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New by-products rich in bioactive substances from the olive oil mill processing

Concepción Romero^a, Eduardo Medina^a, M^a Antonia Mateo^b, Manuel Brenes^{a,*}

^a*Food Biotechnology Department, Instituto de la Grasa (IG-CSIC), Campus University Pablo de Olavide, Ctra. Utrera km 1, 41013-Seville, Spain*

^b*Jaencoop SCA, Avda. de Valencia s/n, 23330-Villanueva del Arzobispo, Jaén, Spain*

*Correspondence to: Manuel Brenes, Food Biotechnology Department, Instituto de la Grasa (IG-CSIC), Campus University Pablo de Olavide, Ctra. Utrera km 1, 41013-Seville, Spain. E-mail brenes@cica.es

Running title: New olive by-products from olive oil mill processing

Abstract

BACKGROUND: Olive oil extraction generates a large amount of residue comprising mainly of the pomace and leaves when using the two-phase centrifugation system. The aim of this study was to assess the content of phenolic and triterpene compounds in the by-products produced in Spanish olive oil mills.

RESULTS: The olive pomace had lower concentrations than 2 and 3 g kg⁻¹ of phenolic and triterpene substances, respectively. The leaves contained a high concentration in these substances although those collected from ground-picked olives had lost most of their phenolic compounds. Moreover, the sediment from the bottom of the olive oil storage tanks did not have a significant amount of these substances. By contrast, the new by-product named olive pomace skin has been revealed as a very rich source of triterpenic acids because this waste can reach up to 120 g kg⁻¹ of these compounds, maslinic acid comprising around 70 % of total triterpenics.

CONCLUSIONS: Among the by-products generated during extraction of olive oil, the olive pomace skin has been discovered as a very rich source of triterpenic acids that can reach up to 120 g kg⁻¹ of the waste. These results will contribute to the valorization of olive oil by-products.

Keywords: olive; phenolic; triterpene; oleuropein; maslinic; oleanolic

40 INTRODUCTION

41 Olive leaves and olive mill pomace are the two main wastes generated during the
42 extraction of olive oil by the two-phase centrifugation system. In Spain, more than 4
43 and 0.2 million tons of olive pomace and leaves are annually produced, respectively.
44 These by-products are used for composting, combustion, animal feed and soil
45 amendment among others.¹⁻⁴ However, they are also rich in bioactive substances such
46 as phenolic compounds and triterpenic acids that could contribute to the revalorization
47 of these by-products.⁵

48 There are many methods and patents to extract phenolic compounds and
49 triterpenic acids from olive leaves because fresh olive leaves may contain up to 70 g kg-
50 ¹ and 20 g kg⁻¹ of phenolic compounds and triterpenic acids respectively; ^{6,7} oleuropein
51 and oleanolic acid being the main phenolic and triterpene substances in this material.⁸
52 These bioactive substances have been analyzed in many fresh olive leaf cultivars during
53 olive maturation, under different agronomic conditions and taking into account many
54 other variables.⁹⁻¹² However, the olive leaf by-product generated in the Spanish olive
55 oil mills comes from two sources, ground-picked or tree-picked olives, which have not
56 been characterized.

57 Hundreds of articles describe the phenolic composition of olive oil mill
58 wastewaters produced during the extraction of olive oil by the three-phase
59 centrifugation system.^{13,14} The characterization of these substances in the two-phase
60 olive pomace has also been reported.¹⁵⁻¹⁷ However, studies on the triterpene
61 composition of this olive pomace, which is named “Alperujo” in Spain, are scarce.^{18,19}

62 Alperujo is a semi-solid waste generated in the Spanish olive oil mills that is
63 stored in large open air ponds for several months until the residual oil is extracted by
64 physical or chemical methods in the oil extracting plants.²⁰ The de-oiled pomace is used
65 for co-generation of electrical power. Currently, large pit fragments are separated from
66 fresh Alperujo in the olive oil mills to use them for combustion. However, pieces of
67 skin and olive pulp are adhered to these pit fragments and they are separated to obtain
68 pits which are free of pulp. Consequently, a new by-product is generated at large scale
69 at the olive oil mills that consists mainly of olive skin together with small pieces of
70 pulp. This by-product is used for combustion or spreading on the soil but it has attracted
71 the attention of enterprises due to its potential content in bioactive substances,

particularly triterpenic acids. It must be highlighted that triterpenic acids are mainly concentrated in the skin of fruits,^{21,22} although no data are available about the composition of this new by-product, rich in the olive skin.

Another by-product produced in the olive oil mills is the sediment that settles at the bottom of the olive oil tanks during the storage of the oil before commercialization. This olive oil lees is composed mainly of fat and water, and it is intended for refining or making soap. The phenolic characterization of the solid and aqueous components of this by-product has been reported but not the composition of the oily phase, particularly triterpenic acids.^{23,24}

Considering the industrial demand for bioactive substances from olive by-products, this study was undertaken to evaluate the content in phenolic and triterpenic compounds of the main by-products generated in the olive oil mills such as Alperujo, leaves, olive oil lees and olive pomace skins.

MATERIALS AND METHODS

Samples

Most samples were obtained from olive oil mill Cooperatives belonging to Jaencoop SCA located in the south of Spain. They were taken between December and January of the 2013/2014 season.

Fresh Alperujo was acquired from three olive oil mill Cooperatives, three samples from each cooperative on three consecutive days, at the beginning of the 2013/2014 harvesting season (December) and another nine samples at the end of the season (January). All samples were transferred to the laboratory and immediately analyzed on the same day without any storage period. The Picual cultivar was processed in the three Cooperatives, and the centrifugation equipment was the two-phase system.

Samples of olive leaves were obtained from one olive oil mill Cooperative at the beginning and end of the 2013/2014 season. In accordance with current practice, olives of the Picual cultivar were harvested and transported to the Cooperative facilities where

101 leaves and small branches were removed. The olive factory has two different olive oil
102 extraction lines for fruits that were tree-picked or ground-picked thereby leaves from
103 these two different types of harvesting were analyzed.

104 Twenty samples of olive pomace skin were obtained from 15 olive oil mill
105 Cooperatives located in the south of Spain that had machines to separate the olive
106 pomace skin from the pit fragments of the pomace. These samples were taken
107 throughout the 2013/2014 harvesting season.

108 The oily sediment of 5 storage tanks located in 5 different olive oil mill
109 Cooperatives was taken from the bottom of the tanks containing virgin olive oil. They
110 were centrifuged at 6000 x g for 5 min (22 °C), and phenolic and triterpenic compounds
111 were analyzed in the oily phase. The virgin olive oil had been preserved for one year in
112 the tanks.

113

114 **Analysis of phenolic compounds in Alperujo and leaves**

115 The extraction of phenolic compounds from Alperujo was based on the
116 methodology reported elsewhere.²⁵ Around 10 g of fresh Alperujo were mixed in an
117 Ultra-Turrax homogenizer (Ika, Breisgau, Germany) with 30 mL of dimethyl sulfoxide
118 (DMSO). After 30 min of resting contact, the mixture was centrifuged at 6000 g for 5
119 min (22 °C) and 0.25 mL of the supernatant were diluted with 0.5 mL of DMSO plus
120 0.25 mL of 0.2 mM of syringic acid in DMSO (internal standard). The extraction of
121 these substances from the leaves was made similarly to the Alperujo as described above,
122 but this time the mixing ratio was 2 g of olive leaf and 30 mL of DMSO.

123 Samples were filtered through a 0.22 µm pore size nylon filter, and an aliquot
124 (20 µL) was injected into the chromatograph. The chromatographic system consisted of
125 a Waters 717 plus autosampler, a Waters 600 pump, a Waters column heater module,
126 and a Waters 996 photodiode array detector operated with Empower2 software (Waters,
127 Mildford, USA.). A 25 cm x 4.6 mm i. d., 5 µm, Spherisob ODS-2 (Waters, Inc.)
128 column at a flow rate of 1 mL min⁻¹ and a temperature of 35 °C, was used in all
129 experiments. Separation was achieved by gradient elution using (A) water (pH 2.5
130 adjusted with 0.15% phosphoric acid) and (B) methanol. The initial composition was
131 90% A and 10% B. The concentration of B was increased to 30% over 10 min and was

maintained for 20 min. Subsequently, B was raised to 40% over 10 min, maintained for 5 min, and then increased to 50%. Finally, B was increased to 60, 70, and 100% in 5-min periods. The initial conditions were reached in 10 min. Chromatograms were recorded at 280 nm.

The evaluation of each compound was performed using a regression curve with the corresponding standard. Hydroxytyrosol, oleuropein, verbascoside, luteolin, luteolin 7-glucoside, rutin, and apigenin were purchased from Extrasynthese S. A. (Genay, France) and tyrosol, caffeic, vanillic and *p*-coumaric acids from Sigma Chemical Co. (St Louis, USA). Hydroxytyrosol-1-glucoside, caffeoyl ester of secologanoside, and comselogoside were quantified using the response factors of hydroxytyrosol, caffeic acid, and *p*-coumaric acid, respectively. Salidroside and ligustroside were quantified using the response factor of tyrosol. Hydroxytyrosol-4-glucoside and the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA) were obtained using a HPLC preparative system.²⁶

Analysis of phenolic compounds in olive oil

They were extracted from the oil with *N,N*-dimethylformamide (DMF).²⁶ Briefly, 0.6 g of oil were extracted with 3 x 0.6 mL of DMF; the extract was then washed with hexane, and N₂ was bubbled into the DMF extract to eliminate residual hexane. Syringic acid (0.2 mM) was employed as internal standard. Finally, the extract was filtered through a 0.22 µm pore size nylon filter and injected into the chromatograph.

The chromatographic system was the same as noted above except that a fluorescence detector was put in series with a DAD detector to monitor all the phenolic compounds. Hydroxytyrosol glycol and 4-ethylphenol were purchased from Sigma (St. Louis, USA), and hydroxytyrosol acetylated, pinorelinol, 1-acetoxypinorelinol, ligustrosideaglycon and the dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol (TyEDA) were obtained using a preparative HPLC system.²⁶

Moisture

162 The water content of Alperujo, leaves and olive pomace skin was determined by
163 weighing 10 g of the organ and then oven drying at 105 °C to constant weight.

165 **Analysis of triterpenic acids in Alperujo, leaves and olive pomace skin**

166 Ten grams of Alperujo or cut leaves were desiccated at 105 °C until weight
167 stabilization. Subsequently, 1 g of dry and triturated Alperujo or leaf was mixed in a 10
168 mL centrifuge tube with 4 mL of methanol/ethanol (1:1, v/v) and vortexed for 1 min,
169 centrifuged at 6000 g for 5 min at 20 °C, and the solvent was separated from the solid
170 phase. This step was repeated six times, and the pooled solvent extract was vacuum
171 evaporated. The residue was dissolved in 2 mL of methanol, which was filtered through
172 a 0.22 µm pore size nylon filter and an aliquot (20 µL) was injected into the liquid
173 chromatograph. The chromatographic system and column were the same as those used
174 for the phenolic compound analysis. The mobile phase (methanol/acidified water with
175 phosphoric acid at pH 3.0, 92:8, v/v) was delivered to the column at a flow rate of 0.8
176 mL min⁻¹ and the eluate was monitored at 210 nm. Oleanolic and maslinic acids were
177 quantified using external standards (Sigma, USA). The extraction of these substances
178 from olive pomace skin was conducted using a procedure similar to that described
179 above for Alperujo and leaf but, this time, the mixing ratio was 0.1 g of olive pomace
180 skin and 4 mL of methanol/ethanol. The residue was dissolved in 10 mL methanol.

182 **Analysis of triterpenic acids in olive oil**

183 The extraction of these substances from olive oil was done using a mixture of
184 methanol/ethanol (1:1).¹⁸ The oil (0.8 g) and the alcoholic solvent (1.6 mL) were
185 vortexed for 1 min, centrifuged at 6000 g for 5 min at 20 °C, and the alcoholic phase
186 was separated from the lipid phase. This step was repeated six times. Subsequently, the
187 pooled alcoholic extract was vacuum evaporated and the residue dissolved in 2.4 mL
188 methanol, which was centrifuged at 6000 g for 5 min at 20 °C. Finally, the solution was
189 filtered through 0.22 µm pore size nylon filter and an aliquot (20 µL) was injected into
190 the liquid chromatograph. The chromatographic system and column were the same as
191 described above.

192 Statistical analysis

193 Statistical comparisons of the mean values for each experiment were performed
194 by one-way analysis of variance (ANOVA), followed by the Duncan's multiple range
195 test ($p < 0.05$) using Statistica software version 8.0 (Stat-Soft, Inc., Tulsa, USA).

197 RESULTS AND DISCUSSION

198 Figure 1 presents the phenolic composition of fresh Alperujo obtained at the
199 beginning and end of the 2013/2014 season. Hydroxytyrosol-4-glucoside,
200 hydroxytyrosol and the dialdehydic form of decarboxymethyl elenolic acid linked to
201 hydroxytyrosol (HyEDA) were the main phenolic compounds detected in this material,
202 followed by verbascoside, tyrosol, salidroside and other substances in minor
203 concentration (see Supporting information, Fig. 1S). This chemical characterization is in
204 agreement with previous studies,^{15,16} and a trend was confirmed to lower concentration
205 of these substances as the harvesting season progressed, except for hydroxytyrosol and
206 tyrosol.¹⁶ It must be noted that the major phenolic compound in fresh olives is
207 oleuropein, which can reach up to 20 g kg⁻¹ in the olive pulp,⁸ whereas the total
208 phenolic content in the Alperujo analyzed was 1-1.3 g kg⁻¹. Taking into consideration
209 that the transfer of phenolic compounds from the olive paste to the oil during the
210 malaxation step is low and the differences between the mass molecular weight of
211 oleuropein (540 u.m.a.) and hydroxytyrosol (154 u.m.a.),^{16,27} most of the phenolic
212 compounds degraded or transformed during the malaxation step. Klen and Vodopivec
213 (2012) estimated that just only half of the initial phenolic content in fresh olives
214 remained in the extracted olive paste. In our case, we estimated that around 80% of the
215 initial amount of phenolic compounds was lost during the extraction process of olive
216 oil. It is well-known that oxidase enzymes act on phenolic compounds, particularly
217 hydroxytyrosol and derivatives, to form quinones and polymers. Besides, esterases and
218 especially β -glucosidase enhance the rupture of the oleuropein bonds during the milling
219 and malaxation steps.

220 With regard to the content of triterpenic acids in Alperujo, the concentration of
221 these substances was rather similar in all the samples obtained from three different olive
222 oil mill Cooperatives regardless of the time of sampling (Figure 2). The mean content

was around 2.5 g kg⁻¹, with maslinic acid being more abundant than oleanolic) (see Supporting information, Fig. 2S). These data are similar to those reported for the concentration of triterpenic acids in fresh olive pulp,⁸ which confirmed the low transfer of these substances to virgin olive oil during oil extraction.²⁸ Therefore, Alperujo was disclosed as a rather good and reliable source of triterpenic acids, although the concentration of these substances in olives is also cultivar dependent.²⁹

Triterpenic acids are abundant in the plant kingdom and play a role in plant defense thereby they are concentrated in the skin of fruits such as olives.^{8,21,30} Hence, the new residue olive pomace skin generated in the olive oil mills must be rich in these substances, and it was confirmed on 20 samples analyzed of this new by-product (Figure 3). The concentration of triterpenic acids was very much higher than found in fresh Alperujo, although a great variability among samples was detected; total triterpenic acids ranged from 40 to 140 g kg⁻¹, with maslinic acid representing around 70% of the total. As it has been commented above, this residue comes from the cleaning of the pit fragments present in the olive pomace that contains pieces of olive skin together with small pieces of pulp. Because the moisture of these samples ranged from 7 to 50%, a correlation between moisture and triterpenic acid concentration was studied but it failed since a higher concentration in triterpenic acids was not correlated with a lower content in moisture. The technology used for the separation of olive skin pomace from the pit fragments is new and there are many different variables such as olive cultivar and agronomic conditions that can be a reasonable explanation for the great variability observed in the samples analyzed. It is also known that the skin of olives is rich in phenolic compounds,²⁵ particularly oleuropein, but the level of phenolic compounds in this new by-product was insignificant (data not shown). The high loss of phenolic compounds in Alperujo occurring during the malaxation step has been commented above, but new oxidation and degradative reactions must undergo at the further steps needed to obtain the olive skin pomace. Overall, a new by-product which is very rich in triterpenic acids has been identified in the olive oil mills.

Leaves are the second by-product in importance generated in the olive oil mills without a valuable use. The chemical characterization of olive leaves has been carried out extensively on leaves picked from the tree.^{8,31} In this study, analyses were performed on leaves obtained from both tree-picked and ground-picked olives as they are currently separated for processing in the Spanish olive oil mills. The concentration

of triterpenic acids in the olive leaves was very high (Figure 4), in particular that of oleanolic acid, which is in line with previous reports.⁸ In addition, no statistical differences were found between ground and tree-picked leaves collected at the beginning of the harvesting season. By contrast, leaves from ground-picked olives collected at the end of the harvesting season showed a statistically higher content in triterpenic acids than those from tree-picked olives. This finding must be related to the lower concentration in moisture of the former leaves (20%) than the latter (38%). It seems that the loss in humidity of the olive leaves does not give rise to a reduction in the triterpenic acid content but enrichment in these substances is originated.

Moreover, leaves from ground-picked olives had a much lower concentration in phenolic compounds than those from tree-picked olives (Figure 5). It seems that oleuropein degraded during desiccation of the leaves on the ground. As leaves lose moisture, cells die, and a contact between phenolic compounds and degradative enzymes occurs giving rise to oxidative and hydrolytic reactions on the oleuropein moiety. Therefore, ground-picked leaves are a good source of triterpenic acids but not for phenolic compounds.

Finally, the sediment of the bottom of the tanks where virgin olive oil is currently stored for months before commercialization was explored as a potential source of bioactive substances. This by-product is mainly composed of oil, followed by water, sugars and other substances. The phenolic and triterpene composition of the oil was analyzed (Figure 6). The phenolic compounds detected in this oil were the same as previously reported in virgin or extra virgin olive oil,²⁶ and the total content ranged between 0.3-0.8 g kg⁻¹, which is a relatively low content for considering their recovery. On the other hand, this oily phase of the sediment was enriched in triterpenic acids during storage of the oil because the concentration of these substances in virgin olive oil is currently lower than 0.1 g kg⁻¹.²⁸ However, these results mean that this by-product does not seem to be a good source of phenolic or triterpene bioactive substances.

CONCLUSIONS

Olive oil mills generate a high amount of by-products that contain valuable bioactive substances whose recovery could contribute to the revalorization of these wastes. This study has explored the presence of phenolic and triterpene compounds in the main wastes produced in the Spanish olive oil mills. Among them, the olive pomace

skin has been discovered as a very rich source of triterpenic acids that can reach up to 120 g kg⁻¹ of the waste, maslinic acid comprising around 70% of the total triterpenics. Alperujo has also been disclosed as an abundant and constant source of triterpenic and phenolic compounds although most of the latter substances are lost during the extracting process. Leaves can be used for the extraction of phenolic compounds, particularly oleuropein, but it has been found that those obtained from ground-picked olives lose their content in these substances. By contrast, triterpenic acids, mainly oleanolic, are very concentrated in this by-product regardless of the place where olives were picked. Finally, the sediment formed in the bottom of the storage tanks of olive oils does not seem to be a good source of bioactive substances.

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Figure captions

Figure 1. Phenolic compounds in Alperujo obtained from three different olive oil mill Cooperatives at the beginning and the end of the harvesting season 2013/2014. Standard deviation of nine samples is drawn on the bars. For each compound, vertical bars with different letters indicate significant differences according to Duncan’s multiple-range test ($p < 0.05$). Hy, hydroxytyrosol; HyEDA, dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol; Hy-4-glucoside, hydroxytyrosol-4-glucoside; others is the sum of vanillic, caffeic and *p*-coumaric acids, luteolin-7-glucoside, rutin, ester of caffeic acid linked to secologanoside and comselogoside.

Figure 2. Triterpenic acids in Alperujo obtained from three different olive oil mill Cooperatives (A, B and C) at the beginning and the end of the harvesting season 2013/2014. Standard deviation of triplicates is drawn on the bars. For each season, vertical bars with different letters indicate significant differences according to Duncan’s multiple-range test ($p < 0.05$).

Figure 3. Concentration of triterpenic acids in 20 samples of olive pomace skin obtained from 15 olive oil mill factories. Standard deviation of duplicates is drawn on the bars.

Figure 4. Triterpenic acids content in leaves obtained from ground-picked and tree-picked olives. Samples were obtained from a local olive oil mill Cooperative, and taken at the beginning and the end of the harvesting season 2013/2014. For each season, vertical bars with different letters indicate significant differences according to Duncan’s multiple-range test ($p < 0.05$).

Figure 5. Phenolic compound content in leaves obtained from tree-picked and ground-picked olives. Samples were obtained from a local olive oil mill Cooperative at the end of the harvesting season 2013/2014. Others are hydroxytyrosol-1-glucoside,

hydroxytyrosol-4glucoside, verbascoside, luteolin-7-glucoside, salidroside, rutin, ester of caffeic acid linked to secologanoside and comselogoside.

Figure 6. Concentration of triterpenic acids and total phenolic compounds in several samples of olive oils obtained from the bottom of the storage tanks after one year of preservation. Samples were from 5 different olive oil Cooperatives (A-E) located in the Jaen province. Standard deviation of triplicates is drawn on the bars. Phenolic compounds quantified were hydroxytyrosol, hydroxytyrosol glycol, hydroxytyrosol acetylated, tyrosol, pinoresinol, 1-acetoxy pinoresinol, 4-ethylphenol, luteolin, apigenin, oleuropein and ligustroside aglycons, and the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol and tyrosol.

Figure 1S. HPLC chromatograms of phenolic compounds in alperujo and olive leaf. 1, hydroxytyrosol, 2, hydroxytyrosol 1-glucoside; 3, hydroxytyrosol 4-glucoside; 4, salidroside; 5, tyrosol; 6, vanillic acid; 7, caffeic acid; IS, internal standard; 8, *p*-coumaric acid; 9, verbascoside; 10, HyEDA; 11, luteolin 6-glucoside; 12, oleuropein; 13, rutin; 14, ester of caffeic acid linked to secologanoside; 15, comselogoside.

Figure 2S. HPLC chromatograms of triterpenic acids in olive leaf (A), alperujo (B) and olive pomace skin (C). 1, maslinic acid; 2, oleanolic acid.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 1S

Figure 2S