Developing an Olive Biorefinery in Slovenia: Analysis of Phenolic Compounds Found in Olive Mill Pomace and Wastewater

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Graphical abstract

Developing an olive biorefinery in Slovenia: Analysis of phenolic compounds found in olive mill effluents

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Abstract

Valorization of olive pomace through extraction of phenolic compounds at an industrial scale has several factors that can have a significant impact on its feasibility. Important factors are the types of phenolic compounds, variation in the compounds and amount of phenolic compounds that are extracted from olive mill effluents. Chemical analysis of phenolic compounds was performed using an HPLC-DAD-qTOF system, resulting in the identification of 45 compounds in olive mill wastewater and pomace where secoiridoids comprised 50 – 60% of the total phenolic content. This study examined three different levels of variation in phenolic content: crops from local farms, processing and seasonal effects. Olive crop varieties sourced from local farms showed high variability, and the highest phenolic content was associated with the local variety "Istrska Belica". During processing, the phenolic content was on average approximately 50% higher during two-phase decanting compared to three-phase decanting and was significantly different. An investigation into the seasonal effects revealed that the phenolic content was 20% higher during 2019 compared to 2018 but was not significantly different. The methods and results used in this study provide a basis for further analysis of phenolic compounds present in the European Union's olive crop processing residues and will inform techno-economic modelling for the development of olive biorefineries in Slovenia.

Keywords: *Olea oleuropea* L., olive mill effluents, pomace, HPLC-DAD-qTOF, phenolic compounds, antioxidant potential

1. Introduction

The production of olive oil in the Istrian region of Slovenia has a long-established tradition dating back to the 4th Century BC (Darovec eand Ermacora, 1998). At the heart of this is the "Istrska belica" cultivar of olives (Istrian white olives), which have been praised for their ability to withstand low temperatures, high oil content, excellent taste, high levels of monounsaturated fatty acids and high levels of biologically active molecules including phenolic compounds, squalene and tocopherols (Lazović et al., 2018; Baruca Arbeiter et al., 2014; Bešter et al., 2008). It has been determined that the levels of phenolic compounds are significantly higher in varieties of "Istrska Belica" when compared to other varieties from within the same location (Bučar-Miklavčič et al., 2016). This high phenolic content contributes to the organoleptic profile of the oil produced from these olives (Bučar-Miklavčič et al., 2016). Phenolic compounds from olives offers a variety of benefits to human health, including a reduction in coronary heart disease risk factors, prevention of several types of cancers and modification of immune and inflammatory responses (Bendini et al., 2007; Bogani et al., 2007; Bulotta et al., 2014).

Modern, industrial olive oil extraction uses a continuous process in which a decanter separates oil from olives using two- or three-phase decanter centrifugation. The two-phase decanter centrifuge generates a waste called alperujo, which is a mixture of pomace, oil and water; the three-phase decanter produces relatively low moisture pomace and olive mill wastewater (OMWW). The pomace contains the remaining olive pulp, skin, stones and water (Niaounakis et al., 2006; Tsagaraki et al., 2007; Di Giovacchino et al., 2002). A destoning process can be incorporated into the process leading to the removal of 70% of the stones. While there are many valuable compounds still present in the pomace (Podgornik et al., 2018; Bandelj et al., 2008; Wang et al., 2010; Cardialli et al., 2012; Rubio-Senent et al., 2012), successful and economically viable extraction methods are still in development. Currently, pomace is used as fertilizer, compost, animal feed or for burning

(Podgornik et al., 2018), but some integrated biorefinery approaches for higher value applications have also been proposed (Romero-García et al., 2014; Scievano et al., 2015). OMWW is the processing water coming from the three-stage method, and it is acidic with high levels of organic pollutants (Kissi et al., 2001). There are currently few uses for this effluent due to variability in the composition, current process limitations in the handling of large volumes and stabilization of oxidation and other natural processes. The high concentration of phenolic compounds from OMWW, produced during processing, can also have a severe environmental impact if they are improperly released. However, there is potential to valorize the phenolic compounds from wastewater and olive pomace. It is important to establish the feasibility of recovering phenolic compounds as an industrial process from olive mill effluents generated through different decanting processes and to determine the effects of yearly variation.

More than 50 different phenolic compounds have been identified in olive pomace with the remaining stones and OMWW that contain mostly simple phenolic compounds, benzoic acid derivates, cinnamic acids derivates, flavonoids, lignans and secoiridoids (Jerman Klen et al., 2015), with the latter molecules found specifically in olives (Ryan et al., 2002; Montedoro et al., 2002). During the olive oil manufacturing process, ligstroside and oleuropein can enter different transformation-reaction pathways involving plant enzymatic and chemical transformation (Rovellini and Cortesi, 2002). When the transformation pathway is reaching its end and the olive oil has already lost its freshness and antioxidative properties after one or two years of storage, depending on the variety, the total phenolic compounds content can be relatively high with higher amounts of simple phenolic compounds such as tyrosol and hydroxytyrosol (Bučar-Miklavčič et al., 2016). The same process of phenolic compounds breaking down into simple phenolic compounds, such as tyrosol and hydroxytyrosol, is expected to occur in olive mill effluents.

Therefore, it is important to identify each phenolic compound, rather than total phenolic content, in order to evaluate the level of phenolic breakdown.

The study's aim was to identify and quantify the phenolic compounds in OMWW and pomace generated from industrial processes to extract olive oil. The first level of variation occurs at the local farms in Slovenian Istria where different varieties of olive crops, such as "Istrska belica", "Leccino", "Buga" and "Maurino", are grown. The second level of variation occurs during processing when different decanting technologies are used to recover the oil. Finally, the third level of variation occurs during different growing seasons. This is the first comprehensive report that has evaluated all three of these parameters in order to establish the feasibility of recovering phenolic compounds from olive mill effluents in a real, state-of-the art industrial environment with all of its boundary conditions, as a means towards valorization of olive residues.

2. Results and discussion

2.1 Identification of phenolic compounds in olive mill wastewater and pomace

Identified compounds in pomace and OMWW samples are presented in Table 1 and Figure 1. In Figure 1, the phenolic compounds identified only in olive mill pomace are presented. All the phenolic compounds identified in olive mill water were also present in pomace samples.

2.1.1 Simple phenolic compounds: Hydroxytyrosol and its derivates

The presence of hydroxytyrosol was confirmed in olive pomace and olive mill water by reference to the retention time of a standard solution (6.2 min). Only one compound was identified as hydroytyrosol glucoside in both pomace and OMMW. Previous reports (Talhaoui et al., 2014, Jerman-Klen et al., 2015) observed two different isomers of hydroxytyrosol glucoside in different olive oil waste production streams, with slightly different retention times. One of them was tentatively identified based on UV-vis spectra characterization as hydroxytyrosol-1-β-glucoside, in contrast to the other one with the slightly different λ_{max} of the B-band at 276 nm, which suggested that the glycosidation occurred at 3′ or 4′ position on the benzene ring (Jerman-Klen et al., 2015).

2.1.2 Benzoic acids

Vanillin was present in the olive mill water and pomace samples and confirmed through reference to a standard solution.

2.1.3 Cinnamic acids

Esters of cinnamic acids, such as verbascoside and β-Methyl-OH-verbascoside, were found in pomace (Jerman-Klen et al., 2015; Mulinacci et al., 2005). However, unlike Jerman Klen et al. (2015), verbascoside was not found in olive mill wastewater. As previously reported (Ryan et al., 1999), during studies on olive fruits, verbascoside may exist as a pair of geometric isomers arising from the caffeic acid moiety or different attachment of the sugar to the aglycone. The presence of verbascoside was confirmed through comparison with the retention time of a standard solution (7.7 min, Figure 1), similar to two β-OH-verbascoside isomers that were found in both pomace and olive mill water (Supplementary Table 1). At 8.1 min, a possible verbascoside isomer was identified; in addition, caffeic acid, a member of a large and varied family of hydrohycinannamoyl conjugates that also includes p-coumaric and ferulic acid derivate (Ellis, 1985), was identified by comparison to previously reported exact mass and fragmentation patterns (Hu et al., 2005). Trans p-coumaric acid 4-glucoside was identified in pomace by exact mass detecting fragments 163 and 119, as previously reported by Jerman Klen et al. (2015). The same fragmentation pattern for p-coumaric acid was previously reported by Araújo et al. (2015).

2.1.4 Flavonoids

Apigenin was determined using a standard both in pomace and OMWW. Luteolin was not identified, in contrast to former studies (Araújo et al., 2015). However, luteolin-4`,7-*O*-diglucoside and three different luteolin-glucosides were identified both in pomace and OMWW, as reported by Jerman Klen et al. (2015). Nevertheless, due to low amounts of luteolin-4`,7-*O*-diglucoside in pomace, the UV absorption maxima of the annotated peak could not be detected.

Based on reported data (Cuyckens and Claeys, 2004 and Jerman Klen et al., 2015), the observed absorption maxima corresponded to three different luteolin-glucosides, tentatively identified as luteolin-7`-*O*-glucoside (retention time 8.3 min), luteolin-4`-*O*-glucoside (8.9 min) and luteolin-3`-*O*-glucoside (9.3 min). However, the latest annotated peak did not have a typical UV absorption maximum at 270 and 340 nm, so it might be the luteolin-3`-*O*-glucoside only in structure. Luteolin rutinoside with typical fragmentation pattern of m/z 593, 447 and 285 eluted before luteolin-4`-*O*-glucoside and after luteolin-7`-*O*-glucoside, as previously reported (Jerman Klen et al., 2015). This compound was present in higher quantities in pomace and in much smaller quantities in OMWW. In OMWW, fragmentation pattern identification was not possible due to the low concentration. In contrast to the literature (Jerman Klen et al., 2015), only one isomer of luteolin rutinoside was found, and this could be attributed to the different column and elution conditions used. The analyses by Jerman Klen et al. (2015) took 88 min per sample, which was infeasible for routine analysis, so, in the current study, the column conditions were modified in order to fully elute the sample in 20 min. However, this can preclude meaningful comparison of phenolic composition based purely on retention times.

2.1.5 Secoiridoids

2.1.5.1 Oleoside

Previous reports (Jerman-Klen, 2015; Talhaoui et al., 2014; Fu et al., 2010) have described the presence of four peaks with the exact mass of oleoside, and a fragmentation pattern characteristic for oleoside was found at retention times 4.8, 5.0, 5.2 and 6.4 min in olive mill pomace. The four peaks had slightly different fragmentation profiles (Supplementary Table 1). The first two peaks determined at 4.8 and 5.0 min might be oleosides only in their structures, as previously suggested

(Jerman-Klen, 2015), due to non-typical UV absorption maxima. However, the third and fourth peaks include typical absorption maxima at 230 nm. In this study it was possible to confirm the previously observed co-elution of the oleoside third peak at 5.2 min with hydroxytyrosol, and the tentative identification of secologanoside, due to absorption maximum at 230 nm and the highest abundance of the fragments 389 and 345. A tentative identification of secologanoside in olive pomace and OMWW was made, in accordance with a previous report (Jerman-Klen et al., 2015).

2.1.5.2 Oleuropein and its derivates

The presence of oleuropein was identified by a pure standard at retention time 9.3. Oleuropein was present in pomace but not in OMWW. At retention times 9.6 and 9.8, two similar compounds were tentatively identified as oleuropein isomers with m/z 539 and similar fragmentation patterns as the oleuropein pure standard (Talhaoui et al., 2014). The last eluted oleuropein isomer was present in OMWW as well.

Demethyloleuropein (molar mass 526.1704 g/mol) was detected in pomace with m/z 571.1693 (M + HCOO), together with m/z 525.1623, along with the same fragmentation pattern (525, 389, 319, 183, 345) and similar relative retention time as reported elsewhere (Jerman Klen et al., 2015). In OMWW, a compound was found at a similar retention time, but it was impossible to identify as demethyloleuropein by the fragmentation pattern due to very low levels.

Oleuropein-aglycone dialdehydes (3,4-DHPEA-EDA) with exact molar masses of 319.1185 (Isomer 1) and 319.1187 (Isomer 2) were tentatively identified at retention times 9.4 and 11.2 min with similar fragmentation patterns as previously reported (Jerman Klen et al., 2015).

196 p-HPEA-EDA (or oleocanthal) has one hydroxyl group less than 3,4-DHPEA-EDA and it is in
197 particular described by Cioffi et al., 2010. Similar retention time and fragmentation pattern for 3,4-
198 DHPEA-EDA was found as previously reported (Jerman-Klen et al., 2015 and Medina et al.,
199 2017).

200 There are twelve possible isomers in various tautomeric forms of oleuropein aglycone already
201 reported in olive oils (Fu et al., 2009). In our study, nine isomers of oleuropein aglycone were
202 found in pomace and one in OMWW, based on exact mass and fragmentation patterns reported
203 previously (Jerman Klen et al., 2015; Fu et al., 2009). The annotated peaks of the oleuropein
204 aglycone did not have the characteristic UV absorption maximum at ~250 nm, but they did have a
205 similar retention time of 10.3 min.

206 *2.1.5.3 Elenolic acid glucoside*

207 Elenolic acid glucoside was previously reported in olive oil process derived matrices, including
208 leaves (Talhaoui et al., 2014; Quirantes-Piné et al., 2013; Fu et al., 2010), olive fruits (Jerman-
209 Klen et al., 2015, Savarese et al., 2007; Obied et al., 2007), olive oil (Jerman-Klen et al., 2015),
210 pomace (Jerman-Klen et al., 2015; Cardoso et al., 2005; Paralbo-Molina et al., 2012) and OMWW
211 (Jerman-Klen et al., 2015). Four different isomers of elenolic acid glucoside have been tentatively
212 identified previously in pomace, but not all four were identified in OMWW (Jerman Klen et al.,
213 2015 and Talhaoui et al., 2014). While in all isomers, the fragments 403, 223 and 179 were found
214 as previously reported (Tahaoui et al., 2014 and Jermam Klen et al., 2015). The fragment with m/z
215 to 223 corresponds to the elimination of hexose, giving rise to m/z 179 by the neutral loss of CO_2
216 (Jerman Klen et al., 2015).

217 *2.1.5.4 Ligustroside*

Ligustroside has one hydroxyl group less than oleuropein, and according to the literature, with comparable elution gradient to our study, it eluted after oleuroside (Jerman Klen et al., 2015; Talhaoui et al., 2014; Obied et al., 2007), as indicated in Supplementary Table 1. The fragmentation pattern of the compound was similar to previous reports (Jerman Klen et al., 2015 Obied et al., 2007; Savarese et al., 2007).

2.1.5.5 Caffeoyl-6-secologanoside and comselogoside

Comselogoside was not found in olive mill water and pomace, while caffeoyl-6-secologanoside was found in both pomace and OMWW with fragmentation pattern and approximate relative retention time as previously reported (Obied et al., 2007; Jerman Klen et al., 2015).

2.1.5.6 Nuzhenide

Based on mass accuracy and fragmentation pattern (Isomer 1: 523, 685, 453, 421, 299 and 223; Isomer 2: 523, 685, 453, 299 and 223), two different isomers of nuzhenide were found in pomace but not in OMWW, which matches previous reports (Obied et al., 2007; Silva et al., 2010). Previously, these compounds have only been found in olive stones (Silva et al., 2010); therefore, it is likely that some of the stones were crushed during processing and ended up in the pomace fraction.

2.2 Quantification of phenolic compounds in pomace

The median, minimum and maximum levels of individual, total phenolic compounds and different groups of phenolic compounds, such as simple phenolic compounds, benzoic acids, cinnamic acid, flavonoids and secoiridoids, together with radical scavenging activity by DPPH, are shown in Table 1. All results are expressed as mg/kg dry weight (dry wt) of pomace sample. Although from the literature it is well known that the phenolic compound concentrations are affected by

agronomic and technological factors, including the cultivar type, raping stage and geographic origin (Bučar-Miklavčič et al., 2016; Cioffi et al., 2010), the total phenolic compounds that varied greatly from 851 mg/kg dry wt to 4473 mg/kg dry wt (Table 1) are in the range as previously reported elsewhere (Podgornik et al., 2018; Mavser et al., 2008; Cioffi et al., 2010). The wide variation of phenolic compounds is consistent with the literature, with the highest levels of total phenolic compounds found in samples from the variety "Istrska belica" (two-phase decanter). The main group of phenolic compounds in pomace was secoiridoids that comprised on average 71% ±7%, with the 3,4-DHPEA-EDA and oleuropein or oleuroside that are eluting at the same times being the most abounded of this kind of compounds. A previous report determined 50-70% of the total phenolic content was attributed to secoiridoids (Cioffiet et al., 2010). These compounds could have useful application in controlling colorectal cancer (Cárdeno et al., 2012), and other applications may be discovered when larger quantities are available.

In contrast to a previous report (Japón-Luján and Luque de Castro, 2007), where simple phenolic compounds were determined as the main phenolic compounds in pomace, both tyrosol and hydroxytyrosol were present at 8% ± 5% of total phenolic compounds in the samples analyzed for this study. The low amounts of simple phenolic compounds and the majority of complex phenolic compounds, such as secoiridioids, identified in our study is promising for potential industrial end-users (e.g., cosmetics and personal care) in applications where antioxidant activity of the extracts is very important (Romero-García et al., 2014). The simple phenolic compounds might be also the end compounds of oxidation pathways of secoiridoids (Gutfinger, 1981; Tsimidou, 1998). In our previous study (Bučar-Miklavčič et al., 2016), it was determined that an increase in tyrosol and hydroxytyrosol and decrease of secoiridoids levels resulted after one and two years of storage for extra virgin olive oil samples. However, in this study, we did not observe any significant

correlation between evaluation of radical-scavenging activity by DPPH assay and the percentage of secoiridoids or simple phenolic compounds for total phenolic compounds in pomace samples.

Several possible levels of variation were identified for the quantify of the phenolic compounds in OMWW and pomace generated from olive oil extraction industrial processes (discussed in the Introduction). However, from the current state-of-the-art industrial point of view (often very difficult to control the input crop) and from a preliminary statistical analysis, the two discussed in sub-sections 2.2.1 and 2.2.2 were chosen for a more detailed investigation and presentation.

2.2.1 Variation in phenolic compound content in olive pomace across different growing seasons

Phenolic compounds are secondary plant metabolites and are synthesized in response to environmental stress factors, including microbial attack, tissue damage, UV rays (Naczk and Shahidi, 2004) and water deficiency in olives, resulting in increased concentrations of these molecules (Petridis et al., 2012). In general, extreme weather conditions can significantly influence the concentrations of phenolic compounds, and it has been determined that the increase in the level of these compounds in extra virgin olive oil, across three years (2011-2013), was strongly influenced by these factors. The oils contain the highest quantity of phenolic compounds in crop year with the highest water deficiency (Bučar-Milavčič et al., 2016). In order to detect seasonal variation of phenolic compounds in Slovenia, pomace samples from three-phase decanter were collected in the crop years 2018 and 2019. The differences in the levels of total phenolic compounds and the main groups of phenolic compounds determined in the pomace samples between the two years are shown in the Figure 2. These two crop years were chosen due to the

variation in weather conditions. In contrast to 2018, the crop year 2019 was unusual; the yields were 50-60% lower in the region than previous years. The season began ten days earlier, and in the beginning of the season, the olives from the variety "Istrska belica" were also present, which is unusual because this is a late season variety. The unusual season was due to increased rainfall in the study region during certain periods of the year (May, July and September) (ARSO, 2020), which allowed the development and spread of the olive fly that greatly affected the olives and final yields.

It was determined that there were no statistically significant differences in total phenolic compounds, simple phenolic compounds, benzoic acids, cinnamic acids and secoiridoids content between the two years. The exception was the marginally significant differences ($p = 0.05$) in levels of flavonoids between the two years. In the case of crop year 2019 (median: 151 mg/kg dry wt), the levels of flavonoids in pomace samples were higher than in crop year 2018 (median: 108 mg/kg dry wt). The fact that there were no observed significant differences between the two years (Figure 2) might be the consequence of different varieties, quality and maturity of olives present in the olive mill when the samples were taken. Analysis of a larger sample range would be necessary to observe the differences between the two years. However, the preliminary results about annual variation of phenolic compounds in pomace samples are promising for further development of biorefinery in Slovenia due to low variation observed between two crop years with very different weather conditions. In order to provide constant quality of raw material, it is necessary to be able to control the factors that influence variability.

2.2.2 Variation in phenolic compound content in olive pomace using different separation (centrifugation) technologies

In contrast to the comparison in total phenolic compound content between crop years, statistically significant differences were observed when two different olive mill separation (centrifugation) technologies were compared ($p = 0.037$).

The levels were higher in pomace samples taken from the two-phase decanter (median: 2970 mg/kg dry wt), compared to the three-phase decanter (median: 1900 mg/kg dry wt), due to the addition of extra water to the olive paste in the latter process, which has a dilution effect and results in dissolved losses of phenolic compounds (Alfei et al., 2013). The two-phase decanter is an extraction system that is also known as "ecologic" or "water saving" as it requires no water addition and reduces wastewater generation up to 80%. The concept of working is similar to that of a three-phase decanter, except that horizontal centrifuge has no, or reduced, requirement for additional water due to superior g values (Niaounakis et al., 2006; Tsagaraki et al., 2007; Di Giovacchino et al., 2002).

There were also significant differences between the main group of phenolic compounds present in pomace, secoiridoids ($p = 0.0374$), with a higher amount in pomace from the two-phase decanter (median: 1990 mg/kg dry wt) compared to three-phase separating decanter (median: 1270 mg/kg dry wt). In addition, significant differences were observed in vanillin content ($p < 0.05$) in pomace from two-phase separating decanter (median: 43 mg/kg dry wt) compared to three-phase separating decanter (median: 6 mg/kg dry wt). The levels of other groups of phenolic compounds, including simple phenolic compounds, cinnamic acids and flavonoids, were not significantly different when the two separation technologies were compared.

This study indicates, for the first time, that the technological approach used in olive mills to separate the different fractions is a critical factor in determining the types and levels of phenolic compounds obtained in the resultant pomace.

2.2.3 Radical scavenging activity by DPPH

Determination of radical scavenging activity, using the DPPH assay, is a suitable method for predicting the inhibition of primary oxidation product formation by natural extracts (Molyneux, 2004; Shwarz et al., 2001). The EC50 value determined in the pomace samples correlates inversely with the concentrations of total phenolic compounds (r_s = -0.8; $p < 0.05$). The inverse correlation is expected because EC50 value is defined as the concentration of substrate that causes 50% loss of DPPH activity (color) (Molyneux, 2004). Spearman Rank correlation is the strongest between the total phenolic compounds and radical scavenging activity by DPPH as compared to the Spearman Rank correlation between each phenolic compound or groups of phenolic compounds determined in the samples and radical scavenging activity by DPPH (Table 1). This confirms the previously reported observation that the antioxidant pattern is usually complex, and it can include synergistic effects of the compounds that are not possible to determine only by the quantification of phenolic compounds by the HPLC-MS method (Schwarz et al., 2001).

3. Conclusions

In this study, 45 compounds were identified in olive mill effluents from Slovenian Istria in different crop years. Secoiridoids were the most abundant of the determined compounds in olive mill pomace, and the end oxidation products of secoiridoids to form simple phenolic compounds were present in smaller amounts. In the first level of variation, examination of phenolic content between crops from different sources of olive crop revealed that the phenolic content showed significant variability, which was dependent on the olive crop variety. The second level of variation examined olive processing to extract oil and revealed significantly more phenolics were associated with the wet pomace after two-phase decanting compared with three-phase decanting that was on average

approximatelly 50% higher. The third level of variation examined seasonal phenolic content and revealed that phenolic content during 2019 was 20% higher than during 2018. However, the differences were not statistically significant. The possible difference between seasons was hidden by the high level of variation in phenolic content occurring between the different varieties of olive crops sourced from the local farms. Further recording and analysis of yearly variations and inclusion of other regionally important varieties of olives could provide a more robust understanding of variations, content and quality of phenolic compounds from mill effluents.

This study reports, for the first time, that the technological approach used in olive mills to separate the different fractions is a critical factor in determining the types and levels of phenolic compounds obtained in the resultant pomace. There is a statistically significant higher level of phenolic compounds obtained in olive pomace when a two-phase decanter system is used. Along with the potential to reduce the environmental burden of olive processing, by minimizing the amount of water required, this information is important from a techno-economic planning perspective and will inform the future development of olive biorefineries in Slovenia that link to a value chain of bio-based products including phenolic compounds.

The knowledge gathered in the presented research is a good platform for understanding the sourcing of olive crop, technological processes of olive milling, and analytical technologies influence on quality and quantity of phenolic compounds found in OMWW and pomace. It allows the industry worldwide a knowledge-based decision making in process change and/or investment for the utilization of phenolic compounds in their side- and waste-streams. The upstream optimization and/or reconfiguration of analysed parameters can allow for either targeting a specific phenolic compound, ensuring consistency and reliability of phenolic compunds output, or increasing the quantity of the downstream phenolic compounds products for a desired

environmental impact improvement, new product development, and ultimately a reliable revenue stream of a particular company

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4. Experiment

4.1 General experimental procedures

The pomace samples were freeze dried by the freeze drier Büchi 1-4 LC plus (Martin Christ, Germany). For concentration of the extracted samples, Büchi Rotavapor R-300 Dynamic (Martin Christ, Germany) was used. Phenolic compounds were characterized using an ultrahigh-pressure liquid chromatography system (HPLC; Agilent 1290 Infinity2 HPLC modules, United States), interfaced with a qTOF mass spectrometer (ESI-QTOF; 6530 Agilent Technologies, United States). HPLC equipment incorporated a Poroshell 120 column (EC-C18; 2.7 µm; 3.0 × 150 mm; Agilent, United States). Radical scavenging activity measured using the DPPH assay was determined at 515 nm by a microplate reader Infinite F200 (Tecan, Switzerland).

Analytical standards such as oleuropein (12247-10MG, Sigma Aldrich), hydroxytarosol (SI-H4291-25MG, Sigma Aldrich), tyrosol (AL-188255-5G, Sigma Aldrich), luteolin (SI-L9283-10MG), verbascoside (V4015-10MG, Sigma Aldrich) and apigenin (SI-SMB00702-5MG, Sigma Aldrich) were used for quantification of phenolic compounds; 2,2-Diphenyl-1-picrylhydrazyl (D9132-250MG, Sigma Aldrich) was used for determination of radical scavenging activity for pomace extracts.

4.2 Samples

A total of 18 pomace samples from olives of *Olea europaea* L. were collected weekly from the beginning olive oil production until the end of the mill production season in 2018 and 2019 (14 October 2018 – 18 November 2018 and 16 October 2019 – 09 November 2019). During crop year 2018, the samples were collected from two olive mills, Franka Marzi and Lisjak (Koper, Slovenian Istria), using different processing technologies (two-phase – Pieralisi FP60 RS ATEX and three-phase decanter centrifuge – Alfa Laval x 4); in 2019, the samples were collected only from three-phase decanter centrifuge (Franka Marzi). During the two-phase decanting process, olives are initially washed, crushed and malaxed (churned), and water is added to a horizontal centrifuge (40–60 L/100 kg fruits weight), separating pomace from the oily must consisting of the vegetable water and oil. Oil, pomace and wastewater are the final products formed at one end of the three-phase decanter. In contrast to three-phase decanter, the two-phase decanter requires no additional water due to the much higher centrifugal speeds, resulting in olive oil and wet olive cake or pomace (Niaounakis et al., 2006; Tsagaraki et al., 2007; Di Giovacchino et al., 2002).

This sampling strategy was used in order to investigate the possible variation in phenolic compounds composition across a number of different olive cultivars (“Maurino”, “Leccino”, “Buga” and “Istrska belica”), reaching maturity at different times during the growing season. In addition to pomace samples, OMWW was also sampled from the mill using three-phase centrifugation. In contrast to the pomace samples, quantification of the phenolic compounds in olive mill samples was not performed due to the unknown exact addition of tap water that varied from 10-25 percent.

Immediately after sampling, the pomace samples were freeze dried (Alpha 1-4, Martin Christ Buchi). Dry pomace and OMWW samples were stored in a freezer (-18 °C) prior to analysis.

4.2.1 Extraction of phenolic compounds

Phenolic compounds were extracted from freeze dried pomace (2g) in methanol / water 80:20 (50 mL, pH 2-HCl) for 30 minutes with stirring at room temperature and then re-extracted with fresh solvent (20 mL) for 15 minutes. The combined extracts were filtered and defatted using hexane (30 mL x 2). The defatted extracts were filtered and concentrated *in vacuo* (1.5 hrs). The residue was reconstituted to 10 mL of methanol and re-filtered through 0.2 µm plastic non-sterile filter. The procedure is described in detail elsewhere by Obied et al. (2008).

The phenolic compounds from olive mill water (15 mL, Batch 4, Franka's olive mill) were defatted using hexane (15 mL). The sample was further extracted with ethyl acetate (15 mL x 3) and then centrifuged (40,000 g, 15 min) and concentrated *in vacuo*. The residue was reconstituted with methanol (10 mL) and then diluted 10 times. The samples were filtered through 0.2 µm 0.2 PA (nylon) filters. The procedure is described by Obied et al. (2008).

4.3 Determination of phenolic compounds by HPLC-DAD-ESI-TOF

Phenolic compounds were characterized by HPLC-ESI-QTOF-MS. An elution gradient of 100% water / formic acid (99.05: 0.5, v/v) (A) towards 100% acetonitrile / methanol (50: 50, v/v) was used over a period of 20 minutes (flow rate: 0.5 mL min; injection volume: 1 uL). A more detailed procedure can be found in Miklavčič et al. (2019) to make the procedure applicable for different column dimensions. The separated phenolic compounds were first monitored using a diode-array detector (DAD) (280 nm) and then MS scans were performed in the m/z range 40-1000 (capillary voltage, 2.5 kV; gas temperature 250 °C; drying gas 8 L/min; sheath gas temperature 375 °C; sheath gas flow 11 L/min). In those conditions, the instruments are expected to provide experimental data with accuracy within ± 3 ppm. All data were processed using Qualitative Workflow B.08.00 and Qualitative Navigator B.080.00 software.

443 The extracts were screened for the range of phenolic compounds previously reported in *O.*
444 *europaea* L. (Jerman Klen et al., 2015; Obied et al., 2007; Savarese et al., 2007; Silva et al., 2010;
445 Talhaoui et al., 2014) and their identification confirmed, based on accurate mass and fragmentation
446 profile with literature data and analytical grade standards (hydroxytyrosol, luteolin, verbascoside,
447 apigenin, oleuropein). While tyrosol cannot be detected by MS because of its high ionization
448 energy, its presence in the extracts was confirmed by comparison with the retention times of the
449 tyrosol standard solution using a DAD.

450 The quantification was performed using calibration graphs prepared using six commercial
451 standards (oleuropein, hydroxytarosol, tyrosol, luteolin, verbascoside, apigenin) by HPLC-DAD
452 and HPLC-ESI-QTOF. Oleuropein and other secoiridoids were quantified with the calibration
453 curve of oleuropein; hydroxytyrosol and hydroxytyrosolhexose isomers with the calibration curve
454 of hydroxytyrosol; tyrosol and tyrosol glucoside were quantified with the calibration curve of
455 tyrosol; apigenin and apigenin derivates were quantified with the calibration curve of apigenin;
456 luteolin and other flavonoids were quantified with calibration curve of luteolin and verbascoside
457 with the calibration curve of verbascoside (Talhaoui et al., 2014). The calibration plots indicated
458 good correlations between peak areas and commercial standard concentrations. Regression
459 coefficients were higher than 0.990. LOQ was determined as the signal-to-noise ratio of 10:1 and
460 varied in the range from 2 mg/kg to 12 mg/kg dried pomace sample. The standard deviation
461 between duplicate was less than 5%.

462 **4.4 Radical scavenging activity measured using DPPH assay**

463 Antioxidant activity of the different extracts was measured in terms of radical-scavenging ability
464 in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay and conducted as reported by Žegura

et al. (2011) with minor modifications. Ethanol was replaced by methanol; tyrosol was used as a standard for positive control instead of ascorbic acid.

Reaction mixtures containing 100 µL of differently diluted extracts and 100 µL 0.2 mM DPPH in methanol were incubated 60 min in darkness at ambient temperature, using 96-well microtiter plates. The decrease of absorbance of the free radical DPPH was measured at 515 nm with a microplate reader. The free radical scavenging activity was calculated as the percentage of DPPH radical that was scavenged and is in detail explained elsewhere (Žegura et al., 2011). EC50 values concentration at which 50% of DPPH radical is scavenged were determined graphically from the curves. Two independent experiments with two replicates each were performed.

4.5 Statistical analysis

All the data obtained were analyzed using STATA13/SE software. The normality of variable distributions was assessed using the Shapiro–Wilk test. Spearman Rank correlation was used for bivariate comparison of the content of phenolic compounds and EC50 (Table 1). The Wilcoxon–Mann–Whitney test was applied for comparison of two different groups. The level of statistical significance was set to $p < 0.05$.

Acknowledgements

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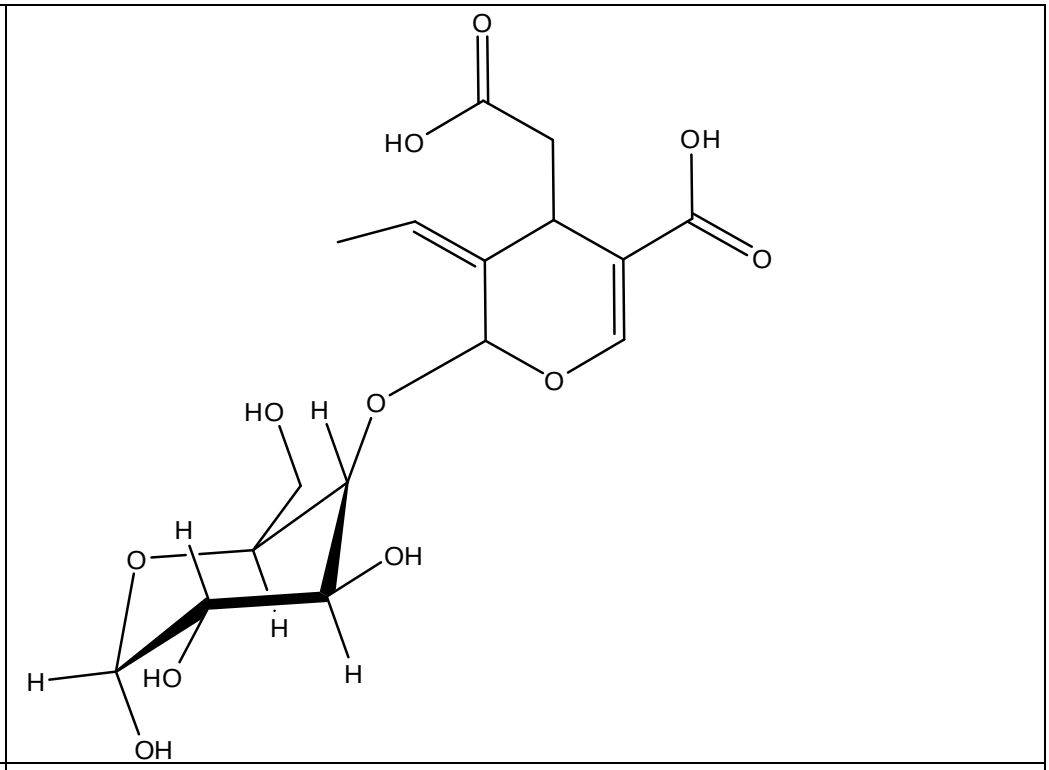
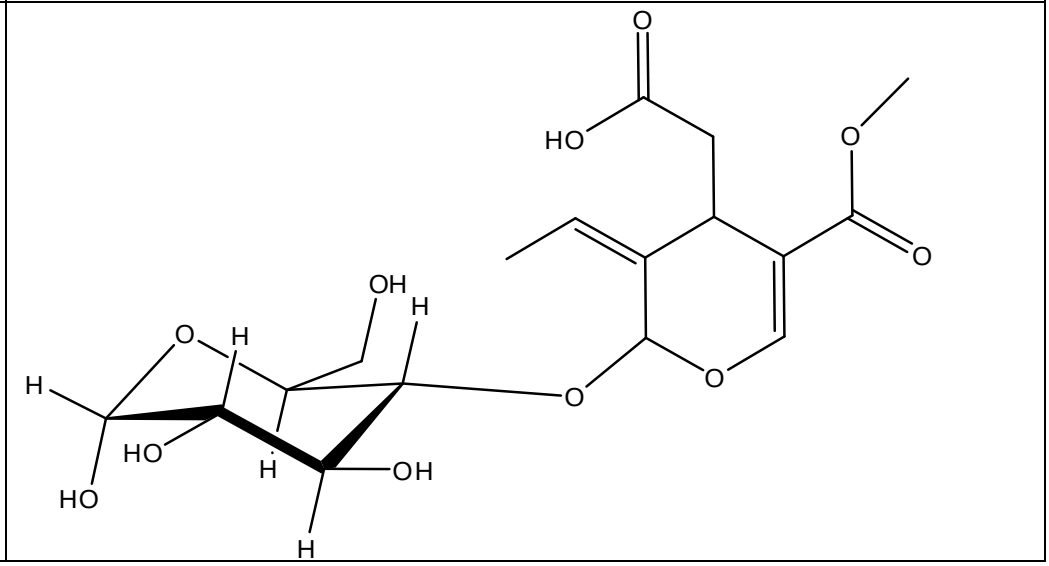
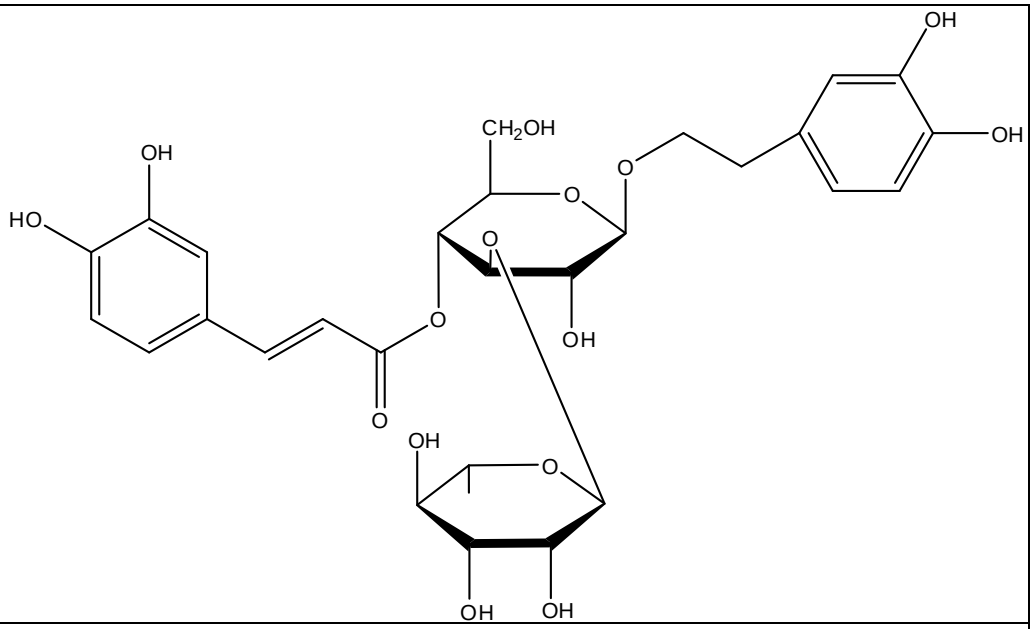
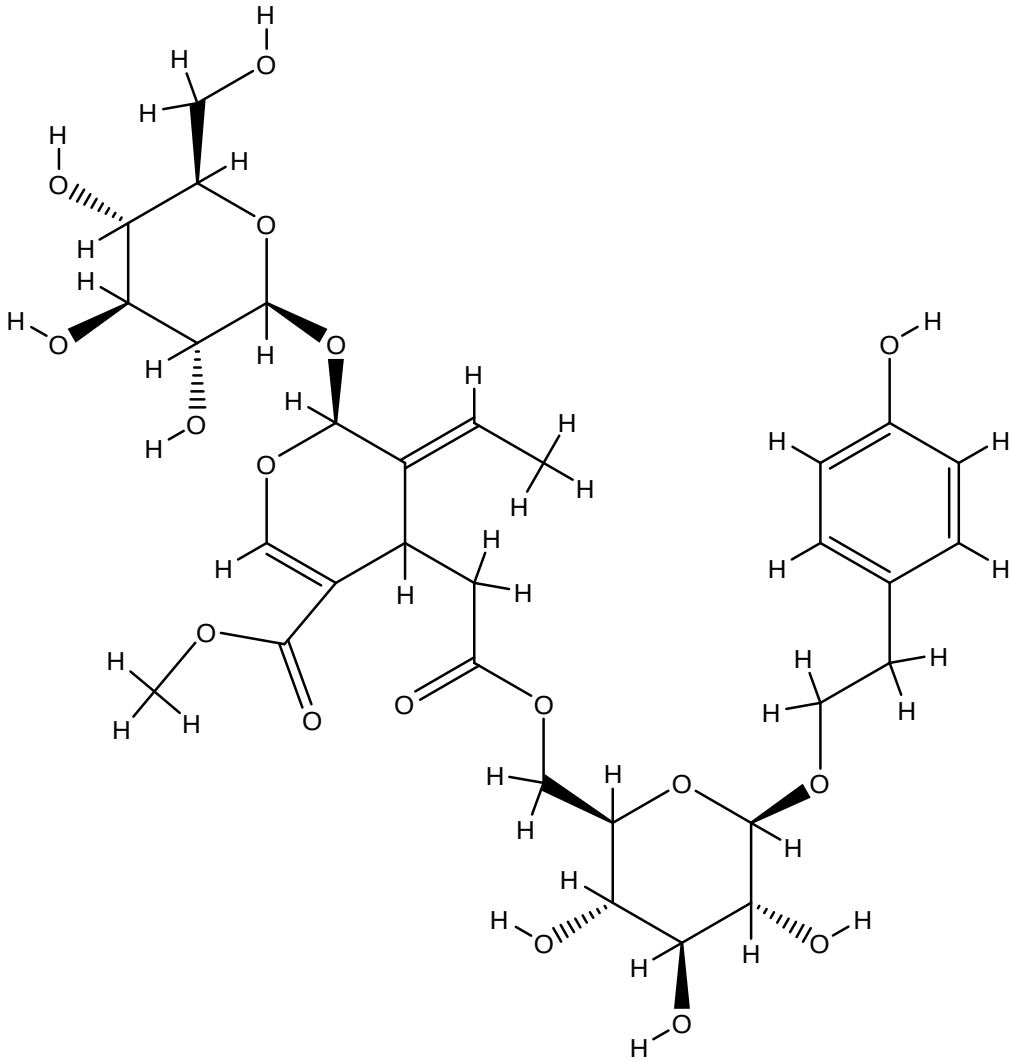
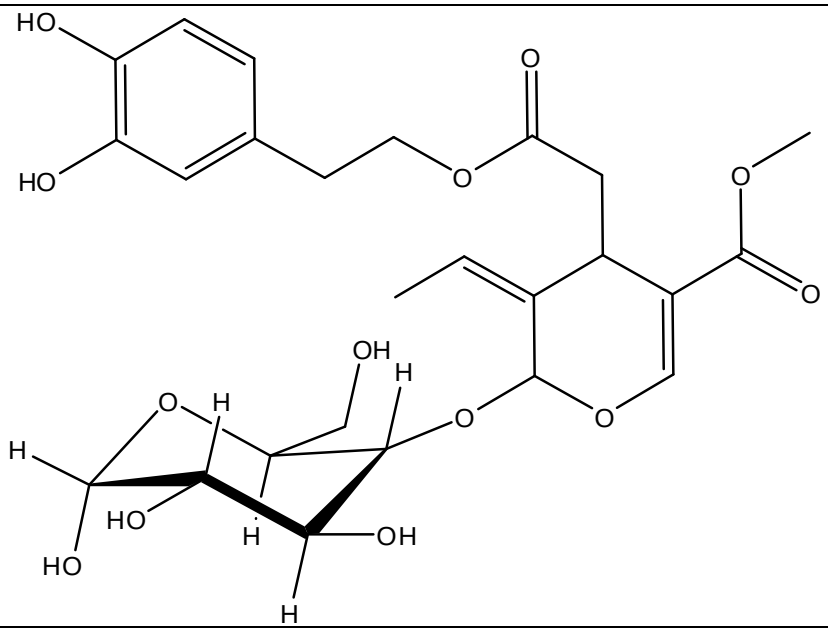
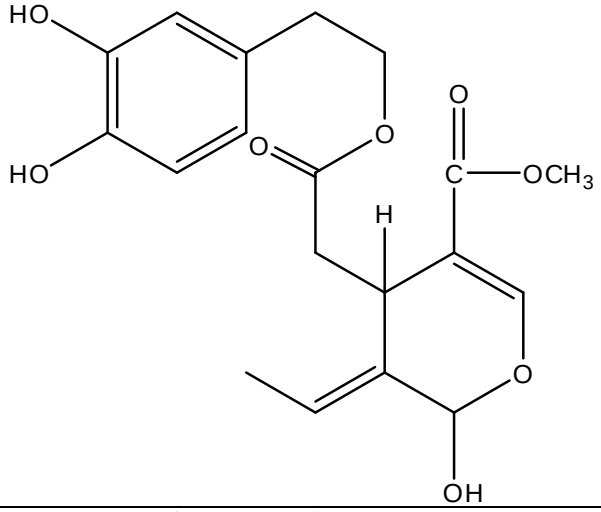
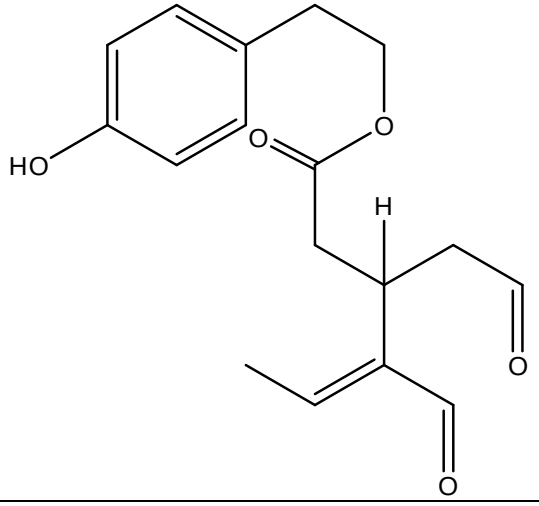
Figures and legends

Oleoside	
Elenolic acid glucoside	

Verbascoside	
Nuzhenide	

Oleuropein	
3,4-DHPEA-EDA	
P-HPEA-EDA	

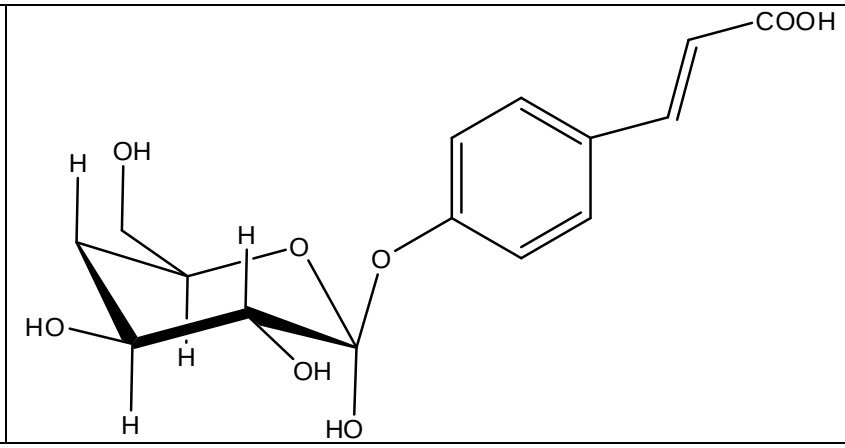
Coumaric acid glucoside	

Figure 1: Phenolic compounds identified only in olive pomace and not in olive mill wastewater.

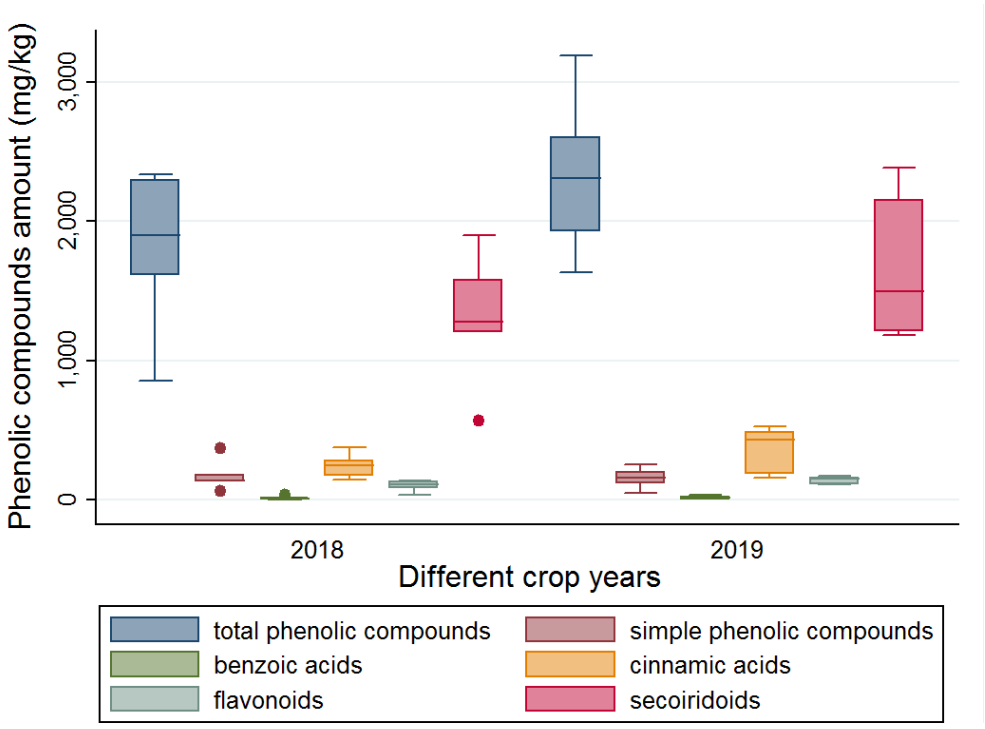


Figure 2: Total phenolic compound and phenolic compound composition according crop years 2018 and 2019.

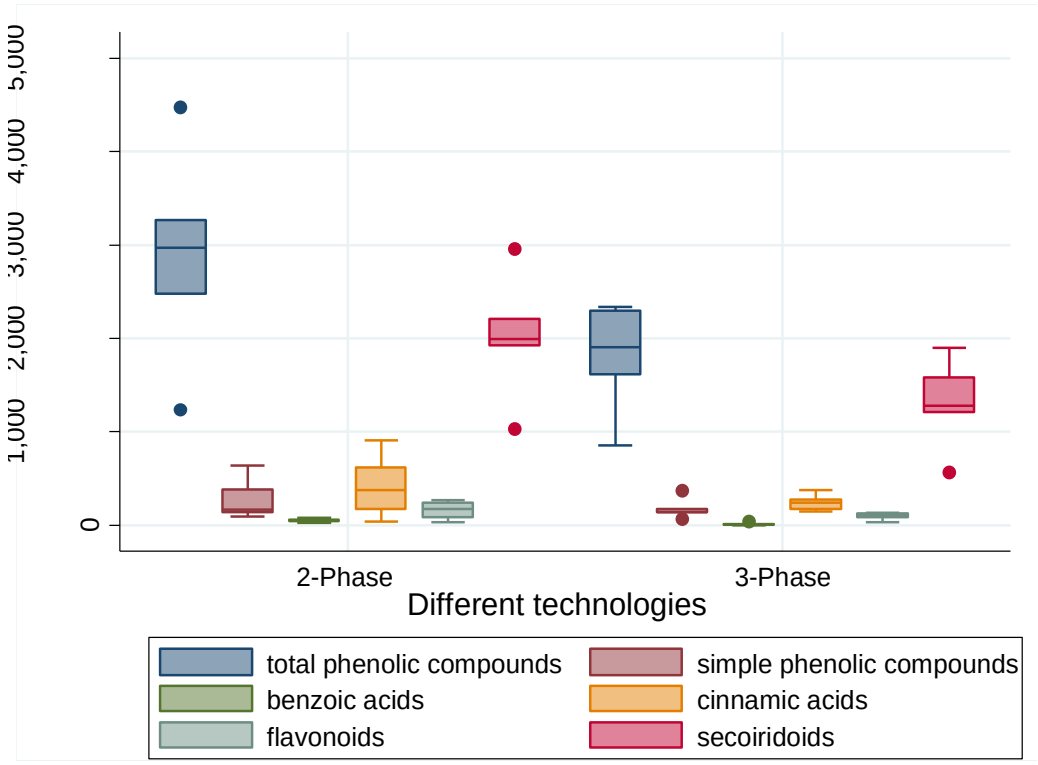


Figure 3: Total phenolic compound and phenolic compound composition according technology used (two-phase separating decanter and three-phase separating decanter).

Tables

Table 1: Median, minimum and maximum levels of each determined phenolic compound; total phenolic compounds; simple phenolic compounds; benzoic acids; cinnamic acids; flavonoids; secoiridoids and radical scavenging activity by DPPH. Eighteen samples were included in all the measurements.

Name of the compound	**Median**	**Min**	**Max**	**r_s DPPH corr. sig. $p < 0.05$**
Oleoside 1** (mg/kg dry wt)	26	13	90	-0.77
Oleoside 2 ** (mg/kg dry wt)	30	<LOQ	46	
Hydroxytyrosol, hydroxytyrosol glucoside, Oleoside 3 (mg/kg dry wt)	115	45	605	-0.70
Elenolic acid glucoside 1 (mg/kg dry wt)	11	<LOQ	76	-0.67
Elenolic acid glucoside 2 (mg/kg dry wt)	<LOQ	<LOQ	24	
Elenolic acid glucoside 3 (mg/kg dry wt)	48	<LOQ	136	-0.66
Tyrosol (mg/kg dry wt)	30	<LOQ	133	
Sacolagonoside (mg/kg dry wt)	98	19	274	
Trans p-coumaric acid 4-glucoside (mg/kg dry wt)	41	<LOQ	150	
Caffeic acid (mg/kg dry wt)	12	<LOQ	97	-0.63

Elenolic acid glucoside 4 (mg/kg dry wt)	14	<LOQ	126	
Luteolin-4`,7-*O*-diglucoside (mg/kg dry wt)	<LOQ	<LOQ	67	
β-OH-verbascoside 1 (mg/kg dry wt)	<LOQ	<LOQ	44	
β-OH-verbascoside 2 (mg/kg dry wt)	64	<LOQ	137	-0.67
Vanilin (mg/kg dry wt)	16	<LOQ	74	-0.67
Verbascoside 1 (mg/kg dry wt)	60	<LOQ	261	
Dimethyloleuropein (mg/kg dry wt)	<LOQ	<LOQ	284	
Rutin (mg/kg dry wt)	39	16	204	
Verbasciside 2 (mg/kg dry wt)	84	<LOQ	405	
Luteolin-7`-*O*-glucoside (mg/kg dry wt)	<LOQ	<LOQ	47	
Luteolin rutinoside (mg/kg dry wt)	20	<LOQ	123	
Nuzhenide 1 (mg/kg dry wt)	14	<LOQ	146	
Luteolin-4`-*O*-glucoside (mg/kg dry wt)	0.1	<LOQ	58	
Caffeoyl-6-secologanoside (mg/kg dry wt)	<LOQ	<LOQ	285	
Nuzhenide 2 (mg/kg dry wt)	123	<LOQ	551	
Luteolin-3`-*O*-glucoside ** (mg/kg dry wt)	7.8	<LOQ	69	
3,4-DHPEA EDA. Oleuroside 2 (mg/kg dry wt)			1981	

	985	293		-0.60
Oleuropein aglycone 2** (mg/kg dry wt)	<LOQ	<LOQ	248	
Oleuropein/Oleuroside 3** (mg/kg dry wt)	<LOQ	<LOQ	55	
Ligstroside (mg/kg dry wt)	<LOQ	<LOQ	162	
Oleuropein aglycone 3 (mg/kg dry wt)	<LOQ	<LOQ	128	
p-HPEA-EDA** (mg/kg dry wt)	<LOQ	<LOQ	91	
Oleuropein aglycone 5** (mg/kg dry wt)	<LOQ	<LOQ	16	
Apigenin (mg/kg dry wt)	5.8	<LOQ	20	-0.66
Oleuropein aglycone 7** (mg/kg dry wt)	<LOQ	<LOQ	154	
3,4-DHPEA EDA (mg/kg dry wt)	<LOQ	<LOQ	52	
Oleuropein aglycone 8** (mg/kg dry wt)	12	<LOQ	30	
Oleuropein aglycone 9** (mg/kg dry wt)	<LOQ	<LOQ	13	
Simple phenolic compounds (m/kg dry wt)	154	45	637	-0.71
Benzoic acids (mg/kg dry wt)	16	<LOQ	74	-0.67
Cinnamic acids (mg/kg dry wt)	265	36	905	-0.60
Flavonoids (mg/kg dry wt)	129	31	266	
Secoiridoids (mg/kg dry wt)	1632	564	2953	-0.72

Total phenolic compounds (mg/kg dry wt)	2317	851	4473	-0.81
Radical scavenging activity by DPPH EC50 (µg/mL)	317	200	1060	

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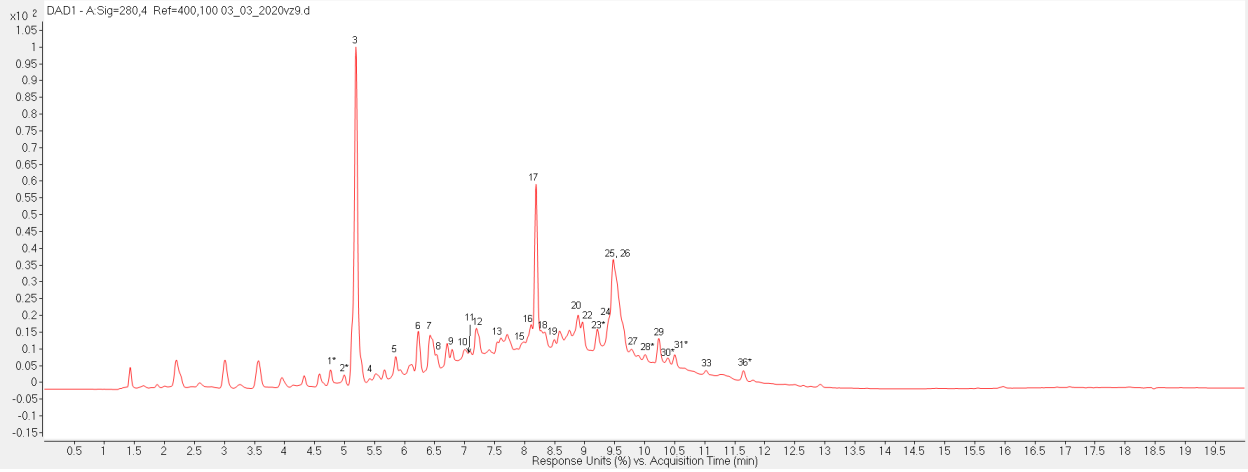
Supplementary material



Supplementary Figure 1: An example of UV chromatogram at 280 nm of olive pomace extract.

Supplementary Table 1: Phenolic compounds found in pomace and in mill water.

Peak number	**Compound**	**Fr.**	**RT**	**Mr Exp.**	**Mr Calc.**	**Diff (ppm)**	**m/z [M]$^-$**	**Fragments**	**Molecular formula**	**UV max (nm)**
1	Oleoside**	P	4.8	390.1159	390.1162	-0.72	389.1089	389, 183, 209, 227	$C_{16}H_{22}O_{11}$	229, 289
2	Oleoside**	P	5.0	390.1163	390.1162	0.13	389.1091	389, 209, 345	$C_{16}H_{22}O_{11}$	255, 290
3	Hydroxytyrosol glucoside	P, W	5.2	316.1148	316.1158	-3.35	315.1071	315, 153, 123	$C_{14}H_{20}O_8$	230, 282
3	Hydroxytyrosol	P, W	5.2	154.0624	154.0630	-3.93	153.0551	123, 153	$C_8H_{10}O_3$	230, 280

3	Oleoside	P	5.2	390.1161	390.1162	-0.4	389.1090	389, 183, 209	$C_{16}H_{22}O_{11}$	200, 230, 280
4	Elenolic acid glucoside – Isomer 1	P	5.4	404.1321	404.1319	0.69	403.1244	403, 223, 179	$C_{17}H_{24}O_{11}$	236
4.1	Elenolic acid glucoside – Isomer 2	P	5.5	404.1320	404.1319	0.29	403.1248	403, 223, 179	$C_{17}H_{24}O_{11}$	235
5	Elenolic acid glucoside – Isomer 3	P	5.8	404.1317	404.1319	-0.43	403.1245	403, 223, 179	$C_{17}H_{24}O_{11}$	233
6	Tyrosol	P, W	6.2	/	/	/	/	/	$C_{10}H_8O_2$	227, 280
7	Secologanoside	P, W	6.3	390.1160	390.3384	-0,49	389.1086	389, 345, 183, 209	$C_{16}H_{22}O_{11}$	230
8	Trans p-coumaric acid 4-glucoside	P	6.5	326.0994	326.1002	-2.49	325.0919	163, 119, 325	$C_{15}H_{18}O_8$	n.d.
9	Caffeic acid	P, W	6.7	180.0433	180.0423	5.55	179.0357	179, 135	$C_{16}H_{22}O_{11}$	230, 289, 330
10	Elenolic acid glucoside Isomer 4	P	7.0	404.1321	404.1319	0.67	403.1249	403, 223, 179	$C_{17}H_{24}O_{11}$	237
11	Luteolin-4`,7-*O*-diglucoside	P, W	7.1	610.1886	610.1898	-1.88	609.1795	609, 447, 285	$C_{27}H_{30}O_{16}$**	n.d.

12	β-OH-verbascoside Isomer I	P,W	7.2	640.2013	640.2003	1.45	639.1927	639, 621, 459, 179, 161	$C_{29}H_{36}O_{16}$	239 283 330
12	β-OH-verbascoside Isomer 2	P, W	7.2	640.2031	640.2003	4.27	639.1935	639, 621, 459, 179, 161	$C_{29}H_{36}O_{16}$	239 283 330
13	Vanilin	W	7.7	152.0477	152.0473	2.5	151.0406	151, 136	$C_8H_8O_3$	235 281 310
14	Verbascoside Isomer I	P	7.7	624.2087	624.2054	5.29	623.2018	623, 461, 161	$C_{29}H_{36}O_{15}$	265, 291, 330
15	Demethyloleuropein	P,W	7.9	526.1704	526.1686	3.33	525.1623*	525, 389, 319, 183, 345	$C_{24}H_{30}O_{13}$	240 280
16	Rutin	P,W	8.1	610.1557	610.1534	3.72	609.1469	609, 300, 179	$C_{27}H_{30}O_{16}$	256 358
17	Verbascoside Isomer II	P	8.2	624.2057	624.2054	0.47	623.1981	623, 461, 161	$C_{29}H_{36}O_{15}$	247 285 331
18	Luteolin-7`-*O*-glucoside	P,W	8.3	448.1014	448.1006	1.76	447.0938	447, 285	$C_{21}H_{20}O_{11}$	255 350
18	Luteolin rutinoside	P, W[x]	8.3	594.1605	594.1585	3.47	593.1533	593, 285, 447	$C_{27}H_{30}O_{15}$	255 350

19	Nuzhenide Isomer 1	P	8.4	686.2392	686.2422	-4.4	685.2334	685, 523, 453, 421, 299, 223	$C_{31}H_{42}O_{17}$	239 277 333**
20	Luteolin-4`-*O*-glucoside	P, W	8.9	448.1010	448.1006	1.06	447.0934	447, 285	$C_{21}H_{20}O_{10}$	285, 330
21	Caffeoyl-6-secologanoside	P, W	8.9	552.1479	552.1479	0.02	551,1406	551, 507, 393, 281, 251, 179, 161	$C_{25}H_{28}O_{14}$	235, 325
22	Nuzhenide Isomer 2	P	9.0	686.2427	686.2422	0.68	685.2365	223, 299, 453, 523, 685	$C_{31}H_{42}O_{17}$	242 280, 330
23	Luteolin-3`-*O*-glucoside**	P, W	9.3	448.1018	448.1006	2.71	447.0939	447, 285	$C_{21}H_{20}O_{11}$	280
24	Oleuropein	P	9.4	540.1844	540.1843	0.26	539.1770	539, 149, 275, 377, 223	$C_{25}H_{32}O_{13}$	233, 282
25	3,4-DHPEA-EDA	P	9.5	320.1269	320.1260	2.77	319.1185	195, 183, 165, 139	$C_{17}H_{20}O_{6}$	237, 282
26	Oleuropein aglycone Isomer 1**	P	9.5	378.1320	378.1315	1.43	377.1245	377, 275, 149, 139, 307	$C_{19}H_{22}O_{8}$	n.d.
27	Oleuropein/Oleuroside	P	9.7	540.1822	540.1843	-3.92	539.1761	377. 539, 275, 149	$C_{25}H_{32}O_{13}$	239

28	Oleuropein aglycone Isomer 2**	P	10.0	378.1328	378.1315	3.44	377.1250	377, 345, 275, 149, 139, 307	$C_{19}H_{22}O_8$	225, 275
28	Oleuropein/Oleuroside **	P,W	10.0	540.1813	540.1843	-5.57	539.1743	275, 539, 149	$C_{25}H_{32}O_{13}$	225, 275
29	Ligstroside	P,W*	10.3	524.1889	524.1894	-0.82	523.1812	523, 223, 101	$C_{25}H_{32}O_{12}$	252, 270, 350
29.1	Oleuropein aglycone Isomer 3	P	10.3	378.1318	378.1315	0.78	377.1240	377, 345, 275, 149, 139, 307	$C_{19}H_{22}O_8$	240, 270
30	p-HPEA-EDA **	P	10.4	304.1312	304.1311	0.38	303.1235	179, 165, 183*, 59*, 137*	$C_{17}H_{20}O_5$	230, 282
30	Oleuropein aglycone Isomer 4 **	P	10.4	378.1321	378.1315	1.64	377.1234	377, 345, 275, 149, 139, 307	$C_{19}H_{22}O_8$	230, 280
31	Oleuropein aglycone Isomer 5 **	P	10.5	378.1314	378.1315	-0.12	377.1240	377, 345, 275, 149, 139, 307	$C_{19}H_{22}O_8$	225 280
32	Oleuropein aglycone Isomer 6	P	10.7	378.1327	378.1315	3.33	377.1242	377, 345, 275, 149, 139, 307	$C_{19}H_{22}O_8$	n.d.
33	Apigenin	P, W	11.0	270.0530	270.0523	0.71	269.0457	269	$C_{15}H_{10}O_5$	239,

										269, 339
34	Oleuropein aglycone Isomer 7	P, W	11.1	378.1322	378.1315	2.02	377.1243	377, 275, 149, 139, 307, 327	$C_{19}H_{22}O_8$	n.d.
35	3,4-DHPEA-EDA	P	11.3	320.1262	320.1260	0.62	319.1187	195, 183, 165, 139	$C_{17}H_{20}O_6$	232, 280
35	Oleuropein aglycone Isomer 8 **	P	11.3	378.1319	378.1315	1.03	377.1242	377, 275, 149, 139, 307, 327	$C_{19}H_{22}O_8$	230 280
36	Oleuropein aglycone Isomer 9 **	P	11.6	378.1315	378.1315	0.06	377.1242	377, 275, 149, 139, 307, 327	$C_{19}H_{22}O_8$	225, 282

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