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Phenolic compounds of 'Galega Vulgar' and 'Cobrançosa' olive oils along early ripening stages

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ABSTRACT

In this study, the lipophilic and hydrophilic phenol composition of virgin olive oils (VOO) obtained from olives from two of the most important Portuguese cultivars ('Galega Vulgar' and 'Cobrançosa'), harvested at different ripening stages and under two irrigation schemes (rain fed and irrigated), was evaluated. Phenolic alcohols (hydroxytyrosol and tyrosol), phenolic acids and derivatives and flavonoids (luteolin and apigenin), as well as tocopherols were quantified. Lipophilic (>300 mg kg^{-1}) and hydrophilic phenols (>600 mg kg^{-1}) were present in high contents in both VOO, for early ripening stages. Gamma-tocopherol content is higher in 'Galega Vulgar' VOO. Total phenols showed a decrease between ripening index 2.5 and 3.5. The dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), also known as oleacein, was the major phenolic compound identified in both oils. The concentration of free hydroxytyrosol and tyrosol in both VOO is very low while their esterified derivatives, like 3,4-DHPEA-EDA and *p*-HPEA-EDA, are much more abundant.

1. Introduction

The benefits of consuming olive oil were traditionally attributed to its high content in oleic acid (Gurr, 2000). However, now it is well known that these benefits may also be ascribed to the phenol compounds of extra virgin olive oil (EVOO) due to their antioxidant, anti-inflammatory and anti-microbial activities. For some activities of EVOO phenolic compounds, the scientific evidence is already strong enough to enable the legal use of health claims on labelling (Martín-Peláez, Covas, Fitó, Kušar, & Pravst, 2013).

Lipophilic and hydrophilic phenols are the most important antioxidants in EVOO. Lipophilic phenols in EVOO are tocopherols, which are molecules with a chroman head (with one phenolic and one heterocyclic ring) and a phytyl tail. The different tocopherols vary in the number of methyl substituents and the patterns of substitution in the phenolic ring. Among them, α-tocopherol is the most abundant (90%) but β- and γ-tocopherols are also present (Beltrán et al., 2010). Claims have been made for the preventive activity of tocopherols against reactive oxygen species (ROS) in biological systems, namely their positive effect on cell aging, some cancer types, immune system maintenance and cardiovascular diseases (Bramley et al., 2000). Moreover, apart from their action as lipid radical scavengers, they also inhibit the photooxidation by reacting with singlet oxygen. Variability in tocopherol contents by crop year is explained by the rainfall levels, showing that oils from drier crop years have higher tocopherol content, in spite of a cultivar-dependent effect (Beltrán et al., 2010). However, the content of tocopherols in virgin olive oils (VOO) is relatively low when compared with several seed oils. In fact, hydrophilic phenols

Abbreviations: ACVA, vanillic acid; APG, apigenin; 3,4-DHPEA, hydroxytyrosol; 3,4-DHPEA-AC, 4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol or oleacein; 3,4-DHPEA-EA, oleuropein aglycone; EA, elenolic acid; EAME, elenolic acid methyl ester; EVOO, Extra Virgin Olive Oil; HYT, hydroxytyrosol; IR, irrigated; LUT, luteolin; MUFA, monounsaturated fatty acids; *p*-HPEA, tyrosol; *p*-HPEA-EA, aldehydic form of elenolic acid linked to tyrosol; *p*-HPEA-EDA, dialdehydic form of elenolic acid linked to tyrosol or oleocanthal; OCUM, *o*-Coumaric acid; PCUM, *p*-Coumaric acid; PDO, Protected Designations of Origin; POD, peroxidases; PPO, polyphenol oxidase; PUFA, polyunsaturated fatty acids; PVP, polyvinylpyrrolidone; RF, rain fed; RI, ripening index; SFA, saturated fatty acids; TYR, tyrosol; VAN, Vanillin; VOO, virgin olive oil.

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are the compounds that most differentiate EVOO from other vegetable oils. The most important phenolic compounds that have been identified in olive oil are phenolic alcohols (hydroxytyrosol (HYT) and tyrosol (TYR)), secoiridoid derivatives, such as the dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) (oleacein), the dialdehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EDA) (oleocanthal), the aldehydic form of elenolic acid linked to tyrosol (p-HPEA-EA), 4-(acetoxyethyl)-1, 2-dihydroxybenzene (3,4-DHPEA-AC), oleuropein aglycone (3,4-DHPEA-EA) and its methylated form (methyl 3,4-DHPEA-EA), phenolic acids and derivatives (such as vanillic acid and vanillin, respectively), lignans (pinoresinol and acetoxypinoresinol) and flavonoids such as luteolin and apigenin (Bendini et al., 2007; Kanakis et al., 2013; Servili & Montedoro, 2002).

Olive oil phenol composition is quite different from that of the olive drupe and of the olive paste (Kanakis et al., 2013). In contrast to olive fruits, olive oil contains neither anthocyanins nor flavonols. During the extraction process, the glycosidic oleuropein, dimethyloleuropein and ligstroside are hydrolyzed by endogenous β-glucosidases to form aldehydic aglycones. The aglycones become soluble in the oil phase, whereas the glycosides remain in the water phase (Servili & Montedoro, 2002). The main source of lignans was demonstrated to be the stone and not the pulp (Oliveras López et al., 2008).

EVOO phenolic compounds play also an important role in organoleptic properties namely in attributes related to bitterness and pungency (Peyrot des Gachons et al., 2011). The phenolic compounds 3,4-DHPEA-EA, *p*-HPEA-EA, 3,4-DHPEA-EDA, *p*-HPEA-EDA, elenolic acid (EA), and elenolic acid methyl ester (EAME) showed high correlations with bitterness and pungency (Dierkes et al., 2012). Moreover, oleocanthal causes a pungency perceived as an unusual irritation in the pharynx, consequence of both the specificity of this molecule for a single sensory receptor and the anatomical restriction of this sensory receptor to the pharynx (Peyrot des Gachons et al., 2011).

Olive endogenous enzymes such as oxidoreductases, polyphenol oxidase (PPO) and peroxidase (POD), which oxidize phenolic compounds may be a biochemical factor affecting the phenol content of VOO (García-Rodríguez, Romero-Segura, Sanz, Sánchez-Ortiz, & Pérez, 2011; Hbaieb et al., 2015).

The ripening stage of olives has a high impact on the oil's yield, quality, stability and sensory characteristics. Irrigation also plays an important role in the productivity of olives and consequently in fruit ripening, and therefore in phenol and volatile composition (Gómez-Rico, Salvador, & Fregapane, 2009). Moreover, when early frosts occur, oils extracted from frosted fruits develop sensory defects (Guillaume, Ravetti, & Gwyn, 2010). So, in the last years a lot of attention has been drawn to the main changes on the characteristics of olives and olive oils along fruit ripening, in order to decide the best harvest time (Dag, Harlev, Lavee, Zipori, & Kerem, 2014; Jiménez, Sánchez-Ortiz, Lorenzo, & Rivas, 2013).

Early ripening has been a recommendation in the center of Portugal (Beira Baixa) for organic olive growing. The predominance of 'Galega Vulgar' cv., which is highly susceptible to pests and diseases, is the main reason for this procedure (Peres et al., 2010). However early ripening corresponds to lower yields, so it is crucial to determine how early the harvest can be, in order to have good quality, high nutritional value and sensory scores and a reasonable yield.

The aim of the present study was to investigate the effect of early harvest corresponding to olive ripening index lower than 4.5, on phenol compound levels in virgin olive oils from 'Galega Vulgar' and 'Cobrançosa' fruits, two of the most important Portuguese cultivars for olive oil extraction, grown in rainfed or irrigated orchards.

2. Materials and methods

2.1. Olives Characterization

Portuguese olive fruits (*Olea europaea* L.) of 'Cobrançosa' and 'Galega Vulgar' cultivars used in this study were produced according to the Integrated Production rules, in Beira Baixa Region (Centre-Interior of Portugal), in two types of farming: rainfed orchard (RF) (39° 49′N, 7° 27′W) and irrigated orchards (IR) (39° 50′N, 7° 42′W). 'Galega Vulgar' orchards have 100–123 trees/ha while 'Cobrançosa' orchards have 200–300 trees/ha. For the irrigated orchards, the irrigation drip system was performed as a function of soil moisture and meteorological conditions and controlled by weekly soil water balance. From measurements of soil moisture, a maximum irrigation quantity was determined as the difference between field capacity and the actual soil water content. This maximum value was then taken as an indication for deciding about the amount of water to be supplied by irrigation. Olive fruits were picked from the beginning of October till the second fortnight of November. The annual accumulated precipitation of the year under study was 737.5 mm, which was very similar to the values reported for the period 1981–2010 in this region (783.2 mm). Their ripening indices (RI) were determined following the guidelines of Estación de Olivicultura y Elaiotecnia, Jaén, Spain (Hermoso, Uceda, Frias, & Beltran, 1997); moisture and fat content (by Soxtec) of the fruits were also evaluated. Only healthy fruits were selected for fruit characterization and for olive oil extraction.

2.2. Enzymatic activity assays

Fruits were destoned with a manual destoner and the kernel was cut with a pipe cutter and the seed removed. Extracts were prepared by homogenizing olive pulp and seeds with cold acetone (−20 °C) in an ultraturrax homogeneizer (2 min), followed by filtration in fiber glass filters, washing the pellet with cold acetone (−20 °C) until total removal of pigments, and by drying samples at room temperature with N_2 (Saraiva, Nunes, & Coimbra, 2007). For enzymatic assay, 0.4 g of acetone powder were suspended in 5 mL of extraction buffer (0.05 M potassium phosphate, pH 6.2 containing 1 M KCl) (Servili et al., 2007) and 2% (w/w) of PVP and stirred for 30 min, 4 °C, 400 rpm; the suspension was centrifuged at 12,000 rpm for 30 min and filtered (0.45 μm). PPO activity was evaluated using catechol (30 mM) as substrate, following the increase in absorbance at 420 nm, during 1 min (Oktay, Kufrevioglu, Kocaçaliskan, & Sakiroglu, 1995). One unit of PPO was defined as the quantity of enzyme that causes the absorbance variation of 0.001 min^{-1} mL^{-1} of enzyme extract, at room temperature. Results were expressed as Ug^{-1} FW (fresh weight).

POD activity was performed following the increase in absorbance at 470 nm (2 min) using 30 mM guaiacol and 4 mM H_2O_2 as substrates (Gajewska, Skłodowska, Słaba, & Mazur, 2006). One unit of POD was defined as the consumption of 1 μmol of guaiacol min^{-1} mL^{-1} of enzyme extract, at room temperature using a molar absorptivity (ε) for tetraguaiacol of 26.6 mM^{-1} cm^{-1}. Results were expressed as Ug^{-1} FW.

2.3. Olive oil extraction

Olive oils were extracted in a laboratory oil extraction system (Abencor analyser; MC2 Ingenieria y Sistemas S.L., Seville, Spain) under optimized conditions (Peres, Martins, & Ferreira-Dias, 2014). The olives were crushed with a hammer mill equipped with a 4 mm sieve at 3000 rpm. Malaxation of the pastes was performed at 27–30 °C, during 30 min, and centrifugation at 3500 rpm for 3 min. After centrifugation, the olive oil was separated by settling

in a graduated cylinder. Water traces in the oil were removed with anhydrous sodium sulfate, filtered through a cellulose filter and stored in amber glass bottles at 4 °C until analysis. From each batch three independent extractions were performed.

2.4. Olive oil characterization

The analysis considered by the European Union as chemical quality criteria (acidity value, peroxide value (PV) and UV specific absorbances (K_{232} and K_{270}) were carried out following the analytical methods described in EEC/2568/91 EU Regulation. Fatty acid methyl esters were evaluated by gas chromatography with flame ionization detector (GC-FID), in a Hewlett Packard 6890, SP column 2380™ Supelco (60 m × 0.25 mm × 0.20 μm). Samples of olive oils were also sensory evaluated by a panel test with more than 10 years of experience in olive oil tasting, according to the methodology of Regulation N° 1343/2013, using a profile sheet with an unstructured scale, adapted from Cerretani, Salvador, Bendini, and Fregapane (2008). Chlorophyll pigments were evaluated by VIS spectroscopy (Pokorny, Kalinová, & Dysseler, 1995). Oxidative stability was measured using a Metrohm Rancimat model 670 (temperature of 120 °C; air flow of 20 Lh^{-1}).

2.5. Phenol composition evaluation

For tocopherol analysis, a solution of oil in hexane (8%, w/v), filtered with Pall Gelman Acrodisc® syringe filters (0.45 μm, 25 mm, GHP membrane) was analyzed by high-performance liquid chromatography (HPLC) in an Agilent 1100 Series chromatograph. Fluorescence detection with excitation set at 290 nm and emission set at 330 nm and a Lichrosorb Si 60 column (250 mm × 4.6 mm × 5 μm), at room temperature, were used. Total phenol compounds were extracted by solid phase extraction (SPE) columns filled with 1 g of octadecyl (C_{18}) material from J.T. Baker and evaluated by VIS spectroscopy, according to the Folin–Ciocalteau method, and the results expressed as gallic acid equivalent (mg GAE kg^{-1}) (Peres et al., 2014).

The profile of phenolic compounds was evaluated by HPLC according to the International Olive Council method with some modifications (IOC, 2009). The phenolic compounds were recovered from olive oil by liquid-liquid extraction using the procedure proposed by Pirisi, Cabras, Cao, Migliorini, and Muggelli (2000). An Agilent 1100 HPLC system, consisting of a degasser, a quaternary pump, an autosampler and a diode array detector (DAD) was used. The stationary phase was a Purospher C18 analytical column (150 mm × 3.9 mm × 4 μm). The mobile phase consisted of solutions of (A) 0.2% H_3PO_4 (v/v), (B) methanol and (C) acetonitrile at a constant flow rate of 1 mL min^{-1}. The gradient program used was the one indicated by the IOC document (IOC, 2009). The quantification of phenolic compounds was carried out using the area values measurements at 280 nm, for gallic acid, hydroxytyrosol, tyrosol, vanillic acid, caffeic acid, vanillin, *o*-coumaric acid; at 320 nm for *p*-coumaric acid and, at 360 nm for luteolin and apigenin. Quantitative assays were achieved using external calibration curves for all the standard phenols. Standards of α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, hydroxytyrosol, tyrosol, vanillic acid, vanillin, cafeic acid, ferulic acid, *o*-coumaric, *p*-coumaric, apigenin, verbascoside, were purchased from Sigma-Aldrich and oleuropein and luteolin from Extrasyntese.

The confirmation of phenolic compounds in VOO samples was achieved with an LC-ESI-MS Agilent 1200 series equipped with a triple quadrupole mass spectrometer Agilent 6400. A Zorbax SB-C18 (50 mm × 4.6 mm i.d. × 1.8 μm particle diameter – Agilent tecnologies) column was used for the separation at a flow rate of 0.7 mL min^{-1}, at 30 °C. The elution was performed by means of a gradient of 0.1% formic acid (eluent A) and acetonitrile (eluent B) as follows: start at 10% B, 20% B at 10 min, 40% B at 40 min, 60% B at 60 min, 90% B at 80 min, at 81 min return to initial conditions and stabilization for 9 min. ESI operated with a nitrogen flow of 10 L min^{-1} at 300 °C. MS detector operated in MS2-Scan scan type in the range 80–1000 Da, and negative mode was selected. The capillary voltage was set to 4.0 kV, the quadrupoles temperatures were 100 °C, fragmentor voltage was 145 V, and cell accelerator voltage was 7 V. Data were acquired and analyzed using Masshunter Workstation Software (version B.04.00) from Agilent technologies. Identification of the phenolic compounds with LC-ESI-MS was obtained by comparison of chromatograms and fragmentation pattern of samples with literature.

2.6. Statistical analysis

Statistical analysis was performed using the software Statistica™", version 6, from Statsoft, Tulsa, OK, USA. Data was analyzed by univariate procedures (ANOVA, Tukey test, $p < 0.05$) in order to identify the differences between the olive oils from both cultivars and the two orchards.

3. Results and discussion

3.1. Olives characterization

The characterization of olives in different ripening dates is presented in Table 1. For each sampling date, the olives from 'Cobrançosa' cultivar had always a lower ripening index than 'Galega Vulgar' fruits (Table1). Also, the olives from the rainfed orchard, presented always higher RI than those from irrigated one, mainly related with the lower tree load of the trees due to water shortage (e.g. 3.2 t/ha *vs* 10 t/ha for RF or IR 'Galega Vulgar' orchards, respectively). Moisture content of the fruits of both cultivars were quite similar, in each harvest date, reflecting the changes related with meteorological conditions, ie., more rain in November. Significantly higher fat contents were achieved in November for all the cultivars and orchards. The olives from the rain fed orchard showed higher fat content, especially for 'Galega Vulgar' in November. A lower tree load (more accumulation of fat and higher ripening index) may explain this difference. A significant increase in oil yield was observed from October to November harvest for both cultivars, except for 'Cobrançosa' in the RF orchard.

3.2. PPO and POD activities of 'Galega Vulgar' and 'Cobrançosa' olives

Phenolic compounds are enzymatically oxidized by PPO, which results in color changes of olive pastes as soon as the rupture of olive fruit tissues begins by crushing. PPO activity was found in the fruit mesocarp (Table 1) but no PPO activity was detected in the seed, which is in agreement with other authors (García-Rodríguez et al., 2011; Servili, Baldioli, Begliomini, Selvaggini, & Montedoro, 2000) and also with our previous results (Peres, Martins, Mourato, & Ferreira-Dias, 2011). PPO mesocarp activity increases with RI and is lower in IR orchards. Olives from RF orchard presented always a higher ripening index. No significant differences were found for PPO activity in Cobrançosa olives in November for both olive orchards, which can show that for this cultivar a stabilization of PPO activity occurs at lower ripening indexes.

The results for POD showed that its activity is detected predominantly in the seed and 'Galega Vulgar' seeds showed higher values. POD activity in the mesocarp was also detected but at very low levels. Only at higher ripening stages, corresponding to November harvests, POD activity values higher than 1 Ug^{-1} FW were

Table 1
Ripening index, moisture, fat content, oxidoreductases activity (PPO and POD) of olive fruits 'Galega Vulgar' and 'Cobrançosa', in two olive orchards (RF – rain fed; IR – irrigated). In each row superscript indexes indicate differences based on Tukey test.

	'Galega Vulgar'				'Cobrançosa'			
Olive orchard	RF		IR		RF		IR	
Harvest month	Oct	Nov	Oct	Nov	Oct	Nov	Oct	Nov
Ripening Index	2.8^{bc}	4.2^{a}	2.1^{c}	3.5^{ab}	2.1^{c}	3.6^{ab}	1.1^{d}	2.7^{c}
Moisture (%)	50.8^{d}	61.6^{a}	51.8^{cd}	62.3^{a}	53.8^{c}	58.1^{b}	53.1^{cd}	59.3^{b}
Fat content (% DW)	36.0^{c}	46.2^{a}	29.1^{d}	38.9^{bc}	37.9^{bc}	41.5^{b}	28.9^{d}	40.9^{b}
PPO mesocarp activity (Ug^{-1} FW)	84.8^{bc}	220.2^{a}	59.8^{c}	124.3^{bc}	111.2^{bc}	147.9^{ab}	44.6^{c}	124.8^{bc}
POD mesocarp activity (Ug^{-1} FW)	<1.0	2.0	<1.0	1.1	<1.0	1.9	<1.0	2.4
POD seed activity (Ug^{-1} FW)	8.4^{ab}	10.1^{ab}	9.8^{ab}	12.7^{a}	7.9^{ab}	7.8^{ab}	6.5^{b}	3.8^{b}

detected. The impact of olive crushing in the VOO phenolic compounds can be related to the different distribution of the endogenous oxidoreductases and phenolic compounds in the pulp and seed of the olive fruit. In fact, the hydrophilic phenols are largely concentrated in the pulp, whereas the seed contains only small quantities of these substances. Concerning tocopherols, they are present in both parts of the fruit, although in higher contents in the seed (Servili et al., 2007). Thus, crushing will promote the contact between seed POD and phenols.

3.3. Olive oil characterization

According to quality criteria defined by the European Union (Regulation (EU) N° 1343/2013) for acidity, peroxide value and UV absorbances, all the samples are classified as "Extra Virgin Olive Oil" (Table 2). Both VOO are characterized by levels of oleic acid higher than 70% and palmitic acid higher than 14%. 'Cobrançosa' olive oils can be distinguished from 'Galega Vulgar' oils by the higher contents of PUFA and in stearic (C18:0) acid. In turn, 'Galega Vulgar' olive oils are characterized by higher contents of oleic (18:1), palmitoleic (C16:1), palmitic (C16:0), and gadoleic (C20:1) fatty acids. Margaric, margaroleic and behenic acids were significantly ($p < 0.05$) lower in rain fed orchards. The levels of linoleic acid and stearic acids for 'Cobrançosa' olive oils also differed between orchards.

3.4. Phenol content of 'Galega Vulgar' and 'Cobrançosa' olive oils

The evolution of total phenols in 'Galega Vulgar' and 'Cobrançosa' VOO along fruit ripening is represented in Fig. 1. Total

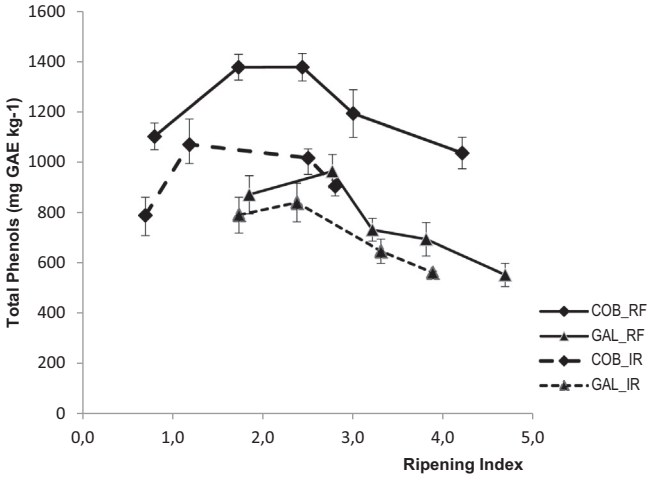

Fig. 1. Evolution of total phenols in early ripening stages for 'Galega Vulgar' and 'Cobrançosa' virgin olive oils in two orchards (RF and IR).

phenols were very high (higher than 500 mg GAE kg^{-1}) in all ripening stages, denoting the high quality of the fruits and the fact that no damaged fruits were processed. 'Cobrançosa' oils showed higher total phenol content than 'Galega Vulgar' for all ripening indexes. Within the same cultivar, olive oils obtained from RF olives have also higher total phenol content than the VOO obtained from irrigated orchards. Also, the decrease in total phenols begins between 2.5 and 3 of ripening index. Such high contents of total phenols

Table 2
Fatty acid composition (%) and quality criteria (acidity, peroxide value and UV absorbances) of 'Galega Vulgar' and 'Cobrançosa' virgin olive oils (mean ± standard deviation), two olive orchards (RF and IR). In each row, superscript indexes indicate differences based on Tukey test.

	'Galega Vulgar'		'Cobrançosa'	
Olive orchard	RF	IR	RF	IR
Miristic acid (C14:0)	0.01 ± 0.000^{a}	0.01 ± 0.001^{a}	0.008 ± 0.000^{a}	0.009 ± 0.001^{b}
Palmitic acid (C16:0)	15.93 ± 0.19^{a}	16.35 ± 0.28^{a}	14.55 ± 0.91^{b}	14.54 ± 0.30^{b}
Palmitoleic acid (C16:1)	2.31 ± 0.11^{a}	2.15 ± 0.15^{a}	1.21 ± 0.17^{b}	1.03 ± 0.04^{b}
Margaric acid (C17:0)	0.10 ± 0.00^{b}	0.13 ± 0.02^{a}	0.11 ± 0.01^{b}	0.16 ± 0.01^{a}
Margaroleic acid (C17:1)	0.28 ± 0.01^{b}	0.35 ± 0.04^{a}	0.22 ± 0.01^{b}	0.25 ± 0.00^{a}
Stearic acid (C18:0)	1.69 ± 0.04^{c}	1.84 ± 0.06^{c}	3.04 ± 0.31^{b}	3.33 ± 0.14^{a}
Oleic acid (C18:1)	74.01 ± 0.30^{a}	73.37 ± 0.21^{a}	70.25 ± 1.61^{b}	70.78 ± 0.14^{b}
Linoleic acid (C18:2)	4.16 ± 0.26^{c}	4.10 ± 0.15^{c}	9.03 ± 1.18^{a}	8.04 ± 0.39^{b}
Linolenic acid (C18:3)	0.66 ± 0.05^{c}	0.69 ± 0.03^{c}	0.75 ± 0.04^{b}	0.89 ± 0.02^{a}
Arachidic acid (C20:0)	0.32 ± 0.06^{a}	0.41 ± 0.09^{a}	0.44 ± 0.08^{a}	0.44 ± 0.03^{a}
Gadoleic acid (C20:1)	0.25 ± 0.01^{b}	0.27 ± 0.02^{a}	0.20 ± 0.00^{c}	0.21 ± 0.01^{c}
Behenic acid (C22:0)	0.10 ± 0.01^{c}	0.11 ± 0.01^{b}	0.11 ± 0.01^{b}	0.13 ± 0.00^{a}
MUFA	76.87 ± 0.24^{a}	76.17 ± 0.30^{a}	71.90 ± 1.54^{b}	72.31 ± 0.14^{b}
PUFA	4.82 ± 0.22^{c}	4.79 ± 0.13^{c}	9.78 ± 1.17^{a}	8.91 ± 0.42^{b}
SFA	18.19 ± 0.17^{c}	18.92 ± 0.42^{a}	18.33 ± 0.59^{bc}	18.70 ± 0.33^{ab}
Acidity (% oleic acid)	0.24 ± 0.04^{b}	0.22 ± 0.04^{b}	0.31 ± 0.02^{a}	0.33 ± 0.05^{a}
Peroxide value (meq O_2 kg^{-1})	5.80 ± 1.57^{bc}	5.16 ± 0.92^{c}	7.93 ± 1.73^{a}	7.14 ± 1.81^{ab}
K_{270}	0.130 ± 0.030^{b}	0.136 ± 0.021^{b}	0.198 ± 0.024^{a}	0.206 ± 0.009^{a}
K_{232}	1.28 ± 0.08^{b}	1.28 ± 0.06^{b}	1.46 ± 0.08^{a}	1.41 ± 0.06^{a}

resulted in high bitter intensity detected by sensory evaluation. The only exception was the VOO obtained from 'Galega Vulgar' with the highest ripening index (>4.0) that showed almost disappearance of the bitter taste (Fig. 2). For 'Cobrançosa' olive oils, high scores of green flavors, as well as quite astringent and pungent notes, were sensory evaluated in all samples, even for ripening index of 4.0. Furthermore, for each cultivar and for each ripening stage, the higher content of phenols has as consequence a high oxidative stability (OS) (Table 3).

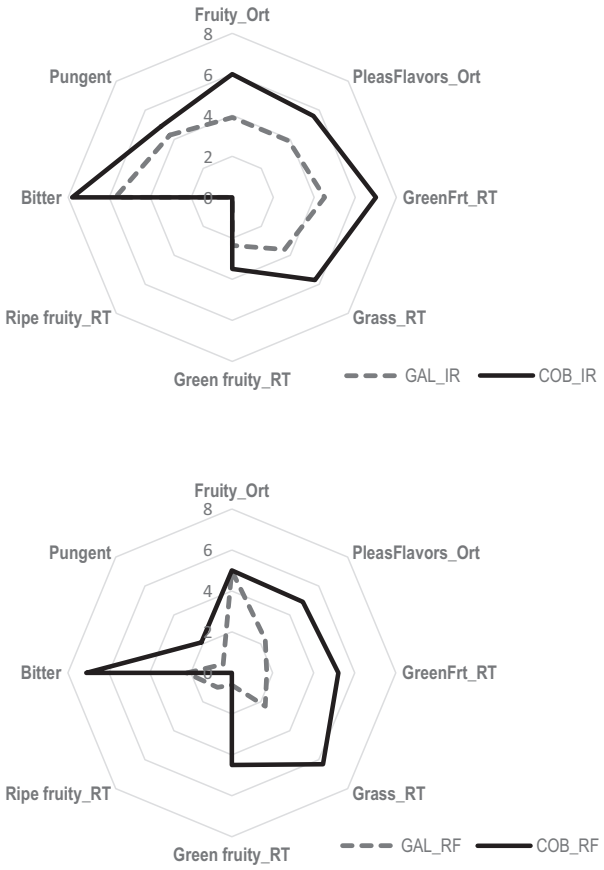

Fig. 2. Sensory profiles of 'Galega Vulgar' and 'Cobrançosa' VOO from olives produced in the orchard IR (Gal_IR and COB_IR, with ripening index of 3.0) and in orchard RF (Gal_RF and COB_RF, with ripening index 4.0). Fruity_ORT – orthonasal olive fruity; PleasFlavors_ORT – other pleasant flavors; GreenFrt_RT – retronasal green fruity; Grass_RT – retronasal grass; Ripe fruity_RT – retronasal ripe flavors.

The phenol compounds quantified in 'Galega Vulgar' and 'Cobrançosa' VOO are presented in Table 3. For both cultivars, α-tocopherol content in VOO decreased during the ripening process while γ-tocopherol showed an increase for the last harvesting dates. This trend is explained by Beltrán et al. (2010) as to be related to the chlorophyll losses in the oil. The presence of high contents of α-tocopherol in early ripening stages (October harvest) represents a good antioxidant protection during the storage of both VOO, without contributing, as hydrophilic phenols do, for bitter taste (Peri, 2014). In the present harvest, γ-tocopherol contents of 'Galega Vulgar' VOO were significantly higher than those of Cobrançosa VOO. This is considered to be a good characteristic for 'Galega Vulgar' VOO, as this compound provides different antioxidant activities in food and *in vitro* studies and showed higher activity in trapping lipophilic electrophiles and reactive nitrogen and oxygen species than the α-tocopherol (Wagner, Kamal-Eldin, & Elmadfa, 2004). Independently of the type of orchard, 'Galega Vulgar' VOO showed always significantly higher oxidative stability than Cobrançosa VOO, extracted from olives with similar RI.

With respect to the hydrophilic phenolic compounds in VOO, their chromatographic assay by HPLC showed a similar profile for both monovarietal oils (Fig. 3). From the 13 identified compounds, only seven of them were possible to identify and quantify due to the lack of standards for the other ones: hydroxytyrosol, tyrosol, vanillic acid, vanillin, *p*-coumaric, luteolin and apigenin (Table 3 and Fig. 3). Oleuropein was not quantified because in 'Cobrançosa' chromatograms had a bad resolution (<1.0). Verbascoside (retention time of 27 min), as expected, was not present in VOO, though it is reported to be present in olive extracts of 'Cobrançosa' olives (Sousa, Malheiro, Casal, Bento, & Pereira, 2014). Both olive oils showed low amounts of phenolic acids and phenolic alcohols, and the prevalent phenolic compound was the dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), confirmed by LC-MS. In some samples of 'Galega Vulgar' oils, this compound corresponds to more than 50 % of total area. It was also confirmed by LC/MS the presence of the secoiridoids *p*-HPEA-EDA, 3,4-DHPEA-EA and 3,4- *p*-HPEA-EA. The phenolic alcohols detected were hydroxytyrosol and tyrosol (Table 3). The first one was present in higher content in 'Cobrançosa' VOO from IR orchard and the second one in 'Galega Vulgar' VOO from RF orchard, but no significant differences between values in early ripening stages were found. Such low contents in hydroxytyrosol and tyrosol are expected in fresh oils, because during the storage of the olive oils, an increase in these phenolic alcohols occurs, which may be explained by the breakdown of the secoiridoids 3,4-DHPEA-EDA, *p*-HPEA-EDA, 3,4-DHPEA-EA, and 3,4-DHPEA-AC (Brenes, García,

Table 3
Tocopherols, quantified phenols, chlorophyll pigments and oxidative stability of 'Galega Vulgar' and 'Cobrançosa' olive oils, in two olive orchards (RF and IR). In each row, superscript indexes indicate differences based on Tukey test (four independent samples per group of olive oils with similar ripening index).

	'Galega Vulgar'				'Cobrançosa'			
Olive orchard	RF		IR		RF		IR	
Harvest month	Oct	Nov	Oct	Nov	Oct	Nov	Oct	Nov
Chlorophyll pigments (mg kg^{-1})	55.54^{ab}	3.01^{c}	76.3^{ab}	9.12^{c}	53.9^{b}	10.6^{c}	79.3^{a}	51.2^{b}
α-Tocopherol (mg kg^{-1})	342.52^{c}	285.07^{d}	393.89^{b}	293.25^{d}	370.69^{bc}	290.25^{d}	448.67^{a}	293.34^{d}
β-Tocopherol (mg kg^{-1})	3.46^{c}	4.27^{bc}	4.90^{ab}	5.16^{a}	3.64^{cd}	3.89^{cd}	4.95^{ab}	3.97^{cd}
γ-Tocopherol (mg kg^{-1})	13.85^{b}	16.00^{b}	9.49^{c}	20.15^{a}	5.99^{de}	8.68^{c}	5.77^{e}	8.18^{cd}
Hydroxytyrosol (mg kg^{-1})	1.20^{b}	1.20^{b}	1.60^{b}	1.48^{b}	1.41^{b}	1.35^{b}	2.43^{a}	3.17^{a}
Tyrosol (mg kg^{-1})	4.28^{a}	3.27^{ab}	1.69^{c}	1.92^{bc}	1.20^{c}	1.33^{c}	2.19^{bc}	2.13^{bc}
Vanillic acid (mg kg^{-1})	0.38^{a}	0.30^{a}	0.33^{a}	0.32^{a}	0.19^{a}	0.25^{a}	0.39^{a}	0.37^{a}
Vanillin (mg kg^{-1})	0.69^{bc}	0.44^{c}	0.62^{bc}	0.56^{c}	0.92^{b}	1.54^{a}	0.75^{bc}	0.73^{bc}
p-Coumaric acid (mg kg^{-1})	0.22^{b}	0.19^{b}	0.26^{ab}	0.22^{b}	0.18^{b}	0.17^{b}	0.20^{b}	0.38^{a}
Luteolin (mg kg^{-1})	0.32^{d}	0.73^{cd}	0.29^{d}	0.43^{d}	1.18^{bc}	2.25^{a}	1.56^{ab}	1.42^{abc}
Apigenin (mg kg^{-1})	0.08^{c}	0.07^{c}	0.07^{c}	0.11^{c}	0.41^{b}	0.65^{a}	0.75^{a}	0.89^{a}
Oxidative stability (h)	42^{a}	33^{b}	38^{a}	33^{b}	32^{b}	23^{c}	25^{c}	27^{c}

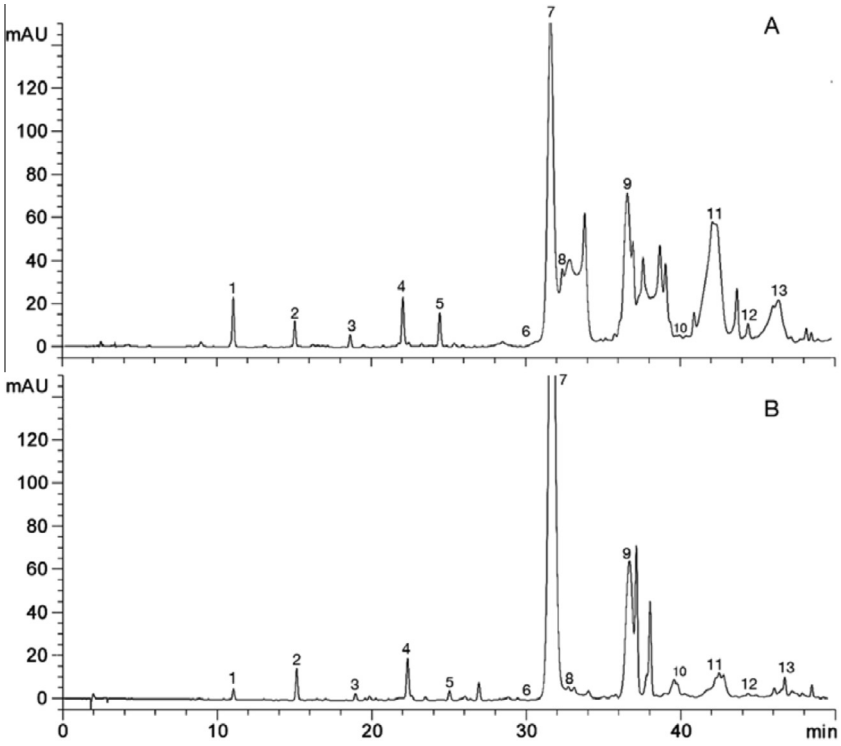

Fig. 3. HPLC chromatograms (at 280 nm) of phenolic extracts of 'Cobrançosa' (A) and 'Galega Vulgar' (B) virgin olive oils, at the same ripening index (RI = 3) in the same orchard (IR). 1 – HYT; 2 – TYR; 3 – ACVA; 4 – VAN; 5 – PCUM; 6 – OCUM; 7 – 3,4-DHPEA-EDA; 8 – OLEU; 9 – *p*-HPEA-EDA; 10 – LUT; 11 – 3,4-DHPEA-EA; 12 – APIG; 13 – *p*-HPEA-EA.

García, & Garrido, 2001). The results obtained for these phenolic alcohols are quite similar to the ones obtained for other Portuguese cultivars ('Negrinha do Freixo' and 'Carrasquinha') in early ripening stages (Garcia, Magalhães, Fregapane, Salvador, & Paiva-Martins, 2012). In what concerns to phenolic acids, no caffeic, ferulic or gallic acids were detected by HPLC-DAD or LC-MS. However, caffeic acid was identified in 'Picual' as well as in 'Moraiolo', 'Frantoio' and 'Leccino' VOO; ferulic and gallic acids were detected in 'Picual' and 'Hojiblanca' olive oils (Rivas, Sanchez-Ortiz, Jimenez, García-Moyano, & Lorenzo, 2013; Servili & Montedoro, 2002). No significant differences ($p > 0.05$) between ripening stages were observed for vanillic acid for all cultivars, orchards and ripening stages. For the phenolic acid derivative, vanillin, higher contents in 'Cobra nçosa' olive oils were found, with significantly higher values ($p < 0.05$) in the olive oils produced from olives from the RF orchard, showing for these oils a significant increase along fruit ripening. This behavior was also observed in the cultivar 'Picudo' (Jiménez et al., 2013). The content of *p*-coumaric acid was higher in 'Cobrançosa' olive oils from IR orchard for higher ripening index. The flavonoids, luteolin and apigenin, were detected in significantly ($p < 0.05$) higher contents in 'Cobrançosa' VOO, but the contents were lower than those reported by Reboredo-Rodríguez, Cancho-Grande, and Simal-Gándara (2014). For all the phenol compounds that was possible to identify in this work, the observed contents were in the range of values referred by "Phenol Explorer", although different cultivar and different modes of olive oil extraction were used (Neveu et al., 2010).

García-Rodríguez et al. (2011) showed that verbascoside, compared to oleuropein and demethyloleuropein, was the preferred substrate for olive POD, which seems to confirm that the best substrates for these enzymes are those having the highest number of hydroxyl groups in the benzoic ring. The same study showed that purified PPO, although active towards both substrates, has slightly higher oxidation rates for verbascoside than for oleuropein and that the highest oxidation rate of VOO secoiridoids by PPO was observed for 3,4-DHPEA-EDA and 3,4-DHPEA-EA (ortho diphenolic secoiridoids) while almost no activity was observed towards monophenolic secoiridoids. In the present study, 'Galega Vulgar' PPO was probably more active towards verbascoside and oleuropein than 'Cobrançosa' PPO, because no verbascoside and low contents of oleuropein were present in 'Galega Vulgar' oils. Higher PPO activity for higher ripening indexes as well as seed POD activity in 'Galega Vulgar' olives may explain the lower phenol content of 'Galega Vulgar' oils *vs.* 'Cobrançosa' oils. However, beta-glucosidase activity can also mask the phenolic glucosides oxidative degradation (Romero-Segura, García-Rodríguez, Sánchez-Ortiz, Sanz, & Pérez, 2012).

Phenols are not the only virgin olive oil compounds with impact on taste and aroma. Bitterness enhanced by the presence of cut grass odorant (e.g. *cis*-3-hexen-1-ol) is an example of taste and smell interactions (Caporale, Policastro, & Monteleone, 2004). The odorants of 'Galega Vulgar' and 'Cobrançosa' VOO evaluated in the present study have already been studied (Peres et al., 2013): in early ripening, the volatile compounds for the cut grass sensations were present, which in conjunction with the high phenol content, explain the very high bitter taste scores given by the panelists (Fig. 2).

4. Conclusion

Phenol composition of VOO can give important information on their quality because it has an important impact on organoleptic evaluation and on the nutritional value of the product. 'Galega Vulgar' and 'Cobrançosa' olive oils in early stages of ripening showed very high contents in phenolic compounds. This study shows that harvesting in different ripening stages, either in rain fed or irrigated orchards, for each cultivar, will produce several types of olive oils from green and pungent oil with high levels of phenol com-

pounds to golden, mild and fruity oil. Therefore, the decision of the harvesting date will allow the production of virgin olive oils with different taste notes and functional value. Moreover, for the production of olive oil with high shelf life, harvesting in early ripening stages can be a good decision. This is particularly important for 'Galega Vulgar' oils that can have improved their nutritional and sensory characteristics, as well as improved shelf life (higher oxidative stability). Especially the activity of PPO can also dictate the profile of phenol compounds in the final olive oil due to different substrate specificity.

However, very early ripening stages can also result in oils with a strong green colour that some consumers are not used to, and for some cultivars, quite astringent and pungent olive oils, that are not balanced at all. From the point of view of the olive grower, a very low yield obtained from very green olives is always another reason for delaying the harvest.

The productivity of the olive orchard, resulting from different agronomical practices can influence the ripening progress and consequently the biosynthesis of the different phenol compounds. In the present study this was more evident in 'Cobrançosa' olive oils that had a different phenol profile in the two orchards studied.

Further studies on these two important cultivars for Portugal, are needed to evaluate the influence of environment and ripening in the phenol content, as well as the influence of other enzymes than PPO and POD, on the composition of the final oil, in order to have a better knowledge for the Protected Designations of Origin (PDO) where they are most closely linked.

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