

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

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1 **Graphical abstract**

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

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25

26

27 **Abstract**

28

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

44

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

47 **1. Introduction**

48

49 The production of olive oil in the Istrian region of Slovenia has a long-established tradition dating  
50 back to the 4th Century BC (Darovec eand Ermacora, 1998). At the heart of this is the “Istrska  
51 belica” cultivar of olives (Istrian white olives), which have been praised for their ability to  
52 withstand low temperatures, high oil content, excellent taste, high levels of monounsaturated fatty  
53 acids and high levels of biologically active molecules including phenolic compounds, squalene  
54 and tocopherols (Lazović et al., 2018; Baruca Arbeiter et al., 2014; Bešter et al., 2008). It has been  
55 determined that the levels of phenolic compounds are significantly higher in varieties of “Istrska  
56 Belica” when compared to other varieties from within the same location (Bučar-Miklavčič et al.,  
57 2016). This high phenolic content contributes to the organoleptic profile of the oil produced from  
58 these olives (Bučar-Miklavčič et al., 2016). Phenolic compounds from olives offers a variety of  
59 benefits to human health, including a reduction in coronary heart disease risk factors, prevention  
60 of several types of cancers and modification of immune and inflammatory responses (Bendini et  
61 al., 2007; Bogani et al., 2007; Bulotta et al., 2014).

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

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

83 More than 50 different phenolic compounds have been identified in olive pomace with the  
84 remaining stones and OMWW that contain mostly simple phenolic compounds, benzoic acid  
85 derivatives, cinnamic acids derivatives, flavonoids, lignans and secoiridoids (Jerman Klen et al.,  
86 2015), with the latter molecules found specifically in olives (Ryan et al., 2002; Montedoro et al.,  
87 2002). During the olive oil manufacturing process, ligstroside and oleuropein can enter different  
88 transformation-reaction pathways involving plant enzymatic and chemical transformation  
89 (Rovellini and Cortesi, 2002). When the transformation pathway is reaching its end and the olive  
90 oil has already lost its freshness and antioxidative properties after one or two years of storage,  
91 depending on the variety, the total phenolic compounds content can be relatively high with higher  
92 amounts of simple phenolic compounds such as tyrosol and hydroxytyrosol (Bučar-Miklavčič et  
93 al., 2016). The same process of phenolic compounds breaking down into simple phenolic  
94 compounds, such as tyrosol and hydroxytyrosol, is expected to occur in olive mill effluents.

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

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

106

## 107 **2. Results and discussion**

### 108 **2.1 Identification of phenolic compounds in olive mill wastewater and pomace**

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

112

#### 113 **2.1.1 Simple phenolic compounds: Hydroxytyrosol and its derivatives**

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

#### 123 **2.1.2 Benzoic acids**

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

126

#### 127 **2.1.3 Cinnamic acids**

128

129 Esters of cinnamic acids, such as verbascoside and  $\beta$ -Methyl-OH-verbascoside, were found in  
130 pomace (Jerman-Klen et al., 2015; Mulinacci et al., 2005). However, unlike Jerman Klen et al.  
131 (2015), verbascoside was not found in olive mill wastewater. As previously reported (Ryan et al.,  
132 1999), during studies on olive fruits, verbascoside may exist as a pair of geometric isomers arising  
133 from the caffeic acid moiety or different attachment of the sugar to the aglycone. The presence of  
134 verbascoside was confirmed through comparison with the retention time of a standard solution  
135 (7.7 min, Figure 1), similar to two  $\beta$ -OH-verbascoside isomers that were found in both pomace  
136 and olive mill water (Supplementary Table 1). At 8.1 min, a possible verbascoside isomer was  
137 identified; in addition, caffeic acid, a member of a large and varied family of hydrohycinannamoyl  
138 conjugates that also includes p-coumaric and ferulic acid derivate (Ellis, 1985), was identified by  
139 comparison to previously reported exact mass and fragmentation patterns (Hu et al., 2005). Trans  
140 p-coumaric acid 4-glucoside was identified in pomace by exact mass detecting fragments 163 and  
141 119, as previously reported by Jerman Klen et al. (2015). The same fragmentation pattern for p-  
142 coumaric acid was previously reported by Araújo et al. (2015).

143

144

#### 145 **2.1.4 Flavonoids**

146

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

152 Based on reported data (Cuyckens and Claeys, 2004 and Jerman Klen et al., 2015), the observed  
153 absorption maxima corresponded to three different luteolin-glucosides, tentatively identified as  
154 luteolin-7'-*O*-glucoside (retention time 8.3 min), luteolin-4'-*O*-glucoside (8.9 min) and luteolin-  
155 3'-*O*-glucoside (9.3 min). However, the latest annotated peak did not have a typical UV absorption  
156 maximum at 270 and 340 nm, so it might be the luteolin-3'-*O*-glucoside only in structure. Luteolin  
157 rutinoside with typical fragmentation pattern of m/z 593, 447 and 285 eluted before luteolin-4'-*O*-  
158 glucoside and after luteolin-7'-*O*-glucoside, as previously reported (Jerman Klen et al., 2015). This  
159 compound was present in higher quantities in pomace and in much smaller quantities in OMWW.  
160 In OMWW, fragmentation pattern identification was not possible due to the low concentration. In  
161 contrast to the literature (Jerman Klen et al., 2015), only one isomer of luteolin rutinoside was  
162 found, and this could be attributed to the different column and elution conditions used. The  
163 analyses by Jerman Klen et al. (2015) took 88 min per sample, which was infeasible for routine  
164 analysis, so, in the current study, the column conditions were modified in order to fully elute the  
165 sample in 20 min. However, this can preclude meaningful comparison of phenolic composition  
166 based purely on retention times.

167

## 168 **2.1.5 Secoiridoids**

### 169 2.1.5.1 Oleoside

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

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

181

#### 182 2.1.5.2 Oleuropein and its derivatives

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

188 Demethyloleuropein (molar mass 526.1704 g/mol) was detected in pomace with m/z 571.1693 (M  
189 + HCOO), together with m/z 525.1623, along with the same fragmentation pattern (525, 389, 319,  
190 183, 345) and similar relative retention time as reported elsewhere (Jerman Klen et al., 2015). In  
191 OMWW, a compound was found at a similar retention time, but it was impossible to identify as  
192 demethyloleuropein by the fragmentation pattern due to very low levels.

193 Oleuropein-aglycone dialdehydes (3,4-DHPEA-EDA) with exact molar masses of 319.1185  
194 (Isomer 1) and 319.1187 (Isomer 2) were tentatively identified at retention times 9.4 and 11.2 min  
195 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 (Talhoui 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 Talhoui 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<sub>2</sub>  
216 (Jerman Klen et al., 2015).

#### 217 2.1.5.4 Ligustroside

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

#### 223 2.1.5.5 Caffeoyl-6-secologanoside and comselogoside

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

#### 227 2.1.5.6 Nuzhenide

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

### 234 **2.2 Quantification of phenolic compounds in pomace**

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

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

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

263 correlation between evaluation of radical-scavenging activity by DPPH assay and the percentage  
264 of secoiridoids or simple phenolic compounds for total phenolic compounds in pomace samples.  
265 Several possible levels of variation were identified for the quantify of the phenolic compounds in  
266 OMWW and pomace generated from olive oil extraction industrial processes (discussed in the  
267 Introduction). However, from the current state-of-the-art industrial point of view (often very  
268 difficult to control the input crop) and from a preliminary statistical analysis, the two discussed in  
269 sub-sections 2.2.1 and 2.2.2 were chosen for a more detailed investigation and presentation.

270

### 271 **2.2.1 Variation in phenolic compound content in olive pomace across different** 272 **growing seasons**

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

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

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

305

### 306 **2.2.2 Variation in phenolic compound content in olive pomace using different** 307 **separation (centrifugation) technologies**

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

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

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

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

### 331           **2.2.3 Radical scavenging activity by DPPH**

332   Determination of radical scavenging activity, using the DPPH assay, is a suitable method for  
333   predicting the inhibition of primary oxidation product formation by natural extracts (Molyneux,  
334   2004; Shwarz et al., 2001). The EC<sub>50</sub> value determined in the pomace samples correlates inversely  
335   with the concentrations of total phenolic compounds ( $r_s = -0.8$ ;  $p < 0.05$ ). The inverse correlation  
336   is expected because EC<sub>50</sub> value is defined as the concentration of substrate that causes 50% loss  
337   of DPPH activity (color) (Molyneux, 2004). Spearman Rank correlation is the strongest between  
338   the total phenolic compounds and radical scavenging activity by DPPH as compared to the  
339   Spearman Rank correlation between each phenolic compound or groups of phenolic compounds  
340   determined in the samples and radical scavenging activity by DPPH (Table 1). This confirms the  
341   previously reported observation that the antioxidant pattern is usually complex, and it can include  
342   synergistic effects of the compounds that are not possible to determine only by the quantification  
343   of phenolic compounds by the HPLC-MS method (Schwarz et al., 2001).

344

### 345           **3. Conclusions**

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

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

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

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

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

## 379 **4. Experiment**

### 380 **4.1 General experimental procedures**

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

389  
390 Analytical standards such as oleuropein (12247-10MG, Sigma Aldrich), hydroxytarosol (SI-  
391 H4291-25MG, Sigma Aldrich), tyrosol (AL-188255-5G, Sigma Aldrich), luteolin (SI-L9283-  
392 10MG), verbascoside (V4015-10MG, Sigma Aldrich) and apigenin (SI-SMB00702-5MG, Sigma  
393 Aldrich) were used for quantification of phenolic compounds; 2,2-Diphenyl-1-picrylhydrazyl  
394 (D9132-250MG, Sigma Aldrich) was used for determination of radical scavenging activity for  
395 pomace extracts.

396

### 397 **4.2 Samples**

398 A total of 18 pomace samples from olives of *Olea europaea* L. were collected weekly from the  
399 beginning olive oil production until the end of the mill production season in 2018 and 2019 (14  
400 October 2018 – 18 November 2018 and 16 October 2019 – 09 November 2019). During crop year  
401 2018, the samples were collected from two olive mills, Franka Marzi and Lisjak (Koper, Slovenian  
402 Istria), using different processing technologies (two-phase – Perialisi FP60 RS ATEX and three-  
403 phase decanter centrifuge – Alfa Laval x 4); in 2019, the samples were collected only from three-  
404 phase decanter centrifuge (Franka Marzi). During the two-phase decanting process, olives are  
405 initially washed, crushed and malaxed (churned), and water is added to a horizontal centrifuge  
406 (40–60 L/100 kg fruits weight), separating pomace from the oily must consisting of the vegetable  
407 water and oil. Oil, pomace and wastewater are the final products formed at one end of the three-  
408 phase decanter. In contrast to three-phase decanter, the two-phase decanter requires no additional  
409 water due to the much higher centrifugal speeds, resulting in olive oil and wet olive cake or pomace  
410 (Niaounakis et al., 2006; Tsagaraki et al., 2007; Di Giovacchino et al., 2002).

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

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

#### 420 **4.2.1 Extraction of phenolic compounds**

421 Phenolic compounds were extracted from freeze dried pomace (2g) in methanol / water 80:20  
422 (50 mL, pH 2-HCl) for 30 minutes with stirring at room temperature and then re-extracted with  
423 fresh solvent (20 mL) for 15 minutes. The combined extracts were filtered and defatted using  
424 hexane (30 mL x 2). The defatted extracts were filtered and concentrated *in vacuo* (1.5 hrs). The  
425 residue was reconstituted to 10 mL of methanol and re-filtered through 0.2 µm plastic non-sterile  
426 filter. The procedure is described in detail elsewhere by Obied et al. (2008).

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

#### 432 **4.3 Determination of phenolic compounds by HPLC-DAD-ESI-TOF**

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

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

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

#### 474 **4.5 Statistical analysis**

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

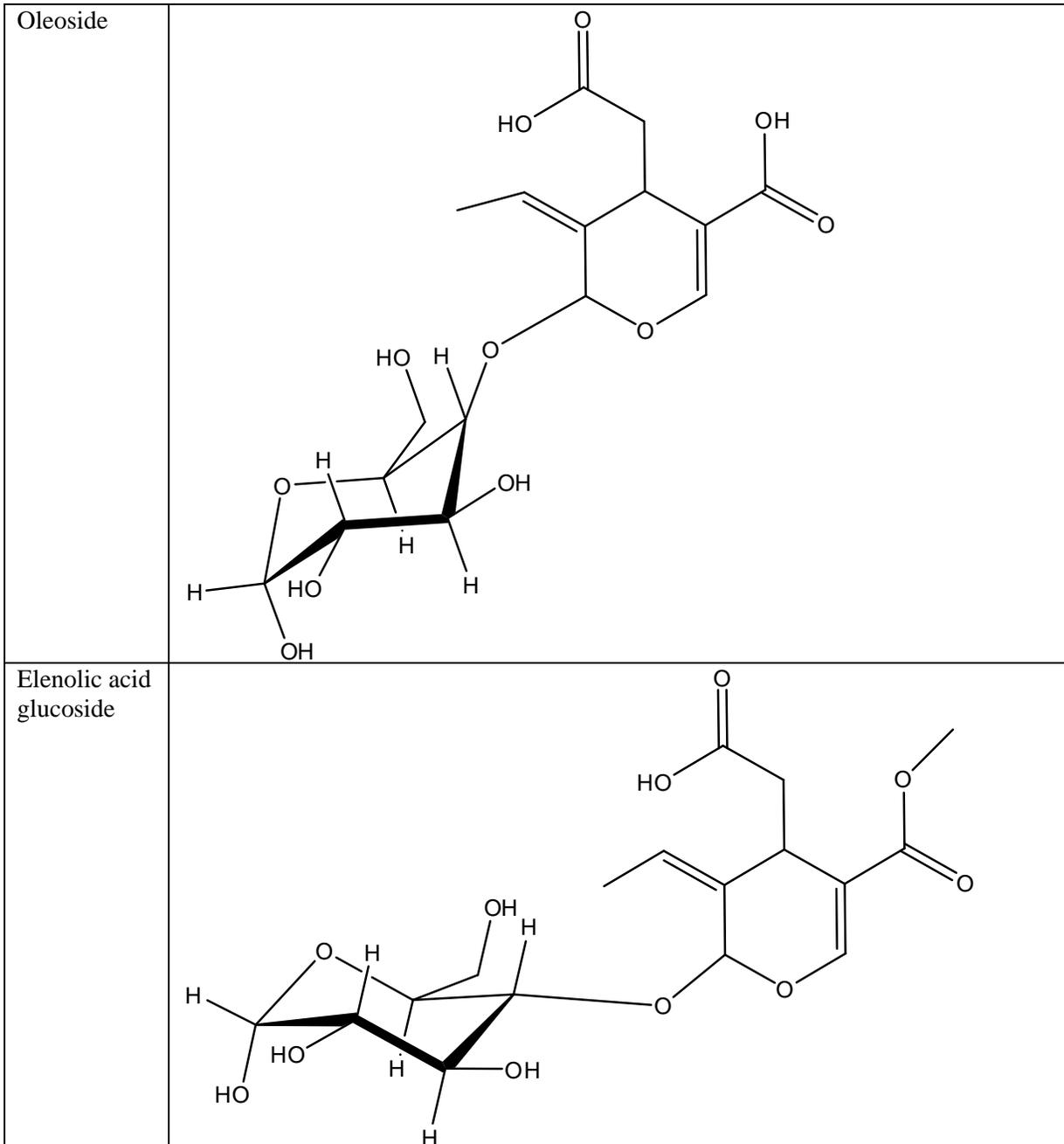
#### 480 **Acknowledgements**

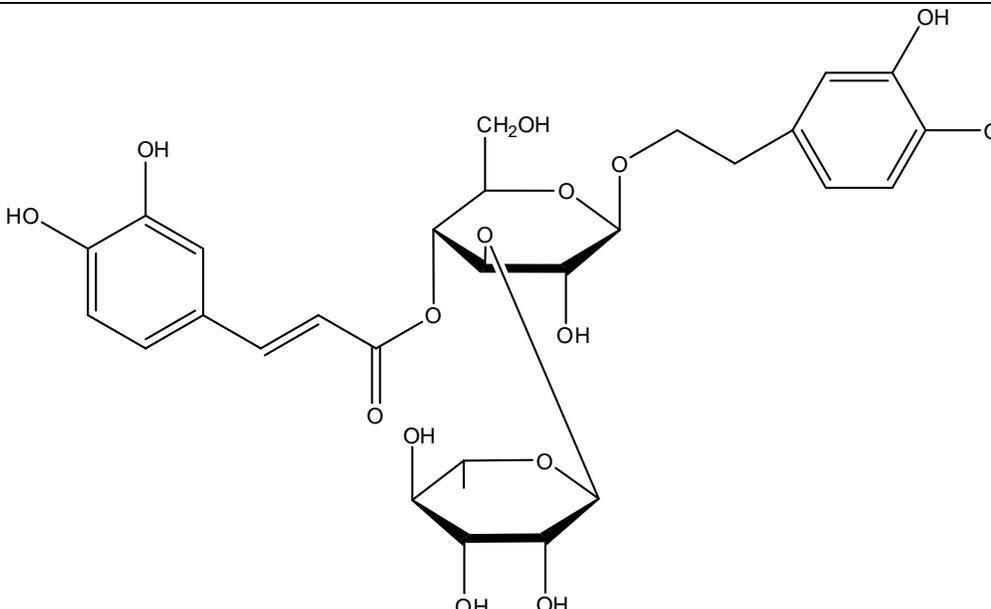
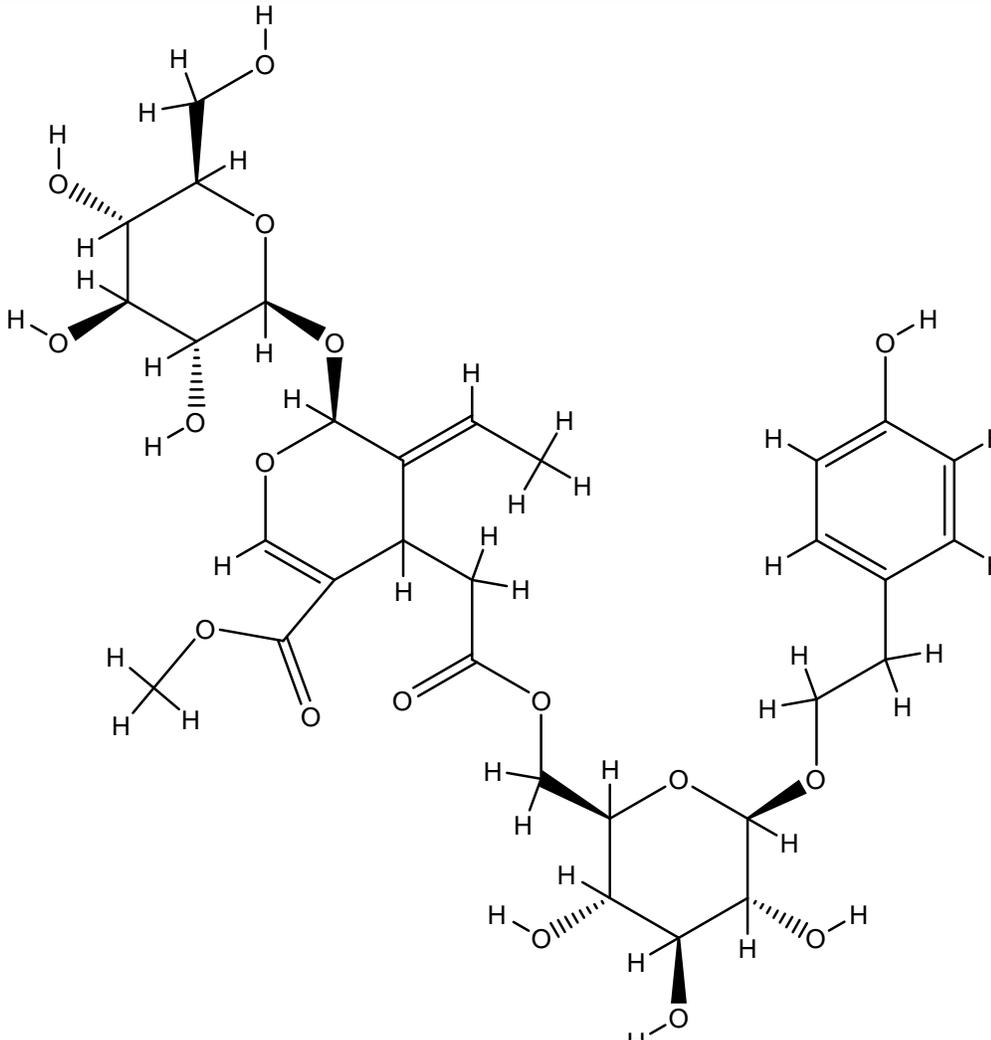
481 This work was supported by the Bio-based Industries Joint Undertaking that provided funding for  
482 the Pro-Enrich project (Grant Agreement No. 792050) under Horizon 2020, the European Union’s  
483 Framework Programme for Research and Innovation, and the Franka Marzi and Lisjak olive mills  
484 (Koper, Slovenian Istria) for provision of samples for this study.

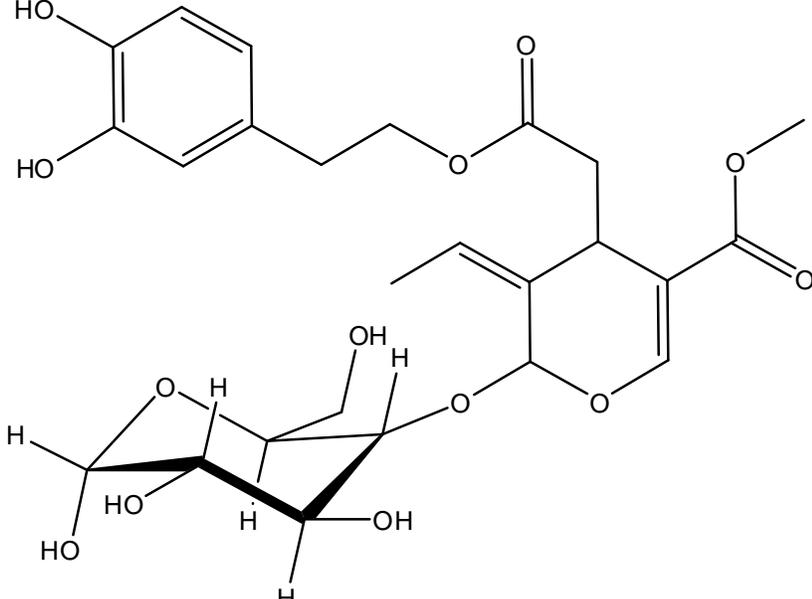
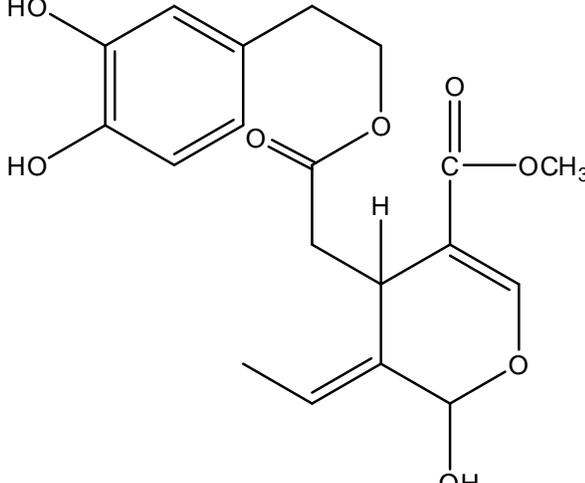
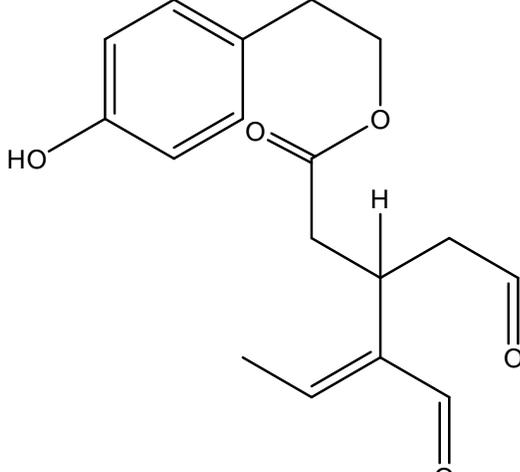
485



## Figures and legends



|                     |  |
|---------------------|--|
| <p>Verbascoside</p> |  <p>The structure of Verbascoside is a complex polyphenolic glycoside. It features a central glucose molecule in its cyclic form, with a gallic acid moiety attached at the C-6 position via an ester linkage. The gallic acid moiety consists of a benzene ring with three hydroxyl groups and a propenoic acid side chain. The glucose molecule also has a hydroxyl group at C-2 and a hydroxymethyl group at C-4. At the C-3 position, there is a 3,4,5-trihydroxybenzyl group attached via an ether linkage.</p> |
| <p>Nuzhenide</p>    |  <p>The structure of Nuzhenide is a complex polyphenolic glycoside. It features a central glucose molecule in its cyclic form, with a gallic acid moiety attached at the C-6 position via an ester linkage. The gallic acid moiety consists of a benzene ring with three hydroxyl groups and a propenoic acid side chain. The glucose molecule also has a hydroxyl group at C-2 and a hydroxymethyl group at C-4. At the C-3 position, there is a 3,4,5-trihydroxybenzyl group attached via an ether linkage.</p>   |

|                      |   |
|----------------------|---|
| <p>Oleuropein</p>    |  <p>The structure of Oleuropein consists of a central glucose molecule in its cyclic pyranose form. The glucose is substituted at the 2, 3, and 6 positions. At C-2, there is a 3,4,5-trihydroxyphenylpropyl chain. At C-3, there is a 4-hydroxyphenyl chain. At C-6, there is a 3,4-dihydroxyphenyl chain. The glucose ring is shown with wedged and dashed bonds to indicate stereochemistry.</p> |
| <p>3,4-DHPEA-EDA</p> |  <p>The structure of 3,4-DHPEA-EDA features a central glucose molecule. It is substituted at C-2 with a 3,4-dihydroxyphenylpropyl chain and at C-3 with a 4-hydroxyphenyl chain. The glucose ring is shown in a chair-like conformation.</p>  |
| <p>P-HPEA-EDA</p>    |  <p>The structure of P-HPEA-EDA features a central glucose molecule. It is substituted at C-2 with a 3,4-dihydroxyphenylpropyl chain and at C-3 with a 4-hydroxyphenyl chain. The glucose ring is shown in a chair-like conformation.</p>  |

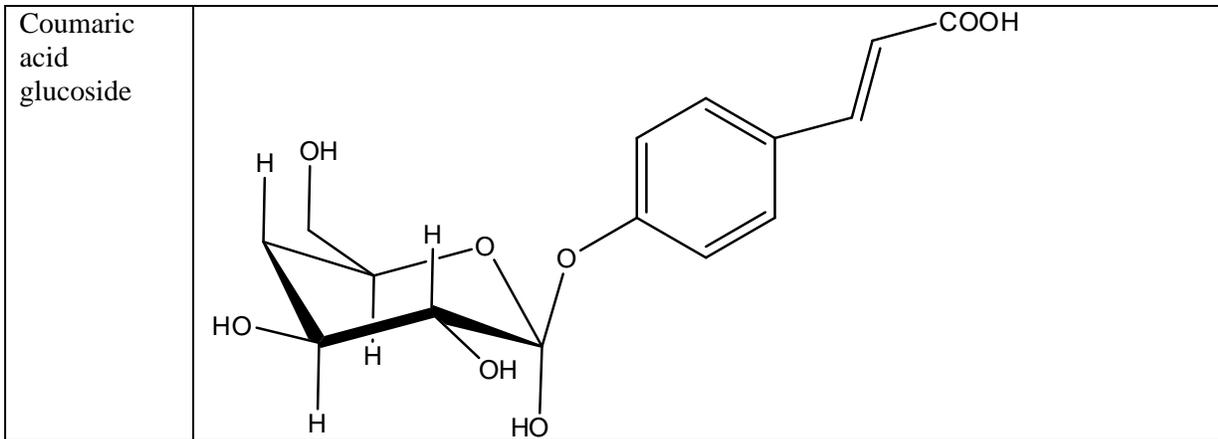


Figure 1: Phenolic compounds identified only in olive pomace and not in olive mill wastewater.

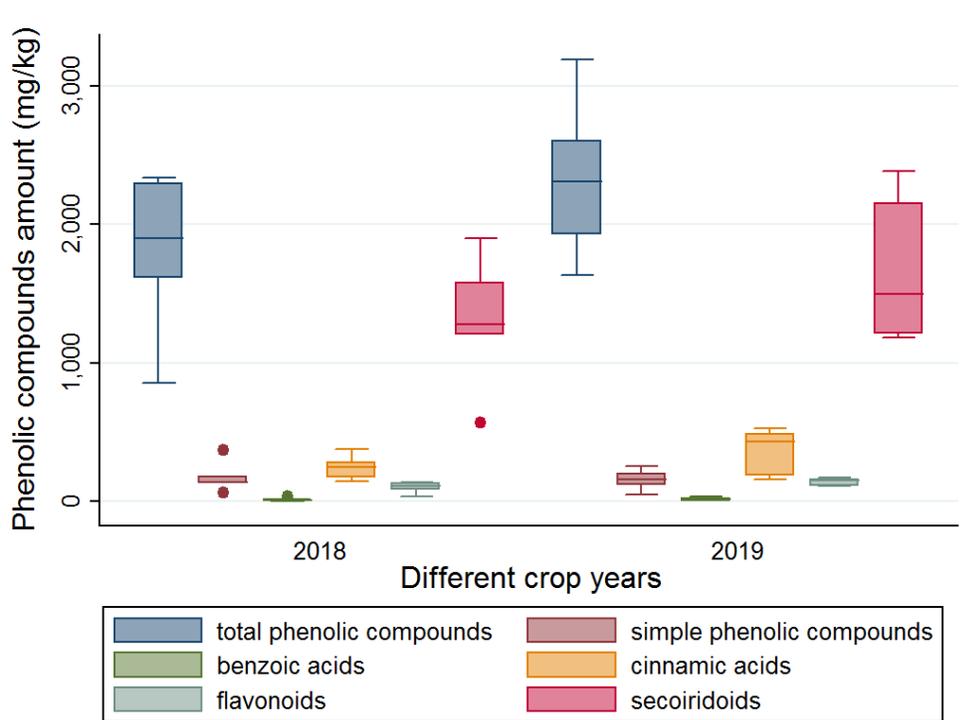


Figure 2: Total phenolic compound and phenolic compound composition according crop years 2018 and 2019.

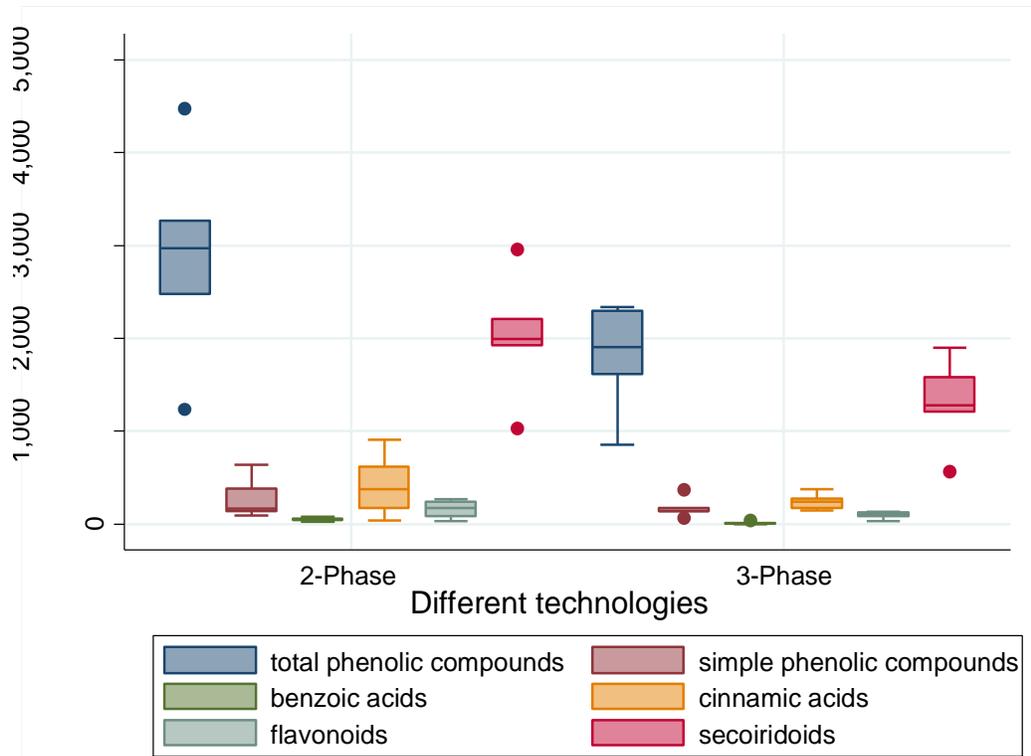


Figure 3: Total phenolic compound and phenolic compound composition according technology used (two-phase separating decanter and three-phase separating decanter).

## 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 | $r_s$<br>DPPH corr.<br>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                                  |

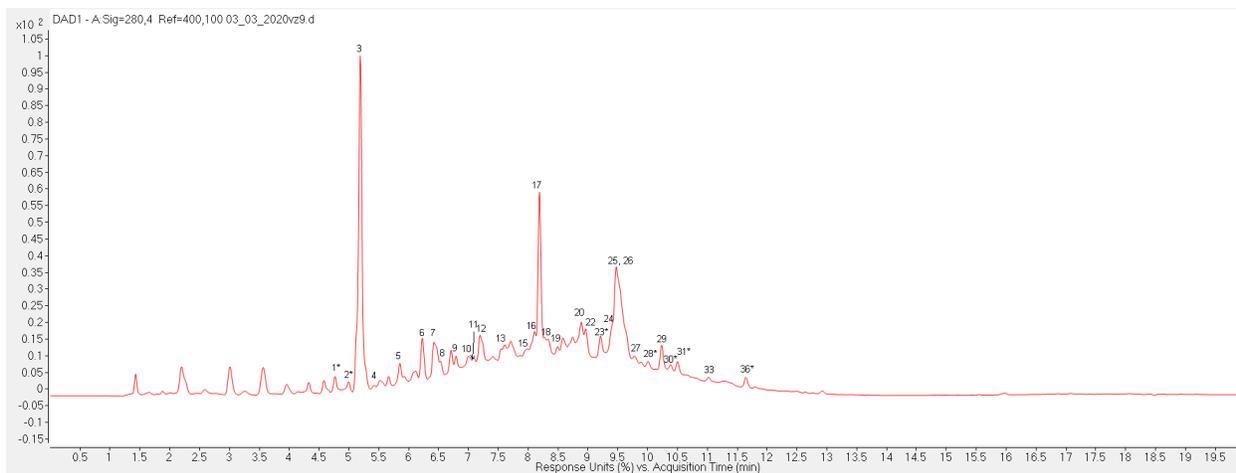
|  |      |      |      |       |
|--|------|------|------|-------|
| Elenolic acid glucoside 4 (mg/kg dry wt)   | 14   | <LOQ | 126  |       |
| Luteolin-4',7-O-diglucoside (mg/kg dry wt) | <LOQ | <LOQ | 67   |       |
| $\beta$ -OH-verbascoside 1 (mg/kg dry wt)  | <LOQ | <LOQ | 44   |       |
| $\beta$ -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  |       |
| Verbascoside 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   |       |
| <b>Simple phenolic compounds (m/kg dry wt)</b> | 154  | 45   | 637  | -0.71 |
| <b>Benzoic acids (mg/kg dry wt)</b>            | 16   | <LOQ | 74   | -0.67 |
| <b>Cinnamic acids (mg/kg dry wt)</b>           | 265  | 36   | 905  | -0.60 |
| <b>Flavonoids (mg/kg dry wt)</b>               | 129  | 31   | 266  |       |
| <b>Secoiridoids (mg/kg dry wt)</b>             | 1632 | 564  | 2953 | -0.72 |

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

487

## 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] <sup>-</sup> | Fragments             | Molecular formula                               | UV max (nm) |
|-------------|--------------------------|------|-----|----------|----------|------------|----------------------|-----------------------|---|-------------|
| 1           | Oleoside**               | P    | 4.8 | 390.1159 | 390.1162 | -0.72      | 389.1089             | 389, 183,<br>209, 227 | C <sub>16</sub> H <sub>22</sub> O <sub>11</sub> | 229,<br>289 |
| 2           | Oleoside**               | P    | 5.0 | 390.1163 | 390.1162 | 0.13       | 389.1091             | 389, 209,<br>345      | C <sub>16</sub> H <sub>22</sub> O <sub>11</sub> | 255,<br>290 |
| 3           | Hydroxytyrosol glucoside | P, W | 5.2 | 316.1148 | 316.1158 | -3.35      | 315.1071             | 315, 153,<br>123      | C <sub>14</sub> H <sub>20</sub> O <sub>8</sub>  | 230,<br>282 |
| 3           | Hydroxytyrosol           | P, W | 5.2 | 154.0624 | 154.0630 | -3.93      | 153.0551             | 123, 153              | C <sub>8</sub> H <sub>10</sub> O <sub>3</sub>   | 230,<br>280 |

|     |   |      |     |          |          |       |          |                       |   |                     |
|-----|---|------|-----|----------|----------|-------|----------|-----------------------|---|---------------------|
| 3   | Oleoside                                  | P    | 5.2 | 390.1161 | 390.1162 | -0.4  | 389.1090 | 389, 183,<br>209      | C <sub>16</sub> H <sub>22</sub> O <sub>11</sub>   | 200,<br>230,<br>280 |
| 4   | Elenolic acid<br>glucoside –<br>Isomer 1  | P    | 5.4 | 404.1321 | 404.1319 | 0.69  | 403.1244 | 403, 223,<br>179      | C <sub>17</sub> H <sub>24</sub> O <sub>11</sub>   | 236                 |
| 4.1 | Elenolic acid<br>glucoside –<br>Isomer 2  | P    | 5.5 | 404.1320 | 404.1319 | 0.29  | 403.1248 | 403, 223,<br>179      | C <sub>17</sub> H <sub>24</sub> O <sub>11</sub>   | 235                 |
| 5   | Elenolic acid<br>glucoside –<br>Isomer 3  | P    | 5.8 | 404.1317 | 404.1319 | -0.43 | 403.1245 | 403, 223,<br>179      | C <sub>17</sub> H <sub>24</sub> O <sub>11</sub>   | 233                 |
| 6   | Tyrosol                                   | P, W | 6.2 | /        | /        | /     | /        | /                     | C <sub>10</sub> H <sub>8</sub> O <sub>2</sub>     | 227,<br>280         |
| 7   | Secologanoside                            | P, W | 6.3 | 390.1160 | 390.3384 | -0.49 | 389.1086 | 389, 345,<br>183, 209 | C <sub>16</sub> H <sub>22</sub> O <sub>11</sub>   | 230                 |
| 8   | Trans p-<br>coumaric acid 4-<br>glucoside | P    | 6.5 | 326.0994 | 326.1002 | -2.49 | 325.0919 | 163, 119,<br>325      | C <sub>15</sub> H <sub>18</sub> O <sub>8</sub>    | n.d.                |
| 9   | Caffeic acid                              | P, W | 6.7 | 180.0433 | 180.0423 | 5.55  | 179.0357 | 179, 135              | C <sub>16</sub> H <sub>22</sub> O <sub>11</sub>   | 230,<br>289,<br>330 |
| 10  | Elenolic acid<br>glucoside<br>Isomer 4    | P    | 7.0 | 404.1321 | 404.1319 | 0.67  | 403.1249 | 403, 223,<br>179      | C <sub>17</sub> H <sub>24</sub> O <sub>11</sub>   | 237                 |
| 11  | Luteolin-4',7-O-<br>diglucoside           | P, W | 7.1 | 610.1886 | 610.1898 | -1.88 | 609.1795 | 609, 447,<br>285      | C <sub>27</sub> H <sub>30</sub> O <sub>16</sub> * | n.d.                |

|    |                                      |                      |     |          |          |      |           |                               |   |                     |
|----|--------------------------------------|----------------------|-----|----------|----------|------|-----------|-------------------------------|---|---------------------|
| 12 | $\beta$ -OH-verbascoside<br>Isomer I | P,W                  | 7.2 | 640.2013 | 640.2003 | 1.45 | 639.1927  | 639, 621,<br>459, 179,<br>161 | C <sub>29</sub> H <sub>36</sub> O <sub>16</sub> | 239<br>283<br>330   |
| 12 | $\beta$ -OH-verbascoside<br>Isomer 2 | P, W                 | 7.2 | 640.2031 | 640.2003 | 4.27 | 639.1935  | 639, 621,<br>459, 179,<br>161 | C <sub>29</sub> H <sub>36</sub> O <sub>16</sub> | 239<br>283<br>330   |
| 13 | Vanilin                              | W                    | 7.7 | 152.0477 | 152.0473 | 2.5  | 151.0406  | 151, 136                      | C <sub>8</sub> H <sub>8</sub> O <sub>3</sub>    | 235<br>281<br>310   |
| 14 | Verbascoside<br>Isomer I             | P                    | 7.7 | 624.2087 | 624.2054 | 5.29 | 623.2018  | 623, 461,<br>161              | C <sub>29</sub> H <sub>36</sub> O <sub>15</sub> | 265,<br>291,<br>330 |
| 15 | Demethyloleurop<br>ein               | P,W                  | 7.9 | 526.1704 | 526.1686 | 3.33 | 525.1623* | 525, 389,<br>319, 183,<br>345 | C <sub>24</sub> H <sub>30</sub> O <sub>13</sub> | 240<br>280          |
| 16 | Rutin                                | P,W                  | 8.1 | 610.1557 | 610.1534 | 3.72 | 609.1469  | 609, 300,<br>179              | C <sub>27</sub> H <sub>30</sub> O <sub>16</sub> | 256<br>358          |
| 17 | Verbascoside<br>Isomer II            | P                    | 8.2 | 624.2057 | 624.2054 | 0.47 | 623.1981  | 623, 461,<br>161              | C <sub>29</sub> H <sub>36</sub> O <sub>15</sub> | 247<br>285<br>331   |
| 18 | Luteolin-7'-O-<br>glucoside          | P,W                  | 8.3 | 448.1014 | 448.1006 | 1.76 | 447.0938  | 447, 285                      | C <sub>21</sub> H <sub>20</sub> O <sub>11</sub> | 255<br>350          |
| 18 | Luteolin<br>rutinoside               | P,<br>W <sup>x</sup> | 8.3 | 594.1605 | 594.1585 | 3.47 | 593.1533  | 593, 285,<br>447              | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | 255<br>350          |

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

|      |  |      |      |          |          |       |          |                                    |   |                     |
|------|--|------|------|----------|----------|-------|----------|------------------------------------|---|---------------------|
| 28   | Oleuropein<br>aglycone Isomer<br>2**     | P    | 10.0 | 378.1328 | 378.1315 | 3.44  | 377.1250 | 377, 345,<br>275, 149,<br>139, 307 | C <sub>19</sub> H <sub>22</sub> O <sub>8</sub>  | 225,<br>275         |
| 28   | Oleuropein/Oleuroside<br>**              | P,W  | 10.0 | 540.1813 | 540.1843 | -5.57 | 539.1743 | 275, 539,<br>149                   | C <sub>25</sub> H <sub>32</sub> O <sub>13</sub> | 225,<br>275         |
| 29   | Ligstroside                              | P,W* | 10.3 | 524.1889 | 524.1894 | -0.82 | 523.1812 | 523, 223,<br>101                   | C <sub>25</sub> H <sub>32</sub> O <sub>12</sub> | 252,<br>270,<br>350 |
| 29.1 | Oleuropein<br>aglycone Isomer<br>3       | P    | 10.3 | 378.1318 | 378.1315 | 0.78  | 377.1240 | 377, 345,<br>275, 149,<br>139, 307 | C <sub>19</sub> H <sub>22</sub> O <sub>8</sub>  | 240,<br>270         |
| 30   | p-HPEA-EDA<br>**                         | P    | 10.4 | 304.1312 | 304.1311 | 0.38  | 303.1235 | 179, 165,<br>183*, 59*,<br>137*    | C <sub>17</sub> H <sub>20</sub> O <sub>5</sub>  | 230,<br>282         |
| 30   | Oleuropein<br>aglycone Isomer<br>4<br>** | P    | 10.4 | 378.1321 | 378.1315 | 1.64  | 377.1234 | 377, 345,<br>275, 149,<br>139, 307 | C <sub>19</sub> H <sub>22</sub> O <sub>8</sub>  | 230,<br>280         |
| 31   | Oleuropein<br>aglycone Isomer<br>5<br>** | P    | 10.5 | 378.1314 | 378.1315 | -0.12 | 377.1240 | 377, 345,<br>275, 149,<br>139, 307 | C <sub>19</sub> H <sub>22</sub> O <sub>8</sub>  | 225<br>280          |
| 32   | Oleuropein<br>aglycone Isomer<br>6       | P    | 10.7 | 378.1327 | 378.1315 | 3.33  | 377.1242 | 377, 345,<br>275, 149,<br>139, 307 | C <sub>19</sub> H <sub>22</sub> O <sub>8</sub>  | n.d.                |
| 33   | Apigenin                                 | P, W | 11.0 | 270.0530 | 270.0523 | 0.71  | 269.0457 | 269                                | C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>  | 239,                |

|    |  |      |      |          |          |      |          |                                    |  |             |
|----|--|------|------|----------|----------|------|----------|------------------------------------|--|-------------|
|    |  |      |      |          |          |      |          |                                    |  | 269,<br>339 |
| 34 | Oleuropein<br>aglycone Isomer<br>7       | P, W | 11.1 | 378.1322 | 378.1315 | 2.02 | 377.1243 | 377, 275,<br>149, 139,<br>307, 327 | C <sub>19</sub> H <sub>22</sub> O <sub>8</sub> | n.d.        |
| 35 | 3,4-DHPEA-<br>EDA                        | P    | 11.3 | 320.1262 | 320.1260 | 0.62 | 319.1187 | 195, 183,<br>165, 139              | C <sub>17</sub> H <sub>20</sub> O <sub>6</sub> | 232,<br>280 |
| 35 | Oleuropein<br>aglycone Isomer<br>8<br>** | P    | 11.3 | 378.1319 | 378.1315 | 1.03 | 377.1242 | 377, 275,<br>149, 139,<br>307, 327 | C <sub>19</sub> H <sub>22</sub> O <sub>8</sub> | 230<br>280  |
| 36 | Oleuropein<br>aglycone Isomer<br>9<br>** | P    | 11.6 | 378.1315 | 378.1315 | 0.06 | 377.1242 | 377, 275,<br>149, 139,<br>307, 327 | C <sub>19</sub> H <sub>22</sub> O <sub>8</sub> | 225,<br>282 |

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