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Study of phenolic composition of olive fruits: validation of a simple and fast HPLC-UV method

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Abstract – A high-performance liquid chromatography (HPLC) method endowed with a gradient elution and a UV detection system was established and validated for the determination of phenolic acids, phenolic alcohols, hydroxycinnamic acid derivatives, flavonoids, secoiridoids and lignans during olive (*Olea europaea* L.) fruit development (green, purple and black olives). Within the test range, the calibration curves exposed a good linear regression (R^2>0.9995). Detection limits ranged between 0.63 and 13.43 mg/L for the detected phenolic compounds. The presented method yielded satisfactory repeatability in terms of retention times and average concentrations of phenolic compounds (RSD < 0.3%). Verbascoside was established as the major phenolic compound in black olives. Oleuropein was established as the dominating phenolic compound in green olives, and its level decreased during maturation. Additionally, this research is the first to experimentally evidence that the flavone luteolin-7-rutinoside is the predominant flavonoid glucoside in black olives, showing the most significant variation with fruit development. The above results validate the method for an easy and fast determination of different classes of phenolic compounds present in olive fruits.

Keywords: phenolic compounds / HPLC-UV / olive fruits / maturation

Résumé – Étude de la composition phénolique des fruits d'olives : validation d'une méthode simple et rapide par HPLC-UV. Une méthode de chromatographie liquide à haute performance (HPLC) dotée d'une élution à gradient et d'un système de détection UV a été établie et validée pour la détermination des acides phénoliques, des alcools phénoliques, des dérivés de l'acide hydroxycinnamique, des flavonoïdes, des sécoiridoïdes et des lignanes durant les différents stades de maturation des fruits d'olive (*Olea europaea* L.). Les courbes d'étalonnage ont montré une bonne régression linéaire (R^2 > 0,9995). Les limites de détection se situent entre 0,63 et 13,43 mg/L pour les composés phénoliques détectés. La méthode présentée a fourni une répétabilité satisfaisante en termes de temps de rétention et de concentrations moyennes des composés phénoliques (RSD < 0,3 %). Le verbascoside a été établi comme le principal composé phénolique des olives noires. L'oleuropéine a été établie comme le composé phénolique dominant dans les olives vertes, et son niveau diminue pendant la maturation. En outre, cette recherche pourrait être considérée comme la première étude fournissant expérimentalement la preuve que la flavone lutéoline-7-rutinoside est le glucoside flavonoïde prédominant dans les olives noires, montrant la variation la plus significative durant le développement du fruit. Les résultats ci-dessus montrent que la méthode validée est adéquate pour une détermination facile et rapide de différentes classes de composés phénoliques présents dans les olives.

Mots clés : composés phénoliques / HPLC-UV / olives / maturation

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Highlights

- Phenolic compositions of olive fruits during maturation were analyzed by a simple HPLC-UV method.
- The HPLC method provided satisfactory repeatability. Detection limits ranged between 0.63 and 13.43 mg/l for the detected phenolic compounds.
- Verbascoside was established as the major phenolic compound in black olives.
- Oleuropein was established as the dominating phenolic compound in green olives.

1 Introduction

Phenolic acids and their derivatives (phenols) are an important group of metabolome, which are produced in the secondary metabolism of several plants *via* the shikimate-henylpropanoid pathway. They are often conjugated with polyamines, sugars or other phenolics. These compounds have a great range of biological functions including anti-microbial, antioxidant, anti-inflammatory, anti-arrhythmic and anti-proliferative effects (Balasundram *et al.*, 2006). In addition to their potential health benefits, phenolic acids and their derivatives are involved in plant defense against herbivores (Uccella, 2000) and pathogens (Konno *et al.*, 1998).

There are several methods for the determination of polyphenols in foods. These include gas chromatography (Ryan and Robards, 1998), capillary gas chromatography-mass spectrometry (Angerosa *et al.*, 1996), high-performance liquid chromatography (HPLC) or HPLC coupled to mass spectrometry (Peng *et al.*, 2016), electrophoresis (Bonoli *et al.*, 2004) and micellar electrokinetic chromatography (Ganzera *et al.*, 2008). Within the last 20 years, the characterization and the separation of phenolic acids and their derivatives have been principally monitored using the HPLC technique (Stalikas, 2007).

The *Olea europaea* olive tree could be considered as the most abundant and important fruit tree in the Mediterranean basin. Virgin and extra virgin olive oils, mechanically extracted from olive fruits, are enormously appreciated by consumers interested in both medicinal and nutritional values of food. The phenolic compositions of olive leaves andolive oil as well as the by-products of olive oil production (*e.g.* wastewater) have been widely studied; nevertheless, other olive parts, such as fruits, have received only limited attention due to limited information about their phytochemical content. The predominant categories of phenolic compounds detected in olive leaves and oil are phenolic alcohols, flavonoids, lignans, phenolic acids and secoiridoids (Quirantes-Piné *et al.*, 2013; Ben Brahim *et al.*, 2017). Also, the most abundant compounds of the afore mentioned family are hydroxytyrosol, tyrosol, oleuropein, verbacoside, apigenin-7-glucoside, luteolin-7-glucoside, luteolin-7-rutinoside, decarboxymethyl oleuropein aglycon, ligstroside aglycon, oleuropein aglycon and decarboxymethyl ligstroside aglycon (Ben Brahim *et al.*, 2017; Taamalli *et al.*, 2012).

Phenolic compounds contained in olive fruits afford numerous healthy benefits related to their antioxidant activity (Malheiro *et al.*, 2014). The main phenolic compounds existing in olive fruits are oleuropein, tyrosol and hydroxytyrosol (Dagdelen *et al.*, 2013). The qualitative and quantitative study of phenolic compounds present in olive fruit is very interesting, and numerous studies have addressed the extraction and their separation (Machado *et al.*, 2013, Crawford *et al.*, 2018). HPLC coupled to diode-array detection (DAD), or UV–Vis was the most common method for the examination of phenolic compounds present in olive fruits, while the run time exceeded 40 min.

The principal objective of this work was to study the predominant phenolic compounds in olive fruits during maturation by the means of an inexpensive, efficient, fast and simple HPLC method with UV detection. This method was used to identify and quantify phenolic acids (p-hydroxybenzoic acid), phenolic alcohols (hydroxytyrosol and tyrosol), hydroxycinnamic acid derivatives (verbascoside), flavonoids (catechin hydrate, quercetin, apigenin, luteolin-7-rutinoside, apigenin-7-glucoside and luteolin-7-glucoside), secoiridoids (oleuropein) and lignan (pinoresinol) that are present also in the olive leaves according to the literature. Actually, up to our knowledge, there are no available studies interested in the simultaneous separation, determination and quantification of these twelve studied components in olive fruits during maturation.

2 Materials and methods

2.1 Equipment and reagents

The study of the phenolic composition was conducted using an Agilent HP 1100 series HPLC system (Agilent Technologies, Inc., Palo Alto, CA, USA) consisting of a quaternary pump solvent delivery system, a variable wavelength UV detector, an auto degasser, and a PC for data processing.

Methanol and acetonitrile (HPLC gradient grade) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Standards of phenolic acids (p-hydroxybenzoic acid; cinnamic acid), phenolic alcohols (hydroxytyrosol and tyrosol), hydroxycinnamic acid derivatives (verbascoside), flavonoids (catechin hydrate, quercetin, apigenin, luteolin-7-rutinoside, apigenin-7-glucoside and luteolin-7-glucoside), secoiridoids (oleuropein) and lignan (pinoresinol) were all provided by Sigma-Aldrich Chemie (Steinheim, Germany). A standard stock solution of these studied components has been prepared by dissolving each phenolic compound at a concentration of 500 μg/mL in an HPLC grade methanol. The working solutions were also prepared by diluting the stock solutions with HPLC grade methanol.

2.2 Materials

In order to study the effect of the maturation process on the phenolic composition of olive fruit extracts, cv. *Chemlali,* olives were harvested from Mourdine, Tunisia (North latitude 35°46′, East longitude 10°33′). The orchard has 15 ha with a

planting density of 12 × 12 m; trees are more than 30 years old. The olive samples were collected at numerous stages of physiological development as reflected by skin coloration (green color, purple color and black color). One kilogram of olive fruit samples was randomly obtained from different parts selected from three separated trees, and immediately transported to the laboratory to avoid compositional changes.

2.3 Extraction of phenolic compounds

A mass of 1.5 g of destoned and mashed olives was placed on a shaker in the presence of 20 mL of methanol at 200 rpm during 24 h. The resulting methanolic extracts were subjected to a centrifugation during 10 min at 5000 rpm, and then filtered through a nylon filter (0.45 μm, Agilent, Palo Alto, CA). Later to the filtration, a clear solution of hydrophilic constituents was obtained and stored in opaque vials at −20 °C until further investigation.

2.4 Chromatographic conditions

The separation of phenolic components from olive fruit extracts was accomplished using HPLC. Separation was achieved by a reversed-phase ZORBAX Eclipse XDB column − C18 (4.6 × 250 mm, 5 μm) from Agilent Technologies. Peak areas were determined at 280 nm for all phenolics. The ambient temperature was controlled and maintained at 20 °C ± 1. The separation of polyphenols was developed using a linear gradient method exploiting 0.2% of sulfuric acid (A) and acetonitrile (B), the sequence began with 15% of B, then the gradient was programmed to obtain 40% of B at 12 min, 60% of B at 14 min, 80% of B at 18 min, 90% of B at 20 min and 100% B at 24 min. The flow rate was set at 0.8 mL/min throughout the gradient, and the injection volume was 10 μL.

The identification of phenolic compounds was developed *via* the comparison of their retention times with the retention times of the injected standard solution under the same operatory conditions. Furthermore, samples were spiked in order to verify the peak identification. Using internal standard calibration, peak area was served for quantification purposes. The internal standard used for the quantification of polyphenols was cinnamic acid.

2.5 HPLC method validation

Linearity was assessed with standard solutions. Standard solutions were injected separately in triplicate into the HPLC system and, for each compound, the calibration curve was separately established by plotting curves of peak areas (y-axis) *versus* concentrations (x-axis). The calculation of the regression equation was developed following the form $y = ax + b$, where y represents the values of peak area and x represents the values of sample concentration. The linearity was measured based on the measurements of the correlation coefficients. The precision of the method was evaluated based on the results for olive fruit samples, and expressed as relative standard deviation (RSD). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the slope of the calibration curve and the standard deviation of the response for each compound.

The LOD and the LOQ were expressed as:

$$LOD = 3.3 \times \sigma/S$$
$$LOQ = 10 \times \sigma/S \text{ '}$$

where σ is the standard deviation of the response, and S is the slope of the calibration curve.

3 Results

3.1 Chromatographic separation

A chromatogram of twelve phenolic compounds of the standard mixture solution as well as the internal standard was shown in Figure 1. As presented in the chromatogram, all the studied phenolic components had responses at 280 nm, where they have been efficaciously separated. Figure 2 showed the peak chromatograms of olive fruit extracts. The comparison of the retention times of those compounds with the retention times of authentic standards lead to their identification. As presented in Figure 2, the identified phenolic compounds belong to different classes: phenolic acids (p-hydroxybenzoic acid), phenolic alcohols (hydroxytyrosol and tyrosol), hydroxycinnamic acid derivatives (verbascoside), secoiridoid (oleuropein), flavonoids (catechin hydrate, quercetin, apigenin, luteolin-7-rutinoside, apigenin-7-glucoside and luteolin-7-glucoside) and lignan (pinoresinol).

3.2 Validation of the HPLC method

The standard solutions relative to each point in the calibration curve were injected in triplicate in order to establish the linearity study. Table 1 summarizes the determination coefficient (R^2) and the regression equations.

Calibration curves were linear over the studied concentration range, and in this range, R^2 values were beyond 0.9995 for all the studied compounds, indicating good linearity over the investigated range. Actually, a constant retention time makes chromatographic peaks readily identifiable and leads to a better evaluation of peak areas. The retention times were checked repeatedly for the detected phenolic compounds during this work and were found to be extremely stable (Tab. 2).

Calculated LOD and LOQ are shown in Table 1. The values determined in this study fluctuated from 0.63 to 13.43 μg/mL and from 1.91 to 40.70 μg/mL, respectively (Tab. 1). The LOD were as follows: 6.86 mg L^{-1} for verbascoside, 2.78 mg L^{-1} for luteolin-7-glucoside, 2.29 mg L^{-1} for apigenin-7-glucoside and 13.43 mg L^{-1} for oleuropein. The LOD of these phenolic compounds obtained by our HPLC-UV method were comparable to that reported in a previous study using the HPLC-DAD method (2.59 mg L^{-1} for verbascoside, 3.92 mg L^{-1} for luteolin-7-glucoside, 1.57 mg L^{-1} for apigenin-7-glucoside and 11.46 mg L^{-1} for oleuropein) (Japón-Luján *et al.*, 2006). Compared with the HPLC-DAD-FLD-MS/MS method, Jerman *et al.* (2010) reported detection limits equal to 2.77, 4.92, 1.48, 0.66 and 1.85 for oleuropein, verbascoside, rutin, luteolin-7-O-glucoside and quercetin, respectively.

The precision of the developed methodology was based on the average phenolic compound concentration and the retention time, and expressed as RSD. The values obtained in this study ranged from 0.1% to 0.3% for the retention time

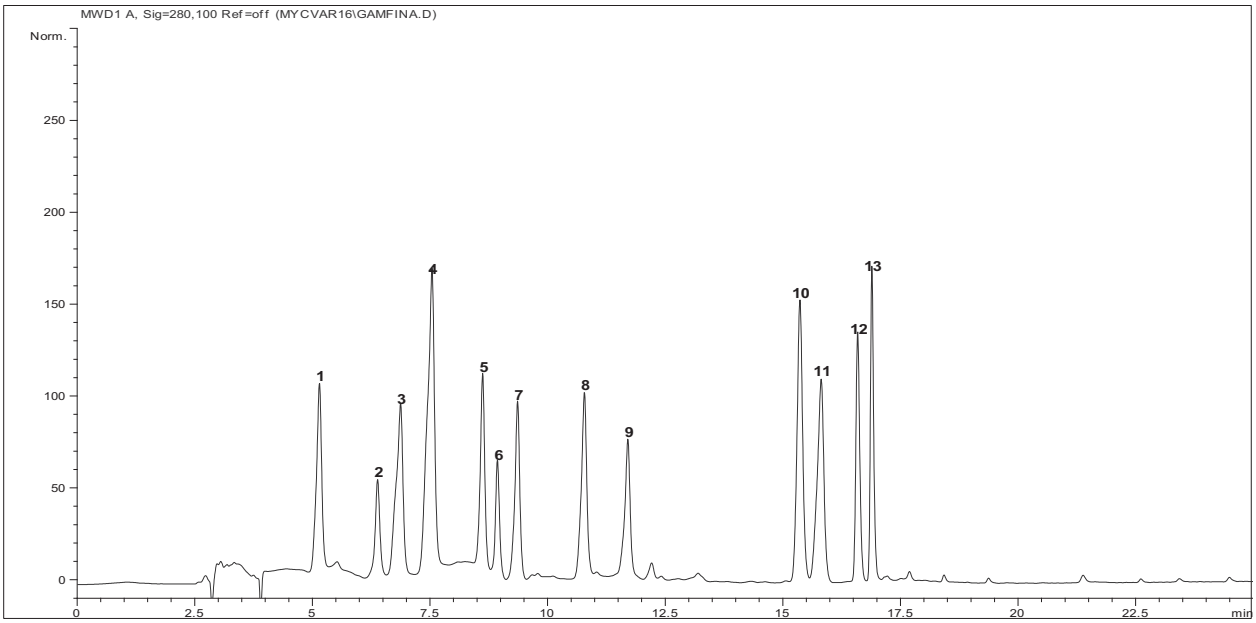

Fig. 1. HPLC-UV chromatogram of the standard mixture solution. 1: hydroxytyrosol; 2: catechin hydrate; 3: tyrosol; 4: p-hydroxybenzoic acid; 5: luteolin-7-rutinoside; 6: verbascoside; 7: luteolin-7-glucoside; 8: apigenin-7-glucoside; 9: oleuropein; 10: quercetin; 11: pinoresinol; 12: cinnamic acid; 13: apigenin.

and from 0.1% to 2.92% for the average concentration (Tab. 2). Therefore, the precision of this methodology is acceptable.

3.3 Phenolic composition of olive fruits

The phenolic compounds detected in olive fruits were hydroxytyrosol, tyrosol, p-hydroxybenzoic, luteolin-7-rutinoside, verbascoside, luteolin-7-glucoside, apigenin-7-glucoside, oleuropein, quercetin and apigenin (Fig. 2). Except for luteolin-7-rutinoside, verbascoside and an unknown compound, whose concentrations tended to increase with olive maturation (Tab. 2), all the studied analytes were in higher concentrations in green olives than in black olives.

The major phenolic compound identified in green olives was oleuropein. The concentration of this compound decreased with fruit development. Its levels varied from 1734 to 490 μg/g in green and black olives, respectively. It is followed by tyrosol, verbascoside and hydroxytyrosol.

Among the identified flavonoid compounds, luteolin-7-rutinoside was the most predominant one; its concentration increased during fruit maturity, from 108.76 μg/g in green olives to 442.13 μg/g in black olives.

The HPLC method used in this work permitted also the detection of an unknown compound at the first part of purple and black olive chromatograms. This compound was absent in green olive extracts (Fig. 2C). Its concentration varied from 227.34 μg/g in purple olives to 466.67 μg/g in black olives.

4 Discussion

Due to the wide range of phenolic compound polarity, numerous gradient elution systems and different types of mobile phases were tested in order to accomplish a proper separation of phenolic compounds, such as methanol, acetonitrile and water (Martí *et al.*, 2015). In the current study, a quantitative and confirmatory HPLC-UV method was validated for linearity, precision and sensitivity. The performance parameters showed the validated method to be adequate for the determination of different classes of phenolic compounds in olives. In this study, the gradient elution conditions followed were: a linear gradient started with 15% of acetonitrile and rose to 40%, 60%, 80% and 90% at 12, 14, 18 and 20 min, respectively, followed by an increase up to 100% of acetonitrile at 24 min. Below these studied gradient conditions, the analytes were eluted in a duration less than 24 min, which means a reduced analysis time with respect to other previously described methods working with an ultraviolet detector, diode-array detector or more powerful techniques (see Tab. 3). Other methods such as that described by Peng *et al.* (2016), despite requiring only 12 min for analyzing phenolic compounds present in olive fruit extracts, involved a considerably higher investment (ultra-high performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UHPLC-Q-TOF/MS). In the same way, Ammar *et al.* (2017) analyzed leaves and wood with stems of 'Chemlali' olive cultivar extracts using hydromethanol by resorting to a very complex procedure involving a reversed-phase HPLC coupled to two detection systems: diode-array detection and quadrupole time-of-flight mass spectrometry in negative ion mode (RP-HPLC-DAD-ESI-QTOF-MS). These sophisticated devices may not be available in all laboratories, especially in developing countries. In consequence, our proposed method has the advantage to be very simple, uncomplicated and fast.

During the past few years, olive trees have greatly attracted researchers as an important source of polyphenols suitable for food and pharmaceutical industries. Based on the literature, the most interesting categories of phenolic componentsin the

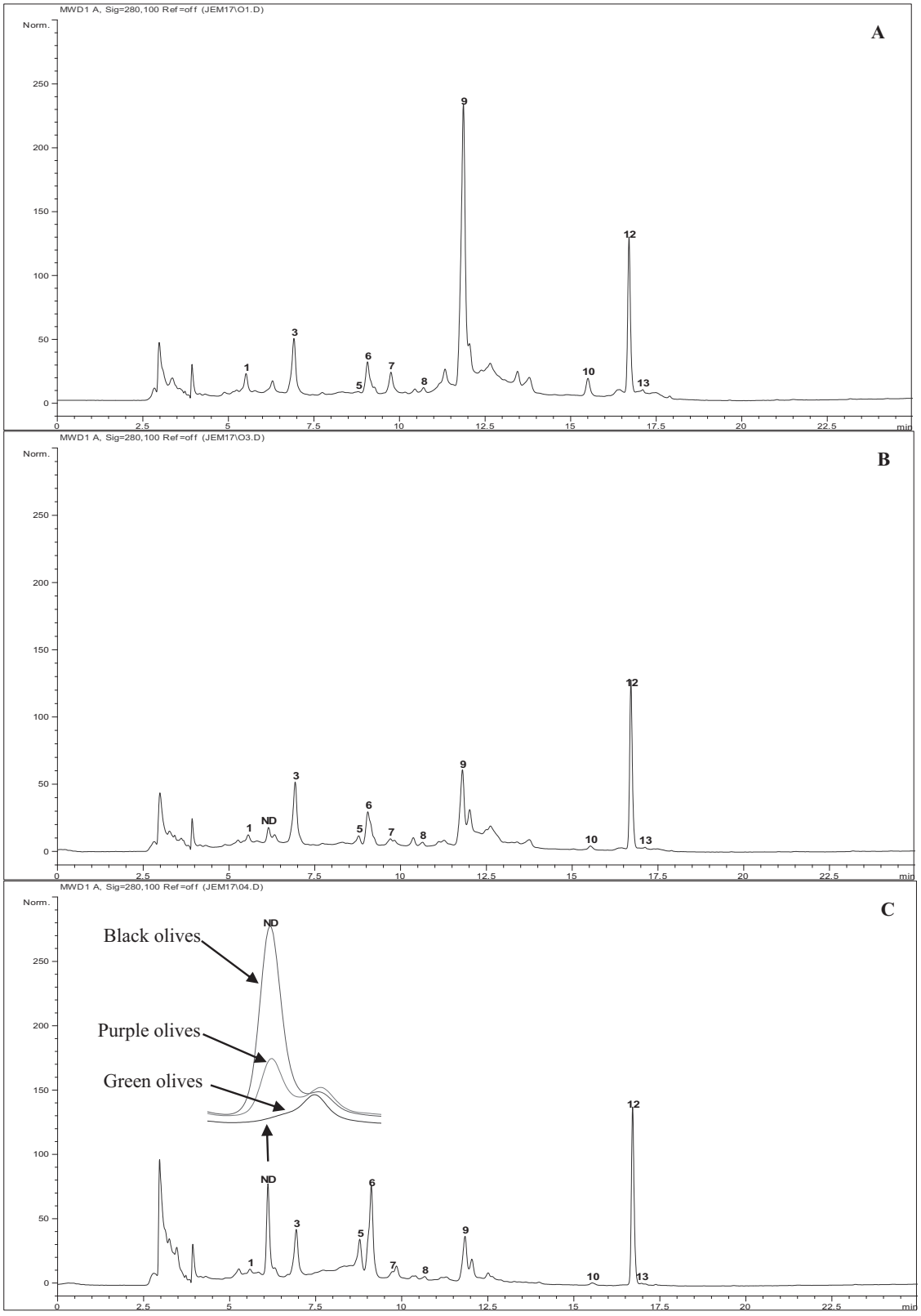

Fig. 2. Typical HPLC-UV chromatograms of green (A), purple (B) and black olives (C). 1: hydroxytyrosol; ND: unknown compound; 3: tyrosol; 5: luteolin-7-rutinoside; 6: verbascoside; 7: luteolin-7-glucoside; 8: apigenin-7-glucoside; 9: oleuropein; 10: quercetin; 12: cinnamic acid; 13: apigenin.

Table 1. Regression equations of the investigated compounds, including the coefficient of determination (R^2), range of calibration, LOD and LOQ.

Compounds	Regression equation	R^2	Range (mg/L)	LOD (mg/L)	LOQ (mg/L)
Hydroxytyrosol	$y = 5871 x + 12$	0.9997	20–100	8.5	25.76
Catechin hydrate	$y = 2740 x + 3$	0.9998	50–200	4.74	14.38
Tyrosol	$y = 3969 x + 3$	0.9995	25–100	3.59	10.89
p-hydroxybenzoic	$y = 8637 x + 2$	0.9997	10–50	1.59	4.85
Luteolin-7-rutinoside	$y = 8337 x + 10$	0.9998	50–250	6.12	18.55
Verbascoside	$y = 6089 x + 6$	0.9998	100–250	6.86	20.79
Luteolin-7-glucoside	$y = 11239 x + 8$	0.9997	20–100	2.78	8.42
Apigenin-7-glucoside	$y = 13916 x + 8$	0.9998	20–100	2.29	6.95
Oleuropein	$y = 6210 x + 20$	0.9997	100–500	13.43	40.70
Quercetin	$y = 10950 x + 1$	0.9996	5–20	0.63	1.91
Pinoresinol	$y = 7193 x + 3$	0.9995	20–100	2.03	6.16
Apigenin	$y = 3990 x + 3$	0.9996	20–100	3.17	9.61

Table 2. Precision of the method according to the mean values of the concentrations of the detected phenolic compounds, calculated as μg/g (mean ± RSD%, $n = 3$) using HPLC-UV, during the maturation of *Chemlali* olive fruits.

Compounds	Retention time (min)	Fruit skin color		
		Green	Purple	Black
Hydroxytyrosol	5,17 ± 0.3	259.51 ± 0.85	202.50 ± 2.69	204.41 ± 0.45
ND	6,15 ± 0.1	–	227.34 ± 1.81	466.67 ± 2.33
Tyrosol	6,86 ± 0.2	810.49 ± 1.78	704.78 ± 1.36	561.53 ± 2.48
p-hydroxybenzoic	7,66 ± 0.3	12.43 ± 2.92	13.39 ± 2.89	12.01 ± 1.88
Luteolin-7-rutinoside	8,60 ± 0.3	108.76 ± 1.82	213.41 ± 1.48	442.13 ± 2.29
Verbascoside	9,05 ± 0.2	357.59 ± 2.07	466.24 ± 1.66	850.72 ± 1.76
Luteolin-7-glucoside	9,49 ± 0.2	43.82 ± 1.84	40.96 ± 2.17	40.17 ± 2.83
Apigenin-7-glucoside	10,85 ± 0.1	105.58 ± 0.16	91.97 ± 1.63	63.17 ± 2.66
Oleuropein	11,76 ± 0.2	1734.34 ± 0.44	631.28 ± 1.46	490.65 ± 1.71
Quercetin	15,48 ± 0.3	97.01 ± 2.62	64.40 ± 1.81	37.10 ± 2.14
Apigenin	17,02 ± 0.1	61.72 ± 1.83	35.32 ± 2.62	14.52 ± 2.41

ND: unknown compound.

different parts of olive tree (*Olea europaea* L.) include phenolic acids, secoiridoids, phenolic alcohols, hydroxycinnamic acid derivatives and flavonoids (Quirantes-Piné *et al.*, 2013; Taamalli *et al.*, 2012; Ammar *et al.*, 2017). These phenolic compounds have been extensively studied in several organs of the olive tree (Quirantes-Piné *et al.*, 2013; Ben Brahim *et al.*, 2017; Ammar *et al.*, 2017). However, other olive tree tissues such as olive fruits have received only limited attention due to the lack of information about their phytochemical content. In this study, the phenolic composition of olive fruits was monitored as a function of fruit color (green, purple and black). This method is supposed to be more adequate for such investigations since diverse colored olive fruits are known to be chemically dissimilar, particularly with respect to phenolic compositions. The decrease in oleuropein concentration with fruit maturation is in accordance with

Table 3. Determination of phenolic compounds in olive oil and different olive tree organs: comparison between the proposed method with previous published researches.

Sample (g)	Extraction solvent	Instrumental technique[a]	Analytes (with elution order)	Analysis time (min)	References
Olive roots (0.5 g) Olive fruits (0.5 g)	Methanol	HPLC-UV Analytical column: ZORBAX Eclipse XDB − C18 (4.6 × 250 mm, 5 μm)	Hydroxytyrosol, catechin hydrate, tyrosol, hydroxybenzoic acid, luteolin-7-rutinoside, verbascoside, luteolin-7-glucoside, apigenin-7-glucoside, oleuropein, quercetin, pinoresinol, apigenin	24	The present method
Olives fruits (0.05 g)	Methanol-water (60:40)	UHPLC-Q-TOF/MS Analytical column: agilent SB-C18 (4.6 × 50 mm, 1.8 μm)	Gallic acid, hydroxytyrosol, methyl gallate, luteolin-7-glucoside,rutin, ellagicacid, oleuropein	12	Ben Brahim *et al.* (2017)
Olive leaves (1 g)	Ethanol-water (59:41)	HPLC-DAD Analytical column: Lichrospher C18 100 (4.6 × 250 mm, 5 μm)	Verbascoside, luteolin-7-glucoside, apigenin-7-glucoside, oleuropein	40	Malik and Bradford (2006)
Olives fruits (2 g)	dimethylsulfoxide	HPLC-DAD Analytical column: agilent eclipse − C18 (4.6 × 250 mm, 5 μm)	Hydroxytyrosol, tyrosol, caffeicacid, verbascoside, luteolin-7-glucoside, rutin, oleuropein, quercetin	45	Konno *et al.* (1998)
Table olive (2 g)	Methanol (2 mM Sodium fluoride)	HPLC-DAD-FLD Analytical column: Gemini-NX C18 (4.6 × 150 mm, 3 μm)	Hydroxytyrosol, tyrosol, catechin, vanillic acid, syringic acid, vanillin, sinapic acid, *o*-vanillin, luteolin-7-glucoside, verbascoside, apigenin-7-glucoside, oleuropein, quercetin luteolin, apigenin	50	Uccella (2000)
Olive oil (5 g)	Methanol-water (60:40)	HPLC/DAD-ESI/MS Analytical column: Spherisorb C18-ODS2 (4.6 × 250 mm,5 μm)	Hydroxytyrosol, tyrosol, vanillic acid, *p*-coumaric acid, ferulic acid, oleuropein, pinoresinol, luteolin, apigenin	50	Ryan and Robards (1998)
Olives fruits (1.5 g)	Methanol	HPLC-DAD Analytical column: Spherisorb C18-ODS2 (4.6 × 250 mm,5 μm)	Hydroxytyrosol, chlorogenic acid, verbascoside, oleuropein, rutin, apigenin-7-glucoside, luteolin	66	Stalikas (2007)
Olives fruits (1.5 g)	Methanol	HPLC-DAD-FLD-MS/MS Analytical column: Spherisorb C18-ODS2 (4.6 × 250 mm, 5 μm)	Oleuropein, verbascoside, quercitrin, luteolin-7-glucoside rutin	70	Obied *et al.* (2008)

[a] HPLC-UV: high-performance liquid chromatography-ultraviolet detector; HPLC-DAD: high performance liquid chromatography-diode array detection; HPLC-DAD-FLD-MS/MS: high-performance liquid chromatography–diode array detection–fluorescence-mass spectrometry; HPLC/DAD-ESI/MS: high-performance liquid chromatography-diode array detection-electrosprayionization-mass spectrometry; UHPLC-Q-TOF/MS: ultra high performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry; HPLC-DAD-FLD: high-performance liquid chromatography-diode array detection-fluorescence.

literature (Machado *et al.*, 2013) due to the hydrolyzing activity of enzymes (Amiot *et al.*, 1986). The diminution in the oleuropein level was accompanied with a diminution in the tyrosol and hydroxytyrosol contents in olive fruits. These results are in conformity with what was reported previously for other olive cultivars (Machado *et al.*, 2013).

Verbascoside is a compound comprising hydroxytyrosol/tyrosol and cinnamyl-derivative moieties (Obied *et al.*, 2008). This molecule content augmented with fruit development. This tendency was contrary to that detected for the secoiridoid oleuropein, supposing that during maturation, in part, the degradation of oleuropein might be related to the intensification in verbascoside concentration. It has been reported that hydroxytyrosol could have resulted from the degradation of oleuropein (Brenes and De Castro, 1998), which then can be transformed into verbascoside. Malik and Bradford (2006) indicated that the increase in verbascoside concentration in completely developed fruits goes in pair with a decline in oleuropein content in mature fruits, and that oleuropein degradation through fruit development could contribute in part to an increase in the level of verbascoside.

The high level of black olive verbascoside, observed in this study, is of a special interest. In fact, verbascoside displayed an extensive biological activity, being a free radical scavenger (D'Imperio *et al.*, 2014). In fact, anti-microbial, anti-fungal and anti-inflammatory properties have been previously described for this phenolic compound (Alipieva *et al.*, 2014).

Flavonoids are an additional interesting class of phenolic compounds which were identified in olive fruits and were also widely represented in olive leaves, buds and flowers (Quirantes-Piné *et al.*, 2013; Taamalli and Abaza, 2013). It has been proved that flavonoids play a primordial role in plant defense against abiotic stress (Alipieva *et al.*, 2014). Many flavonoids were also identified in this study. They included luteolin-7-rutinoside, apigenin-7-glucoside, luteolin-7-glucoside, quercetin and apigenin. All flavonoids were in higher levels in green olives than in black olives, except for luteolin-7-rutinoside. Çakir *et al.* (2006) demonstrated that the antioxidant and radical scavenging properties of anti luteolin-7-rutinoside were higher than luteolin-7-glucoside ones. The radical scavenging activity of luteolin derivatives has been found to be structure-dependent (Orhan *et al.*, 2013). The high level of luteolin-7-rutinoside observed in this study may (i) prevent olive fruits from many diseases, (ii) contribute to the long stability and sensory characteristics of virgin olive oils, and (iii) be involved in pharmacological and biochemical effects, including antioxidant and anti-carcinogenic properties. Moreover, this work is the first to provide experimental evidence that luteolin-7-rutinoside was the predominant flavonoid glucoside in black olives and showed the most significantvariation with fruit development. The nutritional impact of these results is important since olives characterized by a high content of luteolin-7-rutinoside are preferred from a human health perspective. Lopez-Lazaro (2009) indicated that luteolin may prevent neurodegenerative diseases by reducing oxidative stress, and cardiovascular diseases by reducing cholesterol and blood pressure.

5 Conclusion

In spite of the important number of studies interested in the polyphenolic composition of *Olea europaea*, this study offers a new vision concerning the phytochemical composition of olive fruits. The described HPLC method seems to be appropriate for the differentiation and determination of the most known flavonoids together with phenolic acids, phenolic alcohols, hydroxycinnamic acid derivatives and secoiridoids, and consequently can be considered a valid and fast technique for the analysis of this interesting category of natural compounds.

Conflicts of interest

The authors declare that they have no conflicts of interest in relation to this article.

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