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First Profile of Phenolic Compounds from Maltese Extra Virgin Olive Oils Using Liquid-Liquid Extraction and Liquid Chromatography-Mass Spectrometry

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Abstract: This study presents the profile of phenolic extracts from different Extra Virgin Olive Oils (EVOOs) from Malta and is the first study that characterizes the phenolic profile of the Maltese EVOOs Bidni (B) and Malti (M) using liquid-liquid extraction (LLE) and Liquid Chromatography-Mass Spectrometry (LC-MS). The total phenolic content (TPC), *ortho* diphenolic content (TdPC) and flavonoid content (TFC) were determined using the Folin-Ciocalteau assay, the Arnow's assay and the Aluminium Chloride method respectively. Results show that the B variety had the highest TPC, TdPC and TFC. Using LC-MS analysis, over 30 phenolic compounds were identified belonging to different classes of phenolic compounds.

Key words: extra virgin olive oil, phenols, liquid-liquid extraction, Liquid Chromatography-Mass Spectrometry

1 Introduction

The average total world olive oil production for the past ten years has been estimated by the International Olive Oil council to amount to 2,944.5 tonnes per year. Of these, an average of 2,050.8 tonnes per year is produced solely by countries in the European Union. The market for olive oils has increased as they are appreciated as a rich source of phenolic compounds that are associated with various beneficial qualities including antioxidant, anti-inflammatory properties, anti-microbial and anti-cancerous effects[1-4].

Olive oil is composed of an unsaponifiable and a glycerol fraction. The phenolic component is a part of the unsaponifiable fraction and comprises between 0.4 and 5% of the drupe. This fraction also consists of sterols, hydro-carbons and tocopherols. In contrast, the glycerol fraction constitutes around 90 to 99% of the olive fruit. Its components, fatty acids and triacylglycerols make up the bulk of olive oil[5]. The phenolic component contributes to the stability of the oil during processing and storage as well as the organoleptic qualities of the oil[6].

Phenols are subdivided into phenolic acids, phenolic alcohols, ligans, stilbenes, secoiridoids, coumarins, xanthones and flavonoids (**Fig. 1**). In EVOOs the major components of the phenolic fraction are tyrosol, hydroxytyrosol and their derivatives[7]. With respect to secoiridoids, the main components found in EVOOs are the dialdehyde of hydroxytyrosol, together with oleuropein aglycone and ligstroside aglycone[8-11]. The flavonoids are described as structurally diverse and are classified into flavonols, anthocyanins, flavones, isoflavones and flavonones[12]. In

Abbreviations: **B**, Bidni; **C**, Carolea; **CE**, Catechin equivalents; **EVOO**, Extra Virgin Olive Oil; **HCl**, Hydrochloric acid; **I**, Commercial EVOO variety; **KOH**, Potassium hydroxide; **LC-MS**, Liquid Chromatography-Mass Spectrometry; **LLE**, liquid-liquid extraction; **M**, Malti; **PyCE**, Pyrocatechol equivalents; **RAPD**, Random amplification of polymorphic DNA; **T**, Tonda iblea; **TPC**, Total phenolic content; **TdPC**, Total *ortho* diphenolic content; **TFC**, Total flavonoid content.
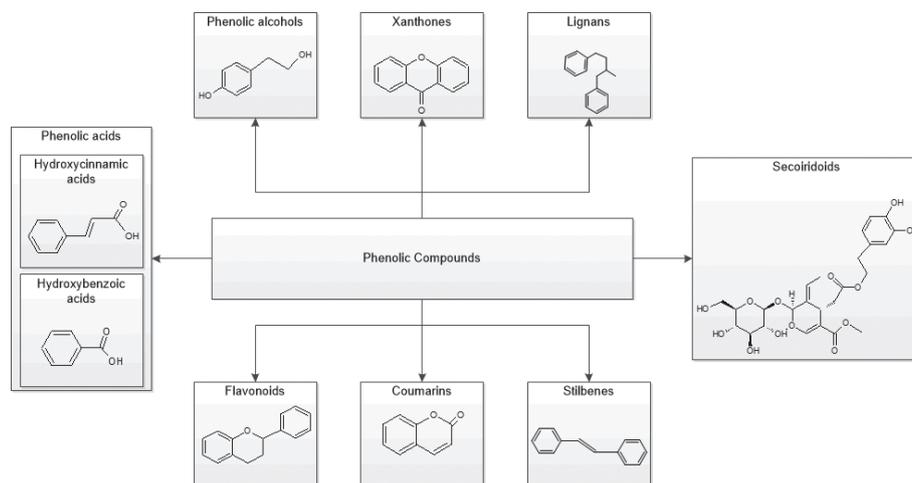
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Fig. 1 The different groups of phenolic compounds and their basic structure.

EVOOs the most common flavonoids are apigenin, luteolin and cyanidins[11, 13]. The phenolic acid sub-group is also characterized by diversity and includes vanillic acid, *p*-coumaric acid, gallic acid, syringic acid as well as caffeic acid[14, 15].

In the distant past, the Maltese Islands had a thriving oil-producing industry[16] but this was eradicated as higher cash-generating crops like cotton became favored. This resulted in the destruction of a number of olive groves and the loss of olive tree germplasm. Recent years have seen belated attempts to regenerate this industry. In the late 1990s, foreign cultivars mainly from Italy and Spain such as Frantoio, Carolea and Nocellara Messinese were introduced to Malta[17]. Successful attempts were also made to revive the industry using Maltese trees regarded as indigenous, such as the Bidni variety. According to the Census of Agriculture, reintroduction programmes of olives have led to a total of 140.3 hectares to be occupied by olive groves, with 72.6 hectares being cultivated for olive oil production.

The Bidni variety is described as having a high pulp to seed ratio, and a marked resistance to diseases and parasites such as the vascular disease-causing fungus, *Verticillium dahliae* and the olive fruit fly *Bactrocera oleae*[16]. Mazzitelli *et al.*[18] analysed the molecular biology of Bidni, Malti and Bajda varieties using random amplified polymorphic DNA (RAPD) and showed that the Bajda shares homology with Italian olive trees as opposed to the other two varieties. Gatt *et al.*[19] studied the cyclooxygenase activity in EVOO derived from the Bidni variety while Lia *et al.*[20] determined the anti-oxidant activity in EVOOs following solid-phase extraction (SPE).

In this study, the polar fraction of EVOOs was separated by LLE and the total phenolic content (TPC), *ortho* diphenolic content (TdPC) and flavonoid content (TFC) were assessed using quantitative assays. LLE is based on the distribution of an analyte between two phases: the aqueous and the organic phase. It is reported to yield a high recovery of secoiridoids[21]. LLE has the advantage of being easy

to operate and it does not require expensive apparatus. Moreover, total phenol recovery rates are very high in LLE and amount to around 93%, making them even higher than those recorded for SPE using diol phase cartridges[22].

The objectives of this study were therefore to characterize the main commercially produced EVOOs from Malta as well as to identify their phenolic profiles using spectrophotometric assays as well as LC-MS analysis.

2 Materials and Methods

2.1 Materials

All monocultivar EVOOs obtained locally were collected at the same stage of harvest and had been subjected to the same irrigation regime. Samples of EVOOs (Bidni, Malti and Carolea) were purchased during the months of October and November in 2012, 2013 and 2015. During 2014, none of these varieties could be collected due to the poor olive harvest. Another monocultivar olive oil, the Barbuto™ from southern Sicily, made using the *Tonda iblea* (T) variety, was purchased in the months of October and November in 2014 and 2015. A fifth EVOO, (I) which is a commercial EVOO, was obtained from a local supermarket in 2013, 2014 and 2015. This is produced from different Tuscany olives, and was chosen solely for comparative purposes, as the only EVOO that was not a monocultivar.

2.2 Reagents

All chemicals used in this study were supplied from Sigma-Aldrich® (St Louis, MO, USA) safe for the absolute ethanol used for phenolic compound stock solution which was supplied from Scharlau (China).

2.3 Methods

2.3.1 Isolation of phenolic compounds from olive oil

The polar fraction of EVOO was obtained using the

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method by Vazquez Roncero *et al.*[23] as described in Papadopoulos and Boskou[24] using LLE. Fifty grams of EVOO were dissolved in 50 mL of hexane and following mixing, phenols were extracted in 30 mL of a 60:40 (v/v) methanol:water mixture three times. The solvent was evaporated under vacuum, using a rotary evaporator at 40℃.

The sample was dissolved in absolute ethanol and stored at −20℃. Phenolic extracts obtained from Bidni, Malti, Carolea, *Tonda iblea* olive oil and the supermarket brand were labelled as B, M, C, T and I respectively.

2.3.2 Determination of the total phenol content using the Folin-Ciocalteu (F-C) assay

Total phenol content was determined using the method of Slinkard and Singleton[25], with a reduction in volumes as described by Waterhouse[26]. Gallic acid was used as the standard. A stock solution of gallic acid was prepared from which different concentrations of the standard were prepared. Twenty microlitres of each gallic acid standard and/or each phenol sample (diluted tenfold) was added to 1.58 mL water and 100 μL of Folin-Ciocalteu reagent. 300 μL of 20% (w/v) anhydrous sodium carbonate solution was added, mixed and heated at 40℃ for 30 minutes. The absorbance of each solution was read at 765 nm using a UV/Visible Spectrophotometer (Pharmacia).

2.3.3 Determination of the total *ortho* diphenolic content using the Arnow's assay

The method used was modified from Woisky and Salatino[27]. Arnow's reagent was prepared by adding 10 g of sodium molybdate dihydrate and 10 g of sodium nitrite in 100 mL of a 1:1 (v/v) Ethanol (Scharlau): water mixture. A stock solution of pyrocatechol was prepared from which different concentrations of the standard were prepared. In a 96 well plate 20 μL of each pyrocatechol concentration and/or each phenol sample (diluted fourfold) was pipetted and to each, 20 μL 1M HCl added, followed by 20 μL of Arnow's reagent. The plate was shaken for 5 minutes at 500 rpm and then incubated at room temperature for 15 minutes and 80 μL of deionised water were added, followed by 40 μL of 1M KOH. The absorbance was read at 370 nm using a UV/Visible microplate reader (SPECTROstar Nano, BMG LABTECH).

2.3.4 Determination of the total flavonoid content using the Aluminium Chloride method

The method used to determine the total flavonoid content of the phenolic extracts was the Aluminium Chloride Colorimetric Method. A stock solution of catechin was prepared from which different concentrations of the standard were prepared. In a 96 well plate 25 μL of each catechin concentration and/or each phenol sample (diluted fivefold) was pipetted and to each, 7.5 μL 10% aluminium chloride, 7.5 μL of a 7% (w/v) sodium nitrite and 80 μL distilled water were added. The plate was shaken for 5 minutes at 500 rpm and then incubated at room temperature for 25 minutes. After this, 100 μL of 1M KOH was added to each well. The absorbance was read at 415 nm using a UV/Visible microplate reader (SPECTROstar Nano, BMG LABTECH).

2.3.5 LC-MS analysis

The apparatus consisted of a Waters, Alliance, 2695 Separations module connected to a Micromass, LCT classic, mass spectrometer. Mass Lynx software, version 4.1 was used for data collection and interpretation. A crude, phenolic extract (2 mg) of sample B was dissolved in methanol (1 mL). An aliquot (20 μL) was injected onto a C18 reverse phase column 50 mm × 2.0 mm operating at 35℃. The solvent gradient was 5% acetonitrile (0.1% formic acid) to 95% acetonitrile (0.1% formic acid) in 30 minutes and then held for 5 minutes. The column was equilibrated at the starting conditions for 10 minutes before analysis of subsequent samples, C, M, T and I.

2.3.6 Statistical analysis

For each sample, all parameters were determined in triplicate. Using the software IBM® SPSS® Statistics Version 21, all data sets were tested for normality using the Shapiro Wilk's test. The Kruskal-Wallis test was selected as the non-parametric test for pairwise comparisons, with significant comparisons having a p-value less than 0.05. The Pearson product-moment correlation coefficient was used to determine correlation between TPC, TdPC and TFC.

3 Results and Discussion

The total phenolic (TPC), *ortho* diphenolic (TdPC) and flavonoid content (TFC) for each of the oils studied are presented in Fig. 2. The highest TPC and TFC were recorded in the EVOO derived from the B variety followed by the EVOO derived from the T variety cultivated in Sicily. The lowest quantity of TPC and TFC were recorded in the supermarket oil variety I. The TPC values for the B EVOO and that of the T variety appear comparable to autochthonous EVOO varieties such as the Italian Tonda di Caligari and Bosana, for which values are quoted to be 261.18 ± 83.83 mg/kg GAE and 355.20 ± 121.34 mg/kg GAE respectively[28].

The TPC is an important parameter in the classification of EVOO as mild, medium or robust where robust reflects the highest TPC. In this respect, mean TPC values for each category differ between both producers and experts, though the difference is the most pronounced for the former category. For experts, EVOO is mild if the TPC is 127 ± 5 mg/kg, while for producers the value is 170 ± 11 mg/kg. It is medium if the TPC is 223 ± 5 mg/kg for experts and 226 ± 7 mg/kg for producers. It is classified as a robust oil if the TPC is 350 ± 9 mg/kg for experts and 291 ± 11 mg/kg for producers. Following this classification, I, M and C appear to be all mild EVOOs, while T and B appear to be medium if classified according to the experts' classification
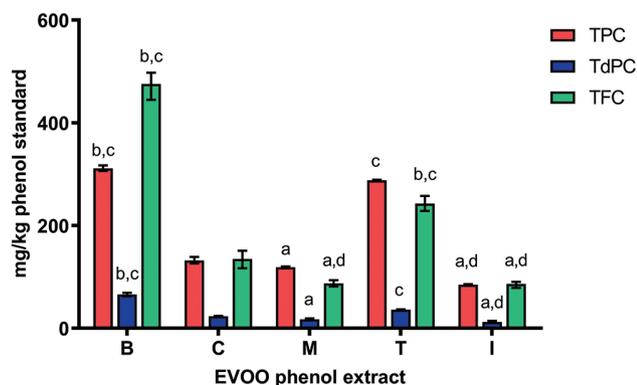
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Fig. 2 The TPC, TdPC and TFC of each EVOO phenol extract. Each value is a mean value where n = 3. Values are presented as mg/kg GAE (Gallic Acid Equivalents), mg/kg PyCE (Pyrocatechol equivalents) and CE (Catechin equivalents) for TPC, TdPC and TFC respectively. Positive and negative errors are presented as the maximum and minimum value difference from the mean. Small letters (B = a, M = b, I = c, T = d) represent statistically significant differences of *p*-values less than 0.05.

or robust (for B) if classified according to producers' values[29].

The link between phenolics and bitterness of EVOOs has long been established and has been attributed to a number of different phenols. While Kiritsakis, García *et al.*, and Soler-Rivas, Espín, and Wichers[30–32] linked this property to oleuropein derivatives, others such as Gutiérrez-Rosales, Perdiguero, Gutiérrez and Olias, and Angerosa *et al.*[8, 33] quoted both oleuropein and ligstroside aglycones and for Tovar *et al.*[34], this property is only a result of ligstroside derivatives.

A number of studies show that the presence of a second hydroxyl group increases the antioxidant activity of EVOOs[5, 35–38]. The TdPC was highest in the EVOO derived from the Bidni variety followed once again by the Sicilian-derived *Tonda iblea* variety. As with the TPC results, the lowest TdPC was reported for the supermarket oil. The TdPC analysed for C, M, I and T was found to be within the range of other oils such as a number of Argentinian VOOs, though these were recorded as caffeic acid equivalents by Laincer *et al.*[39] not pyrocatechol equivalents as in this study, hence direct comparisons are not possible. The varieties are namely Tabelout (19.36 ± 0.97 mg/kg CAE), Blanquette de Gelma (23.37 ± 0.59 mg/kg CAE) and Bouricha (15.65 ± 0.86 mg/kg CAE). The TdPC of B was found to be similar to Portugese monocultivar EVOOs Cobrançosa (56.0 ± 1.50 mg/kg GAE), Madural (48.39 ± 1.00 mg/kg GAE) and the commercial Herdade do Esporão-Galega (59.23 ± 0.60 mg/kg GAE)[40]. In contrast, all TdPC values recorded were less than those quoted as caffeic acid equivalents by

Youssef *et al.*[41] for the Tunisian EVOOs of the varieties Chetoui (282.82 ± 40.95 mg/kg CAE), and Oueslati (185.62 ± 0.20 mg/kg CAE), as well as Portuguese monocultivar EVOOs such as Cordovil de Castelo Branco (263.5 ± 6.40 mg/kg GAE) and Blanqueta (163.9 ± 7.40 mg/kg GAE)[40].

Of all locally derived oils, the EVOO derived from the Malti variety contained the lowest TPC, TdPC and TFC. However, this may be a reflection of the difficulty encountered to separate the organic and aqueous layer separation during LLE, due to the presence of an interphase layer that was recalcitrant to separation. As a result, some phenols may have not been collected in the aqueous layer resulting in a lower TPC. While a number of studies report the TFC of a variety of plant extracts, those investigating the TFC of EVOOs are very limited. The results obtained in this study contrast highly with those reported by Ebrahimi *et al.*[42] as rutin hydrate equivalents for crude olive oils in Iran, who report TFCs of 2.73 mg/g, 3.44 mg/g, 3.53 mg/g, and 3.61 mg/g. However, a clear cut comparison of this data with that of our study is not possible as the values reported by Ebrahimi *et al.*[42] are expressed as rutin hydrate equivalents not as catechin equivalents as in this study. Values reported by Ammar *et al.*[43] also greatly differ from those obtained in this study. Ammar *et al.*[43] record the TFC of a Tunisian Chemlali olive oil, as a value of 14.50 ± 0.29 mg/kg CE.

Figure 3 shows that the TPC, TdPC and TFC were all found to be positively correlated as determined by the Pearson product-moment correlation coefficient. The TPC was found to be positively correlated with both the TdPC and TFC (Pearson's values of 0.906 and 0.896 respectively). There also appears to be a positive correlation between TdPC and TFC (Pearson's value of 0.995). Also, *p*-values confirm that there is a statistically significant correlation between all the variables.

The phenolic profile of each of the EVOOs was analysed using LC-MS analysis where the representative total ion chromatograms obtained through LC-MS analysis of the five EVOO phenolic extracts are shown in Fig. 4. The compounds identified through LC-MS are presented in Table 1 along with the *m/z* of each peak obtained through MS and the compound or fragments responsible for that respective *m/z*.

Figure 4 shows that the total ion chromatogram for all five EVOOs consists of a total of 27 peaks at identical retention times but differing peak heights. This indicates that the EVOO phenolic extracts contain the same phenolic compounds but at differing peak heights. While a number of compounds seem to be present at similar peak heights across the oils, other peaks indicate differences. Such differences correspond to peaks 2, 8, 16, 17, 26 and 27.

In this study, over 30 phenolic compounds were identified belonging to different classes of phenolic compounds. On one hand, the phenolic compounds in the Bidni, Caro-
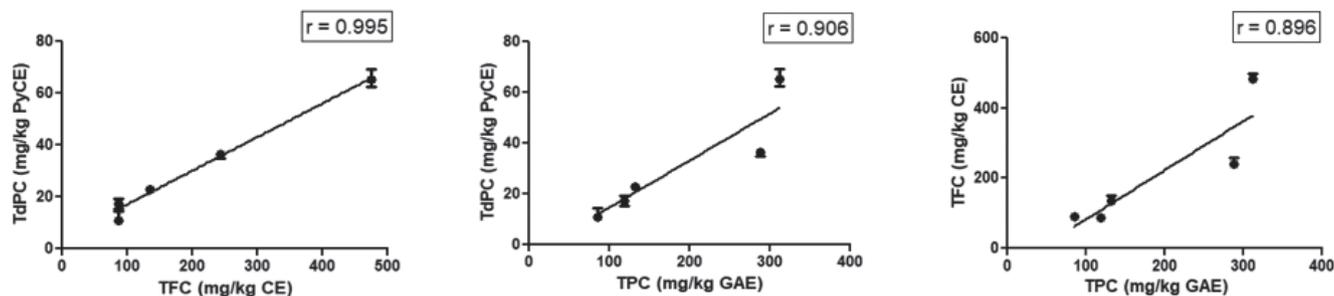
Fig. 3 The Pearson's correlation analysis for TPC, TdPC and TFC. TPC is positively correlated with TdPC $(r = 0.906)$, TPC is positively correlated with TFC $(r = 0.896)$ and TdPC is positively correlated with TFC $(r = 0.995)$. Correlation analysis shows statistically significant differences of p-values less than 0.05.
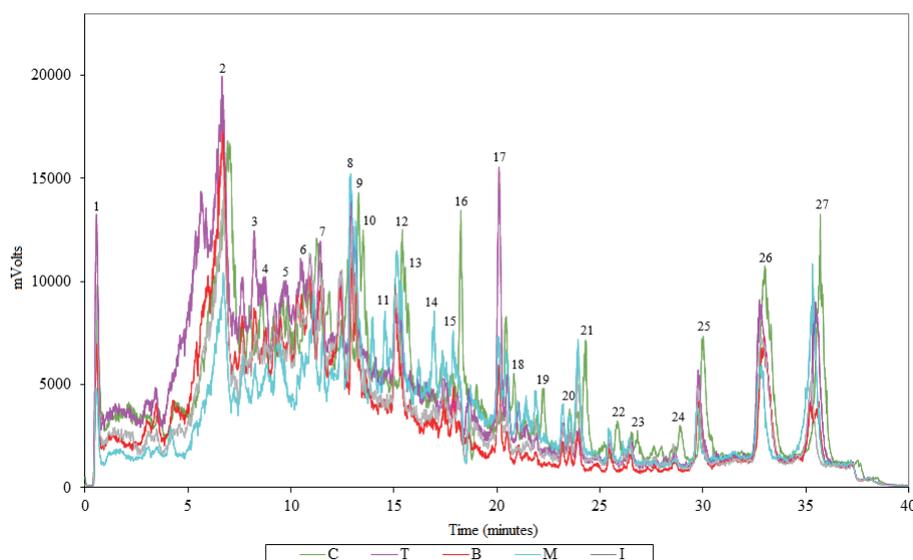

Fig. 4 The total ion chromatogram obtained for the five EVOO crude phenolic extracts using a C18 reverse phase column and a solvent gradient of 5% acetonitrile to 95% acetonitrile.

lea, *Tonda iblea* and the supermarket varieties that were present at the highest concentration were sinaptic acid or hydroxy-elenolic acid and tyrosol glycoside. On the other hand, in the Malti variety, the most prominent phenolic compound was the ligstroside aglycone. Certain compounds such as the closed ring carboxilade demethylated hydroxilade oleuropein form fragment, the 3,4-DHPEA-EDA diglycoside or oleuropein dihydroxytyrosol and the open ring carboxilade dialdehydic oleuropein glycoside fragment were found at very low concentrations uniformly across each of the oils.

Among the first identified phenols from EVOO were phenolic acids and these were identified in a number of studies. A number of common members of this class include sinaptic acid, caffeic acid, gallic acid and vanillic acid[44−46]. It is the former of these compounds that was identified in this study.

With respect to lignans, (+)–pinoresinol, and (+)– 1–acetoxypinoresinol were identified, with all being present as the glycoside forms, and with the former being found as

the free (+)–pinoresinol form, the glycoside form and the tetrameric form. With regards free and tetrameric (+)–pinoresinol, as well as (+)– 1–acetoxypinoresinol glycoside, these were found in the largest amounts in both C and M oils, while the rest were present in similar amounts across all oils. The study by Owen *et al.*[11] quote the former two lignans as the major components of the phenolic EVOO fraction. It has been reported that the concentration of lignans is much higher in olive fruits when compared to olive oil[47].

The flavonoids apigenin and luteolin were identified in this study, and were found to be present in similar amounts in all tested oils. These were first identified from *Olea europaea* by Rovellini *et al.*[48] by HPLC-UV and HPLC-electrospray-MS, and also detected in Portuguese *Olea europaea* leaf cultivars by Meirinhos *et al.*[49] by HPLC-DAD.

The greatest diversity of compounds was seen for the secoiridoid oleuropein and its derivatives, as these amounted to ten different compounds, being both the open and closed ring decarboxilade aldehydic forms, the closed

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Table 1 The compounds identified from LC-MS analysis.

Peak	*m/z*	Compound / Fragment
1	257.2	Hydroxy-Elenolic acid oxidised hydroxyl form
2	225.1	Sinaptic acid or Hydroxy-Elenolic acid
	252.06	Tyrosol Glycoside
3	225.07	Hydroxy-Elenolic acid
4	225.07, 239.08	Hydroxy-Elenolic acid
	361.13	*p*-HPEA-EDA
	393.16	Ligstroside aglycone derivative
5	287.05	Apigenin
6	287.05	Luteolin
7	225.07	Hydroxy-Elenolic acid
	379.13	Methyl-3,4-DHPEA-EA
8	225.08	Product obtained following rearrangement of the decarboxylated form of ligstroside aglycone
	363.14	Decarboxylated form of ligstroside aglycone
9, 10	274.24	Closed ring carboxilade hydroxilade form
11	225.07, 363.12, 495.24	Open ring decarboxilade aldehydic form of oleuropein
	530.27	Open ring decarboxilade aldehydic form of oleuropein glycoside
12	415.23, 432.25	Closed ring carboxilade hydroxilade glycoside form
13	302.31	Hydroxytyrosol elenolic acid dialdehyde (3,4-DHPEA-EDA)
14	346.35	Oleuropein aglycone demethylated derivative
15	277.21, 351.25	10-hydroxy-oleuropein fragment
	552.39	10-hydroxy-oleuropein
16	566.36	10-hydroxy methyl – oleuropein
17	487.3	Closed ring decarboxilade aldehydic oleuropein
	505.31	Closed ring decarboxilade aldehydic oleuropein glycoside
18	487.28	Closed ring decarboxilade aldehydic oleuropein iridoid form
19	258.2	Hydroxy-Elenolic acid oxidised hydroxyl form
	279.21, 395.26	Closed ring carboxilade demethylated hydroxilade oleuropein form fragment
	551.35	Closed ring carboxilade demethylated hydroxilade oleuropein form
20	267.18	Closed ring decarboxilade oleuropein form fragment
	391.27	Closed ring decarboxilade oleuropein form
	515.38	Open ring decarboxilade Aldehydic Oleuropein glycoside form
	539.39	(+) - pinoresinol glycoside
	597.4	(+) – 1- acetoxypinoresinol glycoside
21	357.28	(+) – pinoresinol
	554.44	(+) – 1- hydroxypinoresinol glycoside
22	678.29	3,4-DHPEA-EDA diglycoside or Oleuropein dihydroxytyrosol
23	225.07	Hydroxy-Elenoic acid
	281.19, 398.78, 419.29, 557.44	Open ring carboxilade dialdehydic oleuropein glycoside fragment
24	603.35	Verbascoside
25	512.047	Open ring decarboxilade dialdehydic ligosecoiridoid glycoside fragment form
26	540.49	Oleuropein
27	708.49	Dimeric form of open ring decarboxilade aldehydic ligosecoiridoid form
	1370.96	Tetrameric form of pinoresinol

ring decarboxilade aldehydic glycoside and the iridoid forms, an oleuropein aglycone demethylated derivative, a fragment of 10-hydroxy-oleuropein, 10-hydroxy-oleuropein, 10-hydroxy methyl-oleuropein, the closed ring carboxilade demethylated hydroxilade oleuropein form fragment, and the open ring carboxilade dialdehydic oleuropein glycoside form. Other analysed secoiridoids were the decarboxymethyl oleuropein dialdehyde form (*p*-HPEA-EDA)

and decarboxymethyl ligstroside aglycone dialdehyde form (methyl-3,4-DHPEA-EA). The latter two compounds are amongst the most abundant EVOO phenolic compounds and are a result of enzymatic hydrolysis[50]. These two were present in the lowest amounts in the M variety.

From this study, one can conclude that out of the tested oils, the Bidni variety is the highest in TPC, TdPC and TFC. Using LC-MS analysis, over 30 phenolic compounds were identified, with these belonging to a number of different classes. The components were uniform across the oils.

Supporting Information

This material is available free of charge via the Internet at http://dx.doi.org/jos.70.10.5650/jos.ess20130

References

1) Ahmed, S.; Rahman, A.; Hasnain, A.; Lalonde, M.; Goldberg, V.M.; Haqqi, T.M. Green tea polyphenol epigallocatechin-3-gallate inhibits the IL-1 beta-induced activity and expression of cyclooxygenase-2 and nitric oxide synthase-2 in human chondrocytes. *Free Radical Biol. Med.* **33**, 1097-1105 (2002).

2) Fabiani, R.; De Bartolomeo, A.; Rosignoli, P.; Servili, M.; Montedoro, G.F.; Morozzi, G. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. *Eur. J. Cancer Prev.* **11**, 351-358 (2002).

3) Moreno, J.J. Effect of olive oil minor components on oxidative stress and arachidonic acid mobilization and metabolism by macrophages RAW 264. 7. *Free Radical Biol. Med.* **35**, 1073-1081 (2003).

4) Albishi, T.; John, J.A.; Al-Khalifa, A.S.; Shahidi, F. Antioxidant, anti-inflammatory and DNA scission inhibitory activities of phenolic compounds in selected onion and potato varieties. *J. Funct. Foods* **5**, 930-939 (2013).

5) Tripoli, E.; Giammanco, M.; Tabacchi, G.; Di Majo, D.; Giammanco, S.; La Guardia, M. The phenolic compounds of olive oil: Structure, biological activity and beneficial effects on human health. *Nutr. Res. Rev.* **18**, 98-112 (2005).

6) Di Giovacchino, L.; Sestili, S.; Di Vincenzo, D. Influence of olive processing on virgin olive oil quality. *Eur. J. Lipid Sci. Technol.* **104**, 587-601 (2002).

7) Gómez-Romero, M.; García-Villalba, R.; Carrasco-Pancorbo, A.; Fernández-Gutiérrez, A. Metabolism and bioavailability of olive oil polyphenols. in *Olive Oil - Constituents, Quality, Health Properties and Bioconversions* (Boskou, D. ed.), InTech Europe, pp. 333-357 (2012).

8) Angerosa, F.; D'Alessandro, N.; Konstantinou, P.; Di Giacinto, L. GC-MS evaluation of phenolic compounds in virgin olive oil. *J. Agric. Food Chem.* **43**, 1802-1807 (1995).

9) Angerosa, F.; D'Alessandro, N.; Konstantinou, P.; Di Giacinto, L. Characterization of phenolic and secoiridoid aglycons present in virgin olive oil by gas chromatography-chemical ionization mass spectrometry. *J. Chromatogr. A* **736**, 195-203 (1996).

10) Kuwajima, H.; Uemura, T.; Takaishi, K.; Inoue, K.; Inouye, H. A secoiridoid glucoside from *Olea europaea. Phytochemistry* **27**, 1757-1759 (1988).

11) Owen, R.W.; Mier, W.; Giacosa, A.; Hull, W.E.; Spiegelhalder, B.; Bartsch, H. Phenolic compounds and squalene in olive oils: The concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. *Food Chem. Toxicol.* **38**, 647-659 (2000).

12) Carocho, M.; Ferreira, I.C. The role of phenolic compounds in the fight against cancer–A review. *Anticancer Agents Med. Chem.* **13**, 1236-1258 (2013).

13) Rovelli, P.; Cortesi, N.; Fedeli, E. Analysis of flavonoids from *Olea europaea* by HPLC-UV and HPLC-electrospray-MS. *Riv. Ital. Sostanze Grasse* **74**, 273-279 (1997).

14) Montedoro, G.F.; Servili, M.; Baldioli, M.; Miniati, E. Simple and hydrolyzable compounds in virgin olive oil. *J. Agric. Food Chem.* **40**, 1577-1580 (1992).

15) Mannino, S.; Cosio, M.; Bertuccioli, M. High perfor-

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mance liquid chromatography of phenolic compounds in virgin olive oils using amperometric detection. *Ital. J. Food Sci.* **2**, 150-157 (1993).

16) Borg, J. *Cultivation and Diseases of Fruit Trees in the Maltese Islands*. Malta Government Authority, Malta (1922).

17) Ministry for Agriculture and Rural Affairs. Iz-zejt taz-zebbuga. *Naturalment Malti: Assistenza Teknika tal-Fond Agrikolu Ewropew ghall-Izvilupp Rurali* (2007).

18) Mazzitelli, O.; Calleja, A.; Sardella, D.; Farrugia, C.; Zammit-Mangion, M. Analysis of the molecular diversity of *Olea europaea* in the mediterranean island of Malta. *Genet. Resour. Crop Evol.* **62**, 1021-1027 (2014).

19) Gatt, L.; Zammit-Mangion, M.; Schembri-Wismayer, P. Study of the cyclooxygenase inhibition by polyphenol extracts derived from monocultivar oils from autochtonous olive tree varieties. *Planta Med.* **13**, 1213-1214 (2013).

20) Lia, F.; Farrugia, C.; Zammit-Mangion, M. Determination of antioxidant activity of olive oil phenolic extracts. *9th ISANH Congress on Polyphenol Applications* (2015).

21) Mateos, R.; Espartero, J.L.; Trujillo, M.; Rios, J.J.; Leon-Camacho, M.; Alcudia, F.; Cert, A. Determination of phenols, flavones, and lignans in virgin olive oils by solid-phase extraction and high-performance liquid chromatography with diode array ultraviolet detection. *J. Agric. Food Chem.* **49**, 2185-2192 (2001).

22) Hrncirik, K.; Fritsche, S. Comparability and reliability of different techniques for the determination of phenolic compounds in virgin olive oil. *Eur. J. Lipid Sci. Technol.* **106**, 540-549 (2004).

23) Vazquez Roncero, A.; Janer de Valle, C.; Janer de Valle, M.L. Determination of total polyphenols in oliver oils. *Grasas y Aceites* **24**, 350-357 (1973).

24) Papadopoulos, G.; Boskou, D. Antioxidant effect of natural phenols on olive oil. *J. Am. Oil Chem. Soc.* **68**, 669-671 (1991).

25) Slinkard, K.; Singleton, V.L. Total phenol analysis: Automation and comparison with manual methods. *Am. J. Enol. Vitic.* **28**, 49-49-55 (1977).

26) Waterhouse, A.L. *Current protocols in food analytical chemistry*. John Wiley and Sons, USA (2003).

27) Woisky, R.G.; Salatino, A. Analysis of propolis: Some parameters and procedures for chemical quality control. *J. Apic. Res.* **37**, 99-105 (2015).

28) Tuberoso, C.I.G.; Jerković, I.; Maldini, M.; Serreli, G. Phenolic compounds, antioxidant activity, and other characteristics of extra virgin olive oils from Italian autochthonous varieties *Tonda di Villacidro, Tonda di Cagliari, Semidana,* and *Bosana. Journal of Chemistry*, 1-8 (2016).

29) Gawel, R.; Rogers, D.A.G. The relationship between total phenol concentration and the perceived style of extra virgin olive oil. *Grasas y Aceites* **60**, 134-138 (2009).

30) Kiritsakis, A.K. Flavor components of olive oil—A review. *J. Am. Oil Chem. Soc.* **75**, 673-681 (1998).

31) García, J.M.; Yousfi, K.; Mateos, R.; Olmo, M.; Cert, A. Reduction of oil bitterness by heating of olive (*Olea europaea*) fruits. *J. Agric Food Chem.* **49**, 4231-4235 (2001).

32) Soler-Rivas, C.; Espín, J.C.; Wichers, H.J. Oleuropein and related compounds. *J. Sci. Food Agric.* **80**, 1013-1023 (2000).

33) Gutiérrez-Rosales, F.; Perdiguero, S.; Gutiérrez, R.; Olias, J.M. Evaluation of the bitter taste in virgin olive oil. *J. Am. Oil Chem. Soc.* **69**, 394-395 (1992).

34) Tovar, M.J.; Motilva, M.J.; Romero, M.P. Changes in the Phenolic Composition of Virgin Olive Oil from Young Trees (*Olea europaea* L. cv. Arbequina) Grown under Linear Irrigation Strategies. *J. Agric. Food Chem.* **49**, 5502-5508 (2001).

35) Rice-Evans, C.A.; Miller, N.J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **20**, 933-956 (1996).

36) Fukumoto, L.R.; Mazza, G. Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem.* **48**, 3597-3604 (2000).

37) Lotito, S.B.; Frei, B. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: Cause, consequence, or epiphenomenon? *Free Radical Biol. Med.* **41**, 1727-1746 (2006).

38) Cicerale, S.; Lucas, L.; Keast, R. Biological activities of phenolic compounds present in virgin olive oil. *Int. J. Mol. Sci.* **11**, 458-479 (2010).

39) Laincer, F.; Laribi, R.; Tamendjari, A.; Arrar, L.; Rovellini, P.; Venturini, S. Olive oils from Algeria: Phenolic compounds, antioxidant and antibacterial activities. *Grasas y Aceites* **65**, e001 (2014).

40) Gouvinhas, I.; Machado, J.; Gomes, S.; Lopes, J.; Martins-Lopes, P.; Barros, A.I.R.N.A. Phenolic composition and antioxidant activity of monovarietal and commercial Portuguese olive oils. *J. Am. Oil Chem. Soc.* **91**, 1197-1203 (2014).

41) Youssef, O.; Guido, F.; Daoud, D.; Mokhtar, Z. Effect of cultivar on minor components in Tunisia olive fruits cultivated in microclimate. *J. Hortic. For.* **3**, 13-20 (2011).

42) Ebrahimi, N.; Jahed-Khaniki, G.; Keshavarz, S.; Shariatifar, N.; Nabizadeh, R.; Sadighara, P. Spectrophotometric method for quantification of flavonoids in olive oil supplied from Tehran market of Iran. *Journal of Food Safety and Hygiene* **1**(2), 59-62 (2015).

43) Ammar, S.; Zribi, A.; Ben Mansour, A.; Ayadi, M.; Ab-

delhedi, R.; Bouaziz, M. Effect of processing systems on the quality and stability of Chemlali olive oils. *J. Oleo Sci.* **63**, 311-323（2014）.

44）Montedoro, G.; Servili, M.; Baldioli, M.; Miniati, E. Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. *J. Agric. Food Chem.* **40**, 1571-1576（1992）.

45）Tsimidou, M.; Lytridou, M.; Boskou, D.; Pappa-Louisi, A.; Kotsifaki, F.; Petrakis, C. On the determination of minor phenolic acids of virgin olive oil by RP-HPLC. *Grasas y Aceites,* **47**（3）, 151-157（1996）.

46）Servili, M.; Selvaggini, R.; Esposto, S.; Taticchi, A.; Montedoro, G.; Morozzi, G. Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. *J. Chromatogr. A* **1054**, 113-127（2004）.

47）López, M.J.O.; Innocenti, M.; Ieri, F.; Giaccherini, C.; Romani, A.; Mulinacci, N. HPLC/DAD/ESI/MS detection of lignans from Spanish and Italian *Olea europaea* L. fruits. *J. Food Comp. Anal.* **21**, 62-70（2008）.

48）Rovellini, P.; Cortesi, N.; Fedeli, E. Analisis of flavonoids from *Olea europaea* by HPLC-UV and HPLC-electrospray-MS. *Rivista Italiana Delle Sostanze Grasse* **74**, 273-279（1997）.

49）Meirinhos, J.; Silva, B.M.; Valentão, P.; Seabra, R.M.; Pereira, J.A.; Dias, A.; Andrade, P.B.; Ferreres, F. Analysis and quantification of flavonoidic compounds from Portuguese olive（*Olea europaea* L.）leaf cultivars. *Nat. Prod. Res.* **19**, 189-195（2005）.

50）Montedoro, G.; Baldioli, M.; Selvaggini, R.; Begliomini, A.; Taticchi, A.; Servili, M. Relationships between phenolic composition of olive fruit and olive oil: The importance of the endogenous enzymes. *Acta Hortic.* **586**, 551-556（2002）.