A simple HPLC-fluorescence screening method for rapid determination of oleuropein in olive leaves; cross validation with an UPLC-MS-UV method and analysis of leaves of French olive varieties

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ABSTRACT

Olive leaves are an abundant but under-exploited by-product of the olive oil industry, comprising 10 % of the total mass processed for olive oil. They are a rich source of oleuropein, the most abundant polyphenol in olive leaves, whose bioactive properties are widely documented. If olive leaves are to be seriously considered as a readily-available source of oleuropein, simple analytical methods are needed to identify varieties and growing conditions conducive to its formation. HPLC-fluorescence detection and UPLC-UV-MS methods on different instruments were developed and cross-validated using blank (oleuropein-free) leaves produced in-house to establish matrix standards in the concentration range 0.4 to 4.8 mg/g. For the HPLC-FLD method, mean repeatability and intermediate precision were respectively 3.64% and 4.76% relative standard deviation; the coefficient of determination was generally >0.9999 and linearity was demonstrated by the Fischer test. There was a close linear relationship (R^2 > 0.999) between back-calculated concentrations of both spiked placebos and authentic standards. Analysis of the same spiked placebos by the three chromatographic methods showed no significant differences in the validation results as confirmed by ANOVA. Cross-validation of the three methods indicate that selective quantification can equally be carried out on standard HPLC equipment available to most laboratories or by more sophisticated UPLC-MS techniques in larger structures. The determination of oleuropein in “Olivière” olive variety is reported here for the first time, and leaves collected in 2022 contained about 40 mg/g oleuropein, almost twice the amount found in Picholine and three times more than in other varieties.

KEY WORDS

Olive leaves - Oleuropein – HPLC-fluorescence detection - Validation - Olivière - Picholine

INTRODUCTION

Increasing consumer demand for more sustainable production methods combined with stricter environmental regulations are important drivers in developing strategies for the management of by-products, such as leaves. Olive leaves account for 10 % of the mass of olive oil produced [1], and progress in recycling and valorisation means that they have become a promising source of phenolic compounds with high added value [2].

The majority of polyphenols are found in these by-products and only 0.3 to 1.5% of available phenols are found in olive oil; consequently, extracts of these wastes have enormous potential particularly as nutraceuticals and pharmaceuticals [3]. Oleuropein, (figure 1) is the most abundant phenolic compound in olive leaves [4] and it also occurs in young olives where it gives the bitter taste in immature fruit.

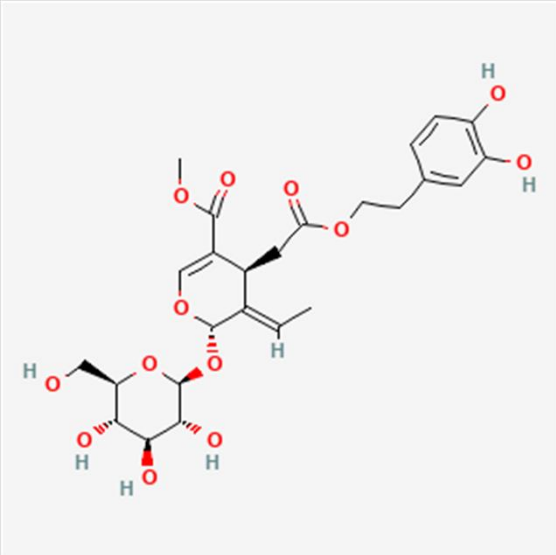

Figure 1

Oleuropein

Different studies have reported varied concentrations of oleuropein in olive oil leaves; for example El and Karakaya found the amount of oleuropein in dry olive leaves to be between 6 and 14 %, and others found 17 to 23 % depending on the time of year at which the leaves are collected [5,6]. Le Tutour found that oleuropein in dry olive oil leaves ranged from 6-9 %, while another study by Topuz and Bayram found oleuropein content to be between 6 and 16% [5,7]. Medina *et al* reported that oleuropein showed high variability in a range of 44 to 108 mg/g, and that it constituted more than 88–94% of total phenolic

compounds in leaves [8]. Parameters such as climate and time of harvest determine the concentration of oleuropein and other biophenols [9,10]; Romani *et al* reported differences in oleuropein amounts depending on cultivar, production area, sampling time, and state of leaves (fresh, dried, frozen or lyophilized) [11].

The antioxidant properties of plant polyphenols form the basis of their various health benefits, and as a result, they are increasingly being considered as constituents of pharmaceuticals [12]. The activities of oleuropein include anti-inflammatory, antioxidant, anti-aging, and anti-bacterial effects [13]. It has also been described as having anti-cancer properties, a topic which has been the subject of several reviews [14–16]. Research has shown that in skin disorders, oleuropein promotes collagen production, inhibits lipid peroxidation post sun exposure and also reduces swelling of skin as we have recently reviewed [17]. It's efficacy in the treatment of metabolic disorders is of growing interest [18], and it has yielded promising results as an anti-viral agent, including SARS-Cov 2 [19]. These and other bioactive properties of oleuropein have been reviewed [20–22].

In view of its antioxidant properties, oleuropein has considerable potential as a food ingredient in preventing oxidation, thereby enhancing the nutritive value of food. However, the production of oleuropein from olive leaves at an industrial scale faces challenges, including its instability in water, decomposing through hydrolysis to form the more apolar aglycone with lower solubility in aqueous systems [23]. Consequently, reliable analytical determination of oleuropein in olive leaves is a pivotal step to identify varieties that could be more seriously considered for large-scale extraction. Furthermore, there is a need to quantify oleuropein in the growing number of nutritional supplements containing olive leaves, particularly given that some products claim to contain concentrations of oleuropein, as high as 40%. It significant that Breakspear and Guillaume found that nutritional supplements on the Australian market vary substantially in their concentrations of oleuropein, whereas Medina *et al* found that only 17–26% of polyphenols diffused to the aqueous phases of olive leaf infusions [8,24].

The most widely used method to determine phenolic compounds in olive fruits and leaves is reversed-phase high/ultra-high performance liquid chromatography (RP-HPLC/UHPLC) coupled to UV [8,24–28] and Mass Spectrometry (MS) [29,30]. In the majority of cases, no validation results were presented, or validation was carried out using only authentic standards [31], one exception being the study by Bertolini *et al* where validation results were reported for the determination of oleuropein in an effervescent formulation using spiked placebos [32]. In some cases, internal standards (IS) were used such as resorcinol or syringaldehyde, though at what stage the IS's were added is unclear [8,26].

One of the main objectives of this study was to develop and validate a rapid and selective method to determine oleuropein in dried olive leaves without internal standardisation. The key elements presented here are the production of an oleuropein-free (blank) olive leaf matrix, a validated HPLC method with fluorescence detection (FLD) using spiked placebos (oleuropein-leaves) and cross validation of the HPLC-FLD method with UPCV-UV-MS methods on a different instrument.

RESULTS AND DISCUSSION

The first objective was to develop an analytical method with a short analysis time using the minimum amount of organic solvents. A 3.5 mm i.d. column allowed a low flow-rate to be used (0.5 ml/min), and increasing column temperature to 40°C reduced run-time and back-pressure in the system. Under these conditions, oleuropein eluted at 5.2 (± 0.1) minutes with a total run time including column re-equilibration of 13 minutes. The excitation and emission wavelengths were optimised for oleuropein at 281 and 316 nm, respectively, and it is worth noting that under these conditions, extremely strong responses are obtained for hydroxytyrosol and tyrosol which may also be determined at the ppb level by this method with elution times of 1.5 and 2.5 minutes, respectively.

Studies on phenol extraction using water at high temperatures demonstrated that it is an efficient method for the recovery of high-value natural bioactive compounds [28]. This high temperature effect could be attributed both to the nature of the matrix and structure of the bioactive compounds to be extracted [33]. Microwave-assisted extraction for oleuropein from olive leaves was first described by

Japón-Luján *et al* in 2006 [34], and Da Rosa described microwave extraction coupled to HPLC-DAD for the determination of oleuropein in olive leaves. The procedure required strict control of extraction conditions as the extraction time was 2-3 minutes, and it was necessary to rapidly cool the samples in an ice bath [35]. However, since this approach has grown in popularity for the extraction of oleuropein from olive leaves [36–38], the starting point for this study was based an extraction method described by Martinez-Navarro, where olive leaves were extracted in water using a domestic microwave at 800 W for 30 seconds [27]. Approximately 100 mg of powdered oleuropein leaves were placed in a test tube with 4 mL of water and microwaved using the conditions described above. Comparaison with the control showed that microwaving was effective in extractng oleuropein from the leaves, but this caused the contents of the test-tubes to rise rapidly, probably due to uneaven heating in the domestic microwave. Even by reducing duration and power setting it was impossible to control the temperature within the test-tubes, and clearly the sample-volume ratios, test-tube/bottle size would need to be optimised and tailored to each expeimental situation.

The next step was to investigate extraction in an ultra-sonic bath as this technique has been described by several authors, though in some cases long extraction times are reported [36,39–41]. Samples (50 mg) were weighed into 10 mL test-tubes with 5 mL water or 70 % aqueous methanol, and placed in the ultrasonic bath at the lowest settings for 30 minutes. Again, it proved difficult to ensure uniform heating; adding ice to the ultra-sonic bath failed to maintain an even temperature, and the variation in heating among samples resulted in poor repeatability of extraction. Furthermore, in some cases, the rapid rise in temperature caused the methanol to evaporate resulting in sample loss. Given this difficulty, the more conventional rotary mixing was compared to ultrasonic extraction. Samples were mixed for 15 minutes on a rotary mixer and then either a further 15 more minutes on the rotary mixer or 15 minutes' sonication. There was little difference between the two extraction methods so rotary mixing was adopted, which has the added advantage of enabling several samples to be extracted simultaneously. Extraction with 100 % methanol with rotary mixing gave greater recovery than with 50% methanol

(Supplemental figure 1). Regardless of the volume used, only 85% of added oleuropein was recovered in a single extraction; (figure 2); most of the remainder was removed in the second extraction, so the final protocol retained was 2 x 2mL extraction of 50 mg powdered leaf which gave total recovery of in excess of 90%. Chromatograms of authentic standards (AS) and spiked standards (at the same concentrations after extraction and dilution) are presented in Supplemental figure 2.

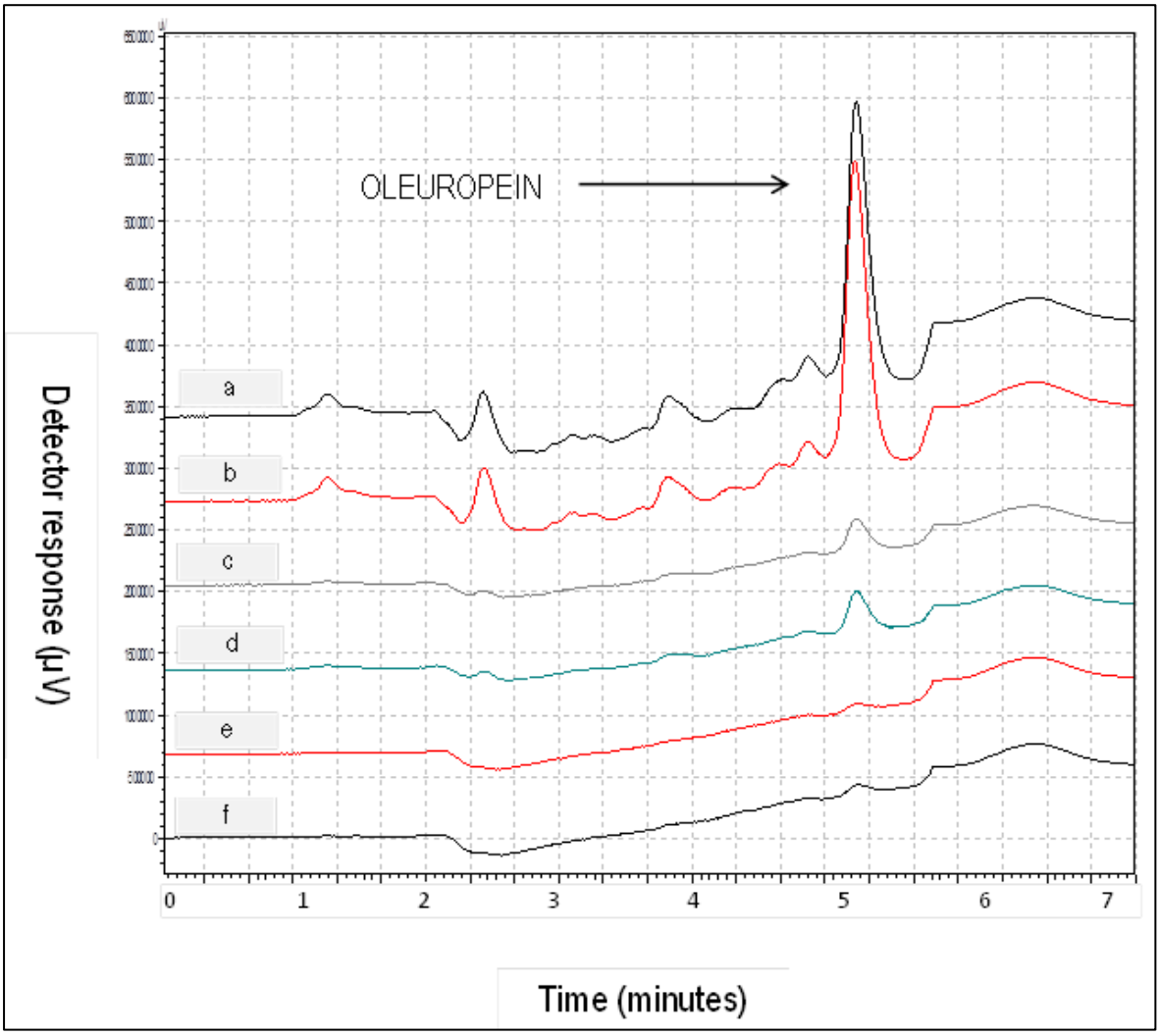

Figure 2

Comparison of ultrasound (US) and rotary extraction (Ext) using.50 mg of sample extracted into 10 mL methanol. HPLC method, chromatographic conditions described in the text.

a– Rotary 1st Ext; b - US 1st Ex.t.: c - Rotary 2nd Ext; d - US 2nd Ext; e – Rotary 3rd Ext; f – Rotary 3rd Ext

Validation results for the HPLC-FLD method are presented in Table 1; those for UPLC-MS and UPLC-UV methods are presented in supplemental Tables S1 and S2, respectively. In the interest of clarity, only back-calculated concentrations are presented rather than detector responses.

Table 1 Validation data HPLC-FLD method

Intra-day precision (repeatability) and inter-day (intermediate precision) and recovery % based on found concentrations (AF mg/g) obtained by back-calculation of the signal on the individual calibration curves.

For intra-day the equation was y = 0.9966x + 0.0565 (R^2 = 1); for inter-day the equation was y y = 0,9941x + 0.0201, (R^2 = 0.9996).

SD: standard deviation; RSD: relative standard deviation (coefficient of variation)

Nominal concentration	**0.4**	**0.8**	**1.6**	**3.2**	**4.8**	**0.4**	**0.8**	**1.6**	**3.2**	**4.8**
Found concentration (AF mg/g)	**Repeatability**					**Intermediate precision (inter-day)**				
AF	0.40	0.79	1.58	3.08	4.81	0.35	0.81	1.80	3.27	4.69
Recovery %	100	99	99	96	100	87	101	112	102	98
AF	0.41	0.80	1.60	3.17	4.82	0.41	0.82	1.66	3.09	4.85
Recovery %	101	100	100	99	100	102	102	104	97	101
AF	0.40	0.81	1.68	3.12	4.83	0.40	0.81	1.68	3.12	4.83
Recovery %	100	101	105	98	101	100	101	105	98	101
AF	0.36	0.77	1.52	3.22	4.74	0.40	0.78	1.61	3.28	4.75
Recovery %	91	96	95	100	99	100	98	100	102	99
AF	0.42	0.85	1.66	3.34	4.76	0.41	0.81	1.60	3.12	4.85
Recovery %	104	106	104	105	99	102	102	100	97	101
Mean AF	0,40	0,81	1,61	3,19	4,79	0,39	0,81	1,67	3,17	4,79
Mean recovery %	99	101	100	100	100	102	101	104	99	100
SD AF	0,02	0,03	0,06	0,10	0,04	0,02	0,02	0,08	0,09	0,07
SD Recovery %	0,05	0,04	0,04	0,02	0,01	0,06	0,02	0,05	0,03	0,01
RSD %	**5.11**	**3.73**	**4.01**	**3.21**	**0.78**	**6.23**	**1.91**	**4.70**	**2.89**	**1.50**

For HPLC-FLD, repeatability, expressed as the the relative standard deviation (intra-day RSD) of was generally less than 5%, and accuracy was 95 to 105%. In addition, the following data were obtained: The mean slope of the detector response vs concentration curve was 3301401 (±81242); RSD 2,46%%. mean coefficient of determination (R^2) 0.9994 (±0,0004). The equation of the curve "mean back-calculated concentration vs nominal concentration" was y = 0,9966x + 0,0565 (R^2 = 1). For intermediate precision, the RSD was less than 6%, and accuracy was between 96 and 102%, exceptions being on day two of the study when recovery was 87% and 112% for the first and third calibration points, respectively. The mean slope of the detector response vs concentration curve was 3250083 (± 121928. RSD, 3.75% with a coefficient of determination (R^2) of 0,9986 (±0,0016). The equation of the mean back-calculated vs nominal concentration was y = 0,9941x + 0,2519 (R^2 = 0,9996). The equivalent values for the UPLC-MS method are mean overall RSD 3,96% (repeatability) and 2.69 % (intermediate precision), and for the UPLC-UV method, 2.88 % and.2.15%, respectively (supplemental tables S1 and S2, respectively). The proximity of the results between the three methods was evaluated by ANOVA; as may be seen in supplemental table S3, the difference in back calculated concentrations among the three methods was not significant.

Linearity was determined by the Fischer test based on residual values, i.e.the differences between nominal and found (back-calculated) concentrations. No significant differences were observed: the function f(Nominal C- found C) exhibits significant linearity (Fisher, p-value < 0.001, R^2 = 0.999) with an intercept of about 0 (Intra-day = -0.080 and Inter-day = -0.106) and a slope of about 1 (Intraday = 1.001 and Interday =1.002). The relationship between detector responses of AS and spiked placebos was measured by analysing four calibration standards and a blank of each set. The concentrations of oleuropein were 0.4, 0.8, 1.6 and 3.2 mg/g for the spiked placebos, which, after extraction (4 ml methanol) and dilution (100 fold in 50% aqueous methanol) result in injection solutions of respectively, 5, 10, 20 and 40 mg/L oleuropein (for 100% recovery). These solutions were compared with AS

solutions at the same concentrations. Five replicates of each concentration were prepared on the same day by the same operator under the same operating conditions.

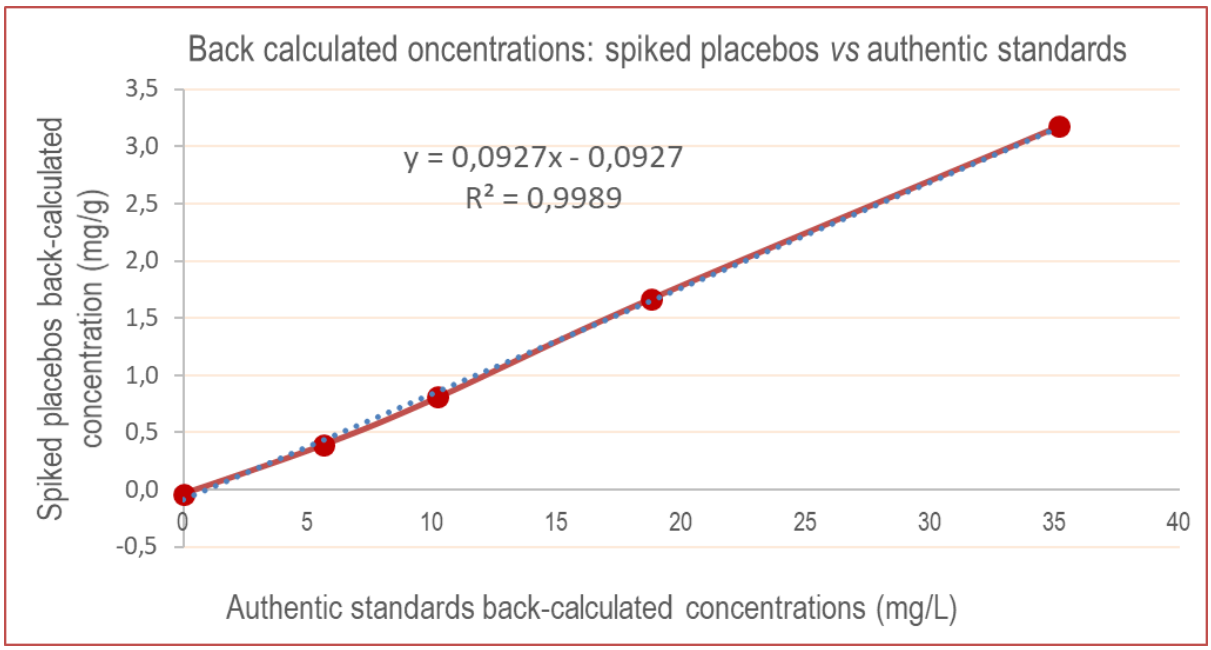

Figure3

Correlation between detector responses for authentic standards and spiked placebo standards

HPLC-fluorescence method

Concentration range: 5-40 mg/L authentic standards and spiked standards (after extraction and dilution)

The AS detector responses vs spiked placebo detector responses were highly correlated (R^2 = 0.9996), and as figure 3 shows, there is a also strong correlation between spiked placebo back-calculated concentratons and AS back-calculated concentratons (R^2 = 0.9989). Similar results were obtained for the UPLC-MS and UPLC-UV methods (data not shown).

Savournin *et al*. reported concentrations of oleuropein in four French varieties (Cailletier, Lucques, Tanche, and Verdale−Picholine hybrid) with Lucques having the highest concentration (12.56−14.16%) [42]. These values were higher than usually reported, and was ascribed to the microwave drying method rather than the extraction procedure. Other authors found between 9% and 14.3% oleuropein in leaves of olive trees depending on the harvest time and agronomic conditions [43] . Notable differences

in the concentration of oleuropein in the leaves of different olive cultivars were found among Mediterranean countries; in a major review article, Talhaoui *et al* reported values varying between 24.7–143.2x10^3 µg/g dry weight (DW) depending on the provenance and time of year when the samples were collected [25]. However, In another review, Özcan *et al.* reported relatively minor differences (31 to 53 mg/g DW) in oleuropein concentrations among 11 olive cultivars originating in France, Spain, Italy and Tunisia [44]. Similar studies found significant differences in oleuropein concentrations among olive varieties of Italian [45] and Portuguese cultivars [46].

There are relatively few publications on oleuropein concentrations in cultivars particular to the South of France where the most common varieties include Picholine, Lucques, Verdale and Negrette. There are no publications on Olivière, another variety widely found in the Languedeoc Region of France. In probably the most relevant publication to this study Savournin *et al.* found that among 14 different olive cultivars, the mean concentration of oleuropein in Picholine leaves was 11.7 *% (w/w), the amount being 13.4 and 10.5 % (w/w) for Lucques and Verdale, respectively [42] .

In addition to cultivars used for the production of olive oil, (Picholine, Olivière, Lucques) analysed in this study, three unidentified cultivars, mainly used as decorative trees, of probably Greek (Bleu Grec) and Spanish (UKN 4) origin were also analysed. The values (mg/g) in table 2 show considerable variation among the cultivars, ranging from a mean concentration of 5.88 mg/g for UNK 3 to 39.31 mg/g for Olivière 2022. These values are not inconsistent with concentrations of oleuropein described above, but are considerably lower than some given elsewhere, as cited in the Introduction section. Such vast differences in reported oleuropein concentrations are probably due to different cultivars (often not specified), harvest times, storage conditions (water content of the leaves is rarely specified), milling/grinding procedures and extraction methods

Table 2

Oleuropein concentration in olive leaves (mg/g dry weight)

	Picholine	Olivière	UNK 1[a]	UNK 2	UNK 3	UNK 4	UNK 4h[b]	Olivière 2021[c]
	14.44	41.32	32.5	16.5	5.96	21.68	21.9	3.85
	15.54	37.92	33.36	19.08	6.06	19	21.46	4.29
	16.22	38.7	36.42	185.6	5.62	21	21.58	3.99
	16.44	39.3	33.3	16.62	-	21.44	21.72	4.36
Mean	15.66	39.31	33.89	59.45	5.88	20.78	21.66	4.12
SD[d]	0.90	1.45	1.73	84.11	0.23	1.22	0.19	0.24
RSD[e] %	5.74	3.70	5.10	141.48	3.92	5.87	0.87	5.92

a: Unknown variety

b: Unknown variety extracted for four hours

c: Olivière leaves stored for 12 months under non-controlled conditions

d: Standard deviation

e: Relative standard deviation (coefficient of variation)

Table 2 also shows that no benefit is gained from increasing extraction time (Bleu Grec 4h), and that the concentration of oleuropein in Olivière variety decreases by almost 90% in dried leaves stored for 12 months. This observation contrasts with that of Martinez-Navarro *et al.* who found that oleuropein was stable for at least six months when leaf powder was stored at any temperature with a relative humidity of 57% [47]. However, it should be pointed out that the 2021 leaf samples in this study were not stored under controlled conditions. Nonetheless, the promising results obtained here for the widely-planted Olivière cultivar paves the way forward for the exploration of this variety as a valuable and abundant source of naturally-sourced oleuropein.

MATERIALS AND METHODS

Reagents and chemicals

All chemicals and reagents were of analytical or HPLC grade or equivalent. Methanol, acetonitrile, acetic acid, dichloromethane and HPLC grade water were obtained from Carlo Erba (Val de Reuil, France), and doubly distilled water was used for washing all consumable materials.

Instrumentation and operating conditions

HPLC-FLD

A Shimadzu (Marne-la-Vallée France) Nexera © chromatograph was used consisting of a DGU-20A50 degassing unit, an LC-AD quaternary pump, a SIL20 A autosampler, an SPDM20A photodiode array detector and Fluorescence detector at λex 281 nm and λem 316 nm. The analytes were separated on an end-capped C_{18} Nucleodur© column 100mm x 3 mm i.d, 3µm particle size. Column temperature was 40°C. Low pressure gradient mobile phase A consisted of water- acetonitrile [95:5] with 0.2% acetic acid; mobile phase B was methanol with 0.2% acetic acid with a flow rate of 0.5 mL/min using a linear gradient program of: 0 min 25% B - 4 min 35% B. Under these conditions oleuropein elutes at 5.2 minutes.

UPLC-UV-MS

Analyses were carried out on a Thermo Ultimate© 3000 instrument with quaternary pump and automatic sampler coupled to an Ultimate 3000 RS type UV spectrophotometer and an ISQ EC mass spectrometer. Data were recorded using Chromeleon 7.2 software (Thermo Scientific). The analytes were separated on a Kinetex© 2.6 µm Biphenyl 100 Å (150 × 2.1 mm) column at 40°C. The mobile phase was methanol acidified with 0.01% formic acid (A) and water acidified with 0.01% formic acid (B) with a flow rate of 0.4 ml/min. The gradient was 0-2 min 75% B, 2-10 min 75-45% B, 10-13 min 45% B, 13-14 min 45-0% B, 14-15 min 0% B, 15-16 min 0-75% B, 16-20 min 100% B. The UV chromatogram was recorded at 280nm and 200nm. The mass spectrometer was operated in negative ion (ESI) mode with the following source parameters are: Sheath Gas Pressure 30 psig; auxiliary Gas Pressure 8 psig; purge gas pressure 0.8 psig, vaporizer temperature 300°C, transfer tube and ion temperature 350°C.

Acquisition was performed in full scan mode (50-800), and for the assay, the spectrum at charge ration m/Z 539 was extracted.

Samples and standards

Olive leaves

Olive leaves of Picholine and Olivière varieties (used for the production of olive oil) were collected in March 2022, in the Hérault department, of the Occitanie region of France (latitude 43.5912356, longitude 3.2583626). The leaves were immediately removed and stored in cardboard boxes protected from light and humidity until analysis in June 2022. Leaves of four other, mainly ornamental varieties, were collected locally and analysed in order to provide additional data for the validation study; These varieties could not be identified, but insofar as could be established, they are Italian or Greek hybrids; in the interest of simplicity, these varieties were named UNK 1, UNK 2, etc.

Preparation of oleuropein-free (blank) olive leaves

After milling and sieving, leaf powder was dried to constant weight with a final water content of 4 to 5%. Approximately 50 mL of methanol was added to 2 g of leaf powder and oleuropein was removed by extraction in an ultrasonic bath at approximately 55°C for 45 minutes. The supernatant was discarded and procedure repeated. The leaves were then dried for two hours at 105°C and maintained in a low-humidity environment before use. The quantity of oleuropein remaining in the blank after double extraction acted as the first point on the calibration curve.

Standards

A stock solution of 250 mg/L oleuropein was prepared in methanol. This solution is stable for 6 months at -20°. Authentic standards at five calibration points (5, 10, 20, 40 and 60 mg/L) were prepared daily in methanol-water [1:1] by serial dilution of the stock solution.

Spiked standards (dried leaf placebos) containing 0.4 0.8 1.6 3.2.and 4.8 mg/g were prepared by adding the appropriate volume of stock solution to 50 mg of blank leaves; the volume was then made up to 2 mL with methanol in five mL haemolysis tubes. The samples were vortex-mixed for 30 seconds and then dried under a stream of nitrogen at 30°C.

Extraction

Two mL methanol were added to the spiked standards (or samples), mixed on a rotary mixer for 30 minutes and then centrifuged at 5000 rpm for 5 minutes. The supernatants were removed and the above process repeated with a second 2 mL methanol. The 2 x 2 mL extracts were combined, diluted 100-fold with methanol-water (50/50) and filtered through a 0.45 µm filter before injection.

Validation

Calibration curves of the spiked standards were obtained from unweighted least-squares linear regression analysis of the data. The slope and intercept of the calibration graphs were determined through linear regression of the peak areas *vs* concentration plot. Individual peak areas were then interpolated on the calibration graphs to determine the found (back calculated) concentrations. The quality of fit was determined using back-calculated-to-nominal concentrations and the Fischer test used confirmed the linearity of the method. Within-day (repeatability) and between-day (intermediate precision) and accuracy were determined by carrying out replicate analyses of the spiked leaf calibration standards. Repeatability was determined by preparing and analysing all five spiked leaf standards five times within a single day, and the intermediate precision was determined by carrying out the same operations for five days over a 10-day period. The precision was given by mean relative standard deviation of the back-calculated concentrations, and the accuracy of the method was evaluated as 100×[mean found concentration/nominal concentration]. Recovery was determined by comparing the back-calculated concentrations and the nominal concentrations and it was expressed as 100 *x* (mean found concentration/nominal concentration].

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1

Oleuropein

Figure 2

Comparison of ultrasound (US) and rotary extraction (Ext) using.50 mg of sample extracted into 10 mL methanol. HPLC method, chromatographic conditions described in the text.

a– Rotary 1st Ext; b - US 1st Ex.t.: c - Rotary 2nd Ext; d - US 2nd Ext; e – Rotary 3rd Ext; f – Rotary 3rd Ext

Figure 3

Correlation between detector responses for authentic standards and spiked placebo standards

HPLC-fluorescence method

Concentration range: 5-60 mg/L authentic standards and spiked standards (after extraction and dilution)

Supplemental Figure 1

Comparison of Water, 50 % Water and Methanol and 100 % Methanol

Extraction time 30 minutes:

Black (a): 100% Methanol

Red (b): 50 % Water and Methanol

Green (c): Water

Supplemental figure 2

Chromatograms of authentic standards and matrix standards (spiked blank leaves). Chromatographic conditions as described in the text

(a): Authentic standard 20 mg/L

(b) Authentic standard 10 mg/L

(c) Spiked standard (placebo) 1.6 mg/g

(d) Spiked standard (placebo) 3.2 mg/g

TABLES

Table 1 Validation data HPLC-FLD method

Intra-day precision (repeatability) and inter-day (intermediate precision) and recovery % based on found concentrations (AF mg/g) obtained by back-calculation of the signal on the individual calibration curves.

For intra-day the equation was y = 0.9966x + 0.0565 (R^2 = 1); for inter-day the equation was y y = 0,9941x + 0.0201, (R^2 = 0.9996).

Nominal concentration	**0.4**	**0.8**	**1.6**	**3.2**	**4.8**	**0.4**	**0.8**	**1.6**	**3.2**	**4.8**
Found concentration (AF mg/g)	**Repeatability**					**Intermediate precision (inter-day)**				
AF	0.40	0.79	1.58	3.08	4.81	0.35	0.81	1.80	3.27	4.69
Recovery %	100	99	99	96	100	87	101	112	102	98
AF	0.41	0.80	1.60	3.17	4.82	0.41	0.82	1.66	3.09	4.85
Recovery %	101	100	100	99	100	102	102	104	97	101
AF	0.40	0.81	1.68	3.12	4.83	0.40	0.81	1.68	3.12	4.83
Recovery %	100	101	105	98	101	100	101	105	98	101
AF	0.36	0.77	1.52	3.22	4.74	0.40	0.78	1.61	3.28	4.75
Recovery %	91	96	95	100	99	100	98	100	102	99
AF	0.42	0.85	1.66	3.34	4.76	0.41	0.81	1.60	3.12	4.85
Recovery %	104	106	104	105	99	102	102	100	97	101
Mean AF	0,40	0,81	1,61	3,19	4,79	0,39	0,81	1,67	3,17	4,79
Mean recovery %	99	101	100	100	100	102	101	104	99	100
SD AF	0,02	0,03	0,06	0,10	0,04	0,02	0,02	0,08	0,09	0,07
SD Recovery %	0,05	0,04	0,04	0,02	0,01	0,06	0,02	0,05	0,03	0,01
RSD %	**5.11**	**3.73**	**4.01**	**3.21**	**0.78**	**6.23**	**1.91**	**4.70**	**2.89**	**1.50**

SD: standard deviation; RSD: relative standard deviation (coefficient of variation)

Table 2

Oleuropein concentration in olive leaves (mg/g dry weight)

	Picholine	Olivière	UNK 1[a]	UNK 2	UNK 3	UNK 4	UNK 4h[b]	Olivière 2020[c]
	14.44	41.32	32.5	16.5	5.96	21.68	21.9	3.85
	15.54	37.92	33.36	19.08	6.06	19	21.46	4.29
	16.22	38.7	36.42	185.6	5.62	21	21.58	3.99
	16.44	39.3	33.3	16.62	-	21.44	21.72	4.36
Mean	15.66	39.31	33.89	59.45	5.88	20.78	21.66	4.12
SD[d]	0.90	1.45	1.73	84.11	0.23	1.22	0.19	0.24
RSD[e] %	5.74	3.70	5.10	141.48	3.92	5.87	0.87	5.92

a: Unknown variety

b: Unknown variety extracted for four hours

c: Olivière leaves stored for 12 months under non-controlled conditions

d: Standard deviation

e: Relative standard deviation (coefficient of variation)

SUPPORTING MATERIAL

Table S1: UPLC – MS Validation data

Intra-day precision (repeatability). inter-day (intermediate precision) and recovery % based on concentrations (AF mg/g) obtained by back-calculation of the signal on the individual calibration curves.

For inter-day the equation of the curve of mean back-calculated concentration *vs* nominal concentration was y = y = 0.9887x + 0.0384 (R^2 = 0.9993); for intra-day the equation was y = 0.9915x + 0.0289 (R^2 = 0.9998)

Nominal concentration	**0.4**	**0.8**	**1.6**	**3.2**	**4.8**	**0.4**	**0.8**	**1.6**	**3.2**	**4.8**
Found concentration (AF mg/g)	**Repeatability**					**Intermediate precision (inter-day)**				
AF	0.41	0.80	1.60	3.17	4.82	0.35	0.81	1.80	3.27	4.69
Recovery %	108	105	102	97	101	124	92	99	101	100
AF	0.40	0.79	1.58	3.08	4.81	0.41	0.82	1.66	3.09	4.85
Recovery %	97	103	98	104	98	109	93	105	95	102
AF	0.40	0.81	1.68	3.12	4.83	0.40	0.81	1.68	3.12	4.83
Recovery %	94	100	105	101	99	92	109	103	96	101
AF	0.36	0.77	1.52	3.22	4.74	0.40	0.78	1.61	3.28	4.75
Recovery %	94	102	104	99	100	96	98	98	101	100
AF	0.42	0.85	1.66	3.34	4.76	0.41	0.81	1.60	3.12	4.85
Recovery %	98	108	105	101	99	107	99	97	103	99
Mean AF	0.40	0.81	1.61	3.19	4.79	0.39	0.81	1.67	3.17	4.79
Mean recovery %	98	104	103	100	99	102	100	104	101	99
SD AF	0.02	0.03	0.06	0.10	0.04	0.02	0.02	0.08	0.09	0.07
SD Recovery %	0.05	0.04	0.04	0.02	0.01	1 .99	0 .12	4 .36	0 .80	0 .85
RSD %	5.11	3.73	4.01	3.21	0.78	6.23%	1.91%	4.70%	2.89%	1.50%

SD: standard deviation; RSD: relative standard deviation (coefficient of variation)

Table S2 Validation data UPLC-UV method

Intra-day precision (repeatability). inter-day (intermediate precision) and recovery % based on concentrations (AF mg/g) obtained by back-calculation of the detector signal on the individual calibration curves.

For Intra-day the equation of the curve mean back-calculated concentration *vs* nominal concentration was y = 1,002x - 0,0068 (R^2 = 0.9998); for inter-day, the equation was y = 1,0051x - 0,0173 (R^2 = 0.9999)

Nominal concentration	**0.4**	**0.8**	**1.6**	**3.2**	**4.8**	**0.4**	**0.8**	**1.6**	**3.2**	**4.8**
Found concentration (AF mg/g)	**Repeatability**					**Intermediate precision (inter-day)**				
AF	0.43	0.81	1.54	3.12	4.87	0.30	0.79	1.52	3.26	4.80
Recovery %	108	101	96	97	101	74	99	95	102	100
AF	0.40	0.77	1.56	3.30	4.75	0.44	0.75	1.68	3.04	4.89
Recovery %	100	96	97	103	99	109	93	105	95	102
AF	0.38	0.79	1.58	3.23	4.79	0.37	0.87	1.64	3.07	4.86
Recovery %	96	98	98	101	100	92	109	103	96	101
AF	0.41	0.85	1.55	3.07	4.90	0.38	0.79	1.58	3.23	4.79
Recovery %	103	106	97	96	102	96	98	98	101	100
AF	0.42	0.87	1.56	3.20	4.80	0.43	0.79	1.55	3.28	4.76
Recovery	106	108	98	100	100	107	99	97	103	99
Mean AF	0.41	0.82	1.56	3.18	4.82	4.78	9.97	19.90	39.71	60.25
Mean recovery	103	102	97	99	100	96	100	99	99	100
SD AF	0.02	0.04	0.01	0.09	0.06	0.69	0.58	0.83	1.42	0.67
SD Recovery %	0.049	0.051	0.009	0.028	0.012	4.43	0.31	0.52	0.71	0.41
RSD %	**4.76**	**5.00**	**0.91**	**2.84**	**1.22**	**14.53**	**5.77**	**4.18**	**3.58**	**1.10**

SD: standard deviation; RSD: relative standard deviation (coefficient of variation)

Table S3 ANOVA of back-calculated oleuropein concentrations (mg/g) of spiked placebos for each of the three methods.

0,4 mg/g

Groupes	*Nombre d'échantillons*	*Somme*	*Moyenne*	*Variance*
Colonne 1	5	1,98589556	0,39717911	0,00088452
Colonne 2	5	2,01945633	0,40389127	0,00038967
Colonne 3	5	1,9847719	0,39695438	0,00041199

Source des variations	*Somme des carrés*	*Degré de liberté*	*Moyenne des carrés*	***F***	*Probabilité*	***Valeur critique F***
Entre Groupes	0,00015537	2	7,7687E-05	**0,1382174**	0,872276	**6,92660814**
A l'intérieur des groupes	0,00674473	12	0,00056206			
Total	0,0069001	14				

0,8 mg/g

Groupes	*Nombre d'échantillons*	*Somme*	*Moyenne*	*Variance*
Colonne 1	5	1,98589556	0,39717911	0,00088452
Colonne 2	5	2,01945633	0,40389127	0,00038967
Colonne 3	5	1,9847719	0,39695438	0,00041199

Source des variations	*Somme des carrés*	*Degré de liberté*	*Moyenne des carrés*	***F***	*Probabilité*	***Valeur critique pour F***
Entre Groupes	0,00015537	2	7,7687E-05	**0,1382174**	0,872276	**6,92660814**
A l'intérieur des groupes	0,00674473	12	0,00056206			
Total	0,0069001	14				

1,6 mg/g

Groupes	*Nombre d'échantillons*	*Somme*	*Moyenne*	*Variance*
Colonne 1	5	4,15049322	0,83009864	0,00060937
Colonne 2	5	4,0822563	0,81645126	0,00166592
Colonne 3	5	4,02565987	0,80513197	0,0009032

Source des variations	*Somme des carrés*	*Degré de liberté*	*Moyenne des carrés*	***F***	*Probabilité*	***Valeur critique pour F***
Entre Groupes	0,00156285	2	0,00078143	**0,73754451**	0,498767	**6,92660814**
A l'intérieur des groupes	0,01271397	12	0,0010595			
Total	0,01427682	14				

HPLC- Fluorescence (colonne 1) UPLC-MS (colonne 2) and UPLC-UV (colonne 3) . Data based on intra-day analysis.

3,2 mg/g

Groupes	Nombre d'échantillons	Somme	Moyenne	Variance
Colonne 1	5	8,23434978	1,64686996	0,00242934
Colonne 2	5	7,77871439	1,55574288	0,0002024
Colonne 3	5	8,03472696	1,60694539	0,00416121

ANALYSE DE VARIANCE

Source des variations	Somme des carrés	Degré de liberté	Moyenne des carrés	***F***	Probabilité	***Valeur critique pour F***
Entre Groupes	0,02086635	2	0,01043318	**4,60765618**	0,032748	**6,92660814**
A l'intérieur des groupes	0,02717176	12	0,00226431			
Total	0,04803811	14				

4,8 mg/g

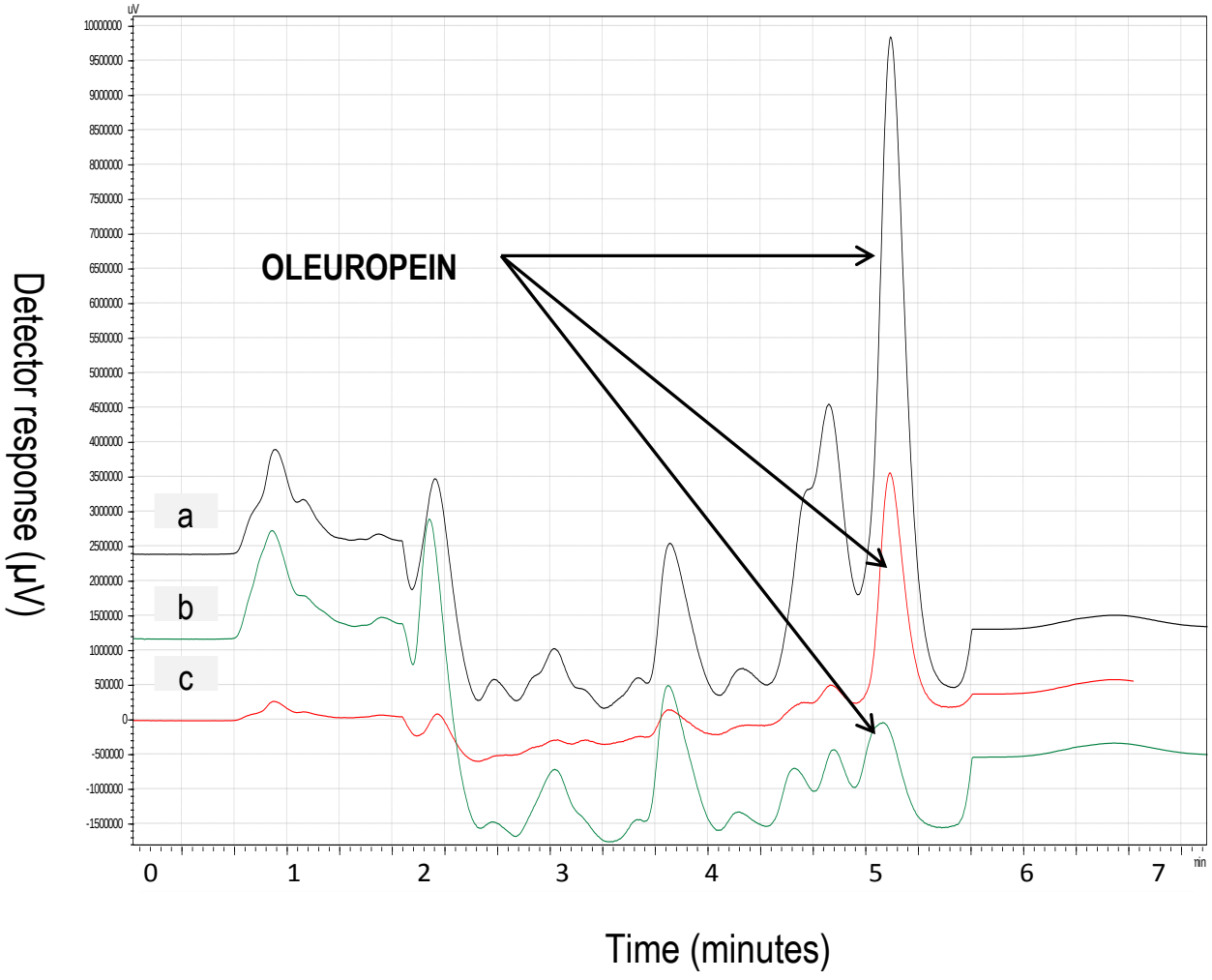
Groupes	*Nombre d'échantillons*	*Somme*	*Moyenne*	*Variance*
Colonne 1	5	23,8552696	4,77105393	0,00321999
Colonne 2	5	24,1151745	4,8230349	0,00343797
Colonne 3	5	23,9508947	4,79017894	0,0014106

Source des variations	*Somme des carrés*	*Degré de liberté*	*Moyenne des carrés*	***F***	*Probabilité*	***Valeur critique pour F***
Entre Groupes	0,00691217	2	0,00345608	**1,28501879**	0,312121	**6,92660814**
A l'intérieur des groupes	0,03227425	12	0,00268952			
Total	0,03918642	14				

Supplemental Figure S1

Comparison of extraction solvents

50 mg blank olive leaves spiked with 45 mg/L oleuropein extracted into 3 mL solvent. A: 100% methanol; B: 50 % methanol- H2O; C:100% H2O. HPLC-fluorescence method; chromatographic conditions described in the text



Supplemental Figure S2

Chromatograms of authentic standards and matrix standards (spiked blank leaves). Chromatographic conditions as described in the text

(a): Authentic standard 20 mg/L

(b) Authentic standard 10 mg/L

(c) Spiked standard (placebo) 1.6 mg/g

(d) Spiked standard (placebo) 3.2 mg/g