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Original Article

Phenolic Profile of Leaves and Drupes in Major Greek Olive Varieties

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Abstract

Leaves and drupes of the major Greek olive varieties 'Koroneiki', 'Lianolia Kerkyras', 'Mastoidis', 'Adramytini', 'Megaritiki', 'Gaidourelia', 'Kalamata', 'Konservolia', 'Chalkidiki' and the Spanish variety 'Arbequina' were collected at different developmental stages during two consecutive years and investigated by HPLC for their phenolic profile and the concentration of the phenolic compounds present. The phenolic compounds identified in year 1 new season leaves were, in declining concentration order, oleuropein, 7-O-glucoside of luteolin and rutin, whereas for those collected in year 2 the main phenolic compounds were oleuropein, rutin, 4-O-glucoside of luteolin and 7-O-glucoside of apigenin. In September - December collected leaves of year 2, oleuropein presented the higher concentration followed by 7-O-glucoside of luteolin, 4-O-glucoside of luteolin and rutin. Regarding green and black drupes for both years, the main phenolic compounds were oleuropein, verbascoside and rutin. Verbascoside was only found in drupes whereas the 7-O-glucoside of apigenin only in leaves. The concentration of oleuropein showed high fluctuations both between different tissues within the same variety and between different varieties within the same tissue. The number of phenolic compounds identified in the phenolic profile of green and black drupes was lower than that of leaves regardless developmental stage or year. Differences were observed in the concentration of phenolic compounds depending on the variety in all tissues. The phenolic profile of new season leaves, green and black drupes was similar for both years.

Keywords: *HPLC, Olea europaea* ssp. *sativa,* oleuropein, phenolic compounds, phenolic concentration

Introduction

The olive plant phenolic content is very complex and can vary both in quality and quantity depending on many factors, such as genotype, tissue type, developmental stage, ripening processes, environmental conditions and cultural practices (Amiot *et al.*, 1989). The level of phenolic compounds is also affected from the biennial bearing (Ryan *et al.,* 2003). Changes in the concentrations of phenolic compounds were observed between varieties of the same species (Romani *et al.,* 1999), as well as within the same variety depending on the developmental stage (Ryan *et al.,* 1999).

Differences in the concentration of phenolic substances can also be caused by a deficiency or excess of a nutrient element. Insufficient supply of the plant with nitrogen usually leads to metabolites composition containing only carbon in the molecule (Gershenzon, 1984; Hamilton *et al.*, 2001). Environmental factors also affect the phenolic content. Drought conditions can cause decrease of the activity of polyphenol oxidase (PPO) in olive trees, leading to increased levels of phenolic compounds in tissues (Sofo *et al.*, 2005). Environmental pollution, soil types (Figueiredo *et al.*, 2008), storage temperatures (Wang & Stretch, 2001), exposure to pathogens or herbicides (Appel, 1993), geographical location (Deidda *et al.,* 1994) and the final process method of the products (Caponio *et al.,* 2001) are also significant factors for the concentration of phenolic substances.

The main phenolic compounds found in olive drupes are: (i) oleuropein and ligustroside, which are the predominant secoiridoids (Amiot *et al.,* 1989; Esti *et al.,* 1998; Romani *et al.,* 1999; Soler-Rivas *et al.,* 2000), (ii) verbascoside, which is the main phenolic acid compound (Romani *et al.,* 1999; Ryan and Robards, 1998; Servili *et al.,* 1999), (iii) tyrosol and hydroxytyrosol, which are the predominant phenolic alcohols (Macheix *et al.,*1990; Mazza and Miniati, 1993; Romero *et al.,* 2002; Ryan and Robards, 1998), and (iv) luteolin 7-O-glucoside, rutin and apigenin 7-O-glucoside, which are the most abundant flavonoids (Amiot *et al.,* 1989; Esti *et al.,* 1998; Romani *et al.,* 1999; Ryan and Robards, 1998). The most

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abundant phenolic compounds in olive leaves are oleuropein, hydroxytyrosol, 7-O-glucoside of luteolin, 7-O-glucoside of apigenin and verbascoside (Benavente-Garcia *et al.,* 2000). Oleuropein, is the main phenolic compound present in plants of the family Oleaceae and can be hydrolyzed either in hydroxytyrosol and elenolic acid glucoside or in oleuropein aglycon and glucose (Manna *et al.*, 2004). During ripening, depending on the developmental stage of olives, qualitative and quantitative changes in the composition of phenolic substances have been observed (Amiot *et al.*, 1989; Esti *et al.*, 1998; Romani *et al.*, 1999).

The aim of the present study, was to identify the phenolic profile and quantify the phenolic compounds present in different olive tissues (leaves and drupes) of the major Greek olive varieties, during different developmental stages for two consecutive years.

Materials and Methods

Plant material

Samples of leaves and drupes of the olive varieties 'Koroneiki', 'Lianolia Kerkyras', 'Mastoidis', 'Arbequina', 'Adramytini', 'Megaritiki', 'Gaidourelia', 'Kalamata', 'Konservolia' and 'Chalkidiki', were collected at different seasons during two consecutive years. New season leaves were collected in April, green drupes in October and black drupes in December for both years. Mature leaves were collected in October and December of the second year. The samples were collected from six years old olive trees kept in pots at the Agricultural University of Athens orchard. Following their collection, all plant material was put in plastic bags, by means of a cooler transported to the laboratory and stored at -80 °C until analysis.

Reagents and reference compounds

The following reagents were used as reference compounds for the HPLC: gallic acid, gentisic acid, tyrosol, catechin, vanillic acid, cafeic acid, chlorogenic acid, syringic acid, epicatechin, p-coumaric acid, feroulic acid, o-coumaric acid, rutin, 7-glucoside of apigenin and quercetin, were obtained from Sigma Aldrich Co. (Steinheim, Germany); hydroxytyrosol, verbascoside, oleuropein, 7-o-glucoside of louteolin, 4-o-glucoside of louteolin and louteolin, were obtained from Extrasynthese Co. (Genay France); m-coumaric acid was obtained from Fluka Co. (Buchs, Switzerland); 2,4 dihydroxybenzoic acid and naringenin were obtained from Alfa Aesar Co. (Ward Hill, USA). Distilled water was used throughout.

Separation, identification and quantification of phenolic compounds

Phenolics were extracted as described by Mitsopoulos *et al.* (2016). The extract was analysed by HPLC. Elution was injected in an analytical HPLC unit (Jasco Corporation, Japan), using a reverse-phase Spherisorb ODS-2/C_{18}, 250×4.6 mm, 5 μm particle size column (Waters Corporation, Milford, USA). The solvent system, according to Cai *et al.* (2004), was acetic acid-water (2.2%) (A) and methanol (B), starting with 95% A and installing a gradient to obtain 70% A at 15 min, 65% A at 40 min, 60% A at 50 min, 55% A at 55 min, 50% A at 60 min, 45% A at 70 min and 100% A at 100 min. The flow rate was 1mL/min and the injection volume 20 μL. The identification of the chromatographic peaks was performed by comparing the retention times of the samples with the corresponding times of the reference compounds and by recording the UV spectra of the peaks in the range of 250-400 nm. Phenolic compounds quantification was accomplished using the external standard method for each of the identified compounds. The data was processed using the Jasco Chrompass Version 1.7.403.1 software.

Results

The phenolic profile and quantification of the identified substances for the various tissues in all ten varieties for two consecutive years was studied using HPLC. Table 1 shows the range of the concentration of the main phenolic compounds for all tissues in each year, while Figs. 1-3 show the representative chromatographs for leaves, green and black drupes, respectively.

According to the results, the phenolic profile for new season leaves collected in April for both years, were comparable with minor variations. Phenolic compounds that were identified in all varieties in new season leaves collected in April of year 1 were oleuropein, ranging from 0.31 mg/gr of fresh tissue (f.t.) ('Megaritiki') to 4.76 mg/gr f.t. ('Konservolia'), 7-O-glucoside of luteolin ranging from 0.19 mg/gr f.t. ('Adramytini') to 1.82 mg/gr f.t. ('Konservolia'), and rutin ranging from 0.14 mg/gr f.t. ('Adramytini' and 'Megaritiki') to 1.41 mg/gr f.t. ('Koroneiki'). In lesser amounts the compounds detected were 7-O-glucoside of apigenin, 4-O-glucoside of luteolin, while chlorogenic acid, m-coumaric acid, and luteolin were found in traces.

In the new season leaves, collected in April of the second year, the phenolic compounds with higher concentrations were oleuropein ranging from 0.59 mg/gr f.t. ('Adramytini') to 9.39 mg/gr f.t. ('Arbequina'), rutin ranging from 0.11 mg/gr f.t. ('Megaritiki') to 1.55 mg/gr f.t. ('Lianolia Kerkyras'), 4-O-glucoside of luteolin ranging from 0.30 mg/gr f.t. ('Adramytini' and 'Gaidourelia') to 1.41 mg/gr f.t. ('Konservolia') and 7-O-glucoside of apigenin ranging from 0.12 mg/gr f.t. ('Adramytini') to 1.10 mg/gr f.t. ('Konservolia'). In lower concentrations hydroxytyrosol, chlorogenic acid, m-coumaric acid, 7-O-glucoside of luteolin and luteolin were found. Fig. 1 presents the chromatograph for the new season leaves collected in year 2 of the variety 'Gaidourelia'. For new season leaves collected during April of both years, an unknown compound (I) was detected with

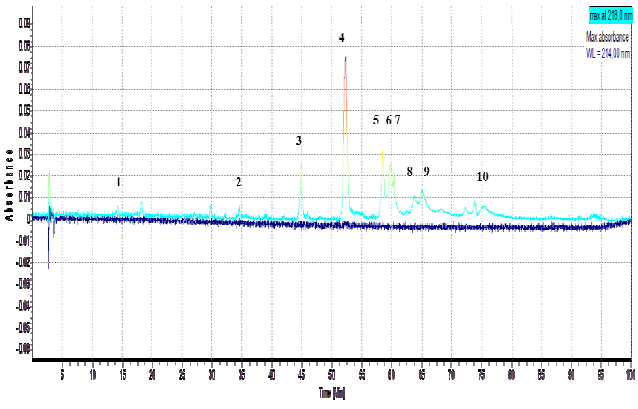

Fig. 1. HPLC chromatograph of April new season leaves of year 2 of variety 'Gaidourelia'. Picks: 1. hydroxytyrosol, 2. chlorogenic acid, 3. μ-coumaric acid, 4. unknown compound (I), 5. oleuropein, 6. 7-O-glucoside of luteolin, 7. rutin, 8. 7-O-glucoside of apigenin, 9. 4-O-glucoside of luteolin, 10. Luteolin

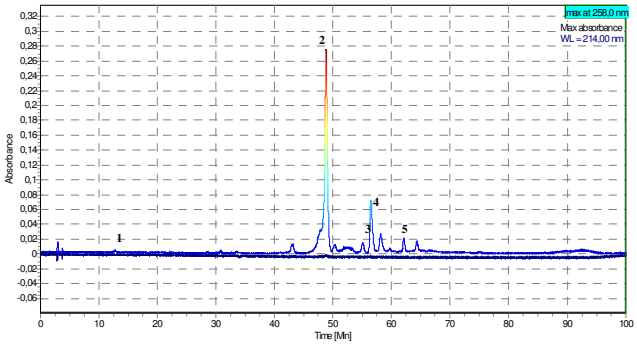

Fig. 2. HPLC chromatograph of green drupes of year 2 of variety 'Lianolia Kerkyras'. Picks: 1. hydroxytyrosol, 2. verbascoside, 3. oleuropein, 4. rutin, 5. 4-O-glucoside of luteolin

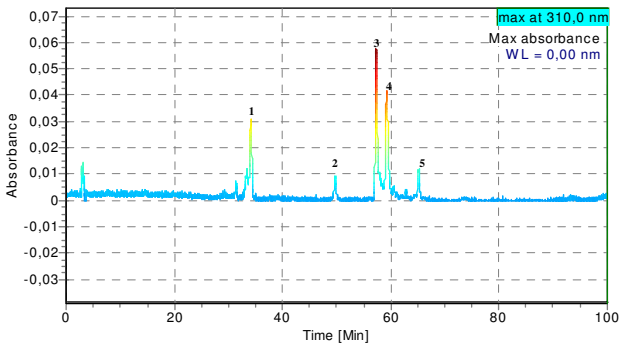

Fig. 3. HPLC chromatograph of black drupes of year 2 of variety 'Koroneiki'. Picks: 1. unknown compound (II). 2. verbascoside, 3. oleuropein, 4. rutin, 5. 4-O-glucoside of luteolin

elusion time 52.92 min and maximum wavelength (λmax: 246 & 290 nm) which could not be identified (Fig. 1, peak 4).

Regarding leaves collected during September and December of year 2, the phenolic profile for all ten varieties was almost identical with small variations. However, it differed from the phenolic profile of new season leaves collected in April of the same year. Compounds like vanillic acid, p-coumaric acid, ferulic acid and coumaric acid were detected in leaves collected in September and December of year 2 but were not detected on new season leaves collected during April of the same year. On the other hand hydroxytyrosol was detected only on new season leaves collected in April of year 2 and not on leaves collected in September - December of the same year.

The compounds with the highest concentration in both September and December of year 2 collected leaves were the same. The phenolic compounds in leaves collected on September of year 2 exhibiting high concentrations were oleuropein, ranging from 1.23 mg/gr f.t. ('Chalkidiki') to 20.31 mg/gr f.t. ('Arbequina'), rutin, ranging from 0.53 mg/gr f.t. ('Chalkidiki') to 1.53 mg/gr f.t. ('Koroneiki'), 4-O-glucoside of luteolin, ranging from 0.66 mg/gr f.t. ('Megaritiki' and 'Gaidourelia') to 1.39 mg/gr f.t. ('Kalamata'), and 7-O-glucoside of luteolin ranging from 0.95 mg/gr f.t. ('Gaidourelia') to 2.01 mg/gr f.t. ('Kalamata'). The phenolic compounds in leaves collected on December of year 2 exhibiting high concentrations were also oleuropein, ranging from 0.25 mg/gr f.t. ('Lianolia Kerkyras') to 8.91 mg/gr f.t. ('Arbequina'), rutin, ranging from 0.40 mg/gr f.t. ('Konservolia') to 1.10 mg/gr f.t. ('Mastoidis'), 4-O-glucoside of luteolin, ranging from 0.73 mg/gr f.t. ('Lianolia Kerkyras') to 1.60 mg/gr f.t. ('Kalamata'), and 7-O-glucoside of luteolin, ranging from 1.16 mg/gr f.t. ('Lianolia Kerkyras') to 2.03 mg/gr f.t. ('Kalamata'). In lesser amounts the compounds found in September leaves were vanillic acid, chlorogenic acid, p-coumaric acid, m-coumaric acid, ferulic acid, o-coumaric acid, 7-O-glucoside of apigenin and luteolin, and in leaves collected in December were chlorogenic acid, p-coumaric acid, ferulic acid, o-coumaric acid, 7-O-glucoside of apigenin and luteolin.

The phenolic profile of year 1 green drupes was almost identical to the phenolic profile of year 2 green drupes. Oleuropein, verbascoside and rutin were the compounds showing the highest concentrations among phenolic compounds in green drupes for both years (Table 1). In green drupes collected on year 1 oleuropein ranged from 0.87 mg/gr f.t.. ('Arbequina') to 5.68 mg/gr f.t. ('Kalamata'), rutin ranged from 0.35 mg/gr f.t.. ('Megaritiki') to 1.51 mg/gr f.t.. ('Lianolia Kerkyras'), and verbascoside ranged from 0.19 mg/gr f.t.. ('Gaidourelia') to 3.90 mg/gr f.t.. ('Mastoidis'). In green drupes collected in year 2 the concentrations ranged, for oleuropein from 0.39 mg/gr f.t. ('Konservolia') to 12.23 mg/gr f.t. ('Lianolia Kerkyras'), for rutin from 0.14 mg/gr f.t.. ('Konservolia') to 0.74 mg/gr f.t. ('Adramytini') and for verbascoside from 0.56 mg/gr f.t. ('Adramytini') to 3.18 mg/grf.t. ('Lianolia Kerkyras'). In smaller amounts the compounds found in year 1 and year 2 green drupes were, hydroxytyrosol, chlorogenic acid, p-coumaric acid, 7-O-glucoside of luteolin, 4-O-glucoside of luteolin and luteolin. The compounds found in smaller quantities in year 2 green drupes were hydroxytyrosol, chlorogenic acid, p-coumaric acid, 7-O-glucoside of luteolin, 4-O-glucoside of luteolin and luteolin. Gallic acid was detected in three varieties ('Konservolia', 'Gaidourelia' and 'Megaritiki') in green drupes of year 1 and was not detected in any of the five varieties ('Koroneiki', 'Lianolia Kerkyras', 'Mastoidis', 'Adramytini', 'Konservolia') in green drupes of year 2. Fig. 2 presents the chromatograph obtained from green drupes of variety 'Lianolia Kerkyras'.

Oleuropein, rutin and verbascoside were the compounds present at the highest concentrations among the phenolic

Table 1. Concentration range of the main phenolic compounds in leaves and drupes at different developmental stages expressed in mg/gr of fresh tissue (f.t.)

Phenolic Compounds	April New Leaves year 1 mg/gr f.t.	April New Leaves year 2 mg/gr f.t.	September Leaves year 2 mg/gr f.t.	December Leaves year 2 mg/gr f.t.	Green Drupes year 1 mg/gr f.t.	Green Drupes year 2 mg/gr f.t.	Black Drupes year 1 mg/gr f.t.	Black Drupes year 2 mg/gr f.t.
verbascoside	-	-	-	-	0.19-3.90	0.56-3.18	0.49-0.94	0.08-2.40
7-O-glucoside of luteolin	0.19-1.82	0.17-0.87	0.95-2.01	1.16-2.03	0.11-0.36	0.23-0.33	0.25-0.72	-
oleuropein	0.31-4.76	0.59-9.39	1.23-20.31	0.25-8.91	0.87-5.68.	0.39-12.23	4.53-9.22	1.41-11.15
rutin	0.14-1.41	0.11-1.55	0.53-1.53	0.40-1.10	0.35-1.51	0.14-0.74	0.11-0.98	0.14-0.91
7-O-glucoside of apigenin	0.08-0.38	0.12-1.10	0.13-0.32	0.15-0.39	-	-	-	-
4-O-glucoside of luteolin	0.24-0.65	0.30-1.41	0.66-1.39	0.87-1.60	0.07-0.21	0.12-0.33	0.08-0.20	0.08-0.12

compounds in black drupes of both years (Table 1). Additionally, 7-O-glucoside of luteolin showed high concentrations in black drupes of year 1 but was not detected in black drupes of year 2 (Table 1). For year 1 black drupes, oleuropein ranged from 4.53 mg/gr f.t. ('Mastoidis') to 9.22 mg/gr f.t. ('Megaritiki'), rutin ranged from 0.11 mg/gr f.t. ('Chalkidiki') to 0.98 mg/gr f.t. ('Megaritiki'), verbascoside ranged from 0.49 mg/gr f.t. ('Mastoidis') to 0.94 mg/gr f.t. ('Lianolia Kerkyras') and 7-O-glucoside of luteolin ranged from 0.25 mg/gr f.t. ('Chalkidiki') to 0.72 mg/gr f.t. ('Mastoidis'). For year 2 black drupes, oleuropein ranged from 1.41 mg/gr f.t. ('Konservolia') to 11.15 mg/gr f.t. ('Koroneiki'), rutin ranged from 0.14 mg/gr f.t. ('Konservolia') to 0.91 mg/gr f.t. ('Koroneiki'), and verbascoside from 0.08 mg/gr f.t. ('Konservolia') to 2.40 mg/gr f.t. ('Lianolia Kerkyras'). Gallic acid, tyrosol, 4-O-glucoside of luteolin and luteolin were the compounds found in smaller quantities in black drupes of year 1, whereas in black drupes of year 2 the compounds with smaller amounts were hydroxytyrosol, p-coumaric acid and 4-O-glucoside of luteolin. Fig. 3 presents a typical chromatograph of black drupes of year 2 of the variety 'Koroneiki'.

Comparing different tissues, verbascoside was detected in high concentrations in green and black drupes for both years while it was absent from the phenolic profiles of leaves in all varieties for both years. On the other hand, 7-O-glucoside of apigenin was found at high concentrations on the phenolic profile of leaves for both years, but not in the phenolic profile of green and black drupes of all varieties for the two years (Table 1).

Discussion

According to the results, the phenolic profile of all varieties and all tissues studied, were comparable between the two years. Verbascoside was found only in the phenolic profile of green and black drupes while it was absent from the phenolic profile of leaves. These findings are in accordance to the literature. Boskou *et al.* (2006) reported similar phenolic profiles in the flesh of marketed table olives to the ones presented here for black drupes. They also found that the main differentiation between the phenolic profile of leaves to that of drupes is the presence of the compound verbascoside only in the latter. Ryan *et al.*, (2002 and 2003) and Giannakopoulou *et al.* (2010) also reached to the same conclusion. On the other hand, Silva *et al.*, (2006), identified the compound verbascoside in the phenolic profile of leaves.

In the present study, differences were observed in the concentration of phenolic compounds depending on the variety in all tissues. In agreement with the findings of the present study, Romani *et al.* (1999), who studied drupes of five Italian olive varieties found wide variation in the concentrations of phenolic compounds depending on the variety. The identified compounds showing the highest concentrations were oleuropein, verbascoside, rutin and hydroxytyrosol. They also identified the presence of 7-O-glucoside of luteolin, 7-O-glucoside of apigenin and 3-glucoside of cyanidin. Vinha *et al.*, (2002), studying drupes collected in November of the varieties 'Cobrancosa', 'Madural' and 'Verdeal', also observed large variations in terms of the amount for each phenolic compound between the three varieties. The phenolic compounds with the higher concentrations detected were oleuropein, hydroxytyrosol, rutin and 7-O-glucoside of luteolin. Verbascoside was present only in two of the three varieties. These results are consistent with the findings of the present study regarding the main phenolic compounds identified and differ only in the concentration of the compound hydroxytyrosol, which in the present study ranged at lower levels compared to those reported in the literature. Esti *et al.* (1998) also reported similar profiles when studying the phenolic profile of drupes from eight Italian varieties for two consecutive years. In addition, in the phenolic profile of leaves, regardless their developmental stage and the year, the number of identified compounds was higher compared to that of green and black drupes. Ryan *et al.*, (2002), reached the same conclusion by studying the phenolic profile of four varieties for new and old leaves as well as of the flesh of drupes. Ryan *et al.*, (2003) studying the phenolic profile of drupes, young and old leaves in the variety 'Hardy Mammoth', during two consecutive years found more phenolic compounds in new and old leaves (10 from a total of 12 identified) than in drupes (7 from 12).

In this study, the concentration of oleuropein, the most important phenolic compound, showed high fluctuations between different tissues within the same variety and between different varieties within the same tissue. A decrease in the amount of oleuropein was observed during the maturation of the leaves while there was no decline during the maturation of the drupes for both years. Concentration of hydroxytyrosol was lower than that of oleuropein. Bouaziz *et al.*, (2010) studying the qualitative and quantitative changes of phenolic compounds in drupes from Tunisia reported that the amount of oleuropein differed depending on the variety. Ryan *et al.* (2002) found that there were differences among the varieties studied in terms of the concentration of the various phenolic compounds; i.e. the concentration of oleuropein for variety 'Roupuda' was almost tenfold compared to the concentration in variety 'Galega'. Amiot *et al.*, (1986 and 1989), Ryan *et al.* (1999), and Soler-Rivas *et al.* (2000) mentioned a reduction in the concentration of oleuropein during ripening of the drupes and concomitant increase in the concentration of hydroxytyrosol.

Conclusion

The phenolic profile and the concentration of the phenolic compounds identified in leaves and drupes of major Greek olive varieties collected at different developmental stages for two consequent years are presented. The phenolic compounds identified in new season leaves of year 1 were, in declining order, oleuropein, 7-O-glucoside of luteolin and rutin, whereas for new season leaves of year 2 the main phenolic compounds were oleuropein, rutin, 4-O-glucoside of luteolin and 7-O-glucoside of apigenin. The phenolic compounds found in September - December leaves of year 2, were oleuropein, 7-O-glucoside of luteolin, 4-O-glucoside of luteolin and rutin. Regarding green and black drupes for both years the phenolic compounds showing the highest concentrations were oleuropein, verbascoside and rutin. Verbascoside was found in higher concentrations in green and black drupes for both years while was absent from leaves in all varieties for both years. The phenolic compound 7-O-glucoside of apigenin was found at high concentrations only in leaves for both years and not in green and black drupes. The phenolic profile of all varieties for each of the tissues studied, for both years, were similar. Differences were observed in the concentration of phenolic compounds depending on the variety in all tissues. The phenolic profile of new season leaves, green and black drupes was similar for both years. Also in the phenolic profile of leaves, regardless developmental stage and year, the number of phenolic compounds identified was higher from the number of phenolic

compounds identified for green and black drupes. The amount of oleuropein, the most important phenolic compound, presented high fluctuations between different tissues within the same variety, and between different varieties within the same tissue.

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