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14
15 **Green-sustainable extraction techniques for the recovery of antioxidant compounds from**
16 **“Citrus limon” by-products**

17
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22
23
24 **Abstract**

25 In this work, optimized techniques of conventional, ultrasound and microwave-assisted extraction
26 were applied for the recovery of antioxidant compounds from lemon by-products (*Citrus limon L.*).
27 Specifically, the effect of solvent, temperature, microwave power, time and their interaction on the
28 extraction was investigated. Among the tested solvents, the hydroalcoholic mixture (ethanol:water,
29 50:50) was the optimal one for all extraction techniques: in particular assisted by ultrasounds at
30 70°C for 30 minutes (total phenolic content: 6.93 mg GAE g⁻¹, total flavonoids: 2.07 mg CE g⁻¹,
31 ABTS assay: 18.36 μM TE g⁻¹). Also, the other techniques allowed to obtain valuable extracts,
32 although with relative lower amounts. The analyses of individual phenols revealed hesperidin and
33 eriocitrin as the main compounds (respectively about 1650 and 1150 mg kg⁻¹) after ultrasound
34 assisted and conventional extraction. Results of this work can be useful to valorise an industrial by-
35 product by sustainable techniques for the high-added value substances recovery.

36
37
38 _____
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43 **KEYWORDS:** Antioxidant activity, Lemon, microwave-assisted extraction, phenolic extract,
44 UHPLC, ultrasound-assisted.

45

46 **Introduction**

47

48 Food industry produces considerable quantity of solid and liquid waste, obtained from the
49 conversion of feedstock into final products, representing a severe environmental issue due to their
50 content of organic substances. On the other hand, interesting perspectives arise from the huge
51 amount of food by-products. Among fruit and vegetables, citrus are one of the world's most
52 abundant fruit crop and the processing of fruits ^[1], such as lemon, produces considerable quantities
53 of by-products, often including useful compounds in their peels, pulps, and seeds ^[2], which could be
54 extracted and utilized as natural antioxidants to avoid oxidation of some foodstuff or may be
55 applied in functional foods formulations ^[3,4]. Among polyphenols, flavonoids are characterized by
56 relevant biological actions, which include antioxidant, anti-inflammatory, anticancer, antiviral, and
57 anti-mutagenic activities ^[5].

58 Extraction represents the first step to get valuable compounds from a food matrix and several
59 optimized techniques can be developed to obtain them from peel wastes, by conventional solvents
60 ^[6], microwave-assisted extraction ^[7,8], assistance with cyclodextrin ^[9], enzymes ^[10], ultrasounds ^[11],
61 and subcritical water ^[12]. Generally, conventional techniques (solid-liquid extractions) have been
62 broadly used to get bioactive compounds from natural matrices, particularly the maceration
63 extraction. This type of extractions can imply many drawbacks such as: use of high temperatures
64 and long extraction times; low selectivity and low extraction yield; high energy input, safety
65 hazards and environmental risks. The application of alternative techniques is useful to reduce the
66 disadvantages and increase the extraction yield ^[13,14]. On the other hand, ultrasound -assisted
67 extraction represents a promising technique to reduce the extraction time and increase the extraction

68 yield and quality, as well as microwave-assisted extraction, which could be considered an
69 alternative extraction technique that combines microwave and traditional solvent extraction.
70 In this context, organic solvents, as ethanol, methanol and their mixture with water are generally
71 used for the extraction of antioxidant compounds from citrus pulp and peel. Although lots of
72 organic solvents, like methanol, are efficient solvents for extraction of polyphenols, ethanol
73 categorized under GRAS (Generally Recognized as Safe) is preferred because of its application in
74 the food system. Water and hydroalcoholic mixture are the most employed solvents in food grade
75 extraction: presence of water in solvent might lead to an increase in the extraction rate since water
76 could be helpful to improve distension of plant material, which allows an increase in the contact
77 surface area between food matrix and solvent ^[15]. Indeed, the yield of antioxidant compounds from
78 plants is correlated to the polarity, solubility, as well as specific extraction parameters such as
79 solvent nature and concentration, temperature, and time ^[16].

80 Furthermore, in citrus peels, phenolic acids are often related to several plant components through
81 ester and glucoside bonds, while flavonoids can be either in the free (aglycones) or bound
82 (glycoside) forms, with the former having higher antioxidant properties compared to the latter. For
83 this reason, applying heat on citrus by-products during the extraction process may boost the
84 liberation of polyphenols by breaking down both ester and glucoside bonds, which tend to be very
85 stable at room temperature ^[17]. The extraction rate depends not only on the nature of the applied
86 solvents and the solvent: sample ratio, but also on the extraction time and temperature and the
87 chemical composition and physical characteristics of the matrices ^[18, 19]. As reported by De Bruno
88 et al. ^[20], a longer extraction time does not always correspond to a higher extraction yield.

89 Therefore, one of the main purposes of this investigation was focused on the effect of the working
90 conditions (solvent, time, and temperature) on the extraction of bioactive compounds from lemon
91 by-products in order to obtain extracts with a high quantity of antioxidant compounds that,
92 consequently, express an equally high antioxidant activity. Moreover, this study aimed to develop

93 environmentally friendly and food grade techniques (conventional, ultrasound and microwave) with
94 higher extraction efficiency.

95

96 **Materials and Methods**

97

98 ***Materials***

99

100 Lemon by-products samples (*Citrus limon (L.) Osbeck*) called also “pastazzo and/or Lemon
101 pomace” were picked during 2018/2019 crop season, at the Agrumaria Reggina company located in
102 Reggio Calabria (Italy). By-products from the extraction of lemon juice and essential oils consisted
103 of lemon peel, pulp and seeds. Afterwards, they were transported to the Food Technology
104 laboratory of the Mediterranea University of Reggio Calabria where they were immediately dried at
105 a temperature of 50°C up to a final moisture content of 12% and stored in polyethylene bags under
106 vacuum to avoid rehydration until subsequent extraction procedures of the bioactive compounds.

107

108 **Chemicals**

109

110 Standards of gallic acid, *p*-coumaric acid, ferulic acid, eriocitrin, narirutin, hesperidin,
111 neohesperidin, naringin were purchased from Merck (Darmstadt, Germany). Apigenin, caffeic acid
112 and rutin were purchased from Extrasynthèse (France). The solvents used for chromatographic
113 analysis (methanol, water, and acetonitrile) were ultra-high-performance liquid chromatography
114 (UHPLC)-MS grade (Carlo Erba, Italy). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
115 diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu's phenol
116 reagent, and Trolox were purchased from Sigma Chemical Co. (USA).

117

118

119 **Experimental procedure**

120

121 The recovery of bioactive compounds has been carried out through three different extraction
122 techniques: conventional solid-liquid extraction, ultrasound-assisted extraction (UAE), and
123 microwave-assisted extraction (MAE). A simplified outline of the experimental procedures was
124 represented in Figure 1.

125 The extractions were performed using two different food grade extraction solvents, water (H₂O) and
126 ethanol: water mixture (EtOH: H₂O, 50:50). Three temperatures (25, 40 and 70°C) were tested. The
127 extraction times for MAE were 5 and 15 minutes, and those for conventional and UAE were: 30, 60
128 and 120 minutes.

129

130

131 *Conventional Solid-Liquid Extraction*

132

133 The extraction was performed according to Papoutsis et al. ^[21], with some modifications.
134 Briefly, 10 g of dried and ground lemon by-products and 50 mL of solvent (1:5, w:v, ratio) were
135 mixed and placed on a heating magnetic stirrer for the desired times and temperatures, monitored by
136 a digital thermometer. Subsequently, samples were centrifuged (NF 1200R, Nüve, Ankara, Turkey)
137 at 5000 rpm for 5 min at 4°C, filtered through a Büchner apparatus with 0.45 µm filter paper and
138 the resulting extracts were made up to volume of 50 mL with the respective extraction solvent. The
139 extracts were then filtered with 0.45 µm nylon filters and stored at -20°C until subsequent analyses.

140

141 *Ultrasound-Assisted Extraction*

142

143 Ultrasound-assisted extraction (UAE) was performed using a Sonoplus Ultrasonic homogenisers,
144 Series 2000.2, HD 2200.2 (BANDELIN, Ultraschall seit 1955), composed of an inox jug with a

145 capacity from 20 to 900 mL, a VS 70 T probe of 13 mm and a maximum permitted amplitude
146 setting of 100%. The ultrasonic generator converts the mains energy input (mains frequency 50 or
147 60 Hz) into high-frequency energy (frequency of 20 kHz), guaranteeing the reproducibility of the
148 process parameters and the validation of the extraction procedure.

149 For the extraction, 20 g of sample and 100 mL of solvent were mixed (1:5, w:v, ratio) and the
150 desired temperature conditions have been achieved through the control and regulation of radiation
151 rate over time, in terms of radiation amplitude (expressed as percentage) and radiation rate per
152 second. The temperature trend was monitored throughout the extraction time with a digital
153 thermometer. It was found that by applying a specific radiation amplitude (ω) and pulsation time, in
154 relation to the frequency of 20 kHz \pm 500 Hz, it was possible to obtain a corresponding temperature
155 for the entire extraction process:

- 156 - 30 minutes at 25°C (ω = 10%, pulsation time on 1s off 15s), 40°C (ω = 20%, pulsation
157 time on 1s off 5s), 70°C (ω = 50%, pulsation time on 1s off 1s);
- 158 - 60 minutes at 25°C (ω = 10%, pulsation time on 1s off 15s), 40°C (ω = 25%, pulsation
159 time on 1s off 5s), 70°C (ω = 50%, pulsation time on 1s off 1s);
- 160 - 120 minutes at 25°C (ω = 10%, pulsation time on 1s off 30s), 40°C (ω = 20%, pulsation
161 time on 1s off 10s), 70°C (ω = 50%, pulsation time on 1s off 1s).

162 Subsequently, each sample was treated as described above for conventional extraction.

163

164 *Microwave -Assisted Extraction*

165

166 The extraction was performed according to Li et al. ^[7], with some modifications. In the Microwave
167 Digestion System (ETHOS EASY, Millestone, Bergamo, Italy) used for the extraction, the thermal
168 conditions have been achieved through control (by easyTEMP thermal sensor - ATC-CE) and
169 regulation of the applied microwave power (Watt), acquiring for each applied power the
170 corresponding temperature. Specifically, it was found that:

- 171 - 250 W corresponds to 25°C;
- 172 - 500 W corresponds to 40°C;
- 173 - 800 W corresponds to 70°C.

174 In brief, 2.5 g of dried lemon by-products were ground and dissolved in 25 mL of solvent extraction
175 (1:10, w:v, ratio), homogenized with ultra-turrax apparatus (IKA T 25, Staufen, Germany) and
176 transferred into PTFE-TFM vessels of 100 mL (SK-15 easyTEMP, high-pressure rotor). The
177 vessels were placed at the centre of the microwave apparatus, heated to the selected temperature in
178 3 minutes and, then, held at temperature for a certain extraction time (5 or 15 minutes), according to
179 the experimental design.

180 After microwave heating, the mixtures in the extraction vessels have been left to cool down to room
181 temperature in 10 minutes. Subsequently, each sample was treated as described above for the other
182 extraction techniques.

183

184 **Analytical Methods**

185

186 *Total Phenolic Content (TPC)*

187

188 TPC was determined according to the method reported by González-Molina et al. ^[22], with
189 appropriate modifications. 0.2 mL of extract were placed inside a 25 mL flask and mixed with 5 mL
190 of deionized water and 1 mL of Folin-Ciocalteu reagent. After 8 minutes, 10 mL of saturated
191 sodium carbonate solution (Na₂CO₃) at 20% (w/v) were added and made up to volume with
192 deionized water. At the same time, the solution used as a blank was prepared, without the addition
193 of the sample. The mixtures were incubated for two hours at room temperature and in the dark.
194 The absorbance of the samples was measured at 765 nm against a blank using a double-beam
195 ultraviolet-visible spectrophotometer (Perkin-Elmer UV- Vis λ2, Waltham, Massachusetts, U.S.)

196 and comparing with a gallic acid calibration curve (concentration between 1 and 10 mg L⁻¹). The
197 results were expressed as mg of gallic acid g⁻¹ (mg GAE g⁻¹ d.w.) of lemon by-products dry weight.

198 Total polyphenol yield was calculated with the following eq.
199

$$\text{TPC Y}_{(mg\ GAE\ g^{-1}\ dw)} = \frac{(\text{TPC} \times V)}{m}$$

200

201 Where: V is the extraction volume and m is the dry weight of lemon by-products (g).

202

203 *Total Flavonoid Content (TF)*

204

205 The total flavonoid content (TF) was quantified on the obtained extracts by the method described by
206 Papoutsis et al. ^[21], with some modifications.

207 In brief, 0.2 mL of extract, 1 mL of deionised water and 0.15 mL of 5% (w/v) NaNO₂ were mixed
208 in a 5 mL flask and incubated at room temperature for 6 minutes. Subsequently, 0.15 mL of 10%
209 (w/v) AlCl₃ were added and incubated at room temperature for 6 minutes. Then, 2 mL of 4% NaOH
210 (w/v) and 0.7 mL of deionised water were added and finally the mixture was made up to volume
211 with deionised water. At the same time, a solution used as a blank was prepared with the same
212 amounts of reagents but without the addition of the sample. The mixture was incubated in the dark
213 for 15 min. The absorbance was measured at 510 nm against a blank using a double-beam
214 ultraviolet-visible spectrophotometer (Perkin-Elmer UV- Vis λ2, Waltham, Massachusetts, U.S.)
215 and comparing with a catechin calibration curve (concentration between 1 and 50 mg L⁻¹). The
216 results were expressed as mg of catechin g⁻¹ (mg CE g⁻¹ d.w.) of lemon by-products dry weight.

217 Total flavonoids yield was calculated with the following eq.
218

219

$$TF Y_{(mg CE g^{-1} dw)} = \frac{(TF \times V)}{m}$$

220

221 Where: V is the extraction volume and m is the dry weight of the lemon by-products (g).

222

223

224

225 **Antioxidant Activity Determination**

226

227 *DPPH Assay*

228

229 The DPPH assay was performed as reported by Brand-Williams et al. ^[23], which is based on the

230 reaction between the DPPH \cdot (2,2-diphenyl-1-picrylhydrazyl) and antioxidant compounds in the

231 samples, resulting in discoloration of the reaction solution due to the disappearance of the radical.

232 In a cuvette, 50 μ L of the extract (aqueous and hydroalcoholic) properly diluted were added to 2950

233 μ L of a 6×10^{-5} M of methanol solution of DPPH \cdot and left in darkness for 30 min at room

234 temperature. The absorbance was measured at 515 nm, against methanol as blank, using a double-

235 beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV- Vis λ 2, Waltham, Massachusetts,

236 U.S.).

237 The results were expressed as μ M Trolox equivalents g^{-1} of lemon by-products dry weight (μ M TE

238 g^{-1} d.w.), comparing with a Trolox calibration curve (from 3 to 18 μ M).

239

240 *ABTS Assay*

241

242 The antioxidant activity of the extracts was determined by ABTS (2,2'-azino-bis acid (3-

243 ethylbenzothiazolin-6-sulfonic acid) assay, a spectrophotometric discoloration method ^[24]. The

244 working solution was prepared by mixing two stock solutions of 7 mM ABTS solution and 2.4 mM

245 potassium persulphate ($K_2S_2O_8$) solution and was incubated at room temperature for 12 hours in the
246 dark to achieve a stable value of absorbance: the reaction between $ABTS^+$ and potassium
247 persulphate determines the direct production of a blue-green chromogen. The resulting $ABTS^+$
248 solution was diluted with ethanol and showed an absorbance of 0.70 (± 0.02) at 734 nm.
249 The reaction mixture was prepared by mixing 25 μL of aqueous extract and 2975 μL of $ABTS^+$
250 solution; the hydroalcoholic extracts required different reaction ratios, such as 10 μL of extract and
251 2990 μL of $ABTS^+$ solution. The absorbance was measured after 6 minutes in the dark at 734 nm
252 using a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV- Vis $\lambda 2$, Waltham,
253 Massachusetts, U.S.).
254 The quenching of initial absorbance was plotted against the Trolox concentration (from 3 to 18 μM)
255 and the results were expressed as μM Trolox equivalents g^{-1} of lemon by-products dry weight (μM
256 TE g^{-1} d.w.).

257

258 **Identification and Quantification of Antioxidant Compounds**

259

260 Identification and quantification of antioxidant compounds was performed in each extract following
261 Romeo et al. ^[25], with some modifications. Chromatographic system consisted in UHPLC
262 PLATINblue (Knauer, Berlin, Germany) provided with a binary pump system, Knauer blue orchid
263 C18 column (1.8 μm , 100 x 2 mm) coupled with a PDA-1 (Photo Diode Array Detector)
264 PLATINblue (Knauer, Berlin, Germany) and Clarity 6.2 software.
265 Extracts were filtered through a 0.22 μm nylon syringe filters (diameter 13 mm) and then 5 μL were
266 injected in the system. The mobile phases used were (A) water acidified with acetic acid (pH 3.10)
267 and (B) acetonitrile; the gradient elution program consisted in 0–3 min, 5% B; 3–15 min, 5%–40%
268 B; 15–15.5 min, 40%–100% B. Ultimately, restoration of the initial conditions was reached during
269 analysis maintaining the column at 30°C. For the quantification of each antioxidant compounds,

270 external standards (concentration between 1 and 100 mg kg⁻¹) were used, and the results were
271 expressed as mg kg⁻¹ of lemon by-products dry weight (mg kg⁻¹ d.w.).

272

273

274 **Antimicrobial Activity of Extracts**

275

276 The three foodborne pathogens *Listeria monocytogenes* (ATCC 13932 strain), *Escherichia coli*
277 (ATCC 8739 strain) and *Salmonella enterica* (wild strain) were used as test organisms to determine
278 antimicrobial activity of the best selected extracts via the agar diffusion method based on the
279 inhibition zones, at the “University of Messina”, Veterinary Science Department. 10 µL of each
280 extract were inoculated onto Petri plates on the surface of solid soft agar with the test organism
281 (TSA, Tryptic Soy Agar, for *L. monocytogenes*; MH, Mueller Hinton Agar, for *E. coli* and *S.*
282 *enterica*) and incubated at 37°C for 24 h. EtOH:H₂O 50% mixture was used as control. Tests were
283 repeated in duplicate. The results were expressed as mm of the halo diameter resulting from the
284 inhibition zone taken as a measure of the antimicrobial activity of the extract.

285

286 **Statistical Analysis**

287

288 All the experimental results were expressed as mean value (n=4) ± standard deviation. SPSS
289 Software (Version 15.0, SPSS Inc., Chicago, IL, USA) was used for data statistical elaboration.
290 Multivariate and One-way analysis of variance (MAVOVA and ANOVA) with Tukey’s post hoc
291 test at p<0.05. Pearson’s correlation test was employed for the determination of correlation
292 coefficients (*r*) among TPC, TF and antioxidant assays (DPPH and ABTS).

293

294 **Results and Discussion**

295

296 *Conventional Solid-Liquid Extraction*

297

298 Multivariate data analysis evidenced a significant difference ($p < 0.01$) between the effect of water
299 and hydroalcoholic mixture when considered jointly on the dependent variables, whereas the tested
300 times affected only TF (Table 1). Consequently, a separate ANOVA was conducted for each
301 dependent variable, with each ANOVA evaluated at an alpha level of 0.05.

302 In this regard, a different TPC extraction yield between the two types of extraction solvents used
303 was highlighted. In particular, the hydroalcoholic one promoted a higher efficiency for TPC than
304 water (Table 2, Fig.2). These results are confirmed by De Bruno et al. ^[20], who reported that the
305 water/ethanol mixture increases phenolic recovery from agricultural by-products.

306 Using solvent W, after 30 and 60 extraction minutes a decrease of TPC was observed with
307 increasing extraction temperature in the aqueous extracts obtained. The reduction could be
308 explained by the possible degradation of phenolic compounds at any considered temperature as the
309 extraction time increases ^[26]. Temperature of 25° C was considered the most appropriate. After 120
310 minutes of extraction with solvent W, the recovery was the lowest without thermal differences. The
311 maximum recovery of total phenolics in water extracts (W) was 2.96 ± 0.11 mg GAE g^{-1} d.w. (30
312 min, 25°C), with higher results than some data reported in literature ^[27].

313 Hydroalcoholic extraction showed different behaviour. The obtained extraction yield is in
314 accordance with literature data related to the phenolic extraction from lemon by-products ^[28]. Our
315 results showed that the recovery of phenolic compounds linearly increased with the increase of
316 extraction temperature at all the considered extraction times (Table 2). Despite the similar ($p > 0.05$)
317 TPC yield after 120 minutes at both 40°C and 70°C, the multivariate statistical analysis evidenced
318 no time effect on TPC values regardless the extraction temperature, as well as on the showed
319 antioxidant activity. As reported by Barrales et al. ^[29], the polyphenols content in ethanol increases
320 as the temperature increases, probably because of the decrease in surface tension, which represents
321 a factor that regulates the penetration of the solvent into the solid matrix. Moreover, higher

322 temperature increases the diffusivity of phenolic compounds into the solvent boosting their
323 transport, even if at 70°C we should approach the boiling point of the W/EtOH azeotropic (78.4°C),
324 which composition is highly dependent on temperature and pressure.

325 About the total flavonoid content (TF), highly significant differences ($p<0.01$) were found among
326 samples at both extraction solvents. It was evident in the hydroalcoholic one, with a maximum total
327 amount of 2.22 ± 0.29 mg CE g^{-1} d.w. in extract obtained at 70°C for 120 minutes. Such value was
328 consistent with that found by Papoutsis et al. [21] by extraction on freeze dried lemon peel.

329 In this study, TPC and TF showed similar patterns of extractability, with significant variations in
330 the interaction effects by the independent variables ($p<0.05$): as the temperature increases, the
331 extraction rate in ethanol 50% increases significantly at the same time.

332 Regarding the antioxidant activity of the extracts, it was tested by two assays, based on DPPH· and
333 ABTS⁺ extinction, which often do not give the same results because of the two different action
334 mechanisms and the two distinct radicals involved. This is the reason why it could be appropriate to
335 perform both antiradical tests [20].

336 The aqueous extracts (W) did not show a significant difference in the expression of antioxidant
337 activity for both assays performed, even if the correlation between polyphenols and flavonoids
338 compounds and the DPPH assay was high, $r=0.92$ and $r=0.83$, respectively. This is possible since a
339 high phenolic content is not necessary characterized by a high antioxidant capacity, which also
340 depends on the structure and interaction among the extracted phenolic compounds.

341 For the conventional solid-liquid extraction, the hydroalcoholic mixture and the highest temperature
342 (70°C), were considered as the most appropriate parameters for the extraction of antioxidant
343 compounds from dried lemon by-products, at any time among those considered, in terms of the
344 highest TPC and TF values (Table 2).

345

346 *Ultrasound - Assisted Extraction*

347

348 The multivariate statistical analysis (Table 1) shows that in ultrasound-assisted extraction all
349 individual independent variables and the interaction of them significantly affected ($p < 0.05$) TPC
350 and TF, as well as the expression of the antioxidant activity performed by ABTS and DPPH assays.
351 The lack of significance of the DPPH assay suggests the absence of interactions among the
352 independent variables in the studied range.

353 Consequently, a separate ANOVA was conducted for each dependent variable, with each ANOVA
354 evaluated at an alpha level of 0.05.

355 Regarding the aqueous extracts (W), there was a highly significant difference ($p < 0.05$) in the
356 extraction yield of TPC and TF, which decreased with the extension of ultrasonic extraction time
357 and temperature (Table 3). The application of high ultrasonic intensity may result in degradation
358 effects. Indeed, the increment in amplitude of ultrasonic radiations had both positive and negative
359 effects on the extraction yield. More precisely, the highest TPC and TF values (5.91 ± 0.20 mg GAE
360 g^{-1} d.w. and 2.30 ± 0.08 mg CE g^{-1} d.w., respectively), were obtained with temperature of 25°C and
361 extraction time of 60 minutes. As described by Papoutsis et al. ^[21], extraction temperature could
362 have a significant negative effect on TPC yields, suggesting that an extraction temperature higher
363 than the optimum leads to a decrease in TPC. The degradation of cell walls may expand as the
364 temperature increases, follow up on release of both phenolic compounds and enzymes involved in
365 polyphenols oxidation (i.e., peroxidase and polyphenol oxidase). Indeed, our results refer that the
366 extraction yield was decreased by a further increment of the process intensity level, up to 70°C for
367 120 minutes, leading to the lowest TPC and TF values, respectively of 1.31 ± 0.04 mg GAE g^{-1} d.w.
368 and 0.25 ± 0.05 mg CE g^{-1} d.w. of lemon by-products.

369 In addition, this aspect could be attributed to the simultaneous effects of the high radiation
370 amplitude and the extended extraction time, producing several temporary hot spots through the
371 collapse of the cavitation bubbles and an increase in temperature and pressure, which leads to the
372 destruction of polyphenols in the UAE process ^[30, 31].

373 On the antioxidant activity showed by the W extracts, there was a high positive linear correlation
374 between polyphenols ($r=0.84$) and flavonoids ($r=0.88$) compounds and the DPPH assay. Otherwise,
375 a lower correlation between TPC and ABTS assay ($r=0.67$) was detected.

376 Contrary to what previously described, in W/EtOH extracts the bioactive compounds extraction
377 yield increased with increasing of temperature and time. Looking at Table 3, there was a clear
378 temperature effect on extractions for 30 minutes, whose increase led to an increment in TPC.

379 Equally, it was noted that the increase of the extraction time beyond 30 minutes at the same
380 temperature led to an increase in the extraction yield of total polyphenols. Extraction temperature
381 and time had a significant positive linear effect on the extraction process ($p<0.05$), implying that
382 higher yields of TPC can be achieved by increasing ultrasonic radiation amplitude and/or extraction
383 time in agreement with Khan et al. [32]. In fact, UAE is an acoustic cavitation extraction technique
384 that combines ultrasound and traditional solvent extraction: sound waves induce intense shear
385 forces that can break the cell walls and allow the solvent to penetrate the plant cells, intensifying the
386 release of constituents [33; 11]. In comparison to water, ethanol is characterized by a higher heating
387 efficiency when applied in an aqueous mixture and it is preferred thanks to its better capacity in
388 solving the phenolic compounds [34].

389 Additionally, high positive correlations were found ($r>0.80$) between TPC and TF content and both
390 antiradical assays. Indeed, high antioxidant capacity value corresponds to a high phenolic content,
391 with a maximum of $21.28\pm 0.29 \mu\text{M TE g}^{-1} \text{ d.w.}$ and $8.25\pm 0.24 \mu\text{M TE g}^{-1} \text{ d.w.}$ of lemon by-
392 products in W/EtOH extracts, for ABTS and DPPH assays, respectively.

393 The greater efficiency of UAE than conventional extraction agrees with results obtained by several
394 authors in the extraction of polyphenols from different matrices [15, 35].

395

396 *Microwave-Assisted Extraction*

397

398 From multivariate data analysis, among the independent variables taken into consideration in this
399 research, the extraction solvent and power showed a highly significant influence on the extraction
400 process. Indeed, the multivariate statistical analysis shows (Table 1) that a different solvent (W or
401 W/EtOH) and the combination of this variable with the extraction power significantly affected
402 ($p < 0.05$) the extraction yield of TPC and TF and the expression of the antioxidant activity (ABTS
403 and DPPH assays). Extraction power also had a significant influence on the dependent variables
404 considered in this study, with the only exception of the total flavonoid content. Contrary to solvent
405 and power variables, the multivariate statistical analysis suggests a lack of significant influence by
406 time, especially on TPC and ABTS variables, and by the interaction of solvent and extraction time
407 on the extraction process in the studied range.

408 Consequently, a separate ANOVA was conducted for each dependent variable, with each ANOVA
409 evaluated at an alpha level of 0.05. Looking at the aqueous extracts (W) obtained by five minutes
410 extraction, there was a high significance difference ($p < 0.01$) between the values within each
411 dependent variable considered, which confirms the influence of microwave power on the extraction
412 process.

413 As reported in Table 4, a similar content of TPC and TF was observed for the extractions carried
414 out at 250 and 500 Watt for 5 minutes, as well as for the expression of antioxidant activity by
415 DPPH assay ($p > 0.05$). However, a reversal was noted when the applied microwave power was
416 increased up to 800 Watt, observing a significant decline in all the studied parameters.

417 By increasing the microwave power over 500 Watt for 5 minutes extraction, the recovery of
418 polyphenols and flavonoids decreased: these results agree with those reported by Shao et al. ^[36]. A
419 different trend was found for the 15 minutes extractions, among which there was no significant
420 difference in terms of TPC regardless of the power applied, as well as for the results of DPPH
421 assay. In contrast to this, a highly significant difference ($p < 0.01$) was determined with regards to
422 the extraction rate of flavonoid compounds and the relative expression of antioxidant activity by
423 ABTS assay, even if without a positive correlation ($r = -0.78$). In this case, although water is a highly

424 polar solvent for the extraction of bioactive compounds, the main inconvenience of its use is the
425 difficulty of determining the high content of water-soluble impurities which interfere with the
426 identification and quantification of target compounds ^[8].

427 Despite a certain correlation between increasing of time and extraction yield was found, few
428 minutes up to 500 Watt of microwave treatment seemed to show the best extraction yield in
429 polyphenols and flavonoids: an overexposure in the microwave encouraged in fact the degradation
430 of the thermolabile compounds, as reported by Nayak et al. ^[37]. According to Rafiee et al. ^[34], the
431 increase in irradiation time up to 15 minutes did not result in improvement in the extraction
432 performance, but sometimes might lead to a decrease in the concentration yield.

433 About the hydroalcoholic extracts (W/EtOH), by increasing the microwave power from 250 to 500
434 Watt with extraction time fixed at 5 minutes, the TPC decreased significantly from 6.74 ± 0.22 to
435 5.56 ± 0.30 mg GAE g⁻¹ d.w. The reduction in TPC was noticed when the optimal conditions
436 determined within the range of variables considered in this study have been exceeded (beyond 250
437 W and 5 minutes). This result was due to the increment in direct effect of microwave energy on the
438 extraction system by the dipolar rotation, which resulted in temperature increase of the system and
439 produced the deterioration of the bioactive substances ^[37].

440 Results indicated that the content of phenolic and flavonoid compounds decreased significantly with
441 power during microwave treatment ($p < 0.05$) and extraction time of 15 minutes did not show any
442 obvious positive effect on extraction yield. In fact, when lemon by-products were treated at 250
443 Watt for 15 min there was a sudden decrease of value, compared with the extraction at 250 Watt for
444 5 minutes, which indicated that longer irradiation time was harmful to the TPC. This suggests that a
445 shorter treatment time is ideal for the release of TPC under microwave irradiation. The highest
446 content of total phenolic content and total flavonoids content was obtained when dried lemon by-
447 products were treated at 250 Watt for 5 minutes, with aqueous-ethanol mixture as extraction
448 solvent. The results suggested that the interaction between the extraction solvent (W or W/EtOH)
449 and microwave power was highly significant ($p < 0.01$) on the extraction efficiency of TPC. Water

450 content, being the most common absorbing phase for microwave energy, shows a crucial role
451 during microwave extraction process ^[38]. Phenolic compounds are polar molecules and the presence
452 of water boosts the polarity index of organic solvents compared with pure solvents, as ethanol,
453 contributing to get higher values for the TPC and antioxidant activity. Moreover, the addition of
454 water not only increases the dielectric constant of the solvent, but also intensifies absorption of
455 microwave energy provoking a higher temperature inside the samples, which causes the fracture of
456 cells and a quick outflow of antioxidant compounds ^[13]. Prolonged extraction time and a high
457 microwave power led to lower TPC values, as a result of the almost certainly damage to phenolic
458 acids by microwave treatment ^[38, 39].

459 Regarding antioxidant capacity of aqueous extracts (W), the same trend of the valuable compounds
460 previously discussed was observed, increasing slightly as microwave power increases up to 500
461 Watt, even if there is no significant difference between the values. After 5 minutes of extraction, the
462 antioxidant activity of aqueous extracts performed by DPPH and ABTS assays was 5.44 ± 0.37 and
463 $8.61 \pm 0.51 \mu\text{M TE g}^{-1} \text{ d.w.}$, respectively.

464 Highest antioxidant capacity of hydroalcoholic extracts (W/EtOH), in both DPPH and ABTS
465 assays, was found after a microwave treatment at 250 Watt for 5 minutes. The expression of
466 antioxidant activity, through both assays, perfectly reflects the values of the reported TPC and TF:
467 correlation coefficients, in each case, were above 0.86, which meant that the increase in the
468 antioxidant activity of the extracts was at least in part due to the increase in extracted valuable
469 compounds. In general, the higher radical scavenging activity of MAE extracts could be explained
470 by the fact that microwave treatment might affect cellular structure due to the sudden and swift
471 increase in temperature and internal pressure, provoking a direct effect of microwaves on
472 molecules, which results in rapid rise of the temperature and fast completion of a reaction ^[37]. In
473 agreement with other authors, MAE could be identified as a fast and reliable method for bioactive
474 compounds extraction from citrus wastes and by-products ^[2, 39].

475 Microwave extractive technique has proved to be more efficient in terms of time saving, with an
476 extraction rate over 6 times faster than conventional and ultrasound assisted extraction. Indeed,
477 MAE is characterised by physical and chemical phenomena that are basically distinct from those of
478 the other two applied extraction techniques. The developed microwave system suggests that this
479 kind of extraction offers clear advantages, such as less time required for the extraction process, as
480 well as being environmentally safe.

481

482 *Comparison of Conventional, Ultrasound (UAE) and Microwave Assisted Extraction (MAE)*

483

484 In Figure 2 the results related to the TPC and TF extraction yield to all the tested samples were
485 reported. It is clearly noticeable that the best extraction solvent used, was the W/EtOH mixture, for
486 all the applied extraction systems (conventional, ultrasounds-assistant and microwave-assistant).

487 The TPC extraction yield ranged between: 19.47 and 40.13 mg GAE g⁻¹ d.w. (conventional); 26.49
488 and 39.56 mg GAE g⁻¹ d.w. (UAE) and 31.37 and 38.48 mg GAE g⁻¹ d.w. (MAE). Regarding total
489 flavonoids, the highest yield was also revealed for hydroalcoholic extracts, particularly in samples
490 subjected to ultrasound-assisted extraction. All these results confirmed that the hydroalcoholic
491 solvent has been found to be the best.

492 The best obtained extracts were compared among them and the main antioxidant properties were
493 presented in Figure 3.

494 By comparing the various extraction techniques, the best antioxidant extracts obtained were
495 selected, not only with reference to the total content of bioactive compounds and related antioxidant
496 activity, but also from the point of view of the cost-effectiveness of the extraction process, in terms
497 of time saving. Indeed, choice of an extraction method would mostly depend on the advantages and
498 disadvantages of the process such as extraction yield, complexity, production cost, time saving,
499 environmental friendliness and safety ^[39]. In this study, for each kind of extraction, the following

500 optimal conditions were identified: 30 minutes at 70°C (conventional), 30 minutes at 70°C (UAE)
501 and 5 minutes at 250 Watt (MAE).

502 Phenolic characterisation of the selected extracts obtained under optimal extraction conditions was
503 performed by UHPLC system (Table 6, Fig.4). The method was developed by the injection of
504 standard solutions at concentration levels. Coefficient of correlation (R^2), regression equations and
505 limits of detection (LOD) and quantification (LOQ) for each antioxidant compound were reported
506 in Table 5.

507 Eleven antioxidant compounds were identified in UAE extract: the chromatographic profile showed
508 different concentrations of individual antioxidant compounds, with hesperidin (1694.98 ± 0.36 mg
509 kg^{-1} d.w.) and eriocitrin (1167.28 ± 0.25 mg kg^{-1} d.w.) as the most abundant flavonoids followed far
510 away by narirutin and ferulic acid. Neohesperidin, naringin and rutin were found in very low
511 amounts when compared to the other flavonoids. Apigenin was the least quantified antioxidant
512 compound. Compared to conventional extraction, UAE allowed a better and more efficient
513 extraction of these bioactive compounds. Indeed, as reported in Figure 2, it can be observed that a
514 30-minute UAE at 70°C extracted an equal amount of TPC and TF as a conventional 120-minute
515 extraction at the same temperature.

516 As shown in Table 6, among all, UAE proved to be the best method that resulted in higher
517 extraction yield of antioxidant compounds from lemon by-products, which was significantly higher
518 than those of conventional and MAE. Even if UAE showed the best effect on the qualitative and
519 quantitative characteristics of extracted bioactive compounds, it is a time-consuming method
520 compared to the shortest process time (5 minutes) of microwave assisted extraction. MAE proved to
521 be an interesting alternative extraction technique for the recovery of antioxidant compounds from
522 lemon by-products through an environmentally green approach. The extraction time required for
523 optimal recovery of antioxidant compounds was significantly less than that required for the other
524 applied techniques, showing to be a more rapid extraction method.

525 In addition, the best selected extracts were subjected to antimicrobial activity analysis.
526 Conventional and UAE extracts showed clear antimicrobial activity against some foodborne
527 pathogens compared to the MAE extract (Table 7, Fig.5). The results of antibacterial activity are
528 often highly correlated to total phenolic content. The results suggest that the bacteria species tested
529 did not show an extremely sensitivity to the lemon by-products extracts, especially *S. enterica*,
530 showing a halo diameter less or slightly more than 6 mm ^[40].
531 The antimicrobial properties of plant extracts are due to the presence of secondary metabolites, such
532 as tannins, flavonoids and phenolic compounds, which are the most relevant active elements against
533 bacteria ^[41]. The absence of antimicrobial activity in the MAE extract might be due to the lack of
534 some valuable compounds, such as apigenin. Indeed, as reported by ^[42], apigenin has the capability
535 to inhibit the growth of *L. monocytogenes*, thanks to its intense antibacterial activity by deactivating
536 microbial adhesion, enzymes and cell transport proteins. A similar effect was detected by ^[43], who
537 reported the contribution of gallic acid in the expression of antimicrobial activity against *E. coli*. As
538 described by the author, the method of biologically active substances extraction is relevant to the
539 final composition of the extract. In general, the UAE was found to be the best in terms of phenolic
540 characterisation and antimicrobial activity.

541

542 **Conclusion**

543

544 In this study, the optimal extraction yield and antioxidant activity values were obtained when the
545 extraction process was performed with the hydroalcoholic solvent (W/EtOH, 50%) for all the
546 techniques applied. The comparative studies revealed that the recovery of antioxidant compounds
547 from lemon by-products using UAE at 70°C for 30 minutes was significantly higher than those of
548 conventional and microwave extraction.

549 Anyway, results obtained by MAE revealed it can be considered a novel time-consuming and high-
550 efficient method in the extraction of many bioactive compounds from various natural matrices.

551 The studied extraction procedures involved an environmentally green approach and it could be
552 generalized to other by-products to obtain antioxidant extracts for the future utilization as
553 ingredients in functional food preparations.

554

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558

559 **Data availability statement:** the data that support this study are available from the corresponding
560 author, [ADB], upon request.

561

562

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696 **Figure caption**

697

698 **Figure 1:** Simplified outline of the experimental procedures.

699 **Figure 2:** The effect of extraction method on total polyphenol yield (TPC yield) and total flavonoid yield (TF yield) of
700 the extracts (a: conventional solid-liquid extraction, b: UAE, ultrasound assisted-extraction, c: MAE, microwave assisted
701 extraction).

702 **Figure 3:** Main antioxidant results of the best extracts. The data are presented as means \pm SD (n=4). TPC: total phenolic
703 content; TF: total flavonoid content; DPPH and ABTS: antioxidant activity assays.

704 C (30', 70°C), conventional solid-liquid extraction for 30 minutes at 70°C; C (120', 70°C), conventional solid-liquid
705 extraction for 120 minutes at 70°C; UAE (30', 70°C), ultrasound-assisted extraction for 30 minutes at 70°C; UAE (120',
706 70°C), ultrasound-assisted extraction for 120 minutes at 70°C; MAE (5', 250W), microwave-assisted extraction for 5
707 minutes at 250W.
708

709 **Figure 4:** Eexample of UHPLC analysis of individual antioxidant compounds in UAE extract (Ultrasound-assisted
710 extraction at 70°C for 30 minutes). Peak designation was: (1) gallic acid, (2) caffeic acid, (3) p-cumaric acid, (4) ferulic
711 acid, (5) rutin, (6) eriocitrin, (7) narirutin, (8) hesperidin, (9) neohesperidin, (10) naringin, (11) apigenin.
712

713 **Figure 5:** Antimicrobial activity results of the best extracts. Conventional: solid-liquid extraction at 70°C for 30 minutes.
714 UAE: Ultrasound-assisted extraction at 70°C for 30 minutes. MAE: microwave-assisted extraction at 250W for 5 minutes.
715 Control: water-ethanol 50% solution.
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Table 1: Multivariate statistical analysis of lemon by-product extracts.

Conventional	TPC	TF	ABTS	DPPH
Solvent	**	**	**	**
Temperature	**	**	**	**
Time	ns	**	ns	ns
Solvent*Temperature	**	**	**	**
Solvent*Time	**	**	**	**
Time*Temperature	**	**	*	*
Solvent*Temperature*Time	**	**	**	ns
UAE	TPC	TF	ABTS	DPPH
Solvent	**	**	**	**
Temperature	**	**	**	**
Time	**	**	**	**
Solvent*Temperature	**	**	**	**
Solvent*Time	**	**	**	**
Time*Temperature	**	**	*	**
Solvent*Temperature*Time	**	**	*	ns
MAE	TPC	TF	ABTS	DPPH
Solvent	**	**	**	**
Power	**	ns	*	**
Time	ns	**	ns	*
Solvent*Power	**	**	**	*
Solvent*Time	ns	ns	ns	ns
Time*Power	**	**	ns	*

Abbreviation: ns, not significant; ** Significance at $p < 0.01$; * Significance at $p < 0.05$.

Conventional: solid-liquid extraction; UAE: Ultrasound-assisted extraction; MAE: microwave-assisted extraction.

TPC: total phenolic content; TF: total flavonoid content; DPPH and ABTS: antioxidant activity assays.

Table 2: Total phenolic content (TPC), Total flavonoid content (TF) and antioxidant activity (DPPH and ABTS assays) values of conventional solid-liquid extraction (W, water, and W/EtOH, hydroalcoholic mixture).

Minutes	°C	TPC (mg GAE g ⁻¹ d.w.)			TF (mg CE g ⁻¹ d.w.)		
		W	W/EtOH	Sign.	W	W/EtOH	Sign.
30	25	2.96±0.11 ^a	3.41±0.10 ^c	**	0.94±0.04 ^{ab}	0.61±0.04 ^c	**
	40	2.52±0.28 ^b	5.42±0.17 ^b	**	0.89±0.03 ^b	1.43±0.04 ^b	**
	70	2.69±0.02 ^{ab}	6.37±0.22 ^a	**	1.07±0.06 ^a	1.84±0.10 ^a	**
	Sign.	*	**		**	**	
60	25	2.24±0.06 ^a	4.92±0.09 ^c	**	0.31±0.06 ^b	1.11±0.05 ^c	**
	40	1.09±0.21 ^b	6.23±0.24 ^b	**	0.49±0.02 ^a	1.58±0.09 ^b	**
	70	1.35±0.58 ^b	6.75±0.25 ^a	**	0.30±0.00 ^b	1.85±0.07 ^a	**
	Sign.	**	**		**	**	
120	25	1.23±0.06	5.01±0.13 ^b	**	0.25±0.03 ^b	1.12±0.04 ^c	**
	40	1.48±0.48	6.86±0.17 ^a	**	0.38±0.05 ^{ab}	1.85±0.10 ^b	**
	70	1.93±0.60	7.03±0.48 ^a	**	0.58±0.24 ^a	2.22±0.29 ^a	**
	Sign.	ns	**		*	**	
Minutes	°C	ABTS (µM TE g ⁻¹ d.w.)			DPPH (µM TE g ⁻¹ d.w.)		
		W	W/EtOH	Sign.	W	W/EtOH	Sign.
30	25	7.65±0.74	14.13±0.79 ^b	**	4.46±0.85	5.13±0.30 ^b	ns
	40	6.72±0.63	20.72±1.82 ^a	**	4.48±0.21	6.75±0.09 ^a	**
	70	7.52±0.57	19.43±0.95 ^a	**	4.22±0.11	6.96±0.24 ^a	**
	Sign.	ns	**		ns	**	
60	25	7.28±1.66	17.02±0.77 ^b	**	4.06±0.46	6.77±0.43	**
	40	6.78±2.32	19.11±0.60 ^a	**	3.61±1.30	7.23±0.11	**
	70	5.54±1.57	19.47±0.70 ^a	**	3.25±1.24	7.08±0.18	**
	Sign.	ns	**		ns	ns	
120	25	5.96±0.24	17.46±0.19 ^c	**	3.30±0.67	6.80±0.22 ^b	**
	40	6.03±0.74	21.50±0.76 ^a	**	3.31±0.73	7.76±0.51 ^a	**
	70	7.45±1.34	19.33±0.89 ^b	**	3.82±0.69	8.27±0.13 ^a	**
	Sign.	ns	**		ns	**	

The data are presented as means ± SD (n=4). Means within a row with different letters are significantly different by Tukey's post hoc test. Abbreviation: Sign., significance; ns, not significant; ** Significance at p<0.01; *Significance at p<0.05.

Table 3: Total phenolic content (TPC), Total flavonoid content (TF) values and antioxidant activity (DPPH and ABS assays) values of Ultrasound assisted extraction (W, water, and W/EtOH, hydroalcoholic mixture).

Minutes	°C	TPC (mg GAE g ⁻¹ d.w.)			TF (mg CE g ⁻¹ d.w.)		
		W	W/EtOH	Sign.	W	W/EtOH	Sign.
30	25	5.13±0.10 ^b	4.64±0.13 ^c	**	1.47±0.09 ^a	1.17±0.04 ^c	**
	40	5.38±0.06 ^a	5.82±0.26 ^b	*	1.55±0.15 ^a	1.77±0.11 ^b	ns
	70	4.14±0.08 ^c	6.93±0.32 ^a	**	1.21±0.04 ^b	2.07±0.11 ^a	**
	Sign.	**	**		**	**	
60	25	5.91±0.20 ^a	6.75±0.34	**	2.30±0.08 ^a	2.04±0.09 ^c	**
	40	3.93±0.19 ^b	6.85±0.21	**	1.59±0.07 ^b	2.23±0.04 ^b	**
	70	2.61±0.06 ^c	6.73±0.21	**	1.03±0.02 ^c	2.40±0.09 ^a	**
	Sign.	**	ns		**	**	
120	25	4.27±0.12 ^a	6.30±0.15 ^b	**	0.95±0.05 ^a	1.97±0.07	**
	40	4.39±0.08 ^a	6.90±0.28 ^a	**	1.01±0.05 ^a	1.98±0.10	**
	70	1.31±0.04 ^b	6.93±0.15 ^a	**	0.25±0.05 ^b	2.13±0.12	**
	Sign.	**	**		**	ns	
Minutes	°C	ABTS (µM TE g ⁻¹ d.w.)			DPPH (µM TE g ⁻¹ d.w.)		
		W	W/EtOH	Sign.	W	W/EtOH	Sign.
30	25	18.67±1.10	14.06±0.02 ^c	**	6.38±0.22 ^a	6.40±0.16 ^c	ns
	40	17.73±1.02	16.37±0.83 ^b	ns	6.16±0.15 ^a	7.13±0.13 ^b	**
	70	16.24±2.12	18.36±0.86 ^a	ns	5.03±0.58 ^b	7.43±0.09 ^a	**
	Sign.	ns	**		**	**	
60	25	10.24±0.94 ^a	19.42±0.63	**	6.26±0.28 ^a	8.25±0.24	**
	40	9.16±0.40 ^a	19.77±0.67	**	5.22±0.62 ^b	7.90±0.94	**
	70	7.03±0.67 ^b	19.62±1.06	**	3.86±0.31 ^c	7.95±0.95	**
	Sign.	**	ns		**	ns	
120	25	9.00±0.53 ^a	19.60±0.46 ^b	**	3.58±0.09 ^a	7.14±0.45	**
	40	5.91±0.34 ^b	20.41±0.55 ^{ab}	**	3.43±0.16 ^a	7.61±0.24	**
	70	3.07±0.44 ^c	21.28±0.29 ^a	**	2.08±0.21 ^b	7.59±0.20	**
	Sign.	**	**		**	ns	

The data are presented as means ± SD (n=4). Means within a row with different letters are significantly different by Tukey's post hoc test. Abbreviation: Sign., significance; ns, not significant; **Significance at p<0.01.

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Table 4: Total phenolic content (TPC), Total flavonoid content (TF) values and antioxidant activity (DPPH and ABS assays) values of Microwave-assisted extraction (W, water, and W/EtOH, hydroalcoholic mixture).

Minutes	Watt	TPC (mg GAE g ⁻¹ d.w.)			TF (mg CE g ⁻¹ d.w.)		
		W	W/EtOH	Sign.	W	W/EtOH	Sign.
5	250	2.94±0.11 ^a	6.74±0.22 ^a	**	0.90±0.04 ^a	2.02±0.13 ^a	**
	500	3.28±0.27 ^a	5.56±0.30 ^b	**	0.98±0.09 ^a	1.62±0.11 ^b	**
	800	2.48±0.09 ^b	5.56±0.27 ^b	**	0.69±0.07 ^b	1.75±0.13 ^b	**
	Sign.	**	**		**	**	
15	250	3.00±0.10	5.62±0.49	**	0.50±0.05 ^b	1.59±0.28	**
	500	3.14±0.54	5.87±0.22	**	0.75±0.08 ^a	1.74±0.12	**
	800	2.56±0.11	5.63±0.19	**	0.89±0.10 ^a	1.69±0.08	**
	Sign.	ns	ns		**	ns	
Minutes	Watt	ABTS (µM TE g ⁻¹ d.w.)			DPPH (µM TE g ⁻¹ d.w.)		
		W	W/EtOH	Sign.	W	W/EtOH	Sign.
5	250	7.45±0.50 ^b	24.08±1.01 ^a	**	5.33±0.46 ^a	12.01±0.30 ^a	**
	500	8.61±0.51 ^a	20.03±0.94 ^b	**	5.44±0.37 ^a	10.55±0.40 ^b	**
	800	6.87±0.66 ^b	21.35±1.09 ^b	**	4.37±0.22 ^b	10.72±0.19 ^b	**
	Sign.	**	**		**	**	
15	250	7.96±0.33 ^a	21.56±0.81	**	5.05±0.27	10.81±0.36	**
	500	7.91±0.66 ^a	21.73±1.94	**	5.06±0.75	10.82±0.31	**
	800	6.37±0.29 ^b	21.99±2.12	**	4.74±0.70	10.41±0.12	**
	Sign.	**	ns		ns	ns	

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The data are presented as means ± SD (n=4). Means within a row with different letters are significantly different by Tukey's post hoc test. Abbreviation: Sign., significance; ns, not significant; ** Significance at p<0.01.

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Table 5: Regression equation, Correlation coefficient (R^2), Limits of detection (LOD) and Limits of quantification (LOQ) in standard solutions detected by UHPLC.

Compounds	Regression equation	R^2	LOD mg kg ⁻¹	LOQ mg kg ⁻¹
Gallic acid	y=150.5x+48.18	0.9997	0.0921	0.7526
Caffeic acid	y=111.97x-84.67	0.9997	0.078	0.2567
p-Coumaric acid	y=114.17x+35.47	0.9999	0.0812	0.3145
Ferulic acid	y=127.18x-72.81	0.9998	0.0774	0.5676
Rutin	y=46.07x-14.44	0.9994	0.09676	0.3209
Eriocitrin	y=41.392x-38.81	0.9994	0.3574	19.082
Narirutin	y=88.81+84.18	0.9998	0.08123	0.7654
Hesperidin	y=54.81x+24.38	0.9991	0.07675	23.452
Neohesperidin	y=115.12x+6.96	0.9999	0.0536	0.8657
Naringin	y=42.17x-20.75	0.9994	0.06756	0.5768
Apigenin	y=100.91x-296.57	0.9966	0.04567	0.2345

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Table 6: Phenolic characterisation of selected hydroalcoholic lemon by-products extracts (mg kg⁻¹ d.w.).

Compounds	Conventional	UAE	MAE	Sign.
Gallic acid	72.92±0.14 ^b	103.97±0.28 ^a	2.08±0.14 ^c	**
Caffeic acid	87.80±0.21 ^a	87.46±0.21 ^a	65.29±0.18 ^b	**
p-Coumaric acid	59.25±0.23 ^a	58.17±0.29 ^b	57.81±0.19 ^b	**
Ferulic acid	134.86±0.24 ^a	134.48±0.32 ^a	85.73±0.19 ^b	**
Rutin	70.71±0.21 ^b	76.96±0.13 ^a	32.76±0.17 ^c	**
Eriocitrin	1129.06±0.22 ^b	1167.28±0.25 ^a	1001.34±0.24 ^c	**
Narirutin	58.50±0.17 ^c	140.89±0.24 ^a	77.21±0.25 ^b	**
Hesperidin	1636.26±0.26 ^b	1694.98±0.36 ^a	780.78±0.04 ^c	**
Neohesperidin	6.11±0.24 ^b	12.45±0.19 ^a	1.87±0.19 ^c	**
Naringin	33.39±0.22 ^b	44.14±0.22 ^a	19.84±0.21 ^c	**
Apigenin	3.43±0.26 ^b	4.29±0.08 ^a	nd	**

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The data are presented as means ± SD (n=3). Means within a row with different letters are significantly different by Tukey's post hoc test. Abbreviation: nd, not detected; Sign., significance; **Significance at p<0.01.

Conventional: solid-liquid extraction at 70°C for 30 minutes; UAE: Ultrasound-assisted extraction at 70°C for 30 minutes; MAE: microwave-assisted extraction at 250W for 5 minutes.

800 **Table 7:** Antimicrobial activity of selected hydroalcoholic lemon by-products extracts. Data are expressed as mm of the halo
801 diameter resulting from the antimicrobial activity of the extract.

	<i>L. monocytogenes</i>	<i>E. coli</i>	<i>S. enterica</i>
	ATCC 13932	ATCC 8739	wild
802 Conventional	7.1±0.1	0.0	4.0± 0.0
803 UAE	7.0±0.0	6.6±0.14	4.0± 0.0
804 MAE	0.0	0.0	0.0
805 Control	0.0	0.0	0.0

806 The data are presented as means ± SD (n=2).

807 Conventional: solid-liquid extraction at 70°C for 30 minutes.

808 UAE: Ultrasound-assisted extraction at 70°C for 30 minutes.

809 MAE: microwave-assisted extraction at 250W for 5 minutes.

810 Control: water-ethanol 50% solution.

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