Antioxidant activity showed by olive pomace extracts

ALESSANDRA DE BRUNO[1*], ROSA ROMEO[1], FRANCESCA L. FEDELE[2], ANDREA SICARI[2], AMALIA PISCOPO[1], and MARCO POIANA[1]

[1] *Department of AGRARIA, University Mediterranea of Reggio Calabria, 89124 Vito ,Reggio Calabria,Italy*

[1] *LINFA S.C.A.R.L., 89900, Vibo Valentia, Italy*

Abstract

In this study, the effects of experimental variables such as type of solvent, ratio sample/solvent and time of extraction have been evaluated to individuate the best results in phenolic recovery by Olive Pomaces (OP) belonging to Carolea and Ottobratica cultivars. Folin-Ciocaulteu procedure and DPPH and ABTS assays were used respectively for total phenol quantification and total antioxidant activity of pomace extracts. The most efficient solvent in extracting phenolic constituents resulted ethanol/water mixture in both olive cultivars. The highest amount of phenolic compounds (171±4 mg of gallic acid 100 g^{-1} of dry pomace) was obtained after extraction at 120 minutes with 2:1 solvent/OP (v/w) of Ottobratica Olive Pomace. The recovery of phenol compounds from olive wastes increases the sustainability of sector, allowing to obtain an extract that could be a suitable alternative for food industry to the use of synthetic antioxidants in order to improve the quality of foods.

---

*Address correspondence to Alessandra De Bruno, Department of AGRARIA, University Mediterranea of Reggio Calabria, 89124 Vito (Reggio Calabria), Italy; Tel.+39 09651694381; Email: alessandra.debruno@unirc.it

30 **KEYWORDS:** ABTS, antioxidant activity, DPPH, olive pomace, phenolic compounds

31

32 ## Introduction

33

34 Agro-food industry generates significant amounts of by-products that are discarded and can be a
35 serious environmental problem. Nevertheless, food by-products are an extraordinary source of
36 bioactive compounds, which can be recovered in order to produce valuable metabolites via
37 chemical and biotechnological processes. Food-related phenolics are getting great interest due to
38 their antimicrobial and antioxidant activity strongly related to cancer prevention, inflammatory
39 disorders and cardiovascular diseases. [1-2]
40 The olive oil industry is very important in Mediterranean countries. The extraction of olive oil
41 generates huge quantities of waste (10 million ton/year), which may have a great impact on land
42 and water environments because of their high phytotoxicity. [3- 4] Olive mill waste (OMW) is a
43 suspension of three phases: water, oil and solids (smashed particles of olive paste and kernel).
44 OMW is a rich source of phenols, as it comprises 98 g per 100 g of the total phenolic content of the
45 olive fruit, and thereby it could be considered a raw material of great potential. The contained
46 organic load ranges from 4 to 16 g per 100 g including phenols, dietary fibers, fats, sugars, volatile
47 acids, nitrogenous and other compounds [5]. In particular, olive pomace (OP) consisted of olive
48 pulp, skin, stones and oil residues. Even if their production is seasonal, its disposal is potentially
49 harmful to environment due to its high moisture content (60–70%) [6].
50 The recovery of phenolic antioxidants by the waste seems achievable to produce substances
51 industrially exploitable as supplemental food. They may be used as chemical preservatives as an
52 alternative to synthetic products. [7] The composition of these waste shows a large variability,
53 depending on several parameters such as cultivar, harvesting time and oil extraction technology. [7]
54 Calabria region is placed at the end of the so called ‘Italian boot’. The olive growing has a long
55 tradition in Calabria with the presence of autochthonous and allochthonous varieties largely

56 cultivated along the region. [9] Among the different representative olive varieties in the region,
57 Carolea and Ottobratica cultivars were considered in this work. Carolea is a polyclonal one and
58 cultivated across most of the region. The main product obtained from Carolea olives is oil (oil yield
59 of about 20-25%) but also table olives are produced. Ottobratica is mainly present in the Tyrrhenian
60 southern area, and with an oil yield of around 18%.
61 Phenols recovery from olive fruit, olive tree leaves or olive mill waste is usually accomplished
62 using organic solvent extraction. [5] Solvent extraction is the most common method used for
63 isolation of phenolic antioxidants. Generally, the method affects differently the recovery of phenols
64 depending on nature of solvents, extraction temperature and time. An extraction solvent system is
65 generally chosen according to the purpose of extraction, polarity of the interested components, and
66 polarity of undesirable components, overall cost, safety and environmental concern. [10]
67 It is well known that extraction conditions and characteristics of the sample can affect the efficiency
68 of the extraction, independently or interactively, and it is generally known that alcohol/water
69 solutions exert a better influence on the extractability of phenolic compounds in comparison to the
70 mono-component solvents. [11]
71 The extraction yield and antioxidant activity of obtained compounds are strongly dependent on the
72 solvent. With regard to olive mill wastes extraction, hydro-alcoholic solutions such as methanol–
73 water or ethanol–water mixtures with different relative concentrations are the most popular choice.
74 Methanol–water mixtures have been used to extract phenols with the highest yield and different
75 polarity. Ethanol possesses a lot of advantages: it is cheap, reusable as well as non-toxic and the
76 corresponding extracts could be utilized directly in the beverage industry. [5]
77 The present study aims to recover the highest amount of phenolic compounds by using of different
78 solvents and times from olive pomace and to evaluate the antioxidant potential of phenolic fractions
79 by using of different assays.
80
81

82 **Materials and methods**

83

84 ***Sampling***

85

86 Olive Pomace samples were obtained during the 2015/2016 crop season from olives samples
87 (Carolea and Ottobratica cv.) processed using a small olive oil press mill of the Company Agrimec
88 Valpesana, Calzaiolo, San Casciano (Florence-Italy) at the laboratory of Food Technologies of the
89 University Mediterranea of Reggio Calabria (Italy).

90

91 ***Characterization of Olive Pomace***

92

93 The samples of olive pomace were promptly analysed for dry matter (Sartorius Moisture analyzer
94 MA37) and fat content. The dry matter (%) of oil pomace was determined by gravimetric method,
95 drying 50 g of sample in an oven at 105° C to constant mass. Fat content was extracted with
96 petroleum ether in a Soxhlet apparatus on 20 g of dry sample performing different number of
97 extraction cycles. The pomace samples were stored at -20°C for further analyses.

98

99 **Extraction of Phenolic Compounds From Olive Pomace (OP)**

100

101 The procedure was carried out using the analytical methodology described by Lafka et al. [12] with
102 some modifications. 5 grams of olive pomace added with n-hexane (5:1 v/w) were homogenized by
103 using of ultra-turrax in order to increase the contact surface. In order to remove fat, a rinsing with n-
104 hexane was carried out for 1 hour in an orbital shaker and at room temperature. This step is
105 commonly performed before phenolic compounds recovery, since fatty acids, triacylglycerols and
106 other non-polar components, such as fat soluble vitamins and pigments, may interfere with phenolic
107 quantification. The samples were filtered using Buchner funnel, and the filtrate was removed. The

108 residues were then extracted at room temperature with different solvents (ethanol and ethanol/water
109 80/20) at five different proportion of solvent volume to sample mass (2:1; 3:1; 4:1; 5:1) for 30, 60
110 and 120 minutes. The resulting extracts were named F1, F2, F3 and F4 for both olive cultivars (Fig.
111 1). The extracts were acidified with HCl (pH 2) and filtered using Buchner funnel.
112 The filtrates were evaporated to dryness in a rotary evaporator at 25°C and the residues were
113 dissolved in methanol. The obtained extracts were filtered using PTFE 0.45 μm syringe filters
114 (diameter 15 mm) and they were kept at -20 °C until subsequent analyses. All extractions were
115 performed in duplicate.
116
117 ***Analytical methods***
118
119 *Total Phenolic Determination*
120
121 The total polyphenol content (TPC) was quantified on the obtained extracts by Folin Ciocalteau
122 method [12] with some modifications. 0.1 mL of the methanolic solution of olive pomace extracts
123 were placed in a 25 mL volumetric flask and mixed with 20 mL of deionized water and 0.625 mL of
124 the Folin Ciocalteau reagent. After 3 minutes. 2.5 mL of saturated solution of Na_2CO_3 (20%) were
125 added. The content was mixed and diluted to volume with deionized water. Thereafter the mixture
126 was incubated for 12 hours at room temperature and in the dark. The absorbance of the samples was
127 measured at 725 nm against a blank using a double-beam ultraviolet-visible spectrophotometer
128 (Perkin-Elmer UV- Vis λ2, Waltham, Massachusetts, U.S.) and comparing with a gallic acid
129 calibration curve (concentration between 1 and 10 mg L^{-1}). The results were expressed as mg of
130 gallic acid 100 g^{-1} of dry pomace.
131
132 *Antioxidant Activity Determination: DPPH˙ and ABTS Assays*
133

134 The total antioxidant activity determination was performed using the Brand-Williams et al. [13]
135 method which is based on the reaction mechanism between the $DPPH^{\cdot}$ (2,2- diphenyl-1-
136 picrylhydrazyl, Carlo Erba, MI, Italy) and the antioxidants present in the samples. 10 μL of OP
137 extracts were added to 2990 μL of a 6 x 10^{-5} M of methanol solution of $DPPH^{\cdot}$ in a cuvette and
138 leaved in the dark for 60 minutes (till stabilization) at room temperature. According to Lafka et al.
139 [12] the decrement of absorbance was determined by a spectrophotometer at 515 nm against
140 methanol as blank and at the temperature of 20°C to eliminate the risk of thermal degradation of the
141 molecules tested. [14]
142 The results were expressed as percentage of inhibition and calculated by applying the following
143 formula:
144 $$\% \text{ Inhibition} = 100 \cdot \frac{(At0 - Ate)}{At0}$$
145 Where *Ate* is the value of absorbance measured after 60 minutes while *At0* is the value of
146 absorbance of $DPPH^{\cdot}$ solution at the initial time.
147 The Trolox Equivalent Antioxidant Capacity (TEAC) method was performed using the method
148 reported by Re et al. [15] This analysis evaluates the capacity of the studied sample to inhibit ABTS
149 radical oxidation, compared with a standard antioxidant (Trolox). The reaction mixture was
150 prepared by mixing 2990 μL of $ABTS^{+}$ and 10 μL of pomace extracts and the absorbance was
151 measured after 6 minutes at 734 nm.
152 The quenching of initial absorbance was plotted against the Trolox concentration (from 1.5 to 24
153 μmol L^{-1}) and the TEAC value was expressed as μmol Trolox g^{-1} of dried olive pomace.
154
155 *Statistical Analysis*
156
157 All experimental results in this study were expressed as mean values ± standard deviation (SD) of
158 four measurements (n= 4). In these single factor experiments, the significant differences ($p < 0.05$)

159 among treatment means were determined by One-way analysis of variance (ANOVA) with Tukey's
160 *post-hoc* test. SPSS Software (Version 15.0, SPSS Inc., Chicago, IL, USA) was used for data
161 processing.
162
163 **Results and discussion**
164
165 ***Fat and Moisture Content of Olive Pomace***
166
167 Ottobratica OP possessed 38% of moisture and 20% of fat, whereas lower amounts were detected in
168 Carolea OP (16% of moisture and 18% of fat). The oil extraction was done using a press laboratory
169 mill, this explains the water and fat content of OP samples, according to literature data. [16] Rajha et
170 al. [17] obtained a higher yield of phenolic compounds by wet pomace than to those by dried pomace,
171 suggesting that probably the largest amount of water promotes greater extractability of the phenolic
172 compounds. The fat content observed in both cultivars was twice the values reported by Fernandez-
173 Bolanos et al. [18] and similar to the fat range (7-19.5%) reported by Alburquerque et al. [19]
174
175 ***Total Polyphenol Content (TPC)***
176
177 Among different extraction methods of phenolic compounds, each one with different efficiency and
178 complexity, the solid–liquid extraction was preferred for its simplicity and convenience. Solvent
179 extraction is the most common method used for isolation of phenolic antioxidants and extraction
180 yield is strongly dependent on the solvent. [10] In order to develop an effective (both qualitatively
181 and quantitatively) extraction, different parameters were optimized: solvent nature, solvent to
182 sample ratio, time of extraction. Ethanol was selected as the most appropriate solvent for the
183 extraction of phenolic compounds from olive mill residues and for the production of extracts with
184 high phenol content and high antioxidant activity, as reported by Lafka. [12]

185 The results showed that the mixture of a percentage of water to ethanol increase the extraction
186 efficiency, in both studied cultivars respect the only ethanol as solvent.
187 TPC of different samples ranged from 57 to 171 mg 100 g^{-1}. The obtained values were higher than
188 results reported by Lafka et al., [12] who used a similar procedure. This could be related to the acid
189 hydrolysis applied in this study after the extraction. The phenolic compounds are linked by ester
190 and glycosidic bonds to matrix components. The acid hydrolysis allowed breaking of these bonds
191 and so increased the recovery of phenolic compounds. [20] In table 1 the TPC of different fractions
192 (F) from Carolea and Ottobratica OP are shown. Ethanol/water (80/20, v/v) showed the significant
193 highest phenolic yield ($p<0.05$) in comparison to the pure ethanol, after all the extraction times.
194 Spigno et al. [23] reported that the addition of small quantity of water to organic solvent usually
195 creates a more polar medium which increases the extraction efficiency of polyphenols. Moreover,
196 Chew et al. [24] observed that binary-solvent system was more useful and favorable in the extraction
197 of phenolic compounds from matrix plant-based, compared to mono-solvent system. Fraction 1
198 (120 min of extraction) showed the lowest values. Hydro-alcoholic mixture is usually used as
199 solvent to their high selectivity for phenol compounds. [21-22] From multivariate data analysis,
200 significant differences were found among samples, solvent/sample ratio, extraction times and used
201 solvents. The best recovery of phenolic compounds was achieved with a solvent/sample ratio of 5:1
202 (F4) with hydro-alcoholic mixture for Carolea cv, while the ethanolic extraction allowed to obtain
203 the maximum amount with the solvent /sample ratio of 3:1 (F2) regardless of the extraction time.
204 Therefore, the results obtained in these conditions were lower compared to obtained results with
205 ethanol-water. Extraction with only ethanol did not allow obtaining of high levels of total phenols.
206 In most cases, the best extraction time was 120 minutes, particularly in Ottobratica cv. The longest
207 extraction time does not always correspond to the best extractability, in fact the samples F1- F4
208 (Carolea cv, EtOH/H_2O), and F2-F4 (Ottobratica cv, EtOH) showed the highest value at 30 minutes.
209 The most abundant TPC was obtained in OP of Ottobratica cultivar, as shown in table 1. The reason
210 for this higher phenolic yield observed might be due to the water content of samples. Also in this

211 case the hydro-alcoholic mixture allowed to obtain a high yield of phenolic compounds, achieving
212 higher values than those observed in Carolea cultivar. Moreover, no significant differences were
213 detected between different solvent/pomace ratio after 120 minutes of extraction in Ottobratica OP.
214
215 ***Evaluation of the Antioxidant Activity***
216
217 The antioxidant activities of the different phenolic fractions were tested using two assays, based on
218 DPPH˙ and ABTS extinction. Data available in the literature on DPPH˙ and ABTS assays indicate
219 that they are not always well correlated. Moreover, they do not often give the same results because
220 two different action mechanisms and two different radicals are involved. It is for this reason that
221 both assays were considered. [25]
222 The antioxidant capacity of extracts was also sensitive to the different solvent system used. In
223 DPPH assay, the absorbance decreases as a result of a color change from purple to yellow as the
224 radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH-H
225 molecule. [26] The data of percentage inhibition were shown in Figures 3 and 4.
226 The analyzed samples were processed statistically by means of their ratio and time of extraction.
227 The radical scavenging activity of olive pomace extracts by ethanol/water (80/20) was significantly
228 ($P<0.05$) higher than those obtained by ethanol. In the Carolea OP extracts (Fig. 2), extraction time
229 of 30 minutes was the most suitable for the maximum extraction of the antioxidant compounds
230 regardless on the solvent/sample ratio, with values of about 80% of inhibition, and this cultivar
231 showed the highest antioxidant capacity.
232 Different trend was shown in Ottobratica OP (Fig. 3): the highest percentage of inhibition (greater
233 than 60%) was observed after 120 minutes of extraction with ethanol/water. As regards the
234 extraction with only ethanol, there were not significant differences on total antioxidant activity
235 between 60 and 120 minutes of extraction.

236 In contrast with the total phenol content, Carolea cultivar showed a higher antioxidant capacity.
237 According to literature data that indicate that these assays are not always well correlated and they
238 do not often give the same results because it deal with two different action mechanisms using two
239 different radicals. [27]
240 The other method employed to measure the antioxidant capacity of the OP extracts was TEAC. It is
241 based on the ability to scavenge the ABTS radical cation, a chromophore with characteristic
242 absorption at 734 nm, converting it into a colorless product. The antioxidant activity of each
243 phenolic extract was calculated by relating the decrease in absorbance induced by the sample to that
244 of Trolox and was expressed both as total antioxidant activity. The results of TEAC in OP samples
245 showed a different trend compared to that of obtained by DPPH assay. A higher extraction time
246 (120 minutes) was needed to obtain about 50 μM Trolox g^{-1} in Ottobratica OP (Fig. 5). Moreover,
247 the extraction with a solvent/sample ratio of 2:1 allowed the obtaining of the greatest values by
248 Tukey *post-hoc* test results ($p<0.05$). This is a relevant observation because the use of high solvent
249 volume makes uneconomical the extraction procedure. Carolea OP showed lower values of TEAC
250 compared with Ottobratica OP (Fig.4), with the difference that the best antioxidant activity was
251 observed in the samples extracted with both solvents for 120 minutes (Fig.5).
252 Whilst the antioxidant activity, in particular that expressed by TEAC method, increased in OP
253 extracts as function of time, no significant increase related to the extraction period was observed for
254 their phenolic yield, making the extraction procedure time-consuming and uneconomical.
255

256 **Conclusions**

257

258 The aim of the present research was to get a better insight into optimization of solvent extraction of
259 antioxidants from Olive Pomace of two cultivars diffused in the South of Calabria, investigating
260 some variables which were selected on the basis of the available literature about the same subject.

261 All olive pomace extracts contained significant amounts of polyphenolic compounds and showed
262 antioxidant activity. However, obtained values differed significantly depending on the solvent
263 polarity. In both investigated cultivars, mixture of ethanol and water was significantly ($P<0.05$)
264 more efficient in extracting of phenolic constituents compared to mono-component solvent system
265 at all extraction time. This occurred for the increased solvation provided by the presence of water.

266

267 **Acknowledgements**

268

269 This work was supported by the grant of MIUR (Ministry of Education, University and Research),
270 Project PON 03PE_00026_1: LINFA – Laboratorio pubblico-privato per la ricerca e l'INnovazione
271 nella Filiera olivicolA

272

273 **References**

274

275 1) Fernández, M.A.; Epino M.; Gomez, F.J.V.; Silva, M.F. Novel approaches mediated by tailor-
276 made green solvents for the extraction, of phenolic compounds from agro-food industrial by-products.
277 Food Chem. **2018**, *239*, 671–678.
278 2) Romero, C.; Medina, E.; Mateo, M.A.; Brenes, M. New By-products rich in bioactive substances
279 from the olive oil mill processing. J Sci Food Agric. **2018,** *98(1),* 225-230.
280 3) Roig, A.; Cayuela, M.L.; Sánchez-Monedero, M.A. An overview on olive mill wastes and their
281 valorization methods. Waste Manag. **2006**, *26*, 960–969.
282 4) Dammak, I.; Neves, M.; Isoda, H.; Sayadi, S.; Nakajima, M. Recovery of polyphenols from olive
283 mill wastewater using drowning-out crystallization based separation process. Innov. Food Sci.
284 Emerg. Technol. **2016**, *34*, 326–335.
285 5) Galanakis, C. M.; Tornberg, E.; Gekasc, V. Recovery and preservation of phenols from olive waste
286 in ethanolic extracts. J. Chem. Technol. Biotechnol. **2010**, *85*, 1148–115.

287 6) Lavecchia, R.; Zuorro A. Evaluation of Olive Pomace as a Source of Phenolic Antioxidants for
288 the Production of Functional Cosmetics. IJAER. **2015**, *10(14),* 34405-34409.
289 7) Giuffrè, A.M.; Sicari, V.; Piscopo, A.; Louadj, L. Antioxidant activity of olive oil mill wastewater
290 obtained from different thermal treatments. Grasas Aceites **2012**, 209-213.
291 8) Di Lecce, G.; Cassano, A.; Bendini, A.; Conidi, C.; Giorno, L.; Gallina Toschi, T. Characterization
292 of olive mill wastewater fractions treatment by integrated membrane process. J. Sci. Food Agric.
293 **2014**, *94*, 2935–2942.
294 9) Piscopo, A.; De Bruno, A; Zappia, A.; Ventre, C.; Poiana, M. Characterization of monovarietal
295 olive oils obtained from mills of Calabria region (Southern Italy). Food Chem. **2016**, *213*, 313–318.
296 10) Tan, M. C.; Tan, C.P.; Ho, C.W. Effects of extraction solvent system, time and temperature on
297 total phenolic content of henna (Lawsonia inermis) stems. Int. Food Res. J. **2013**, *20*(6), 3117-3123.
298 11) Bucić-Kojić, A.; Planinić, M.; Tomas, S.; Jokić, S.; Mujić, I.; Bilić, M.; Velić, D. Effect of
299 Extraction Conditions on the Extractability of Phenolic Compounds from Lyophilised Fig Fruits
300 (Ficus Carica L.). Pol. J. Food Nutr. Sci. **2011**, *61* (3), 195-199.
301 12) Lafka, T.I.; Lazou, A.E.; Sinanoglou, V.J.; Lazos. E.S. Phenolic and antioxidant potential of olive
302 oil mill wastes. Food Chem. **2011**, *125*, 92–98.
303 13) Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of free radical method to evaluate antioxidant
304 activity. Lebensm Wiss Technol. **1995**, *28*, 25–30.
305 14) Bondet, V.; Brand-Williams, W.; Berset, C. Kinetics and mechanism of antioxidant activity using
306 the DPPH free radical method Lebensm Wiss Technol. **1997**, *30*, 609-615.
307 15) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant
308 activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med.
309 **1999**, *26*, 1231–1237.
310 16) Moral, P. S.; Méndez, M. V. R. Production of pomace olive oil. Grasas aceites, **2006**, *57*(1), 47-
311 55.

312 17) Rajha, H.N.; Ziegler, W.; Louka, N.; Hobaika, Z.; Vorobiev, E.; Boechzelt, H.G.; Maroun, R.G.
313 Effect of the Drying Process on the Intensification of Phenolic Compounds Recovery from Grape
314 Pomace Using Accelerated Solvent Extraction. Int J Mol Sci. **2014**, *15*(10), 18640-18658.
315 18) Fernández-Bolanõs, J.; Rodriguez, G.; Gomez, E.; Guillen, R.; Jimenez, A.; Heredia, A.;
316 Rodriguez, R. Total recovery of the waste of two-phase olive oil processing: Isolation of added value
317 compounds. J. Agric. Food Chem. **2004**, *52*, 5849-5855.
318 19) Alburquerque, J. A.; Gonzalvez, J.; Garcia, D.; Cegarra, J. Agrochemical characterization of
319 “alperujo”, a solid byproduct of the two-phase centrifugation method for olive oil extraction.
320 Bioresource Technol. **2004**, *91*, 195-200.
321 20) Araújo, M.; Pimentel, F. B.; Alves, R. C.; Oliveira, M. B. P. P. Phenolic compounds from olive
322 mill wastes: Health effects, analytical approach and application as food antioxidants. Trends Food
323 Sci Technol. **2015**, *45*(2), 200–211.
324 21) Alu’datt, M. H.; Alli, I.; Ereifej, K.; Alhamad, M.; Al-Tawaha, A. R.; Rababah, T. Optimisation,
325 characterisation and quantification of phenolic compounds in olive cake. Food Chem. **2010**, *123*(1),
326 117–122.
327 22) Klen, J.T.; Vodopivec, M. B. The fate of olive fruit phenols during commercial olive oil
328 processing: Traditional press versus continuous two- and three-phase centrifuge. LWT - Food Sci.
329 Technol. **2012**, *49*(2), 267–274.
330 23) Spigno, G.; Tramelli, L.; De Faveri, D.M. Effects of extraction time, temperature and solvent on
331 concentration and antioxidant activity of grape marc phenolics. J Food Eng **2007**, *81*, 200-208.
332 24) Chew, K. K.; Khoo, M. Z.; Ng, S. Y.; Thoo, Y. Y.; Aida, W. M. W.; Ho, C. W. Effect of ethanol
333 concentration, extraction time and extraction temperature on the recovery of phenolic compounds and
334 antioxidant capacity of Orthosiphon stamineus extracts. Int Food Res J. **2011**, *18*(4), 1427-1435.
335 25) Rubio-Senent F.; Rodríguez-Gutiérrez G.; Lama-Muñoz A.; Fernández-Bolaños J. Phenolic
336 extract obtained from steam-treated olive oil waste: Characterization and antioxidant activity. LWT
337 - Food Sci Technol. **2013**, *54*, 114-124.

338 26) Bandoniene, D.; Murkovic, M.; Pfannhauser, W.; Venskutonis, P. R.; Gruzdiene, D. Detection
339 and activity evaluation of radical scavenging compounds by using DPPH free radical and on-line
340 HPLC–DPPH methods. Eur. Food Res. Technol. **2002**, *214*, 143–147.

341 27) Rubio-Senent, F.; Rodríguez-Gutiérrez, G.; Lama-Muñoz, A.; Fernández-Bolaños, J. Phenolic
342 extract obtained from steam-treated olive oil waste: Characterization and antioxidant activity. Food
343 Sci Technol. **2013**, *54*, 114-124.

344 **FIGURE CAPTIONS**

345

346 **Figure 1**. Schematic overview of the experimental plan

347 **Figure 2.** Results of DPPH assay on Olive Oil Pomace extracted with different solvents: (EtOH)
348 Ethanol and (EtOH/H_2O) Ethanol/Water (Carolea cultivar). Antiradical activity values expressed as
349 % inhibition. The data are presented as means ± SDs. The different letters indicate significantly
350 different results ($p < 0.05$).

351 **Figure 3.** Results of DPPH assay on Olive Oil Pomace extracted with different solvents: ((EtOH)
352 Ethanol and (EtOH/H_2O) Ethanol/Water (Ottobratica cultivar). Antiradical activity values expressed
353 as % inhibition. The data are presented as means ± SDs. The different letters indicate significantly
354 different results ($p < 0.05$).

355 **Figure 4.** Results of ABTS assay on Olive Oil Pomace extracted with different solvents: (EtOH)
356 Ethanol and (EtOH/H_2O) Ethanol/Water (Carolea cultivar). Antiradical activity values expressed as
357 μM Trolox g^{-1}. The data are presented as means ± SDs. The different letters indicate significantly
358 different results ($p < 0.05$).

359 **Figure 5.** Results of ABTS assay on Olive Oil Pomace extracted with different solvents: (EtOH)
360 Ethanol and (EtOH/H_2O) Ethanol/Water (Ottobratica cultivar). Antiradical activity values expressed
361 as μM Trolox g^{-1}. The data are presented as means ± SDs. The different letters indicate significantly
362 different results ($p < 0.05$).

363

364

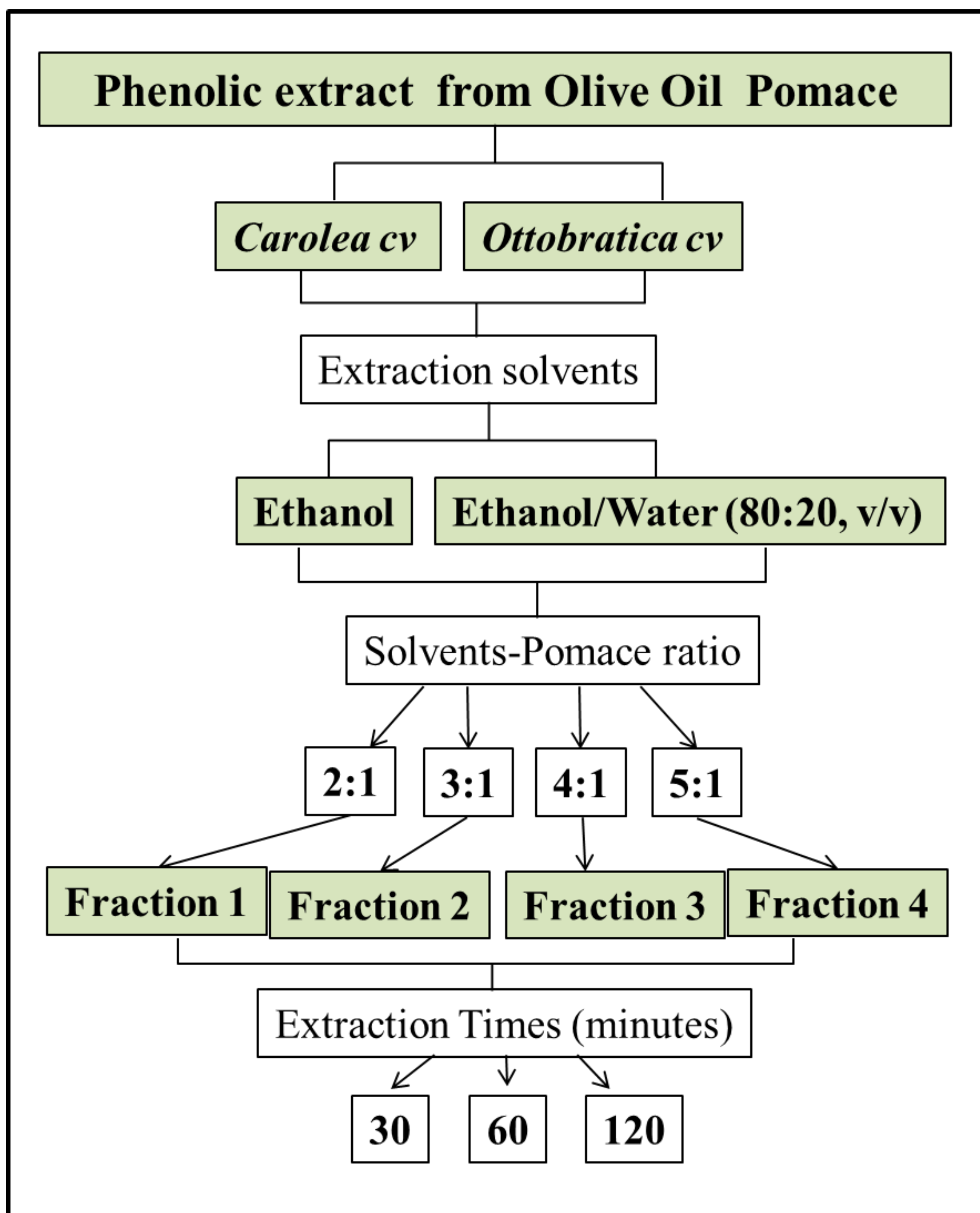
365

366

367

368

369



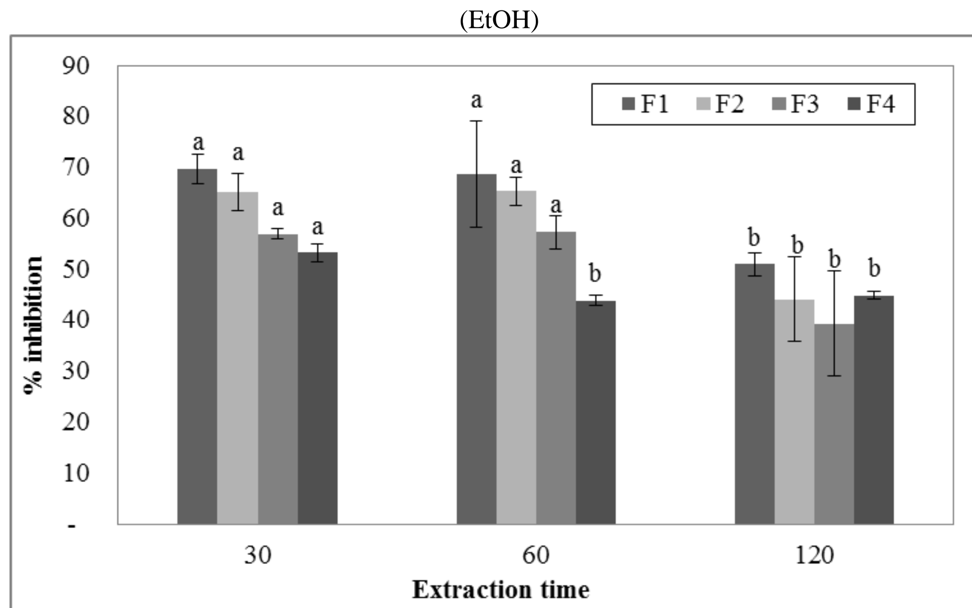
370

371 Fig. 1

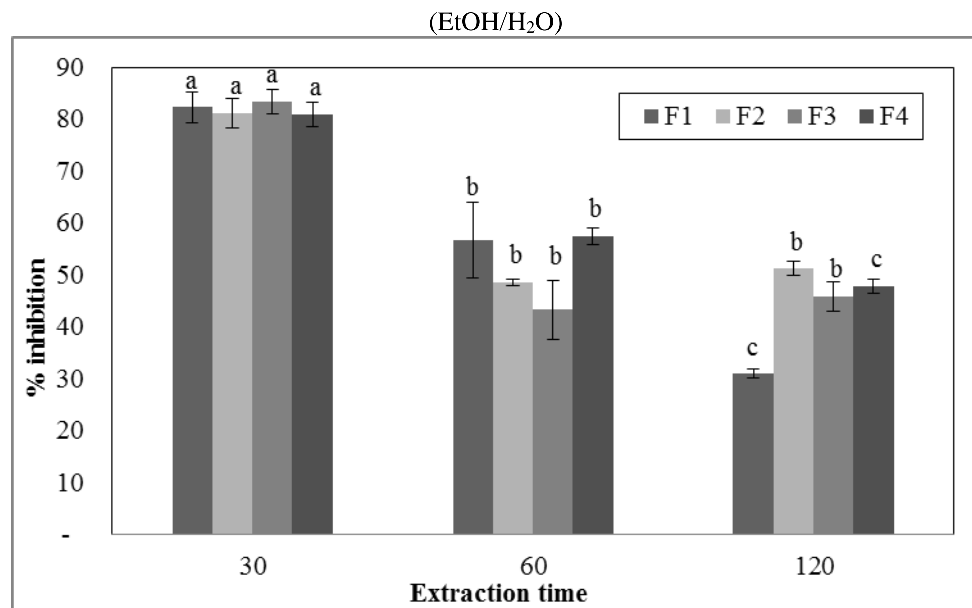
372

373

374 (EtOH)



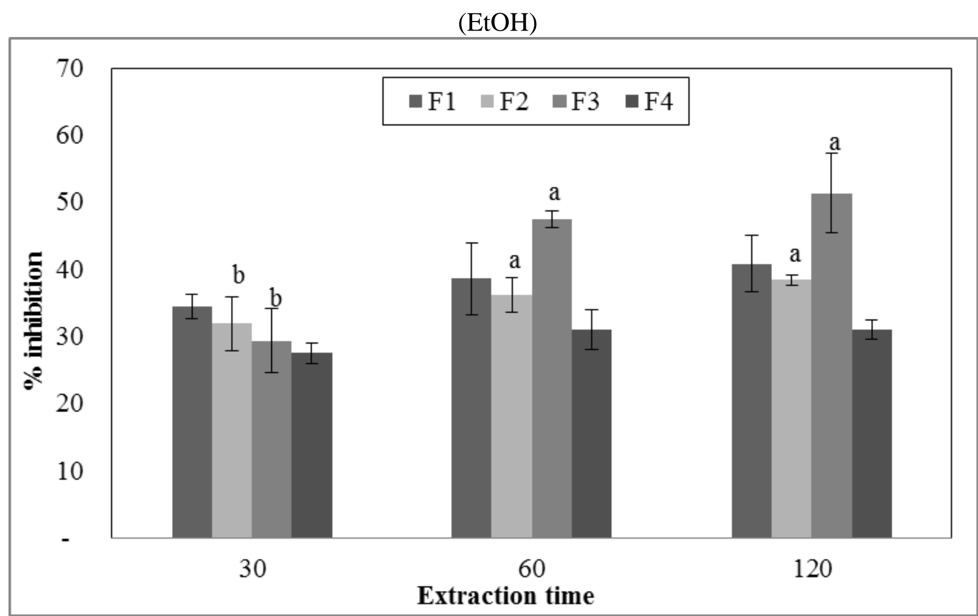
375
376
377
378 ($EtOH/H_2O$)



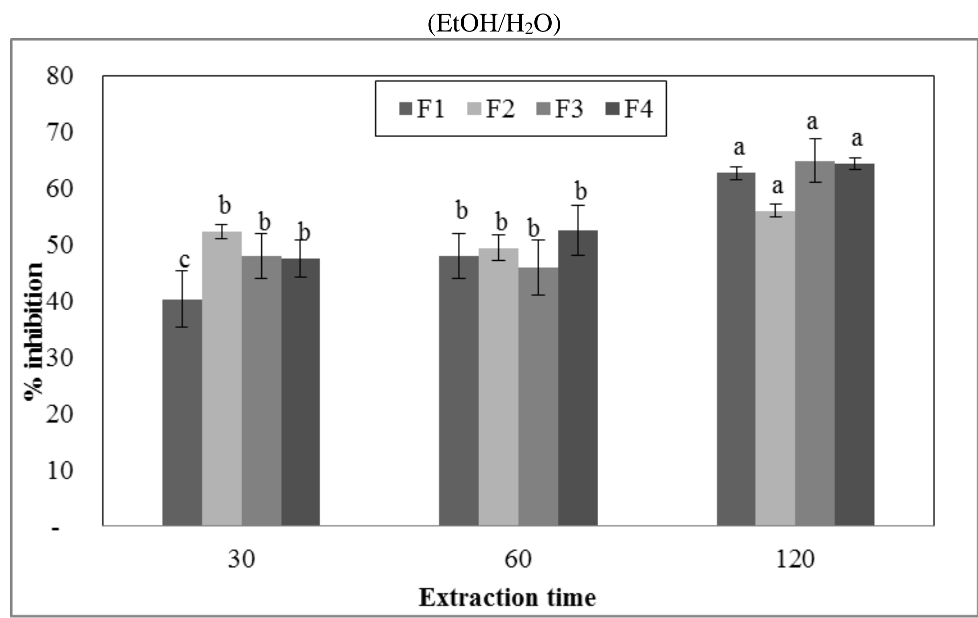
379
380
381
382 Fig. 2
383
384
385

386

387 (EtOH)



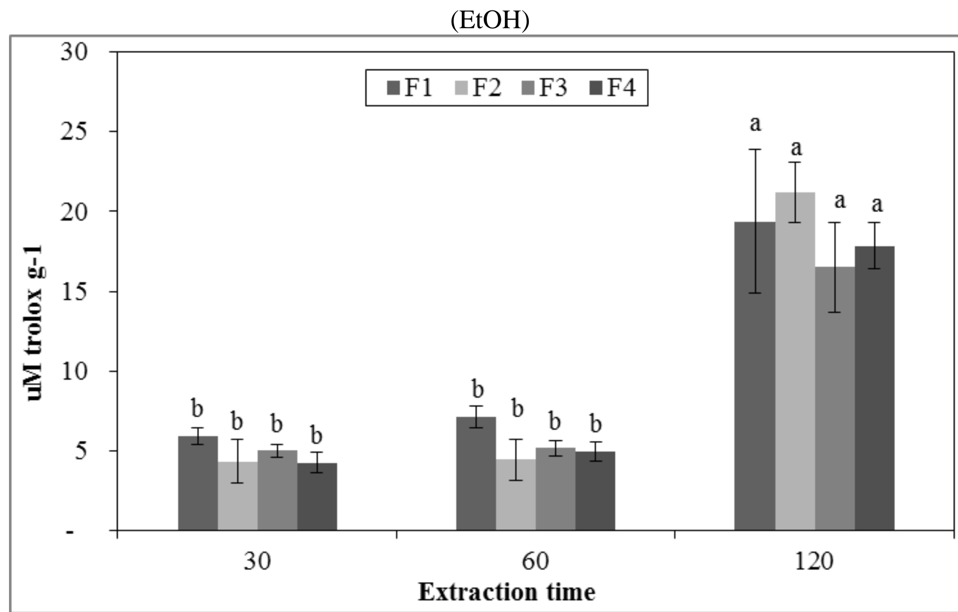
388
389
390
391 (EtOH/H_2O)



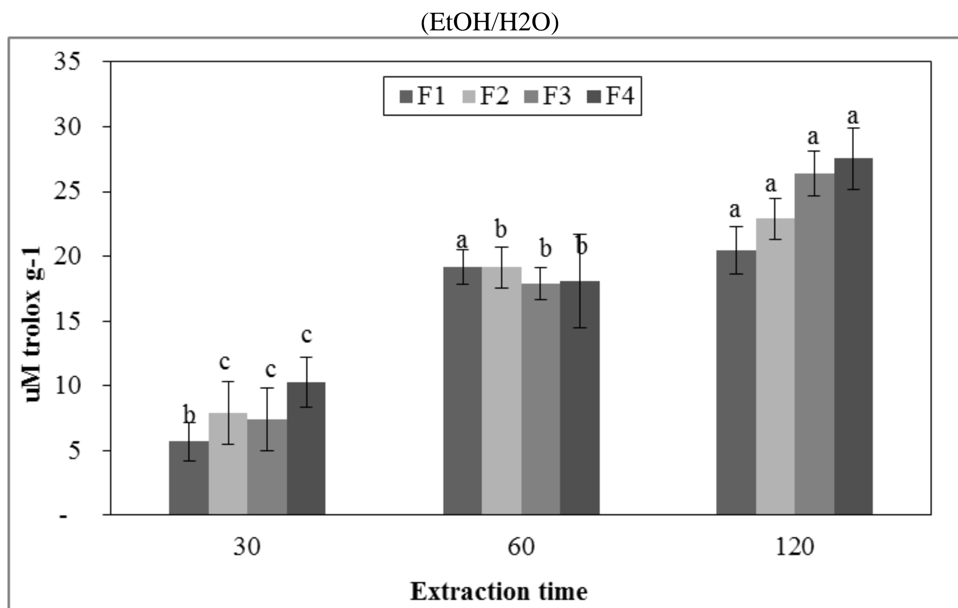
392
393
394
395 Fig. 3

396
397

398 (EtOH)



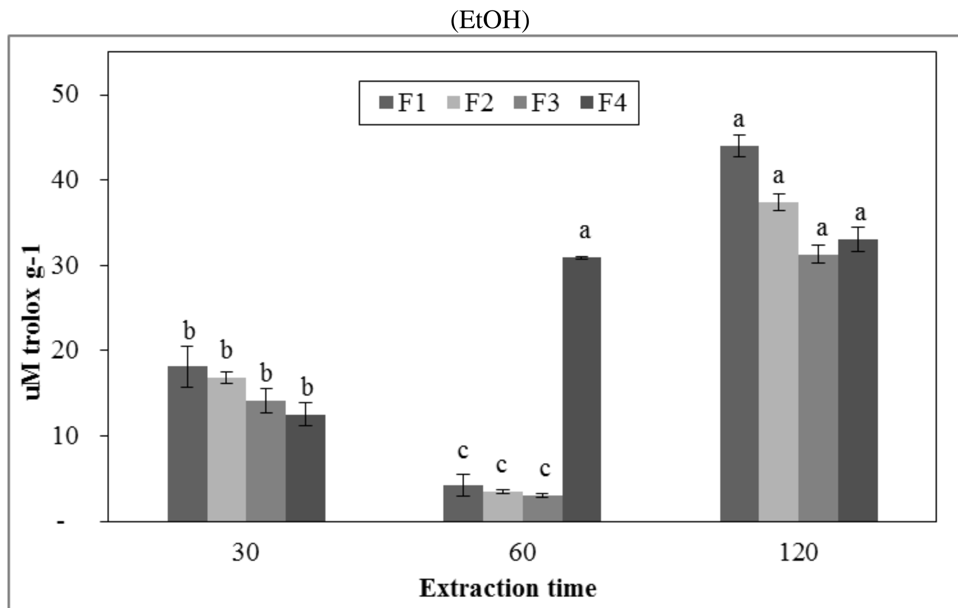
399
400
401
402 (EtOH/H2O)



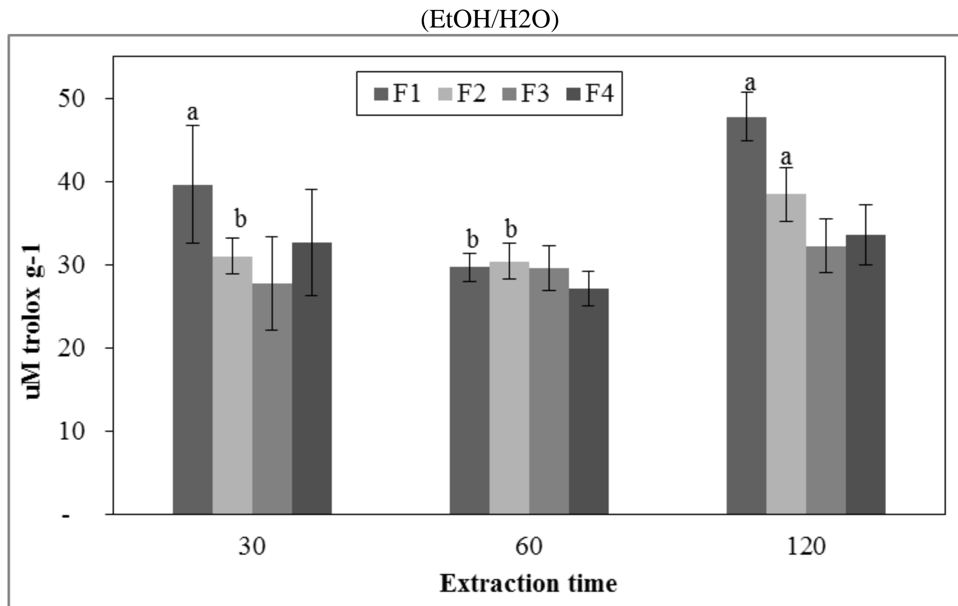
403
404
405
406
407 Fig. 4

408
409

410



411
412
413
414



415
416
417
418
419 Fig. 5

420
421

Table 1: Total Phenolic Compounds from different Extracts of Olive Oil Pomace of two Cultivars, Carolea and Ottobratica.

Cultivar	Solvent	Solvent/Sample	30 min	60 min	120 min	sign.
Carolea	**Ethanol**	F1	79 ± 6^{aB}	93 ± 11^{aB}	101 ± 11^{A}	*
		F2	80 ± 6^{a}	87 ± 2^{a}	90 ± 7	**n.s.**
		F3	63 ± 5^{bB}	81 ± 2^{aB}	88 ± 4^{A}	**
		F4	57 ± 1^{bC}	71 ± 2^{bB}	92 ± 1^{A}	**
		sign.	**	**	**n.s.**	
	Ethanol/Water	F1	104 ± 4^{cAB}	125 ± 24^{abA}	82 ± 2^{cB}	**
		F2	107 ± 1^{cB}	108 ± 2^{abB}	113 ± 3^{bA}	*
		F3	117 ± 3^{bA}	99 ± 13^{bB}	128 ± 3^{aA}	**
		F4	131 ± 5^{aA}	136 ± 0^{aA}	124 ± 1^{aB}	**
		sign.	**	**	**	
Ottobratica	**Ethanol**	F1	78 ± 16^{AB}	59 ± 8^{bB}	88 ± 7^{bA}	*
		F2	98 ± 11^{A}	72 ± 4^{aB}	99 ± 1^{aA}	**
		F3	83 ± 7^{A}	70 ± 1^{aB}	92 ± 3^{abA}	**
		F4	83 ± 4^{A}	69 ± 2^{abB}	74 ± 3^{cB}	**
		sign.	**n.s.**	*	**	
	Ethanol/Water	F1	123 ± 3^{bB}	115 ± 6^{bB}	157 ± 7^{A}	**
		F2	139 ± 3^{abB}	125 ± 1^{aC}	171 ± 4^{A}	**
		F3	125 ± 15^{bB}	128 ± 5^{aAB}	153 ± 18^{A}	*
		F4	147 ± 12^{aA}	123 ± 1^{aB}	159 ± 2^{A}	**
		sign.	*	**	**n.s.**	

The data are presented as means ± SDs. ** Significance at $P < 0.01$. * Significance at $P < 0.05$; n.s. not significant. By Tukey's multiple range test, small letters show differences among extracts at each extraction time and bold letters show differences among extraction time in each extracts.