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 effects1-4.

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 oil5. The phenolic component contributes to the stability of the oil during processing and storage as well as the organoleptic qualities of the oil6.

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 derivatives7. With respect to secoiridoids, the main components found in EVOOs are the dialdehyde of hydroxytyrosol, together with oleuropein aglycone and ligstroside aglycone8-11. The flavonoids are described as structurally diverse and are classified into flavonols, anthocyanins, flavones, isoflavones and flavonones12. 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.
The most common flavonoids are apigenin, luteolin and cyanidins. 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.

In the distant past, the Maltese Islands had a thriving oil-producing industry 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. 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 para-sites such as the vascular disease-causing fungus, Verticillium dahliae and the olive fruit fly Bactrocera oleae. Mazzitelli et al. 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. studied the cyclooxygenase activity in EVOO derived from the Bidni variety while Lia et al. 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. 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.

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 monocultivár.

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
method by Vazquez Roncero et al. as described in Papadopoulos and Boskou 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°C.

The sample was dissolved in absolute ethanol and stored at −20°C. Phenolic extracts obtained from Bidni, Malta, 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-Ciocalteau (F-C) assay

Total phenol content was determined using the method of Slinkard and Singleton, with a reduction in volumes as described by Waterhouse. 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-Ciocalteau reagent. 300 µL of 20% (w/v) anhydrous sodium carbonate solution was added, mixed and heated at 40°C 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. 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). 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 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.

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.
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 values 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.

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 linked this property to oleuropein derivatives, others such as Gutiérrez-Rosales, Perdiguero, Gutiérrez and Olias, and Angerosa et al. quoted both oleuropein and ligstroside aglycones and for Tovar et al., 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, and the TdPC was highest in the EVOO derived from the Bidni variety followed once again by the Sicilian-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 variety of Argentinian VOOs, though these were recorded as caffeic acid equivalents by Laincer et al. 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 Portuguese monocultivar EVOOs Covrançosa (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). In contrast, all TdPC values recorded were less than those quoted as caffeic acid equivalents by Youssef et al. 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).

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. 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. are expressed as rutin hydrate equivalents not as catechin equivalents as in this study. Values reported by Ammar et al. also greatly differ from those obtained in this study. Ammar et al. 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-
leu, *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. It is the former of these compounds that was identified in this study.

With respect to lignans, (+)-pinoresinol, and (+)–1-acetoxy-pinoresinol 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-acetoxy-pinoresinol 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.* 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.

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 Meirinhos *et al.* 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

*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.
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                                              |
| 3    | 252.06            | Tyrosol Glycoside                                                                    |
| 4    | 225.07, 239.08    | Hydroxy-Elenolic acid                                                                |
| 5    | 225.07, 239.08    | Hydroxy-Elenolic acid p-HPEA-EDA                                                     |
| 6    | 287.05            | Apigenin                                                                             |
| 7    | 225.07            | Hydroxy-Elenolic acid Methyl-3,4-DHPEA-EA                                            |
| 8    | 287.05            | Luteolin                                                                             |
| 9    | 225.07            | Product obtained following rearrangement of the decarboxylated form of ligstroside aglycone |
| 10   | 274.24            | Decarboxylated form of ligstroside aglycone                                          |
| 11   | 225.07, 363.12, 495.24 | Open ring decarboxylate aldehydic form of oleuropein                                   |
| 12   | 530.27            | Open ring decarboxylate aldehydic form of oleuropein glycoside                       |
| 13   | 415.23, 432.25    | Closed ring carboxylate hydroxylade form of oleuropein                               |
| 14   | 302.31            | Hydroxytyrosol elenolic acid dialdehyde (3,4-DHPEA-EDA)                              |
| 15   | 346.35            | Oleuropein aglycone demethylated derivative                                          |
| 16   | 225.07, 363.12, 495.24 | 10-hydroxy-oleuropein fragment                                                       |
| 17   | 552.39            | 10-hydroxy-oleuropein                                                               |
| 18   | 566.36            | 10-hydroxy methyl – oleuropein                                                      |
| 19   | 487.3             | Closed ring decarboxylate aldehydic oleuropein                                       |
| 20   | 505.31            | Closed ring decarboxylate aldehydic oleuropein glycoside                             |
| 21   | 487.28            | Closed ring decarboxylate aldehydic oleuropein iridoid form                          |
| 22   | 258.2             | Hydroxy-Elenolic acid oxidised hydroxyl form                                         |
| 23   | 279.21, 395.26    | Closed ring carboxylate demethylated hydroxylade oleuropein form fragment            |
| 24   | 551.35            | Closed ring carboxylate demethylated hydroxylade oleuropein form                     |
| 25   | 267.18            | Closed ring decarboxylate oleuropein form fragment                                   |
| 26   | 391.27            | Closed ring decarboxylate oleuropein form                                            |
| 27   | 515.38            | Open ring decarboxylate Aldehydic Oleuropein glycoside form                          |
| 28   | 539.39            | (+) – pinoresinol                                                                    |
| 29   | 597.4             | (+) – 1-acetoxypinoresinol glycoside                                                 |
| 30   | 357.28            | (+) – pinoresinol                                                                    |
| 31   | 554.44            | (+) – 1-hydroxypinoresinol glycoside                                                 |
| 32   | 678.29            | 3,4-DHPEA-EDA diglycoside or Oleuropein dihydroxytyrosol                             |
| 33   | 225.07            | Hydroxy-Elenolic acid                                                               |
| 34   | 281.19, 398.78, 419.29, 557.44 | Open ring carboxylate dialdehydic oleuropein glycoside fragment                       |
| 35   | 603.35            | Verbascoside                                                                        |
| 36   | 512.047           | Open ring decarboxylate dialdehydic ligosecoiridoid glycoside fragment               |
| 37   | 540.49            | Oleuropein                                                                           |
| 38   | 708.49            | Dimeric form of open ring decarboxylate aldehydic ligosecoiridoid form               |
| 39   | 1370.96           | Tetrameric form of pinoresinol                                                       |

ring decarboxylate 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 carboxylate demethylated hydroxylade oleuropein form fragment, and the open ring carboxylate dialdehydic oleuropein glycoside form. Other analysed secoiridoids were the decarboxymethyl oleuropein dialdehyde form (p-HPEA-EDA)
and decarboxymethyl ligstoside 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. 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.

Author Contributions
Lucienne Gatt performed phenolic extractions, the TPC, TdPC and TFC assays, and wrote the manuscript. Simon J. Thorpe and Lucienne Gatt performed LC-MS analysis. Frederick Lia performed compound identification. Marion Zammit-Mangion and Pierre Schembri-Wismayer supervised the work and reviewed the manuscript.

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Supporting Information
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