HPLC Analysis with Fluorescence Detection of Chlorophyll Degradation Products Pheophytins and Pyropheophytin in Virgin Olive Oil

Application Note

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Abstract

This application note shows a simple and rapid method for the analysis of the chlorophyll thermal degradation products pheophytins (PP) and pyropheophytin a (PPP) in extra virgin olive oil (EVOO) by modifying the photomultiplier (PMT) of a fluorescence detector (FLD). By exchanging the standard PMT with a red-sensitive Hamamatsu R928HA PMT, the raw increase in signal was approximately 120-fold. In addition, an overall improvement of 180-fold was observed during analysis due to reduced baseline noise. A straightforward direct analysis of diluted EVOO was performed without further sample preparation in this method, which provides less solvent consumption and shorter LC cycle times, with good resolution, over the traditional International Standards Organization (ISO) solid phase extraction (SPE)/ultraviolet detector (UV) method.

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Introduction

EVOO is obtained from olives (*Olea europaea* L.) through a mechanical procedure. Chlorophylls are the natural green pigments existing in EVOO, and are prone to degrade to pheophytins and pyropheophytin as a result of processing and storage. Chlorophylls, particularly, can be affected by acid conditions and heating during fruit milling and paste beating, thus greater accessibility between components and the generalized release of the acid compounds produce pheophytinization in great extension [1]. This leads to the use of the degradation products of chlorophylls as one of the quality parameters for EVOO. The ISO method of PPP analysis was adopted by Australia and New Zealand as part of olive oil standards in 2010, but has yet to be adopted in any other country. With the obtained values, it can be determined if an EVOO was properly stored or was adulterated with refined olive oil.

The traditional SPE/UV method in ISO 29841 includes several steps in sample preparation that are very solvent-consuming and time-consuming. In this modified FLD method, an isopropanol diluted olive oil sample was ready for the HPLC injection. A quaternary solvent gradient method was used to include a fourth strong solvent wash (tetrahydrofuran) on a quaternary gradient pump, which eliminated the need to premix any solvents and also greatly reduced the oil residues on the column from previous analysis. Furthermore, the FLD was enhanced more than 180× by exchanging the standard PMT with one more sensitive in the red region.

Experimental

Instrument configuration

ISO 29841 SPE/UV method
An Agilent 1290 Infinity Binary LC system, controlled by ChemStation (Rev. C.01.03 [44]), and consisting of the following modules was used. A diode array detector (DAD) was used as the UV detector in the original ISO method:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A)

Modified FLD method
An Agilent 1260 Infinity Quaternary LC system, controlled by ChemStation (Rev. C.01.03 [44]), and consisting of the following modules was used:

- Agilent 1260 Infinity Quaternary Pump (G4204A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Fluorescence Detector (G1321B), equipped with Hamamatsu R928HA PMT

Sample preparation

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

ISO SPE/UV method
Approximately 300 mg of olive oil sample was dissolved in 1 mL of petroleum ether and vortexed to mix them well. The sample solution was applied onto the 1,000 mg/6 mL silica cartridge (Phenomenex, Torrance, CA, USA) and rinsed twice with 1 mL portions of petroleum ether. As soon as the solvent had drained to the top of column packing, the nonpolar substances were eluted twice with 5 mL petroleum ether/diethyl ether (90:10, v/v) and discarded. Then, the pheophytin fraction was eluted with 5 mL of acetone and collected in a 10-mL glass tube covered with foil (protected from light). The solvent was evaporated to dryness on a rotary evaporator at max 20 °C, and the residue was dissolved in 1 mL acetone for further HPLC analysis [2].

Modified FLD method
A 200 mg amount of olive oil sample was dissolved in 1 mL of reagent isopropanol by vortex for 15 seconds.

LC conditions

ISO SPE/UV method

The LC chromatographic conditions of the ISO 29841 SPE/UV method are listed in Table 1. The original 4.6 × 250 mm C18 column was modified for a 3.0 × 100 mm C18 column in this situation. An isocratic method was used.

Table 1. Chromatographic Conditions of ISO SPE/UV Method

Stationary phase	Agilent ZORBAX Eclipse Plus C18 column, 3.0 × 100 mm, 1.8 µm
Eluting mobile phase	Water/methanol/acetone (4:36:60, v/v/v)
Flow rate	0.5 mL/min
Volume injected	5 µL
Detector	Diode array detector, 410 nm

Modified FLD method

The sample was run with the LC system using the chromatographic conditions listed in Table 2, and following the quaternary solvent gradient shown in Table 3.

Table 2. Chromatographic Conditions of Modified FLD Method

Stationary phase	Agilent ZORBAX Eclipse Plus C18 column, 3.0 × 100 mm, 1.8 µm
Mobile phase	Gradient shown in Table 3
Volume injected	5 µL
Detector	A red-sensitive FLD, excitation 430 nm/emission 670 nm

Table 3. Quaternary Solvent Gradient of Modified FLD Method

Time (min)	A Water %	B Tetrahydrofuran %	C Methanol %	D Acetone %	Flow rate (mL/min)
0	4	0	76	20	0.5
4	4	0	36	60	0.5
14	4	0	36	60	0.5
14.2	0	100	0	0	1.0
20.9	0	100	0	0	1.0
21	4	0	76	20	0.5
25	End of test, ready to inject new sample				

Results and Discussion

Figure 1 shows an overlay chromatogram for the two ISO sample preparation and detection schemes, SPE/UV (in blue) and dilution/FLD (in red), using the modified chromatographic conditions newly developed for the dilution/FLD variation. The obtained PPP values from both methods were 15% (calculated based on Equation 1). Both sample separation methods showed good resolution on HPLC, and resolution of pheophytin a and pheophytin a' is significantly improved over the specified ISO method chromatographic conditions.

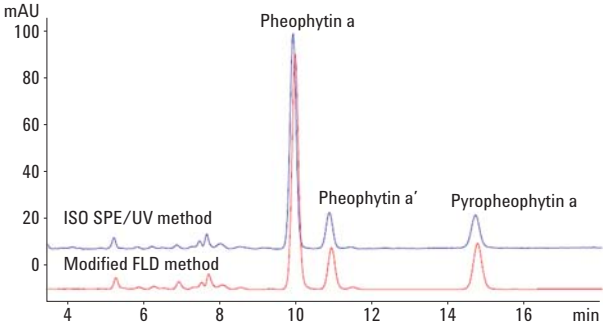

Figure 1. An overlay chromatogram of both sample preparation methods (SPE and simple dilution) of an extra virgin olive oil with 15% PPP content in chlorophyll a degradation products. Conditions were those used for the modified fluorescence method.

The sample preparation time of the ISO SPE/UV method is 30 minutes, with an HPLC analysis time of 40 minutes or longer. With the modified UHPLC/FLD method, on the quaternary system with a 3.0 × 100 mm C18 column, the dilution step is brief and the analysis time is reduced to 25 minutes. Solvent consumption and total analysis time for the UHPLC analysis are 40% or less when compared to the standard ISO conditions. It represents a significant reduction in analysis cost and time, and has demonstrated improved sensitivity for low level samples.

$$\% PPP = 100 \times Appp/(App + Appp)$$

Where
% PPP = % pyropheophytin a content in olive oil sample
Appp = peak area of pyropheophytin a
App = total peak area of pheophytin a and pheophytin a'

Equation 1. Calculation of the PPP content in olive oil.

When a lower PPP content EVOO sample was analyzed, the UV detection was insufficient in quantification as shown in Figure 2. A recently harvested EVOO sample was analyzed under both conditions. The PPP value gained from the ISO SPE/UV method was 0, which was theoretically inaccurate since the decarbomethoxylation from pheophytins to pyropheophytin occurs once olives are processed to oil. However, a small amount of PPP (6%) was still detectable under the modified FLD method.

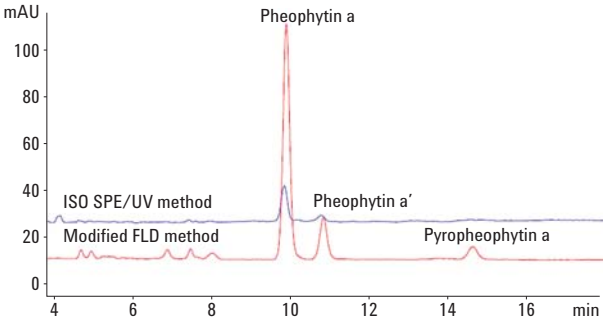

Figure 2. An overlay chromatogram of both methods of an EVOO with 6% PPP content in chlorophyll a degradation products.

Figure 3 further demonstrates the high selectivity and sensitivity of modified FLD method. An extra light olive oil was analyzed by both methods. Extra light olive oil is a blend of mostly refined olive oil and virgin olive oil. Thus, it has considerably low PPP content. As shown in Figure 3, the response signal under a DAD method was extremely low, with a high background noise. This prevented the PPP content quantification for the EVOO portion in this extra light olive oil. Conversely, with the modified FLD method, signals as low as 0.15 LU could be detected, and the PPP content was able to be further quantified by peak areas.

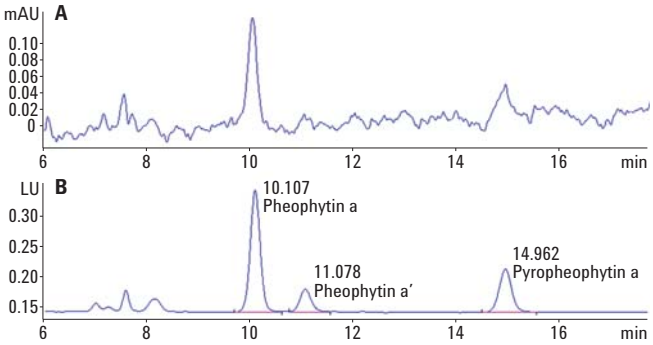

Figure 3. Separation of the degradation products of chlorophyll a under both methods for the same extra light olive oil sample, ISO SPE/UV method (A) and modified FLD method (B).

Replicate analyses using the specified gradient and column with two different PMTs are summarized in Table 4. The raw increase in signal with the Hamamatsu R928HA was approximately 120-fold. The further improvement, due to reduced baseline noise, gave an overall improvement of 180-fold.

Table 4. Summary of Improved Results after Changing the Photomultiplier Tube to the Suggested Agilent Alternate

Pheophytin a, a', and pyropheophytin detection
Summary of modified FLD analysis excitation 430 nm/emission 670 nm

PMT	Standard	R928HA
Peak area total*	6.8	854.5
Average Signal/Noise	34.8	6847.5

* Combined response of pheophytin a, pheophytin a' and pyropheophytin

Conclusions

The modified FLD method has been shown to be a promising option to the traditional ISO SPE/UV method. In this case, low level PPP samples can be measured when the photomultiplier is exchanged from the standard PMT to the red-sensitive R928HA model. This results in a substantial cost savings in time, labor, SPE materials, and solvent consumption in any laboratory needing to analyze large numbers of samples.

References

1. D. Hornero-Méndez, B. Gandul-Rojas, and M.I. Mínguez-Mosquera "Routine and sensitive SPE-HPLC method for quantitative determination of pheophytin a and pyropheophytin a in olive oils" *Food Research International* **38**, 1067-1072, doi:10.1016/j.foodres.2005.02.022 (2005).

2. Vegetable fats and oils - Determination of the degradation products of chlorophylls a and a' (pheophytins a, a', and pyropheophytins), ISO 29841:2009.

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