

Article

A First Description of the Phenolic Profile of EVOOs from the Maltese Islands Using SPE and HPLC: Pedeo-Climatic Conditions Modulate Genetic Factors

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Abstract: Achieving economic sustainability in the olive oil production sector is a challenge. This is particularly so for small scale producers who are faced with pressing, production and marketing costs that relative to overall sales, minimise profits. In this study we aimed to describe the phenolic profile of extra virgin olive oils (EVOOs) derived from the Maltese islands. The polar fractions from EVOOs from nine indigenous (six Bidni and three Malti), one historically acclimatized tree (Bajda), 12 locally-grown but foreign cultivars and 32 foreign EVOOs were extracted using SPE (solid phase extraction), separated using HPLC analysis at 280 nm and 320 nm and identified using mass spectrometry. Application of ANOVA and Tukey post hoc hypothesis testing for analysis of variance on the peak areas identified a significantly higher concentration of p-coumaric acid, tyrosol acetate, DHPEA-EDA and oleocanthal in EVOOs derived from indigenous or historically acclimatized cultivars. Imported but locally grown cultivars showed differences when compared to the same cultivar grown in other countries, confirming that pedo-climatic conditions modulate genetic factors.

Keywords: Maltese islands; olive culture; sustainability; phenolic compounds; extra virgin olive oils

1. Introduction

The inhabited Maltese Islands consist of a set of three small islands, just under 316 km² in area (Malta 247.5 km², Gozo 67.1 km² and Comino 2.8 km²), isolated in the central Mediterranean Sea [1]. Typical of the region bordering the Mediterranean basin, the islands exhibit conditions favorable for olive (*Olea europaea* L. subsp. *europaea*) cultivation. Historical records and cultural references abound, with olive pollen having been reported in samples from the Neolithic and Phoenician periods [2], a Bronze Age pit [3] as well as from a detailed study of a core from the Burmarrad region [4]. Remnants of olive cultivation may be evidenced by the remains of ‘rustic villas’ where processing of olives occurred during the Roman occupation [5], while today towns and valleys still bear place-names associated with olives and cultivation, e.g., Zejtun, Zebbug and Wied Ghajn Zejtuna. Despite the optimal geo-climatic conditions on the islands, down-sizing and abandonment of the sector in response to rationalization of land use for maximal financial or agronomic returns has been a feature of the industry [6].

The local olive culture industry offers an interesting case-study as the revival may be traced to a recent commercial initiative, the Project for the Revival of the Indigenous Olive (PRIMO). This focused on the grafting of the ‘Bidni’ tree where 30,000 trees were subsequently planted. The ‘Bidni’ variety is named after the place where the trees were found growing (Bidnija, Malta). The tree shows vigorous growth and is characterized by a small, almost elliptical, violet drupe and is markedly resistant to the fruit-fly *Bactrocera oleae*. The small-sized drupe is difficult to harvest but results in an oil of excellent

quality [6]. Other trees that form part of the nascent industry are the Maltese olive 'Malti' and the white olive or 'Bajda'. The former shows vigorous growth, with a violet drupe that is also elliptical but blunt at the lower extremity. The tree is highly productive and the drupe is used both for oil and table purposes. The 'Bajda' olive tree is smaller and produces a pale green drupe and is mostly used for the table. The project PRIMO has served as a strong impetus and over the last two decades, olive trees such as Carolea, Frantoio, Leccino, Pendolino and the table varieties Uovo di Piccione and Bella di Spagna have become widely cultivated [7]. Currently olive groves cover a total of 128.5 hectares [7] equivalent to 8.88% of permanent crop cover or 1.3% of total agricultural land.

Achieving economic sustainability for the olive oil sector is a challenge [8–10], but more so locally, given the low availability of agricultural land for olive tree cultivation and the severe conflict over land use. Absorption of production, harvesting, pressing and marketing costs from a small volume of high quality olive oil to generate sustainable profits is also difficult. A niche market based on strong product valorization may permit premium olive oil prices and help to offset some of the additional costs identified above. Honojosa-Rodriguez et al. [10] argues that reduction of production cost is also an essential step and that a strategy focused on achieving quality can help to achieve 'competitive and marketing advantages'.

The ability to distinguish olive cultivars is a critical preliminary step for traceability, valorization and breeding programs [11] where genetic sequences are often favored over biochemical markers as they are independent of terroir. DNA based methods are effective in allowing a traceability chain to be established between tree and oil [12]. In a first approach, random amplified polymorphic DNA (RAPD) and microsatellite analysis [11,13] were used to identify marker sequences specific to local varieties. Both studies showed that indigenous olive trees occupied a distinct group. Presently further studies using a larger number of local varieties are ongoing. As yet, there are no papers in the published domain describing the organoleptic, biochemical and nutritional characteristics of Malta-derived olive oils with the exception of a published description of their antioxidant properties [14]. Characterization of these properties would help to identify uniqueness, while preventing adulteration or mislabeling of local extra virgin olive oils (EVOOs) while ensuring the growth of the local industry.

Several methods have been developed to characterize phenols from olive oils including high pressure liquid chromatography (HPLC) separation in conjunction with UV-vis, mass spectrometry (MS) and nuclear magnetic resonance (NMR) [15–18]. Extraction of phenolic compounds may be achieved using liquid–liquid extraction (LLE) or solid phase extraction (SPE) [19]. LLE is reported to yield a higher recovery of secoiridoids under certain conditions, but is more laborious and uses higher volumes [19]. SPE methods are well characterized and include conditioning of cartridges with hexane, application of small volumes of olive oil, followed by washing with hexane to remove non polar fractions and elution with methanol or acetonitrile [20,21]. Many separation methods on HPLC have been described using a variety of solvents and separation profiles [22–24].

In this study the aim was to describe the phenolic profile of Maltese-derived extra virgin olive oils (EVOOs), in so doing leading to future variable-characterization that may help to establish their authenticity and reducing the potential for adulteration with cheaper oils (both olive and seed oils) or mislabeling by foreign imported oil.

2. Method

2.1. EVOO Sample Collection and Extraction

In this paper specific metabolites belonging to the polyphenolic class of compounds that could be used as biochemical markers were studied. The EVOOs for this study were derived from nine indigenous (six Bidni and three Malti), one historically acclimatized tree (Bajda) and 12 locally-grown but foreign cultivars. Indigenous and foreign but locally grown EVOOs were collected directly from olive presses following expert identification of drupes between late September to November of the harvest seasons 2014–2016 (bar 1 sample dating to 2013). Thirty-two foreign EVOOs were also

purchased over the same time period 2014–2016 and included in the study (Table S1). These oils were sought with the demarcation EVOO as well as the protected designation of origin (PDO) in order to ensure traceability of the product. Samples were collected from different oil producers so as to cover a representative sample of the Maltese islands in terms of pedological conditions and pressing equipment, all olive presses used cold press technique. EVOOs were stored at 4 °C in the absence of light prior analysis, i.e., stored using standard methods in line with methods used in published papers where such methods are recorded, to assure the integrity of the analysis, then preheated to 35 °C in a water bath for an hour and mixed to ensure homogeneity. Phenolic compounds from monocultivar EVOOs were extracted using solid phase extraction (SPE) on diol-bonded phase cartridges (Analytical Columns SolGel-1ms™, Croydon, England). Of EVOO samples 3.5 g were applied under vacuum (not more than 15 mmHg) to the cartridge, washed with hexane to remove apolar components (2 × 6 mL) and twice with hexane/ethyl acetate (85:15, *v/v*; 8 mL) based on the method described in Mateos et al. (2001) [19]. The phenolic fraction was eluted with methanol (20 mL) filtered and concentrated to half of the initial volume under vacuum and then to dryness using a nitrogen evaporator at 45 °C to ensure complete removal of solvent.

2.2. Polyphenol Separation

All solvents were HPLC grade. HPLC analysis was carried out on an HPLC (Waters, Milford, MA, USA) using a 20 µL injection volume on a Symmetry® C18 analytical column (250 × 4.6 mm i.d.) with a particle size of 5 µm (Woodford, Santry, Dublin, Ireland). The mobile phases were degassed and consisted of (A) water: Acetic acid (98:2, *v/v*) and (B) methanol: Acetonitrile (1:1, *v/v*) at a constant flow rate of 1 mL/min. Elution was performed using the following solvent program: 80% (A): 20% (B) 0–30 min; 70% (A): 30% (B) 30–45 min; 50% (A): 50% (B) 45–55 min; 40% (A): 60% (B) 55–65 min; 100% (B) 65–75 min and then 80% (A): 20% (B) for the final 4 min as a post-equilibration step. The column temperature was set to 35 °C whilst the sample chamber was set to 10 °C to prevent phenolic degradation. UV detection was carried out at 280 nm and 320 nm. Compounds were identified using peak identification through retention time followed by analysis of molecular fragments by MS. Method transfer from HPLC was carried to UPLC MS/MS.

2.3. UPLC MS/MS Analysis

An *m/z* scan was carried out for every eluting peak, which allowed detection of the parent molecular ion. For each of these a separate multiple reaction monitoring (MRM) experiment to identify the most stable fragment followed using IntelliStart™ function in MassLynx™ (Waters, Milford, MA, USA). The UPLC system consisted of an AcQuity™ UPLC equipped with Waters binary pump system (Milford, MA, USA) using an AcQuity UPLC™ BEH C18 column (1.7 µm, 100 mm × 2.1 mm i.d.). During the analysis, 1.7 µL of sample was injected, the column was kept at 35 °C and the flow rate was 0.2 mL/min using 2% acetic acid as solvent A and 1:1 methanol/acetonitrile as solvent B. The elution program was as follows 80% (A): 20% (B; 0 min); 80% (A): 20% (B; 5.5 min); 70% (A): 30% (B; 6.5 min); 50% (A): 50% (B; 20 min); 40% (A): 60% (B; 26 min); 25% (A): 75% (B; 30 min) and 80% (A): 20% (B; 31.6 min).

The UPLC system was coupled to a TUV detector AcQuity UPLC™ and a TQDTM mass spectrometer equipped with a Z-spray electrospray ionization (Waters, Milford, MA, USA). The software used was MassLynx 4.1. Ionization was performed by electro-spray (ESI) in the negative mode and the data was collected in the selected ion recording mode (SIR) mode.

For the detection and identification of phenolic compounds present in olive oils, a phenolic sample was infused at a rate of 50 µL/min for 10 min. Nitrogen was used as the nebulizing and desolvation gas. The MS conditions were capillary potential 3.0 kV, extractor cone voltage 30 V, RF lens voltage 0.1 V, source temperature 130 °C, desolvation temperature 300 °C and desolvation gas flow rate 500 L/h. Data were collected by use of the MassLynx 4.0 software resident in a personal computer. In order to confirm the identity of the compounds MRM transitions parameters were optimized by using the IntelliStart

tool of the MassLynx software, which consisted in automatically detecting the major fragments and optimizing cone voltages and collision energies.

3. Results

The phenolic profile of indigenous Maltese EVOOs (Bidni, Malti) and foreign but locally grown EVOOs is presented, where a summary of the peaks identified using SPE followed by HPLC analysis at 280 nm and 320 nm and MS are presented in Table 1 (see also Supplementary Material Figure S1 and Table S2). A total of 32 peaks were observed where p-coumaric acid (peak 6), Tyrosol acetate (peak 10), DHPEA_EDA (peak 15), oleocanthal (peak 17) and an unknown, possibly an aldehydic form of oleuropein glycone (peak 28) were identified in EVOOs derived from indigenous or locally grown but foreign trees. Conversely, phenolic acids such as p-hydroxybenzoic acid, o-coumaric acid and syringic acid were present either at very low or undetectable concentrations. With respect to the Italian EVOOs luteolin (peak 20), followed by pinoresinol (peak 19) and an unknown, possibly decarboxymethyl-ligstroside aglycone (peak 21) featured prominently, while in the French EVOOs an unknown compound possibly 10-hydroxy-oleuropein aglycone (peak 26) and another unknown compound, possibly an isomer of the former compound (peak 27) were distinguished. Pinoresinol (peak 19) and the oleuropein aglycone 3,4 DHPEA-EA were noticeable in the Greek EVOOs at 320 nm. Similarly peak 23 identified as a possible hydroxy-decarboxymethyl-ligstroside aglycone was observed in the Spanish EVOOs.

Table 1. Peak number, retention times and phenolic compounds at 280 nm.

Peak Number	Retention Time (RT)	Phenolic Compound
Peak 1	3.0–3.45	Gallic Acid
Peak 2	4.0–4.27	Hydroxytyrosol
Peak 3	4.9–5.06	Protocatechuic acid
Peak 4	5.3–5.9	Tyrosol
Peak 5	7.0–7.51	Syringic Acid
Peak 6 *	8.89–9.0	p-coumaric acid
Peak 7	10.9–11.5	Vanillin
Peak 8	11.8–12.2	o-coumaric acid
Peak 9	13.1–13.6	Ferulic acid
Peak 10 *	14.8–15.2	Tyrosol Acetate
Peak 11	17.5–18.0	Hydroxytyrosol Acetate
Peak 12	19.2–19.9	3-methoxycinnamic acid
Peak 13	24.9–25.5	Unk1 Verbascoside/dialdehydic form of oleuropein aglycone
Peak 14	39.1–41.0	Oleuropein
Peak 15 *	42.8–43.1	3,4 DHPEA-EDA
Peak 16 *	44.7–45.5	Unk2 Hydroxy-decarboxymethyl oleuropein aglycone
Peak 17 *	47.3–47.8	Oleocanthal
Peak 18	48.9–49.6	Cinnamic Acid/Acetoxypinoresinol
Peak 19	51.7–52.3	Pinoresinol
Peak 20	53.1–53.5	Luteolin
Peak 21	53.8–54.1	Unk3 Decarboxymethyl-ligstroside aglycone
Peak 22	54.5–55.5	3,4 DHPEA-EA
Peak 23	56.7–57.3	Unk4 Hydroxy-decarboxymethyl-ligstroside aglycone
Peak 24	57.6–58.3	p-HPEA-EA
Peak 25	59.0–61.0	Apigenin
Peak 26	61.5–62.1	Unk7 10-Hydroxy-oleuropein aglycone
Peak 27	62.5–62.8	Unk8
Peak 28 *	63.4–64.3	Unk9 Aldehydic form of oleuropein aglycone
Peak 29	65.5–66.6	Unk10
Peak 30–32	42.8–43.1	3,4 DHPEA-EDA and its oxidized forms

* Marked presence in indigenous and foreign but locally grown trees.

A univariate statistical analysis on the distinct peak areas showed that EVOOs derived from locally grown cultivars and indigenous cultivars had a significantly higher concentration of the polyphenol *p*-coumaric acid (peak 6, significant at the 95% confidence level) when compared to EVOOs derived from other Mediterranean countries (Table 2). Likewise, application of an ANOVA and Tukey post hoc hypothesis testing for the analysis of variance on the peak areas of tyrosol acetate (peak 10) showed a significantly higher level in EVOOs derived from indigenous or historically acclimatized cultivars such as Bidni, Malti and Bajda, when compared to EVOOs derived from foreign varieties. Similar results were also obtained on comparing DHPEA-EDA (peak 15), oleocanthal (*p*-HPEA-EDA) (peak 17) and an aldehydic form of oleuropein aglycone (peak 28).

Table 2. Peak areas and their corresponding compounds, which were found to vary significantly under an ANOVA statistical test across the three classes of extra virgin olive oils (EVOOs) studied.

	Peak6 <i>p</i> -Coumaric Acid	Peak10 Tyrosol Acetate	Peak15 3,4 DHPEA-EDA	Peak17 Oleocanthal <i>p</i> -HPEA-EDA	Peak28 Unk9
Indigenous (<i>n</i> = 9)	$3.81 \times 10^5 \text{ a} \pm$ 3.54×10^4	$1.20 \times 10^5 \text{ a} \pm$ 8.75×10^4	$2.62 \times 10^6 \text{ a} \pm$ 4.81×10^4	$1.56 \times 10^6 \text{ a} \pm$ 3.28×10^4	$1.00 \times 10^6 \text{ a} \pm$ 2.25×10^4
Foreign but locally grown (<i>n</i> = 12)	$9.80 \times 10^5 \text{ b} \pm$ 6.71×10^4	$3.57 \times 10^4 \text{ c} \pm$ 3.87×10^4	$4.27 \times 10^6 \text{ b} \pm$ 5.37×10^4	$1.37 \times 10^5 \text{ c} \pm$ 1.14×10^4	$6.26 \times 10^5 \text{ b} \pm$ 4.87×10^4
Foreign (<i>n</i> = 32)	$9.30 \times 10^4 \text{ a} \text{ *} \pm$ 1.13×10^4	$2.95 \times 10^4 \text{ c} \pm$ 2.63×10^4	$1.16 \times 10^6 \text{ a} \pm$ 6.52×10^4	$1.23 \times 10^5 \text{ c} \pm$ 1.02×10^5	$2.84 \times 10^5 \text{ b} \pm$ 1.70×10^5

Superscript letters in the same column represent statistically distinct homogeneous subsets as determined by an ANOVA and post hoc Tukey analysis at a 5% confidence level, same letters followed by an * indicate homogeneous subsets at a 10% confidence level.

4. Discussion

In this paper a first description of the phenolic profile of Maltese derived EVOOs using SPE and HPLC is presented. Phenolic profiles of Maltese EVOOs (both indigenous and foreign but locally grown) derived during the three harvest seasons of 2014–2016 were studied. 3,4 DHPEA-EDA, and oleocanthal (*p*HPEA-EDA) were markedly present in the indigenous EVOOs rather than in the foreign varieties. As these EVOOs were extracted using the same mechanical and recovery processes, the presence of these compounds appears to reflect a marked occurrence in the indigenous EVOOs. The tyrosol/hydroxytyrosol ratio was observed to be similar to EVOOs from other Mediterranean countries for other varieties [25]. The presence of these phenols is attributed to the partial hydrolysis of their derivatives, while the higher antioxidant activity of hydroxytyrosol when compared to tyrosol make it more susceptible to degradation [26]. The presence of pinoreosinol was not noticeable, while trans-cinnamic acid was observed to co-elute with 1-acetoxypinoreosinol. These compounds are the main components of the phenolic fraction, which is derived from olive seed and are noticeably absent from the drupe pulp [27]. Hence their presence in oil reflects crushing and admixing of the olive pits during the crushing process rather than inherent variations in the drupe and hence in the EVOO.

Direct comparison with the foreign EVOOs is difficult either because the phenolic profile for a particular EVOO is not available in the published literature or due to differences in the extraction processes, separation methods and elution profiles used. For example the UV detection in this study was carried out at 280 nm and 320 nm in line with the absorption of polyphenols. In this study the Italian EVOOs were rich in the flavonoid luteolin that is known to increase with fruit ripening and pinoreosinol but these latter phenolic compounds were negligibly present in local EVOOs. The relatively low levels of oleuropein in all EVOOs tested are correlated to the action of β -glucosidases that result in the formation of secoiridoid aglycones.

The results confirm the organoleptic qualities reported for certain Maltese EVOOs. The presence of the phenolic compound in Table 1 such as oleocanthal supports the known pungent, burning sensation associated with the Bidni variety [23].

Notably the HPLC chromatograms of foreign and indigenous/local EVOOs (Figure 1) showed diversity in terms of a quantitative variation in their phenolic profile rather than a qualitative one (for example tyrosol acetate, p-coumaric acid). Variations in polyphenol expression may arise due to the harvesting period and ripeness, which reportedly affects an increase in hydroxytyrosol, tyrosol and luteolin, the extraction system [28], oxygen concentration and temperature during the malaxation process [29–32], removal of stones [33] storage conditions [34] and not least irrigation regimes [35]. However there are important factors that affect the phenolic fingerprint and are independent of processing, such as the combination of climate and pedological factors [36] as well as cultivar genotype. Compounds such as 3,4 DHPEA-EA, the aldehydic form of oleuropein aglycone and particularly DHPEA-EDA are produced by the activation of endogenous β -glucosidases the expression of which reflects cultivar genotype. These compounds that have been ascribed protective functions and contribute to EVOO bitterness [37–39] were markedly present in both indigenous and foreign but local varieties. As these phenolic compounds were recorded across indigenous as well as in foreign but locally derived EVOOs (e.g., Carolea) this indicates that the pedo-climatic conditions contributed strongly to the expressed phenolic phenotype, particularly since the ripening stages, i.e., the harvesting period, the conditions of oil extraction (uniquely due to few oil presses) and irrigation treatment based on a low water regime typical for the arid island were uniform [39]. Interestingly low water levels are correlated with oils with increases in DHPEA-EDA and 3,4 DHPEA-EA as well as elevated bitterness and pungency and this may explain their marked presence in indigenous and acclimatized EVOOs [39]. Ultimately the presence of these biochemical markers in the locally derived EVOOs offers an encouraging indication that a tool derived from variable data derived from multiple separation and structural pattern recognition techniques in conjunction with DNA markers may be possible [39–41]. This will have important implications on developing methods to ensure authenticity and allowing sustainability contribution of the olive industry to the economy.

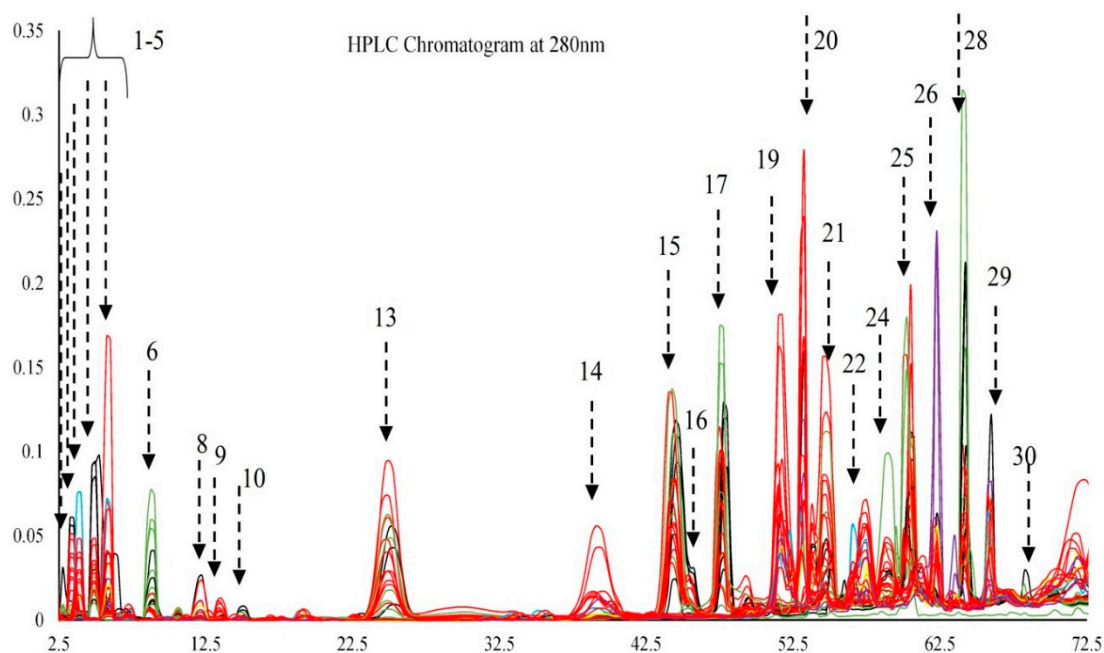


Figure 1. Cont.

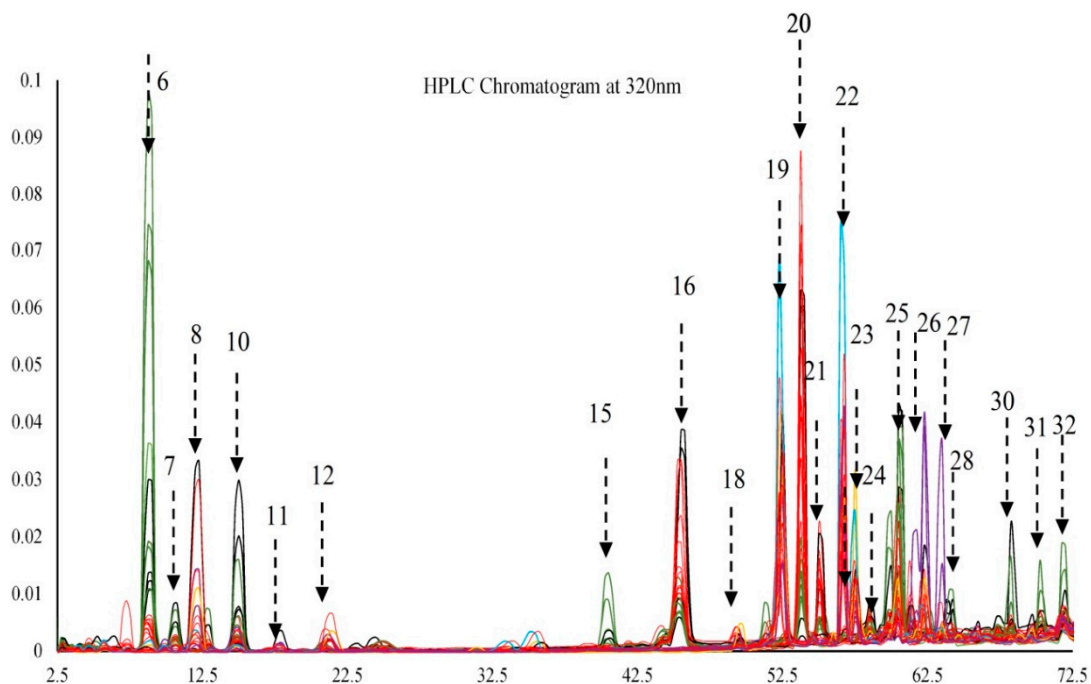


Figure 1. Phenolic chromatogram observed at 280 nm and 320 nm obtained for all the monocultivar EVOOs studied. Different colors represent olive oils derived from different geographical origin. Black = indigenous Maltese cultivars; green = foreign cultivars that are locally grown; red = Italian cultivars; blue = Greek origin; purple = French origin and yellow = Spanish origin.

5. Conclusions

This paper achieves a profile based on the separation of polyphenols in EVOOs; for both indigenous and foreign but locally derived EVOOs from the Maltese islands. Evidence that ultimately certain polyphenols were dependent on the pedo-climatic conditions and not solely on the genetic component of the originating olive tree is also presented.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2077-0472/9/5/107/s1>, Figure S1: The recorded selected ions pertaining to different compounds, Table S1: The cultivars used in this study and their country of origin, Table S2: MRM method development for the most common minor compounds present in EVOO.

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Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Cassar, L.; Conrad, E.; Schembri, P.J. The Maltese Archipelago. In *Mediterranean Island Landscapes: Natural and Cultural Approaches*; Vogiatzakis, I.N., Pungetti, G., Mannion, A.M., Eds.; Springer: Dordrecht, The Netherlands, 2008; pp. 297–322.
2. Fenech, K. *Human-Induced Changes in the Environment and Landscape of the Maltese Islands from the Neolithic to the 15th Century AD as Inferred from a Scientific Study of Sediments from Marsa, Malta*; BAR International Series 1682; Archaeopress: Oxford, UK, 2007; p. 155.
3. Godwin, H. Appendix I, Report on a Pollen Sample from the Late Borg in-Nadur Cistern at Tal-Mejtin. In *Report on the Working of the Museum Department for the Year 1961*; Department of Information: Valletta, Malta, 1961; p. 8.

4. Gambin, B.; Andrieu-Ponel, V.; Médail, F.; Marriner, N.; Peyron, O.; Montade, V.; Gambin, T.; Morhange, C.; Belkacem, D.; Djamali, M. 7300 Years of vegetation history and climate for nw malta: A holocene perspective. *Clim. Past* **2016**, *12*, 273–297. [[CrossRef](#)]
5. Bonanno, A. *Malta Phoenician, Punic and Roman*; Midsea Books Ltd.: Santa Venera, Malta, 2005.
6. Borg, J. *Cultivation and Diseases of Fruit Diseases in the Maltese Islands*; Malta Government Printing Press: Marsa, Malta, 1922.
7. National Agricultural Policy 2018–2028, Parliamentary Secretary for Agriculture, Fisheries and Animal Rights. Available online: <https://agriculture.gov.mt/en/agric/Pages/nationalAgriPolicy.aspx> (accessed on 5 February 2019).
8. Gomez-Limon, J.; Riesgo, J. Sustainability assessment of olive groves in Andalusia: A methodological proposal. *New Medit* **2012**, *11*, 39–49.
9. Menozzi, D. Extra-virgin olive oil production sustainability in northern Italy: A preliminary study. *Br. Food J.* **2014**, *116*, 1942–1959. [[CrossRef](#)]
10. Hinojosa-Rodriguez, A.; Parra-Lopez, C.; Carmona-Torres, C.; Sayadi, S. Protected Designation of Origin in the olive growing sector: Adoption factors and goodness of practices in Andalusia, Spain. *New Medit* **2014**, *13*, 2–12.
11. di Rienzo, V.; Sion, S.; Taranto, F.; D’Agostino, N.; Montemurro, C.; Fanelli, V.; Sabetta, W.; Boucheffa, S.; Tamendjari, A.; Pasqualone, A.; et al. Genetic flow among olive populations within the Mediterranean basin. *PeerJ* **2018**, *6*, e5260. [[CrossRef](#)] [[PubMed](#)]
12. Pasqualone, A.; Montemurro, C.; di Rienzo, V.; Summo, C.; Paradiso, V.; Caponio, F. Evolution and perspectives of cultivar identification and traceability from tree to oil and table olives by means of DNA markers. *J. Sci. Food Agric.* **2016**, *96*, 3642–3657. [[CrossRef](#)] [[PubMed](#)]
13. 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. Evolut.* **2014**, *62*, 1021–1027. [[CrossRef](#)]
14. Lia, F.; Zammit-Mangion, M.; Farrugia, C. Determination of antioxidant activity of olive oil phenolic extracts. In Proceedings of the ISANH 9th World Congress on Polyphenols, St Julians, Malta, 30 September 2015.
15. Karkoula, E.; Skantzari, A.; Melliou, E.; Magiatis, P. Direct measurement of Major Secoiridoid Derivatives in Olive Oil by Quantitative ¹H NMR. Establishment of a New Index for the Characterisation of Extra Virgin Olive Oils. *J. Agric. Food Chem.* **2012**, *60*, 11696–11700. [[CrossRef](#)]
16. Karkoula, E.; Skantzari, A.; Melliou, E.; Magiatis, P. Quantitative Measurement of Major Secoiridoid Derivatives in Olive Oil Using qNMR. Proof of the Artificial Formation of Aldehydic Oleuropein and Ligstroside Aglycon Isomers. *J. Agric. Food Chem.* **2014**, *62*, 600–607. [[CrossRef](#)]
17. Hrnčirik, K.; Fritsche, S. Comparability and reliability of different techniques for the determination of phenolic compounds in virgin olive oil. *Eur. J. Lipid Sci. Technol.* **2004**, *106*, 540–549. [[CrossRef](#)]
18. Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A.; Montedoro, G. High-performance liquid chromatography evaluation of phenols in olive fruit, virgin olive oil, vegetation waters, and pomace and 1D- and 2D-nuclear magnetic resonance characterization. *J. Am. Oil Chem. Soc.* **1999**, *76*, 873–882. [[CrossRef](#)]
19. Mateos, R.; Espartero, J.; Trujillo, J.; León-Camacho, M.; Alcludia, 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.* **2001**, *49*, 2185–2192. [[CrossRef](#)] [[PubMed](#)]
20. Bendini, A.; Bonoli, M.; Cerretani, L.; Biguzzi, B.; Lercker, G.; Gallina, T. Liquid-Liquid and Solid Phase Extractions of Phenols from Virgin Olive oil and their Separation by Chromatographic and Electrophoretic Methods. *J. Chromatogr. A* **2003**, *985*, 425–433. [[CrossRef](#)]
21. Pirisi, F.; Cabras, P.; Falqui, C.; Migliorini, M.; Mugelli, M. Phenolic Compounds in Virgin Olive Oil. Reappraisal of the Extraction, HPLC Separation and Quantification Procedures. *J. Agric. Food Chem.* **2000**, *48*, 1191–1196. [[CrossRef](#)] [[PubMed](#)]
22. Bakhouch, A.; Lozano-Sánchez, J.; Beltrán-Debón, R.; Joven, J.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Phenolic characterization and geographical classification of commercial Arbequina extra-virgin olive oils produced in southern Catalonia. *Food Res. Int.* **2013**, *50*, 401–408. [[CrossRef](#)]

23. Gutierrez-Rosales, F.; Rios, J.J.; Gomez-Rey, M.L. Main Polyphenols in the Bitter Taste of Virgin Olive Oil. Structural Confirmation by On-Line High Performance Liquid Chromatography Electrospray Ionisation Mass Spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 6021–6025. [[CrossRef](#)] [[PubMed](#)]
24. De la Torre-Carbot, K.; Jauregui, O.; Gimeno, E.; Catellote, A.; Lamuela-Raventos, R.; Lopez-Sabater, M. Characterisation and Quantification of Phenolic Compounds in Olive Oils by Solid-Phase Extraction, HPLC-DAD and HPLC-MS/MS. *J. Agric. Food Chem.* **2005**, *53*, 4331–4340. [[CrossRef](#)] [[PubMed](#)]
25. Tsimidou, M.; Lytridou, M.; Boskou, D.; Pappa-Louisi, F.; Kotsifaki, A.; Petrakis, C. On the determination of minor phenolic acids of virgin olive oils by RP-HPLC. *Gracas Y Aceites* **1996**, *47*, 151–157. [[CrossRef](#)]
26. Baldioli, M.; Servili, M.; Perretti, G.; Montedoro, G. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. *J. Am. Oil Chem. Soc.* **1993**, *73*, 1589–1593. [[CrossRef](#)]
27. Owen, R.; Mier, W.; Giacosa, A.; Hull, W.; Spiegelhalder, B.; Bartsch, H. Olive oil consumption and health, the possible role of antioxidants. *Food Chem. Toxicol.* **2000**, *38*, 647–659. [[CrossRef](#)]
28. Amarinte, P.; Catalano, R.; Amarinte, G.; Montel, G.; Dugo, V.; Lo Turco, L.; Baccioni, D.; Fazio, A.; Mattei, A.; Maretti, F. Estrazione da paste denocciolate. *Olivo Olio* **2001**, *43*, 48–55.
29. 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* **2004**, *1054*, 113–127. [[CrossRef](#)]
30. Servili, M.; Esposto, S.; Fabiani, R.; Urbani, S.; Taticchi, A.; Mariucci, F.; Selvaggini, R.; Montedoro, G.F. Phenolic compounds in olive oil: Antioxidant, health and organoleptic activities according to their chemical structure. *Inflammopharmacology* **2009**, *17*, 76–84. [[CrossRef](#)] [[PubMed](#)]
31. Taticchi, A.; Esposto, S.; Veneziani, G.; Urbani, S.; Selvaggini, S.; Servili, M. The influence of the malaxation temperature on the activity of polyphenoloxidase and peroxidase and on the phenolic composition of virgin olive oil. *Food Chem.* **2013**, *136*, 975–983. [[CrossRef](#)]
32. Servili, M.; Taticchi, A.; Esposto, S.; Urbani, S.; Selvaggini, R.; Montedoro, G. Influence of the decrease in oxygen during malaxation of olive paste on the composition of volatiles and phenolic compounds in virgin olive oil. *J. Agric. Food Chem.* **2008**, *56*, 10048–10055. [[CrossRef](#)] [[PubMed](#)]
33. Servili, M.; Taticchi, A.; Esposto, S.; Urbani, S.; Selvaggini, R.; Montedoro, G. Effect of olive stoning on the volatile and phenolic composition of virgin olive oil. *J. Agric. Food Chem.* **2007**, *55*, 7028–7035. [[CrossRef](#)] [[PubMed](#)]
34. Morelló, J.R.; Motilva, M.J.; Tovar, M.J.; Romero, M.P. Changes in commercial virgin olive oil (cv Arbequina) during storage, with special emphasis on the phenolic fraction. *Food Chem.* **2004**, *85*, 357–364. [[CrossRef](#)]
35. Famiani, F.; Proietti, P.; Farinelli, D.; Tombesi, A. Oil Quality in Relation to Olive Ripening. *Acta Hort.* **2002**, *586*, 671–674. [[CrossRef](#)]
36. Ranalli, A.; Mattia, G.; Pattumi, M.; Proietti, P. Quality of virgin olive oil as influenced by origin area. *Grasas Y Aceites* **1999**, *50*, 249–259. [[CrossRef](#)]
37. Sindona, G.; Caruso, A.; Cozza, A.; Fiorentini, S.; Lorusso, B.; Marini, E.; Nardi, M.; Procopio, A.; Zicari, S. Anti-Inflammatory Effect of 3,4-DHPEA-EDA [2-(3,4 -Hydroxyphenyl) ethyl (3S, 4E)-4-Formyl-3-(2-Oxoethyl)Hex-4-Enoate] on Primary Human Vascular Endothelial Cells. *Curr. Med. Chem.* **2012**, *19*, 4006–4013. [[CrossRef](#)]
38. Gill, C.; Boyd, A.; McDermott, E.; McCann, M.; Servili, M.; Selvaggini, R.; Taticchi, A.; Esposto, S.; Montedoro, G.; McGlynn, H.; et al. Potential anti-cancer effects of virgin olive oil phenols on colorectal carcinogenesis models in vitro. *Int. J. Cancer* **2005**, *117*, 1–7. [[CrossRef](#)] [[PubMed](#)]
39. Campestre, C.; Angelini, G.; Gasbarri, C.; Angerosa, F. The compounds responsible for the sensory profile in monovarietal virgin olive oils. *Molecules* **2017**, *22*, 1833. [[CrossRef](#)] [[PubMed](#)]
40. Ben-Ayed, R.; Kamoun-Grati, N.; Rebai, A. An Overview of the Authentication of Olive Tree and Oil. *Compr. Rev. Food Sci. Food Saf.* **2013**, *12*, 218–227. [[CrossRef](#)]
41. Aparicio, R.; Morales, M.T.; Aparicio-Ruiz, R.; Tena, N.; Garcia-Gonzalez, D.L. Authenticity of Olive Oil: Mapping and Comparing Official Methods and Promising Alternatives. *Food Res. Int.* **2013**, *54*, 2025–2038. [[CrossRef](#)]

