Injection volume:	20 µL	
Temperature TCC:	RT	20 °C
FLD:	Ex: 430 nm, Em: 670 nm	
Peak width:	> 0.05 min (1 s resp. time) (9.26 Hz)	

Table 1
Chromatographic conditions.

Results and Discussion

Figure 2 shows a chromatogram of chlorophyll a and its degradation products pheophytin a, pheophytin a', and pyropheophytin. Due to the lack of pheophytin and pyropheophytin standards, chlorophyll a was used to produce these degradation products. Pheophytin was generated by acidification of the chlorophyll standard using formic acid. Additional heating over 100 °C further produced pyropheophytin a.

The epimers chlorophyll a' (respectively pheophytin a') were generated by isomeric relocation. Due to the decarbomethoxylation reaction, no isomers were found for the pyropheophytins.

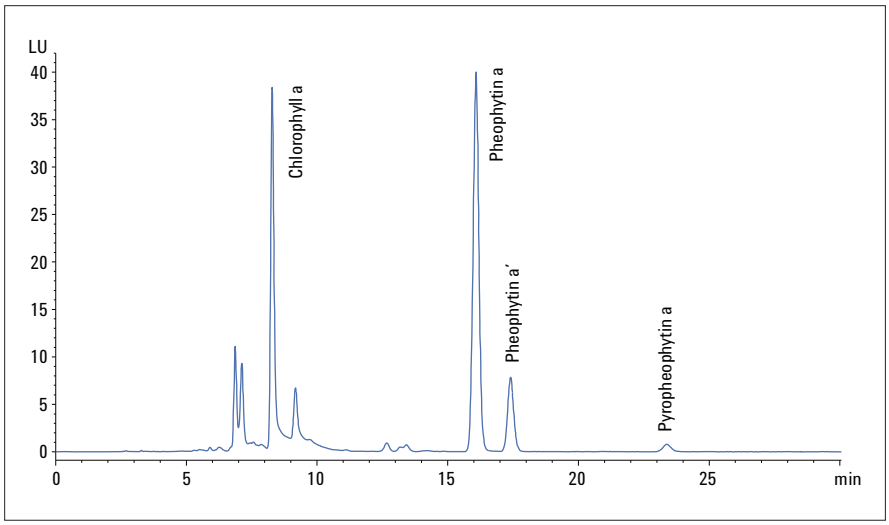

Figure 2
Separation of chlorophyll a and its degradation products.

Seven olive oils were analyzed for chlorophyll degradation products after solid phase extraction (SPE). Figure 3 shows the separation of chlorophyll degradation products in virgin olive oil (same brand) from olives from the same region, but from two different harvesting years, 2011 and 2012. The percentage of pyropheophytin is substantially higher (11.5%) in the virgin olive oil from 2011 compared to the one from 2012 (1.5%), (Table 3). Pyropheophytin is not present in freshly extracted oils, but its concentration increases during storage depending on conditions such as light and temperature[6].

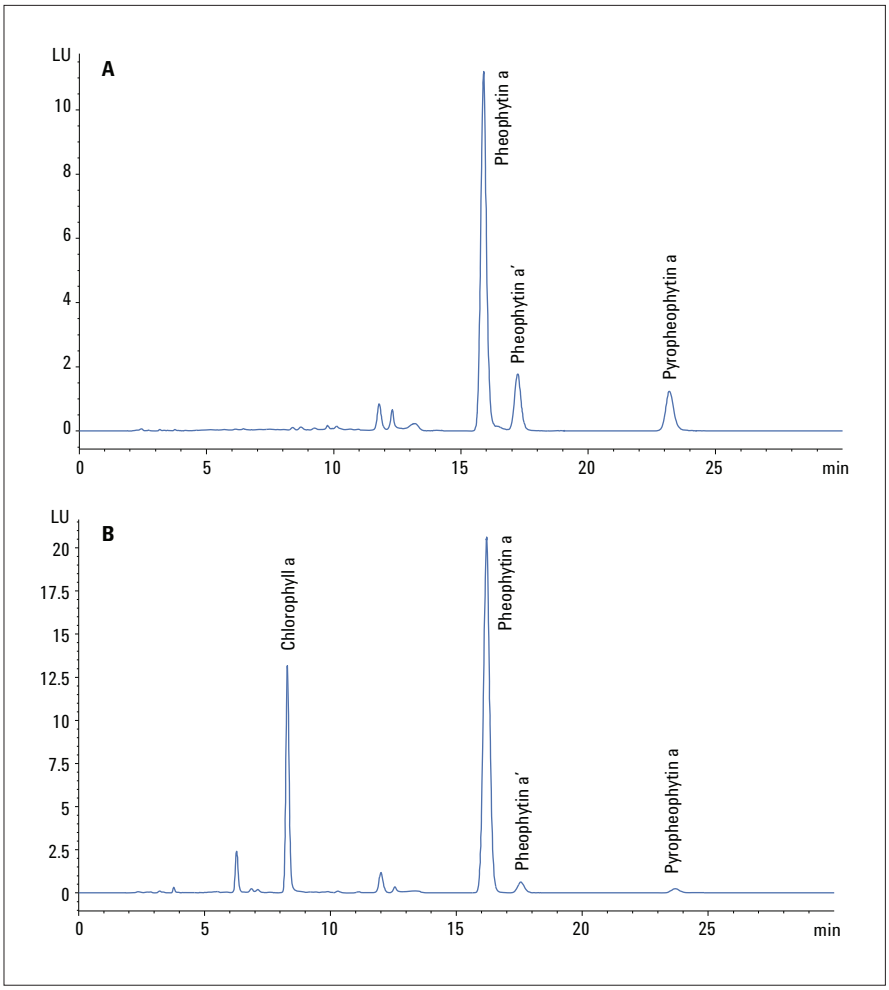

Figure 3
Separation of chlorophyll a and its degradation products in virgin olive oil of the same brand, harvest 2011 (A) and harvest 2012 (B).

Harvest	2011	2011	2011	2011/2012	2011/2012	2012	n.k.
Olive oil	Virgin olive oil 1	Virgin olive oil 2	Virgin olive oil 3	Virgin olive oil 4	Virgin olive oil 5	Virgin olive oil 6	Partly refined olive oil
% pyropheophytin	13.54	11.50	13.10	14.76	8.12	1.46	18.03

Table 3
Relative content of pyropheophytin in seven olive oils.

Figure 4 shows the separation of chlorophyll a degradation products from partly refined olive oil with a higher percentage of pyropheophytin compared to the virgin olive oils. Pyropheophytin develops slowly during storage of the olive oils, but this reaction is amplified to a great extent if the oil is exposed to heat treatment during refining processes.

All relative contents of pyropheophytin a to pheophytin a in seven olive oils were calculated using Formula 1. This formula is independent of absolute quantities and can be directly calculated from the ratio of the corresponding peak areas. Table 3 summarizes the relative contents of pyropheophytin a in seven olive oils. Virgin olive oil from 2012 showed a significantly lower amount of pyropheophytin a compared to the virgin olive oils from 2011 or 2011/2012 (virgin olive oil, produced from an olive mix of the harvest of 2011 and 2012) or the refined olive oil.

To accelerate the analysis time of the chlorophyll degradation products in olive oil, a short 50-mm column with sub-2 µm particles was used (Agilent ZORBAX RRHT SB-C18, 4.6 × 50 mm, 1.8 µm). Figure 5 shows a short run of the analysis of the fresh virgin olive oil in 8 minutes, producing great resolution between the pheophytins a and a′ and pyropheophytin a.

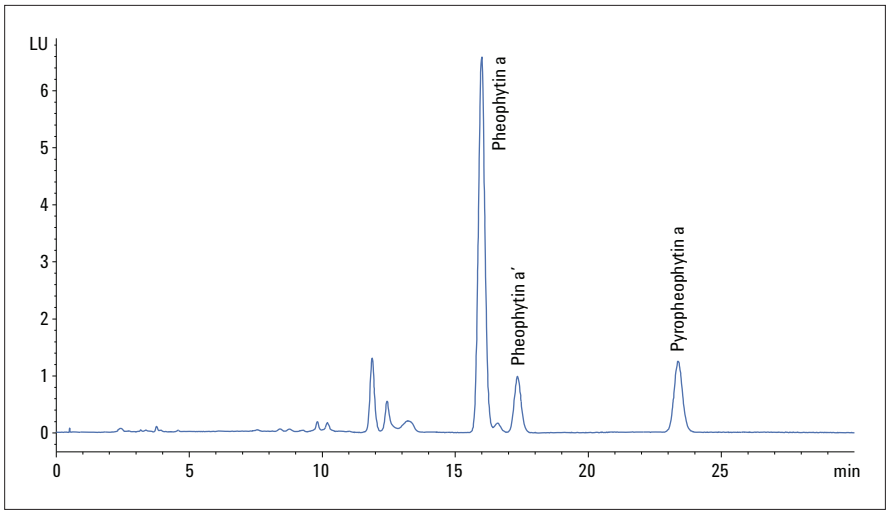

Figure 4
Separation of chlorophyll a degradation products from partly refined olive oil.

$$\%\ pyroa = 100 \times \left(\frac{pyro_a}{pheo_a + pyro_a} \right)$$

% pyroa = % pyropheophytin in sample
$pyro_a$ = Peak area of pyropheophytin a
$pheo_a$ = Sum of peak areas of pyropheophytin a and a′

Formula 1
Calculation of the relative content of pyropheophytin.

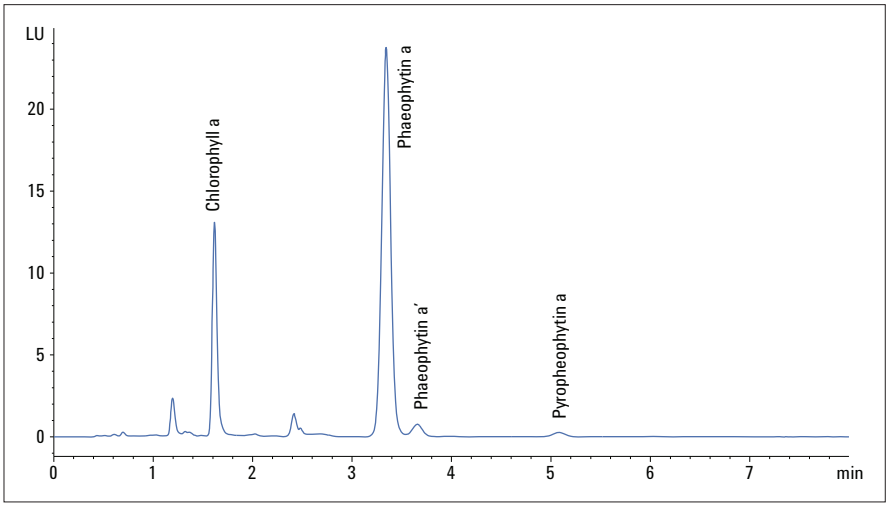

Figure 5
Short analysis of chlorophyll degradation products in relatively fresh virgin olive oil.

Summary and Conclusion

Seven olive oils were analyzed for the chlorophyll degradation products pheophytin and pyropheophytin after sample preparation according to ISO 29841:2009(E). Pyropheophytin is not detectable in fresh virgin olive oils, but develops slowly during storage of the olive oil. This decarbomethoxylation reaction is boosted by heat treatment of the oil, for example during refining processes. Indeed, pyropheophytin was found in about 1.5 year old virgin olive oils (8–13.5%) or partly refined olive oil (18%). In contrast, in relatively fresh virgin olive oil (ca. 3–4 month old) only a minor amount (1.5%) of pyropheophytin was detected. In addition to the long run of 30 minutes, an 8-minute analysis was carried out using a short 50-mm column, still obtaining great resolution using sub-2 µm particles.

The Agilent 1260 Infinity LC with fluorescence detection is an optimum solution for the determination of the chlorophyll degradation products in olive oils. With this method, virgin olive oil can be analyzed for proper storage conditions or potential refining processes.

References

1.
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