1 **Functional ingredient from avocado peel: microwave-assisted extraction, characterization and**
2 **potential applications for the food industry**

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18

19 **Abstract**

20 Avocado peel is a by-product obtained in high amounts in the food industry with no further
21 applications despite its richness in bioactive compounds. In this context, an efficient “green”
22 microwave assisted extraction (MAE) was optimized to maximize the extraction of bioactive
23 polyphenols. Moreover, the phenolic composition of the developed green avocado extract was
24 characterized by HPLC coupled to MS analysers and the potential appli- cations for the food industry
25 were studied assaying different bioactivities. Thus, the matriX metalloproteinases inhibition, the
26 antioXidant capacity and the antimicrobial activity against gram-positive and gram-negative bacteria,
27 yeast and mold were tested. The results pointed out both, high matriX metalloproteinases inhibitory
28 capacity and antioXidant activity of avocado peel MAE extract. These findings suggest the potential
29 food industry applications of this extract as natural food preservative, functional food ingredient or
30 nutraceuticals with anti- oxidant and anti-aging activities

31

32 **Keywords:**

33 Avocado peel; microwave assisted extraction; matrix metalloproteinases inhibition; antioxidant
34 activity; food ingredient

35

36 **1 Introduction**

37 Avocado (*Persea americana* Mill.) is a very nutritious fruit, contain high levels of unsaturated fatty
38 acids, vitamins, minerals, proteins and fibre (Rodríguez-Carpena, Morcuende, Andrade, Kylli, &
39 Estévez, 2011). It is native to Mexico and Central America, although nowadays, it is cultivated in
40 almost all tropical and subtropical regions worldwide (Rodríguez-Carpena et al., 2011). Among their
41 principal varieties, 'Hass' is the most popular grown and imported type (Saavedra et al., 2017).
42 Avocado is commonly consumed as fresh fruit, nevertheless an amply variety of industrial products
43 have appeared recently, such as guacamole, frozen pieces of pulp or avocado oil (Saavedra et al.,
44 2017). In this sense, the industrial processing of avocados generates a large amount of by-products,
45 such as peels and seeds, which should be processed as residues with high cost for the industry. As an
46 example, 13 % of the mass of each avocado is only peel, which nowadays represents tons of wastes
47 discarded with no further application unless for animal feed (Kosińska, Karamać, Estrella,
48 Hernández, Bartolomé, & Dykes, 2012; Rodríguez-Carpena et al., 2011; Wang, Bostic, & Gu, 2010).

49 Nowadays, the current climatic and environmental situation makes a change in production model
50 imperative, extensive to the agro-food industry. This change is translated into a sustainability policy,
51 represented by the application of circular economy in production processes. This new production
52 model in the food industry has two aspects: on the one hand, the use of all the wastes generated during
53 processing for obtaining other value-added products; and on the other, the improvement of
54 conservation methods to reduce food waste. In fact, the search of new natural antioxidant compounds
55 obtained from by-products is a new line of investigation with high impact on the food industry
56 (Oswell, Thippareddi, & Pegg, 2018).

57 Moreover, nowadays the consumer awareness of the relation between nutrition and health are
58 promoting a new trend based on natural and functional foods and nutritional supplementation for
59 improved health benefits. In this scenario, avocado extracts have demonstrated numerous biological

60 activities, e.g., antimicrobial, antioxidant, anti-inflammatory or anticancer properties (Jimenez et al.,
61 2020). Nevertheless most biological activities were associated with avocado seeds instead of skin
62 (Araújo, Rodriguez-Jasso, Ruiz, Pintado, & Aguilar, 2018). Despite the limited information regarding
63 peel bioactivity, its composition suggests that it could possess numerous pharmacological activities.
64 Indeed, some studies have pointed out recently interesting antioxidant and mosquito larvicidal
65 activities (Louis, Pushpa, Balakrishna, & Ganesan, 2020; Melgar et al., 2018).

66 Therefore, the present research focus its main objective in the use of avocado peel by-product as a
67 source of bioactive compounds and exploring different functional properties which could be of
68 interest for the food industry. For that purpose, Microwave Assisted Extraction (MAE), a modern
69 extraction technique, which is known as an environmental-friendly process, due to its remarkable
70 reduction in the use of solvents and , has been applied (Liazid, Guerrero, Cantos, Palma, & Barroso,
71 2011). Compared to conventional methods and other advanced extraction techniques applied to
72 avocado skin (Kosińska et al., 2012; Morais et al., 2015, López-Cobo, Gómez-Caravaca, Pasini,
73 Caboni, Segura-Carretero, & Fernández-Gutiérrez, 2016; Wang et al., 2010; Figueroa, Borrás-
74 Linares, Lozano-Sánchez, Quirantes-Piné, & Segura-Carretero, 2018; Rodríguez-Carpena et al.,
75 2011), MAE is characterized by time-saving and high efficiency processes due to the use of
76 microwave energy (Trujillo-Mayol, Céspedes-Acuña, Silva, & Alarcón-Enos, 2019). This
77 electromagnetic field applied directly to the sample increases the cell breakdowns and the consequent
78 release of substances to the solvent at less aggressive extraction conditions, minimising the
79 degradation of sensitive compounds (Eskilsson & Björklund, 2000). Thus, in the present study
80 solvents compatible with MAE and future pharmaceutical and food industry applications were used,
81 such as ethanol, water or mixtures of both, all Generally Recognized as Safe (GRAS) for their use in
82 these industries (Routray & Orsat, 2012). In addition the MAE extraction parameters with higher
83 impact in the extraction efficiency of bioactive compounds (solvent composition, temperature, time

84 and solvent-sample ratio) were optimized by a Response Surface Methodology (RSM). As far as we
85 are concerned, until now, the effects of extraction parameters in a microwave environment on the
86 concentration of bioactive compounds extracted from avocado skin have not been previously
87 reported.

88 The aims of the study were: a) the application of RSM to optimize the MAE extraction conditions for
89 recovering polyphenol from avocado peel using GRAS solvents; b) assessment of the influence of
90 these extraction parameters on the individual bioactive compound concentrations by HPLC-MS; and
91 c) in vitro screening of the activities of avocado peel MAE extract (matrix metalloproteinases
92 inhibition, antioxidant capacity and antimicrobial activity). As a result, the present research could be
93 of interest to the food industry due to the development and evaluation of potential applications of
94 avocado peel ingredients. This could minimize economic and environmental costs of waste
95 management, delving into the search of new antioxidant ingredients for food preservation and new

96 functional ingredients with healthy beneficial properties for consumers

97 ## 2 Material and methods

98 ### *2.1 Chemicals and reagents*

99 All reagents and solvents were of analytical or MS grade. For extraction, ultrapure water was obtained
100 with a Milli-Q system (Millipore, Bedford, MA, USA) and absolute ethanol (EtOH), methanol
101 (MeOH) and acetone was purchased from Fisher Scientific (Leicestershire, UK). To measure the total
102 phenolic content (TPC), Folin–Ciocalteu reagent, sodium carbonate and gallic acid were purchased
103 from Sigma-Aldrich (Steinheim, Germany). Regarding HPLC analysis, LC–MS grade acetonitrile
104 was purchased from Fisher Scientific (Leicesterchire, UK), formic acid was supplied by Sigma-
105 Aldrich (Buchs, Switzerland) and ultrapure water was obtained with a Milli-Q system describes
106 above. Standards compounds used for the quantification were: (+)-catechin, chlorogenic acid, (−)-

107 epicatechin, neohesperidin (internal standard), quercetin-3-β-glucoside and rutin supplied by Sigma-
108 Aldrich (Steinheim, Germany) and procyanidin dimers A2 and B2 from Extrasynthese (Genay Cedex,
109 France). For identification purposes, citric acid, syringic acid, naringenin, quercetin, kaempferol, 4-
110 hydroxybenzoic acid and quinic acid were supplied by Sigma-Aldrich (Steinheim, Germany), Fluka
111 Chemika (Buchs, Switzerland) and Acros Organics (Geel, Belgium). Finally, for assessing biological
112 activities, all reagents to prepare the microbial culture mediums (Luria-Bertani broth: LB broth, violet
113 red bile agar: VRBA, yeast extract, peptone, glucose, and european microbiological agar) were
114 supplied by CondaPronadisa - Condalab (Torrejón de Ardoz, Madrid); chloramphenicol,
115 cycloheximide, dimethyl sulfoxide (DMSO),2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate
116 (ABTS), sodium acetate, acetic acid, ferric chloride, 2,4,6-tripyridiltriazine (TPTZ), phosphate buffer
117 solution (1M, pH 7.4), fluorescein, 2,2‘-azobis(2-amidinopropane) dihydrochloride (AAPH) and Trolox
118 were purchased from Sigma-Aldrich (Steinheim, Germany); and the matrix metalloproteinases
119 (MMP) inhibitor Profiling Kit, Florometric RED (BML-AK308) was supplied by Enzo (Farmingdale,
120 NY, USA).

121 *2.2 Samples*

122 Fresh avocado fruits (*Persea americana* Mill.) variety 'Hass' were used for the optimization of
123 bioactive compounds extraction. The samples were kindly supplied by the commercial group La
124 Caña, Miguel García Sánchez e Hijos, S.A. (Motril, Spain). Avocadoes were store at room
125 temperature until they reached ready-to-eat ripeness. Then, the peels were manually separated from
126 the flesh, cleaned under continuous flow of tap water and cut into 4-cm squares. Immediately, the
127 chopped peels were dried until a moisture content of < 10 %, according to the optimized conditions
128 described in a previous study (Figueroa, Borrás-Linares, et al., 2018). Afterwards, the dried peels
129 were powdered (average particle size of 0.5 mm) in an ultra-centrifugal mill ZM 200 (Retsch GmbH,

130 Haan, Germany). The material was stored at room temperature and protected from light until its
131 extraction and analysis.

132 *2.3 Experimental design*

133 Response surface methodology (RSM) with a central composite design (CCD) was used in order to
134 optimize the extraction of phenolic compounds from avocado peel (Table 1). The design variables
135 were temperature (50 – 130 °C), time (5 – 45 min), ethanol-water mixtures (0 – 100 % EtOH) and
136 solvent-sample ratio (10 – 50 mL/g), while the response variable was total phenolic content (TPC)
137 expressed as mg gallic acid equivalent per gram of dry peel (mg GAE/g peel dm). The levels of
138 independent variables were selected based on values obtained in preliminary experiments. Two
139 extractions were carried out at each experimental point to assess reproducibility. The effects of
140 unexplained variability in the observed response due to extraneous factors were minimized by
141 randomizing the order of experiments. To verify the suitability of the quadratic equation for predicting
142 the optimum TPC, the verification experiment was carried out under optimum conditions.

143 *2.4 Microwave-assisted extraction (MAE)*

144 MAE was performed in a Multiwave 3000 SOLV instrument (Anton Paar, Graz, Austria), equipped
145 with two standard magnetrons of 850 W delivering up to 1500 W microwave power, a magnetic stirrer
146 device and a pressure/temperature sensor. In addition, the temperature in the system was measured
147 using an optic fibre temperature sensor. For each extraction of MAE experimental design, the specific
148 quantity of avocado peel powder was mixed with a pertinent amount of selected solvent mixture
149 according to experimental design (Table 1). After cooling, samples were centrifuged at a relative
150 centrifugal force (RCF) of 12,499 for 15 min at 4°C in a Sorvall ST 16 R centrifuge (Thermo
151 Scientific, Leicestershire, UK) and the supernatants were evaporated to dryness at 35 °C in a Savan
152 SC250EXP Speed-Vac (Thermo Scientific, Leicestershire, UK). The extracts were stored at −20 °C
153 until further use.

154 *2.5 Conventional solid-liquid extraction (SLE)*

155 In order to compare the overall performance of MAE, a previously optimized convectional extraction
156 was carried out using different solvents. Briefly, 0.5 g of avocado peel was extracted with 15 mL of
157 solvent: EtOH-water (20:80, v/v) and mixtures of EtOH, MeOH or acetone with water (80:20, v/v).
158 The solutions were maintained in agitation at room temperature during 1 hour. For removing the
159 solvent, the aforementioned evaporation process (Section 2.4) was used. The final SLE extracts were
160 stored at -20 °C until the analyses.

161 *2.6 Determination of total phenolic content (TPC)*

162 Total phenolic content (TPC) of the avocado peel extracts was used as independent variable for the
163 optimization of MAE. In this sense, this content was determined by Folin–Ciocalteu assay, with slight
164 modifications. Briefly, the extracts were reconstituted in an appropriated volume of its correspondent
165 extraction solvent for obtaining solutions at a concentration range of 1.0 – 1.5 mg/mL. Then, aliquots
166 of 10 µL of diluted extract were mixed with 600 µL of water, followed by the addition of 50 µL of
167 undiluted Folin-Ciocalteu reagent. After 10 min, 150 µL of 2% (w/v) Na_2CO_3 were added, and at that
168 time the volume was made up to 1.0 mL with water. After 2 h of incubation at room temperature in
169 darkness, 200 µL of the mixture was transferred into a well of a 96-well microplate. A Synergy Mx
170 Monochromator Based Multi-Mode Micro plate reader (BioTek Instruments, Winooski, VT, USA)
171 was used to measure the absorbance at 760 nm. TPC was calculated based on the calibration curve of
172 gallic acid (5–150 µg/mL) prepared in the same manner. The results were expressed as mg of gallic
173 acid equivalents (GAE)/g peel dry mass (dm). All determinations were performed in triplicate.

174 *2.7 Qualitative and quantitative characterization of optimum MAE peel extract by high-*
175 *performance liquid chromatography coupled to electrospray ionization time-of-flight and*
176 *quadrupole-time-of-flight mass spectrometry (HPLC-ESI-TOF/QTOF-MS)*

177 The extract obtained with the optimized condition was reconstituted with the same solvent used in
178 the extraction at a concentration of 10 mg/mL and filtered with regenerated cellulose syringe-filters
179 of 0.2 μm pore size (Millipore, Bedford, MA, USA). Analyses were performed using an Agilent 1200-
180 RRLC system (Agilent Technologies, Palo Alto, CA, USA) of the Series Rapid Resolution coupled
181 to a microTOF™ mass analyser (Bruker Daltonik GmbH, Bremen, Germany). The coupling was
182 performed with an electrospray ionization (ESI) interface operating in negative ionization mode
183 showing the molecular ions $[M-H]^-$. The HPLC system was equipped with a vacuum degasser, a
184 binary pump, an autosampler and a thermostated column compartment. Compounds were separated
185 using a Zorbax Eclipse Plus C18 (4.6×150 mm, 1.8 μm particle size) column with its corresponding
186 guard column of the same packaging (4.6 × 5 mm, 1.8 μm) (Agilent Technologies, Palo Alto, CA,
187 USA). The temperature of the column was maintained at 25°C and the injection volume was 10 μL.
188 Acidified water (0.1 % formic acid, v/v) and acetonitrile were used as mobile phases A and B,
189 respectively. The elution gradient was conducted at a constant flow rate of 0.8 mL/min, as follows: 0
190 min, 95 % A; 30 min, 75 % A; 35 min, 65 % A; 40 min, 5 % A; 50 min, 95 % A. Finally, these initial
191 conditions were kept for 5 min at the end of each analysis to equilibrate the system before the
192 subsequent injection.

193 In order to ensure stable ionization conditions, the effluent from the HPLC column was reduced using
194 a "T" type splitter before being introduced into the mass analyser (split ratio 1:3) to provide a stable
195 spray and, consequently, reproducible results. Detection was carried out considering a mass range of
196 50-1000 *m/z*. The ionization parameters were: capillary voltage, 4500 V; drying gas temperature,
197 190°C; drying gas flow, 9 L/min; nebulizing gas pressure, 2.0 Bar; and end plate offset, −500 V. The
198 values of transfer parameters were: capillary exit, −150 V; skimmer 1, −50 V; hexapole 1, −23 V, RF
199 hexapole, 199 V; skimmer 2, −22.5 V; the trigger time was set to 53 μs (50 μs for transfer time and
200 3 μs for pre-pulse storage time).

201 External mass spectrometer calibration was carried out with a sodium formate cluster solution (5 mM
202 sodium hydroxide and water-2-propanol 1:1 (v/v) with 0.1% (v/v) of formic acid) in quadratic plus
203 high-precision calibration (HPC) regression mode. The mixture was injected at the beginning of each
204 run using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected
205 to the interface and all the spectra were calibrated before identification. Because of the compensation
206 for temperature drifts inside the instrument, this external calibration provided accurate mass values
207 better than 5 ppm.

208 The data was acquired with the software HyStar 3.2 whereas the data treatment was carried out with
209 Data Analysis 4.0, both from Bruker Daltonik GmbH (Bremen, Germany). This software provided a
210 list of possible elemental formulae by using the Generate Molecular Formula™ Editor, which uses a
211 CHNO algorithm providing standard functionalities such as minimum/maximum elemental range,
212 electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison
213 of the theoretical with the measured isotopic pattern (Score and Sigma-Value™) for increased
214 confidence in the suggested molecular formula. It is important to add that even with very high mass
215 accuracy (<1 ppm) many chemically possible formulae may be obtained, depending upon the mass
216 regions considered, and thus high mass accuracy (<1 ppm) is not in itself enough to exclude sufficient
217 candidates with complex elemental compositions. The use of isotopic abundance patterns as a single
218 further constraint removes more than 95% of false candidates. This orthogonal filter can reduce
219 several thousand candidates down to a small number of molecular formulas.

220 The identification of compounds was also supported by fragmentation analysis performed using an
221 Agilent 1260 HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent
222 6540 Ultra High Definition (UHD) Accurate Mass Q-TOF equipped with a Jet Stream dual ESI
223 interface. This chromatographic runs were carried out according to the previously described analytical

224 method with slight modifications applying different collision energies (10, 20 and 40 eV). In this
225 case, the data was acquired and treated with the software Agilent Mass Hunter.

226 Thus, the tentative compound identification was performed by the molecular formula and
227 fragmentation patterns provided by the softwares in combination with the information reported in
228 literature and from data bases such as MetFrag, MassBank, SciFinder, Scopus or SciDirect. In the
229 cases when commercial standards were available the compound identification were achieve by the
230 comparison of retention time, exact mass and isotopic distribution of the tentative analyte with those
231 of authentic standards. In this sense, the confidence level of identification 1 was ascribed to
232 substances for which the commercial standard was available, while for the rest of analytes which
233 could be confirmed by their fragments this level of confidence was assigned to 2. In the cases for
234 which the identification were not supported by commercial standards or fragmentation analysis, this
235 indicator was maintained at level 3, following the guidelines provided by Schymanski et al. (2014).

236 Calibration curves of (+)-catechin, chlorogenic acid, (-)-epicatechin, procyanidin A2, procyanidin
237 B2, quercetin and rutin were prepared from LOQ to 120 mg/L, respectively, at 9 concentration levels.
238 In this sense, neohesperidin was used as internal standard (istd) at a concentration of 10 mg/L. All
239 calibration curves showed good linearity among different concentrations, and the determination
240 coefficients (R^2) were higher than 0.99 in all cases. The concentrations were determined by obtaining
241 the relative area of each compound (compound area/internal standard area) and by interpolation in
242 the corresponding standard calibration curve. The compounds for which no commercial standard was
243 available were tentatively quantified on the basis of calibration curves from other compounds with
244 structural similarities. It should be taken into account that the response of the standards can be slightly
245 different from that of the analytes found in the extract, and consequently the quantification of these
246 compounds is only an estimation of their actual concentrations although it can be considered a useful
247 approximation.

248 The validation of the proposed method was performed with linearity, sensitivity, and precision
249 parameters. **Supplementary Table 2** shows the limits of detection (LODs) and quantification
250 (LOQs), calibration range, calibration equations, and regression coefficient (R^2) for all the used
251 standards. The LODs and LOQs for individual compounds in standard solutions were also calculated
252 as S/N = 3 and S/N = 10, respectively, where S/N is the signal-to-noise ratio. The repeatability of the
253 method was measured as the relative standard deviation (RSD, %) in terms of concentration. An
254 avocado-peel extract was injected several times (n = 6) on the same day (intraday precision) and 3
255 times on 2 consecutive days (interday precision, n=12). The intraday repeatability of the developed
256 method for all analytes ranged from 0.86 to 8.31%, whereas the interday repeatability ranged from
257 1.11 to 9.73%.

258 *2.8 Assessment of potential biological activities*

259 Further analyses were performed in three replicates of the avocado peel MAE extract obtained under
260 optimum conditions to study *in vitro* its potential applications in the food, cosmetic and
261 pharmaceutical industries.

262 *2.8.1 Antimicrobial activity*

263 The antimicrobial activity was assessed by the agar disk diffusion method. The avocado peel dried
264 extracts were diluted in H_2O:EtOH:DMSO (2:1:1, v/v/v) to a concentration of 100 mg/mL, filtered
265 through cellulose acetate filters (0.22 μm) and tested against five microbial strains from Research and
266 Development Functional Food Centre (CIDAF) collection, including two Gram
267 positive (*Staphylococcus epidermidis* and *Enterococcus faecalis*), two Gram negative (*Escherichia*
268 *coli* and *Enterobacter hormaechei*), one yeast (*Kluyveromyces marxianus*), and one mold
269 (*Galactomyces candidus*). To prepare active cultures for inocula, *Staphylococcus epidermidis* and
270 Gram negative strains were grown at 37°C in LB broth, *Enterococcus faecalis* strain at 30 ºC in Man,
271 Rogosa & Sharpe (MRS) broth, whereas yeast and mold strains were incubated at 28°C in Yeast

272 extract, Peptone & Dextrose (YPD) broth. Afterward, a 100 µL aliquot of active bacteria, yeast or
273 mold cultures containing >10^6 colony forming units per mL (cfu/mL) was spread onto the surface of
274 LB, MRS or YPD agar when required, to create a microbial lawn and then left to dry. Sterile filter
275 paper disks (diameter = 5 mm) were impregnated with 20 µL of the extract and left to dry before
276 being placed on each inoculated agar. In addition, H_2O-EtOH-DMSO (2:1:1, v/v/v) was employed as
277 negative control and chloramphenicol (0,025 mg/mL) and cycloheximide (1 mg/mL) were used as
278 positive controls for bacteria, as well as yeast and mold strains, respectively. The plates inoculated
279 with bacteria were incubated at 37 °C or 30ºC when required for 24 h, and yeast and mold trains were
280 incubated at 28°C for 48 and 72h, respectively. After incubation, the antimicrobial activity was
281 determined by measuring the clear zone (zone of grown inhibition) around each paper disk. All assays
282 were performed in triplicate. The measures included the disk diameter and were expressed in mm.

283 *2.8.2 Total Antioxidant Capacity (TAC)*

284 The TAC was assessed using three different commonly used methods, as previously described
285 (Figueroa, Borrás-Linares, Lozano-Sánchez, & Segura-Carretero, 2018a). In all the TAC assays,
286 measurements were made in triplicate.

287 *2.8.2.1 ABTS assay*

288 This method assesses the ABTS radical cation ($ABTS^{+\bullet}$) scavenging activity of samples mainly due
289 to their single-electron transfer ability (. Briefly, the $ABTS^{+\bullet}$ stock solution was prepared by mixing
290 7mM aqueous ABTS solution with 2.45 mM potassium persulfate. After 12–24 h in darkness at room
291 temperature, the $ABTS^{+\bullet}$ solution was diluted with H_2O-EtOH (1:1, v/v) to adjust its absorbance value
292 to 0.70 ± 0.02 at 734 nm. A volume of 20 µL of diluted samples was then mixed with 200 µL
293 $ABTS^{+\bullet}$ working solution in a 96-well microplate and the decay in absorbance after 30 min at 25 °C

294 was monitored. A standard curve with Trolox was prepared for expressing the antioxidant activity as
295 mmol of Trolox equivalents per gram of dry extract (mmol Eq T/g DE).

296 *2.8.2.2 Ferric Reducing Antioxidant Power (FRAP) assay*

297 This method determines the reducing capacity of samples based on single-electron transfer
298 mechanism. First, the FRAP reagent was prepared according to Benzie and Strain (1996). Then, 20
299 µL of diluted samples were mixed on a 96-well plate with 125 µL of freshly prepared FRAP reagent
300 and incubated for 5 min at 37 °C. FRAP values were calculated measuring the absorbance at 593 nm
301 on the microplate reader and using $FeSO4 \cdot 7H2O$ as standard. The results were expressed as mmol of
302 Fe(II) equivalents per gram of dry extract (mmol Eq Fe(II)/g DE).

303 *2.8.2.3 Oxygen Radical Absorbance Capacity (ORAC) assay*

304 To assay the capacity of the MAE extract to scavenge peroxyl radicals through its hydrogen atom
305 transfer ability, a validated ORAC method was used (Ou, Hampsch-Woodill, & Prior, 2001) with
306 some modifications (Figueroa, Borrás-Linares, et al., 2018a). Briefly, 30 µL of diluted samples were
307 mixed with fluorescein and AAPH, which were used in the final assay mixture (210 µL total volume)
308 at 40 nM and 19 mM, respectively. Several dilutions of Trolox (2.5–100 µM) were used to construct
309 the calibration curve. The experiments were conducted at 37 °C and under pH 7.4 conditions with a blank
310 sample in parallel. The fluorescence was measured at 485 and 520 nm as excitation and emission
311 wavelengths, respectively. A regression equation between the Trolox concentration and the net area
312 of the fluorescence decay curve was used in order to obtain the final ORAC values, which were
313 expressed as mmol of Trolox equivalents per gram of dry extract (mmol Eq T/g DE).

314 *2.8.3 Matrix metalloproteinases (MMPs) inhibiting activity*

315 The capacity to inhibit the activity of four MMPs was assessed using the MMP inhibitor Profiling Kit
316 (BML-AK308, Enzo, Farmingdale, NY, USA), following the fluorometric method described by the
317 supplier. Briefly, the assays were directly carried out in a 96-well black microplate. The enzyme
318 concentrations in the assays were: MMP1 (128 U/mL), MMP2 (11 U/mL), MMP3 (12.6 U/mL), and
319 MMP7 (12.8U/mL). The extracts were tested against all the enzymes at a final concentration of 150
320 mg/L in the assay, and the potent broad-spectrum MMP inhibitor N-Isobutyl-N-(4-
321 methoxyphenylsulfonyl)-glycyl hydroxamic acid (NNGH) was used as a positive control at a final
322 concentration of 0.5 mg/L. After 30 min of incubation at 37 °C, the substrate (OmniMMP^{TM}RED)
323 was added (final concentration = 0.75 µM) and the increase in fluorescence (Ex/Em=545/576nm)
324 was followed during 10 min of reaction. Calculations were performed according to the kit protocol
325 to obtain the initial reaction velocities. Results were expressed as % of remaining enzymatic activity
326 with respect to the negative control (extract solvent = 100%).

327 3 Results and discussion

328 *3.1 MAE Optimization*

329 Extraction is the most important step for recovering the highest amount of target compounds from
330 the sample matrix. In general, the efficiency of this operation is influenced by multiple parameters,
331 such as temperature, time, solvent composition and solvent-sample ratio, among others, and their
332 effects may be either independent or interactive. Furthermore, response surface methodology (RSM)
333 has shown to be a powerful tool in the optimization of extraction procedures principally due to the
334 possibility of evaluating the interaction effect between the variables on the response (Bezerra,
335 Santelli, Oliveira, Villar, & Escaleira, 2008). In this sense, a RSM was conducted to study the impact
336 of all MAE parameters combined together on TPC of avocado peel extracts. The measured TPC of
337 avocado extracts varied from 18.1 to 68.8 mg GAE/g peel dm, which corresponds to the following

338 extraction conditions: 90 and 130 °C, 100 and 50 % (v/v) of EtOH, respectively, and 25 min and 30
339 mL/g as solvent-sample ratio for both.

340 In order to define MAE conditions which allow maximize the TPC, all the possible variable
341 combinations (Table 1) were statistically analysed using an approach called least squares method, a
342 multiple regression technique that provides a mathematical model to a set of experimental data
343 generating the lowest possible residual (Bezerra et al., 2008). The predicted response Y for the TPC
344 of avocado peel could be expressed by the following second-order polynomial equation:

345 $$Y\ (TPC) = 1.90722 - 0.68391x_1 + 0.50250x_2 + 1.18233x_3 + 1.64651x_4 + 0.00387x_1^2$$
346 $$- 0.02330x_2^2 - 0.00996x_3^2 - 0.02466x_4^2 + 0.00759x_1x_2 + 0.00001x_1x_3$$
347 $$+ 0.00262x_1x_4 - 0.00399x_2x_3 + 0.01066x_2x_4 - 0.00699x_3x_4$$

348 where x_1 is temperature (°C), x_2 is time (min),x_3 is % EtOH in ethanol-water mixtures and x_4 is
349 solvent-sample ratio (mL/g).

350 The analysis of variance was statistically significant and suggested that at least one of the parameters
351 of the model can explain the experimental variation for TPC. Nevertheless, it is important to check
352 the fitting of the RSM mathematical model to make sure that it is reliable in the prediction of MAE
353 conditions for TPC of avocado peel. In this sense, the suitability of the model was investigated
354 through the lack of fit test ($p > 0.05$), indicating that the model could adequately fit the experimental
355 data. Furthermore, the coefficient of determination (R^2) of the model was 95 %, indicating that at
356 least 95 % of the actual values were matched with the predicted values proposed by the mathematical
357 model.

358 Table 1 shows the TPC values for the avocado peel MAE extracts obtained by applying the evaluated
359 extraction conditions. Figure 1 shows the response surface graphs for those variables and interactions
360 that were significant ($p \leq 0.05$). Furthermore, the analysis of variance of the regression models is

361 presented in Table 1S. Regarding the temperature of extraction (x_1), a linear effect was detected as
362 expected, which confirms that the increase in temperature improves the TPC recovery. These could
363 be due to the fact that at higher temperature the solvent viscosity decreases, increasing its mobility
364 and solubility, thus enhancing the extraction efficiency of target compounds (Pimentel-Moral, Borrás-
365 Linares, Lozano-Sánchez, Arráez-Román, Martínez-Férez, & Segura-Carretero, 2018; Veggi,
366 Martinez, & Meireles, 2013). Furthermore, the increasing temperature may also cause a cellular
367 pressure build up which could result in a breakdown of the cell walls and the subsequent release of
368 the target compounds into the surrounding solvent (Proestos & Komaitis, 2008; Routray et al., 2012).
369 Nevertheless, for safety reasons, the maximum temperature evaluated was 130 °C. In addition, several
370 studies described a degradation of thermo-sensitive phenolic compounds with temperatures above
371 130 °C (Liazid et al., 2011; Xiao, Han, & Shi, 2008).

372 On the contrary, there was no linear effect for the extraction time (x_2), while the quadratic effect ($p<$
373 0.001) was highly significant. Indeed, there was an increase in TPC with time up to an exposure of
374 39 min, and then the TPC started decreasing. These could be due to a degradation of thermolabile
375 compounds. Similar results were found when MAE was applied to extract phenolic compounds from
376 different vegetable matrix, such as flavonoids from Radix astragali (Xiao et al., 2008), anthocyanins
377 from grape skins (Liazid et al., 2011) and phenolic acids from citrus mandarin peels (Hayat et al.,
378 2009).

379 Concerning solvent composition, it could be observed that this parameter was highly significant on
380 the extraction of TPC. The linear effect ($p< 0.001$) was positive, whereas the quadratic effect ($p<$
381 0.001) was negative, indicating that there was a maximum in the TPC at 36 % EtOH. In contrast, the
382 TPC was lower when 100 % of pure solvent was applied. Similar results were found in an earlier
383 study where a concentration of 46% EtOH was found as optimal for extracting phenolic compounds
384 from avocado peel using a pressurized liquid extraction system (Figueroa, Borrás-Linares, et al.,

385 2018). Moreover, similar results have been obtained for other matrices, in fact several authors have
386 encountered that a concentration of EtOH between 32 and 37% is optimal for extracting phenolic
387 compounds from dried fruits and herbs (Li, Li, Lin, Zhang, Zhao, & Li, 2017).

388 Finally, concerning to solvent-sample ratio, in Table 1 it can be observed that at the beginning the
389 TPC increased with the increase of this variable, and after that a slight reduction was observed with
390 ratios greater than 44 mL/g. Similar results were reported by Rezaei, Rezaei, Haghighi, and Labbafi
391 (2013) using MAE technique for the extraction of polyphenol content from apple pomace. In this
392 sense, the increase of the TPC at low to moderate levels of solvent-sample ratio could be explained
393 due to the degree of cell wall breakage of the cell membrane of raw materials is greater with increasing
394 solvent quantity (Karami, Emam-Djomeh, Mirzaee, Khomeiri, Mahoonak, & Aydani, 2015). On the
395 other hand, once the polyphenols located in the matrix have been extracted from the matrix to the
396 extract, a further increase in the solvent-sample ratio could not provide an improvement in the
397 extraction efficiency (Rezaei et al., 2013).

398 Therefore, the optimal MAE conditions for maximizing the extraction efficiency were: temperature
399 of 130 °C, extraction time of 39 min, ethanol concentration of 36 % and solvent-sample ratio of 44
400 mL/g, which provided a predicted TPC value of 72.04 mg GAE/g peel dm. Verification experiments
401 under the above mentioned conditions were carried out to confirm the accuracy of the model. Three
402 replicates of the optimal point were prepared and analysed, and the experimental result (73.2 ± 3.8
403 mg GAE/g peel dm) did not report significant differences ($p < 0.05$) compared to the predicted value
404 (CV=1.13 %). Because of the low absolute error values obtained by the comparison between observed
405 and predicted values, the proposed model could be used to predict the response variable (TPC).

406 Thus, the higher TPC observed for this optimum avocado peel MAE extract in comparison with the
407 conventional SLE extracts assessed in this study is noteworthy. In this sense a TPC of 9.5 ± 0.2 mg
408 GAE/g peel dm was obtained for the maceration with a mixture of EtOH-H_2O (20:80, v/v), followed

409 by the TPC obtained for MeOH-water (80:20, v/v) = 5.36 ± 0.1 mg GAE/g peel dm, EtOH-H_2O
410 (80:20, v/v) = 5.0 ± 0.1 mg GAE/g peel dm and acetone-water (80:20, v/v) = 4.61 ± 0.1 mg GAE/g
411 peel dm. This fact also highlights the potential of MAE to efficiently extract phenolic compounds of
412 avocado peel by-product.

413 *3.2 Identification of polar compounds in avocado peel extracts by HPLC-ESI-TOF/QTOF-MS*

414 A representative example (optimized extraction condition) of the base peak chromatograms of
415 avocado peel extracts obtained by MAE resulting from the HPLC-ESI-TOF-MS method described
416 above is shown in Figure 2, where the peaks are numbered according to their elution order. The
417 detected compounds were characterized by the comparison of their retention time and MS and
418 MS/MS spectras provided by the mass analysers with those of authentic standards when available
419 and the information suitable on the literature. TOF/QTOF-MS instrumentation with excellent mass
420 resolution and mass accuracy in combination with true isotopic pattern and fragmentation analysis,
421 is the perfect choice for molecular formula determination of small molecules using the editor Smart
422 Formula™ (García-Villalba et al., 2010). Proposed compounds with their retention time,
423 experimental *m/z*, calculated *m/z*, molecular formula, error, score, miliSigma and MS/MS fragments
424 (m/z and relative abundance) are compiled in Table 2.

425 The use of MAE and HPLC-MS with TOF and QTOF analysers revealed the presence of a wide
426 variety of polyphenols and other polar compounds. A total of 53 compounds were tentatively
427 identified and classified in different families: organic acids, phenolic acids, flavonoids, catechins,
428 procyanidins and other polar compounds. Procyanidins were the chemical group with the highest
429 number of compounds detected in the sample (25 compounds). To the best of our knowledge, no
430 other publication covers such a number of procyanidins compounds in avocado peel (Figueroa,
431 Borrás-Linares, Lozano-Sánchez, & Segura-Carretero, 2018b; Figueroa, Borrás-Linares, et al., 2018;

432 López-Cobo et al., 2016; Rodríguez-Carpena et al., 2011; Saavedra et al., 2017; Trujillo-Mayol et al.,
433 2019; Wang et al., 2010).

434 *3.2.1 Organic acids*

435 According to the elution order and MS data, two compounds were characterized as organic acid.
436 Peaks 1 and 2, with RT 1.96 and 2.34 min and *m/z* = 191.0566 and 191.0191 were identified as quinic
437 acid and citric acid, respectively, by their comparison with standards. These substances were
438 previously reported in avocado peel (Figueroa, Borrás-Linares, et al., 2018b).

439 *3.2.2 Phenolic acids and phenolic alcohol derivatives*

440 Concerning the phenolic acids, five compounds were found in the avocado peel MAE extracts. The
441 compound 4-hydroxybenzoic acid (RT = 12.36 min and *m/z* = 137.025) was unambiguously
442 identified by comparison with the commercial standard. Moreover, peak 3 was identified as syringic
443 acid by comparing their molecular formula, mass spectra and order of elution found in the literature
444 (Figueroa, Borrás-Linares, et al., 2018a). Besides, peaks 4 (RT = 10.06 min), 10 (RT = 13.76 min)
445 and 12 (RT = 14.84 min) with the same precursor ion *m/z* 353.087 were tentatively identified
446 according to their elution order as 3-O-caffeoylquinic, 5-O-caffeoylquinic and 4-O-caffeoylquinic,
447 respectively (Figueroa, Borrás-Linares, et al., 2018b). Moreover, the presence of 5-O-caffeoylquinic
448 acid was confirmed by co-elution with the respective commercial standard.

449 Regarding to phenolic alcohol derivatives, compound 8, with *m/z* 431.156 and molecular formula
450 $C_{19}H_{28}O_{11}$ was identified as tyrosol-glucosyl-pentoside, previously described in this vegetable matrix
451 (Figueroa, Borrás-Linares, et al., 2018b).

452 *3.2.3 Flavonoids*

453 Sixteen flavonoids were tentatively identified belonging to classes such as flavonols, flavanones and
454 flavones. In this sense, the following flavonoids previously found in avocado peel were also

455 confirmed in MAE sample (Figueroa, Borrás-Linares, et al., 2018b; Figueroa, Borrás-Linares, et al.,
456 2018): two isomers of quercetin-diglucoside (peaks 21 and 22, at 20.24 min and 20.52 min,
457 respectively, with *m/z* 625.131), quercetin-O-arabinosyl-glucoside (peak 26, at 22.11 min and *m/z*
458 595.127), luteolin 7-O-(2″-O-pentosyl)hexoside (peak 33, at 25.05 min and *m/z* 579.138), quercetin
459 glucuronide (peak 35, at 25.34 min and *m/z* 477.065), multinoside A (peak 38, at 26.86 min and *m/z*
460 609.144), quercetin-xylosyl-rhamnoside (peak 42, at 28.65 min and *m/z* 579.137) and kaempferol-O-
461 glucosyl-rhamnoside (peak 45, at 29.82 min and *m/z* 593.154). Moreover, the compounds rutin,
462 quercetin-3-β-glucoside, quercetin, naringenin and kaempferol were unambiguously identified by
463 comparison with their commercial standards.

464 Additionally, two signals with *m/z* 565.231 (peak 31 and 46) were observed at 24.15 and 30.75 min.
465 These compounds showed a molecular formula of $C_{28}H_{38}O_{12}$. According to Kosińska et al. (2012), it
466 was tentatively identified as quercetin derivatives. Finally, the peak 41 with *m/z* 433.085 and a
467 molecular formula of $C_{20}H_{18}O_{11}$ was tentatively identified as quercetin-3-O-arabinoside.

468 *3.2.4 Catechins and procyanidins*

469 Catechin, epicatechin and epicatechin gallates are major catechins with dietary importance for human
470 health (Yilmaz, 2006). In these sense, (+)-catechin (RT = 14.43 min and *m/z* = 289.074) and (-)-
471 epicatechin (RT = 18.23 and *m/z* = 289.074) were unambiguously identified since its retention time
472 and MS data matched with their commercial standards.

473 Procyanidins, another important group of oligomeric compounds in avocado peel formed from
474 catechin and epicatechin molecules, have also been characterized in these MAE extracts. Examination
475 of mass spectra and elution profile of compounds in avocado peel revealed the presence of nine
476 procyanidin dimers A and thirteen procyanidin dimers B. The presence of procyanidin A2 (peak 40)

477 was confirmed by co-elution with the respective standard. Moreover, two procyanidin trimers A and
478 one procyanidin trimer B were tentatively identified.

479 *3.3 Quantification of individual phenolic compounds in avocado peel MAE extract by HPLC-ESI-*
480 *TOF-MS*

481 Once the MAE process was optimized, the phenolic compound identified in avocado peel extract
482 obtained under the optimized MAE conditions was quantified by HPLC-ESI-TOF-MS. The
483 quantitative information is shown in Table 3 expressed as mg/100 g peel dm and μg/g extract.
484 Moreover, total content for the different families and all phenolic compounds in avocado peel was
485 tentatively calculated as the sum of the individual compound concentrations. Condensed tannins were
486 the major components of the polyphenol fraction in avocado peels (793 mg/100 g peel dm),
487 accounting the 58% of the sum of phenolic compound concentrations determined individually by
488 HPLC-ESI-TOF-MS. This value was higher that the concentration of procyanidins found in an extract
489 of the same sample obtaining by pressured liquid extraction (269.05 mg/100 peel dm) (Figueroa,
490 Borrás-Linares, et al., 2018). As mentioned before, procyanidins have attracted increasing attention
491 in the fields of nutrition, health and medicine largely due to their potent antioxidant capacity and
492 antimicrobial activity (Gu, House, Wu, Ou, & Prior, 2006; Tang, Xie, & Sun, 2017; Wang et al.,
493 2010). Furthermore, some research suggests that these phytochemicals may modulate immune
494 function and platelet activation (Hammerstone, Lazarus, & Schmitz, 2000; Mao, Powell, Van de
495 Water, Keen, Schmitz, & Gershwin, 1999). In this sense, avocado peel contains a significantly larger
496 amount of procyanidins compared with grape seed extract (6,387 μg/g extract) (Cádiz-Gurrea,
497 Borrás-Linares, Lozano-Sánchez, Joven, Fernández-Arroyo, & Segura-Carretero, 2017), but
498 somewhat less than natural cocoa powder (48,700 μg/g extract) (Gu et al., 2006). Within this family,
499 the major compounds consist of dimer and trimer of procyanidin type B. Nevertheless, high
500 concentrations of procyanidins type A were also found (Table 3). In this sense, the presence of

501 procyanidin type A could provide additional health benefits to these avocado by-products. In fact,
502 Howell, Reed, Krueger, Winterbottom, Cunningham, and Leahy (2005) suggest that the presence of
503 procyanidin type A in cranberry may enhance both *in vitro* and urinary bacterial anti-adhesion
504 activities and aid in maintaining urinary tract health.

505 Although only two catechins were identified, their concentration amounted to 22% of the total sum
506 of phenolic compounds found in the avocado peel extract. In particular, (+)-catechin was the main
507 compound (237 ± 2 mg/100 g peel dm), following by its isomer (-)-epicatechin (59 ± 1 mg/100 g peel
508 dm). These values were significantly higher than those found by Morais et al. (2015) in conventional
509 extracts of the same variety of avocado (0.171 and 0.129 mg/100 g peel dm for (+)-catechin and (-)-
510 epicatechin, respectively). These results confirm the potential of the MAE technique vs. dynamic
511 maceration for the extraction of catechins. Moreover, peel extracts exhibited similar concentration of
512 catechin than grape seed extract (7,747 ± 496 µg/g extract) (Cádiz-Gurrea et al., 2017) and higher
513 concentration than *Theobroma cacao* extract (4,203.1 ± 586.3 µg/g extract) (Cádiz-Gurrea, Lozano-
514 Sanchez, Contreras-Gámez, Legeai-Mallet, Fernández-Arroyo, & Segura-Carretero, 2014), products
515 recognized as a source of catechins. Thus, avocado peel extract could be a rich source of catechins,
516 which are commonly known as strong antioxidants with powerful benefit to human health such as:
517 protective effect against degenerative diseases, control of common oral infections, decreases liver
518 damage, liver inflammation, liver triglyceride, etc. (Rasouli, Farzaei, & Khodarahmi, 2017).

519 Furthermore, five phenolic acids and one phenolic alcohol derivatives were also quantified in avocado
520 peel. Its concentration represents 11% of the total sum of phenolic compounds. The 4-O-
521 caffeoylquinic acid and 5-O-caffeoylquinic acid, were the most abundant phenolic acids in avocado
522 peel extract, with concentrations of 1,120 ± 50 and 950 ± 40 µg/g extract, respectively. These phenolic
523 acids, in particular chlorogenic acid isomers, have been reported that exert health benefits in the
524 management of obesity, cardiovascular diseases, type 2 diabetes mellitus, and metabolic syndrome

(Bento-Silva et al., 2020). In addition, hydroxycinnamic acid derivatives serve as precursor molecules for stilbenes, chalcones, flavonoids, lignans and anthocyanins, all of them with potent bioactive activities (Gutiérrez-Grijalva, Picos-Salas, Leyva-López, Criollo-Mendoza, Vazquez-Olivo, & Heredia, 2018).

Finally, the flavonoid group represents 9% of the total polyphenolic compounds present in the avocado peel extract with a concentration of 3,787 µg/g extract. Multinoside A, quercetin-diglucoside (isomer 1) and quercetin-O-arabinosyl-glucoside were the most abundant flavonols (Table 3). Furthermore, the potential of MAE as extraction technique of avocado peel flavonoids was ratified. For instance, the concentration found for quercetin-O-arabinosyl-glucoside (13 ± 1 mg/100 g peel dm) was higher than the value (8.04 ± 0.6 mg/100 g peel dm) reported by Kosińska et al. (2012) who used dynamic maceration as extraction method.

3.4 Bioactivities and potential applications of avocado peel MAE extract

The optimum MAE extract obtained from avocado peel was further studied for potential applications in food, cosmetic and pharmaceutical industries by evaluating *in vitro* some of its biological activities (antimicrobial, antioxidant and anti-ageing). These properties are mainly due to its content of flavonoids and phenolic acids since their functional groups can perform scavenging actions on free radicals, metal chelating activities, inhibition or activation of enzymes, interactions with cell signalling pathways and changes in gene expression patterns, among other bioactivities, depending on the structure of each phenolic compound (Daglia, 2012; de Lima Cherubim, Buzanello Martins, Oliveira Fariña, & da Silva de Lucca, 2020; Heim Jr, 2002; Sang et al., 2006).

First, the disk diffusion method was conducted to screen the antimicrobial activity of the optimum avocado peel MAE extract (100 mg/mL) against several pathogen, opportunistic and spoilage microorganisms. However, only moderate antimicrobial effects against the Gram-positive bacterial

548 strains were observed (zone of inhibition diameter 2-3 times lower than chloramphenicol at 0.025
549 mg/mL - positive inhibitory control) (Table 4). These results agree with those reported by Rodríguez-
550 Carpena et al. (2011), who observed zones of inhibition of similar diameter in extracts obtained from
551 avocado peels using SLE extraction with acetone/water (70:30 v/v), as well as higher effectiveness
552 of these extracts against Gram-positive than Gram-negative bacteria, probably due to the differences
553 between their bacterial outer membranes. Therefore, despite the optimum MAE extract from avocado
554 peel could be used as a preservative in food and cosmetics against certain Gram-positive bacteria, the
555 MAE extraction was unable to extract bioactive compounds from this by-product with higher
556 antimicrobial activity than that reported for other extracts of avocado peel obtained by conventional
557 extraction techniques, (Rodríguez-Carpena et al., 2011). In fact, a greater antimicrobial potential has
558 been reported for extracts obtained from other avocado tissues, such as pulp and seeds (Rodríguez-
559 Carpena et al., 2011), and also from other fruit by-products rich in tannins (Widsten, Cruz, Fletcher,
560 Pajak, & McGhie, 2014).

561 In contrast, a high TAC was observed in the avocado peel extract by antioxidant assays based on
562 single-electron transfer (ABTS: 1.34 mmol Eq T/g DE, and FRAP: 2.66 mmol Eq Fe(II)/g DE) and
563 hydrogen atom transfer (ORAC: 3.02 mmol Eq T/g DE) (Table 4), which indicates that optimized
564 MAE using GRAS solvents is able to extract many antioxidant compounds with significant reducing
565 and antiradical capacities. In fact, the comparison of present results with those obtained in methanolic
566 extracts of different parts of avocado (peel, seed coat and seeds) (Ortega-Arellano, Jimenez-Del-Rio,
567 & Velez-Pardo, 2019) and of oven-dried peels from avocado and other tropical fruits (pineapple,
568 banana, papaya, passion fruit, watermelon and melon) (Morais et al., 2015) confirms the interest of
569 avocado peel as a raw material to obtain natural antioxidant ingredients for industrial applications in
570 foods, nutraceuticals and cosmetics.

571 Finally, the high MMP inhibitory capacity observed in the avocado peel MAE extract at relatively
572 low concentrations (150 µg DE/mL in the assay) must be highlighted (Table 4). MMPs are
573 extracellular proteases that specifically cleave a wide variety of substrates, including basement
574 membrane and extracellular matrix components such as several types of collagens, elastin,
575 fibronectin, gelatine, proteoglycans, etc. In fact, these enzymes are involved in many different
576 processes, both normal and pathological, with increased MMP activity (induced by certain radical
577 species) being related to premature skin ageing (de Lima Cherubim et al., 2020), and aberrant MMP
578 expression being noted in cancer, inflammation, arthritis, and periodontal disease, among others
579 (Butler & Overall, 2009). However, several polyphenols and flavonoid-rich plant extracts have been
580 shown to regulate the activity and expression of MMPs (Dell'Agli, Canavesi, Galli, & Bellosta, 2005;
581 Pientaweeratch, Panapisal, & Tansirikongkol, 2016). The results presented in Table 4 showed that
582 the optimized MAE extract of avocado peel was able to inhibit the four MMP assessed, being
583 particularly effective against MMP7 (matrilysine), MMP2 (gelatinase A) and MMP1 (collagenase-1)
584 in comparison with the chemical compound used as unspecific inhibitory control (NNGH, 0.5
585 µg/mL). The differences observed in the inhibition of the different enzymes must be due to the
586 different bioactive compounds present in the extract. In fact, a large number of compounds that can
587 act as MMP inhibitors have been described and some of the structural characteristics necessary to
588 present such inhibitory action have been deduced and are present in certain phenolic compounds: *a)*
589 a functional group capable of binding the catalytic Zn^{2+} (Zinc-Binding Group, ZBG), e.g. the 3-
590 hydroxyflavon structure and the hydroxamic acid and carboxylic acid groups; *b)* at least one
591 functional group capable of forming a hydrogen bond with the backbone of the enzyme, e.g. hydroxyl
592 groups; *c)* one or more side chains that can establish van der Waals interactions with the enzyme's
593 subsites, which mainly determines the affinity, and therefore, the selectivity of the inhibitors between
594 the different MMPs (especially interesting is the region known as the S1 site, as it is rather particular
595 of each MMP). In addition, hydrophobic interaction between the benzene ring of polyphenol and

596 MMPs could also result in the conformational changes leading to dysfunctional enzymes
597 (Pientaweeratch et al., 2016; Zapico et al., 2011). As far as we are concerned, this is the first time that
598 the MMP inhibitory capacity of an avocado peel extract is investigated, supporting the great interest
599 of this polyphenol-rich extract for cosmetic and pharmaceutical applications, as it might be able to
600 prevent skin ageing and the pathologies in which MMP increased activity has been described.

601 **4 Conclusions**

602 The RSM was successfully used to optimize the condition of polyphenols extraction from avocado
603 peel by MAE. All extraction factors selected had an effect on the TPC. The optimal MAE conditions
604 were temperature of 130 °C, extraction time of 39 min, ethanol concentration of 36 % and solvent-
605 sample ratio of 44 mL/g. The maximum TPC under these optimal MAE conditions was in good
606 agreement with the predicted TPC. Furthermore, approximately eight-fold higher TPC was observed
607 with the proposed MAE optimum conditions compared to the conventional solid-liquid extraction
608 using different combinations of solvents. In addition, fifty-three polar compounds were tentatively
609 identified in avocado peel under the optimum MAE conditions. Among them, dimers and trimers of
610 procyanidin (type A and B) were the most abundant phenolic compounds. Regarding potential
611 applications of the optimized avocado peel MAE extract, the high MMP inhibitory capacity at
612 relatively low concentrations -and the high antioxidant capacity suggest its interest for the food
613 industry as antioxidant ingredient with preserving properties or for the formulation of functional foods
614 and nutraceuticals with antioxidant and anti-aging activities.

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628 **Author Contributions:**

629 JGF conducted the extraction and analytical experiments and prepared the manuscript. IBL supported
630 the experimental work and revised the data and manuscript. JLS supervised the extraction process
631 and revised the manuscript. RDPG conducted the biological activity assays and collaborated in the
632 manuscript preparation. JAC conducted the microbial assays and revised the manuscript. ASC
633 designed the experiments and supervised the manuscript.

634 **Conflicts of Interest:**

635 The authors declare no conflict of interest.

636 **References:**

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820 **Table and Figure Captions**

821 **Table 1.** Central composite design applied for avocado peel phenolic compounds extraction.

Temperature (°C)	Time (min)	Solvent (% EtOH)	Solvent-sample ratio (mL/g)	TPC (mg GAE/g peel dm)
90 (0)	25 (0)	50 (0)	10 (-1)	33.6
70 (-0.5)	15 (-0.5)	25 (-0.5)	20 (-0.5)	30.5
110 (+0.5)	15 (-0.5)	25 (-0.5)	20 (-0.5)	37.1
70 (-0.5)	35 (+0.5)	25 (-0.5)	20 (-0.5)	30.8
110 (+0.5)	35 (+0.5)	25 (-0.5)	20 (-0.5)	42.7
70 (-0.5)	15 (-0.5)	75 (+0.5)	20 (-0.5)	29.7
110 (+0.5)	15 (-0.5)	75 (+0.5)	20 (-0.5)	36.5
70 (-0.5)	35 (+0.5)	75 (+0.5)	20 (-0.5)	23.5
110 (+0.5)	35 (+0.5)	75 (+0.5)	20 (-0.5)	42.0
90 (0)	25 (0)	0 (-1)	30 (0)	31.8
90 (0)	5 (-1)	50 (0)	30 (0)	38.9
50 (-1)	25 (0)	50 (0)	30 (0)	46.4
90 (0)	25 (0)	50 (0)	30 (0)	51.1
90 (0)	25 (0)	50 (0)	30 (0)	49.8
90 (0)	25 (0)	50 (0)	30 (0)	48.3
90 (0)	25 (0)	50 (0)	30 (0)	47.3
90 (0)	25 (0)	50 (0)	30 (0)	46.9
90 (0)	25 (0)	50 (0)	30 (0)	45.7
90 (0)	25 (0)	50 (0)	30 (0)	45.7
130 (+1)	25 (0)	50 (0)	30 (0)	65.8
90 (0)	45 (+1)	50 (0)	30 (0)	42.2
90 (0)	25 (0)	100 (+1)	30 (0)	18.1
70 (-0.5)	15 (-0.5)	25 (-0.5)	40 (+0.5)	36.5
110 (+0.5)	15 (-0.5)	25 (-0.5)	40 (+0.5)	46.0
70 (-0.5)	35 (+0.5)	25 (-0.5)	40 (+0.5)	39.1
110 (+0.5)	35 (+0.5)	25 (-0.5)	40 (+0.5)	59.1
70 (-0.5)	15 (-0.5)	75 (+0.5)	40 (+0.5)	27.8
110 (+0.5)	15 (-0.5)	75 (+0.5)	40 (+0.5)	40.8
70 (-0.5)	35 (+0.5)	75 (+0.5)	40 (+0.5)	32.5
110 (+0.5)	35 (+0.5)	75 (+0.5)	40 (+0.5)	42.4
90 (0)	25 (0)	50 (0)	50 (+1)	46.4

R-cuad. = 95.52% R-cuad.(ajusted) = 91.60%
Optimum extraction conditions: 130 °C, 36 % EtOH, 39 min, solvent-sample ratio 44 mL/g
Optimized desirability 0.9868

Response	**Predicted**	**Observed**	**CV (%)**
TPC (mg GAE/g peel dm)	72.04	73.2 ± 3.8	0.98

822

823 **Table 2.** Identification of bioactive compounds in the MAE peel extract of avocado by HPLC-ESI-

824 TOF/QTOF-MS.

Peak Number	Retention time (min)	*m/z* experimental [M-H]-	*m/z* calculated [M-H]-	Molecular formula	Error (ppm)	Score	mSigma	Proposed compound
1	1.96	191.0566	191.0561	$C_7H_{12}O_6$	-2.5	100	19.7	Quinic acid
2	2.34	191.0191	191.0197	$C_6H_8O_7$	3.4	100	11.9	Citric acid
3	8.80	197.0452	197.0455	$C_9H_{10}O_5$	1.8	100	23.1	Syringic acid
4	10.06	353.0891	353.0878	$C_{16}H_{18}O_9$	-3.5	100	43.5	3-O-caffeoylquinic acid
5	11.79	443.1949	443.1923	$C_{21}H_{32}O_{10}$	-5.9	100	26.9	Penstemide
6	12.35	577.1365	577.1351	$C_{30}H_{26}O_{12}$	-2.4	100	36.1	Procyanidin dimer B (isomer 1)
7	12.36	137.0257	137.0244	$C_7H_5O_3$	-9.0	100	4.7	4-hydroxybenzoic acid
8	12.43	431.1566	431.1559	$C_{19}H_{28}O_{11}$	-1.6	100	3.1	Tyrosol-glucosyl-pentoside
9	13.37	577.1330	577.1351	$C_{30}H_{26}O_{12}$	3.8	100	7.3	Procyanidin dimer B (isomer 2)
10	13.76	353.0875	353.0878	$C_{16}H_{18}O_9$	0.8	100	1.0	5-O-caffeoylquinic acid
11	14.43	289.0733	289.0718	$C_{15}H_{14}O_6$	-5.2	100	2.6	(+)-Catechin
12	14.84	353.0869	353.0878	$C_{16}H_{18}O_9$	2.7	100	30.5	4-O-caffeoylquinic acid
13	15.00	577.1320	577.1351	$C_{30}H_{26}O_{12}$	5.5	100	28.3	Procyanidin dimer B (isomer 3)
14	16.64	577.1279	577.1351	$C_{30}H_{26}O_{12}$	12.5	100	37.6	Procyanidin B2
15	17.45	577.1266	577.1351	$C_{30}H_{26}O_{12}$	4.6	100	12.3	Procyanidin dimer B (isomer 4)
16	18.23	289.0735	289.0718	$C_{15}H_{14}O_6$	-5.8	100	6.3	(-)-Epicatechin
17	18.45	577.1259	577.1351	$C_{30}H_{26}O_{12}$	16.0	100	18.5	Procyanidin dimer B (isomer 5)
18	19.20	863.1840	863.1829	$C_{45}H_{36}O_{18}$	-1.3	100	127.0	Proc. trimer A (isomer 1)
19	19.60	577.1270	577.1351	$C_{30}H_{26}O_{12}$	14.0	100	10.8	Procyanidin dimer B (isomer 6)
20	20.11	865.2045	865.2044	$C_{45}H_{38}O_{18}$	-6.8	100	15.3	Proc. trimer B (isomer 1)
21	20.24	625.1345	625.1410	$C_{27}H_{30}O_{17}$	10.5	100	9.0	Quercetin-diglucoside (isomer 1)
22	20.52	625.1317	625.1410	$C_{27}H_{30}O_{17}$	9.3	100	7.9	Quercetin-diglucoside (isomer 2)
23	21.14	577.1287	577.1351	$C_{30}H_{26}O_{12}$	11.2	100	14.8	Procyanidin dimer B (isomer 7)
24	21.73	577.1304	577.1351	$C_{30}H_{26}O_{12}$	8.3	100	50.8	Procyanidin dimer B (isomer 8)
25	21.96	863.1822	863.1829	$C_{45}H_{36}O_{18}$	0.8	100	119.6	Proc. trimer A (isomer 2)
26	22.11	595.1274	595.1305	$C_{26}H_{28}O_{16}$	5.2	92	15.3	Quercetin-O-arabinosyl-glucoside
27	22.61	609.1485	609.1461	$C_{27}H_{30}O_{16}$	-3.9	100	6.7	Rutin
28	22.79	577.1287	577.1351	$C_{30}H_{26}O_{12}$	11.2	100	15.5	Procyanidin dimer B (isomer 9)
29	23.45	441.1790	441.1766	$C_{21}H_{30}O_{10}$	-5.5	100	25.3	(1′S, 6′R)-8′-hydroxyabscisic acid β-D-glucoside
30	23.64	575.1136	575.1195	$C_{30}H_{24}O_{12}$	10.2	100	137.4	Proc. dimer A (isomer 1)
31	24.15	565.2310	565.2291	$C_{28}H_{38}O_{12}$	-3.5	100	8.5	Quercetin derivative (isomer 1)
32	25.03	463.0915	463.0882	$C_{21}H_{20}O_{12}$	-7.1	100	8.3	Quercetin-3-β-glucoside
33	25.05	579.1383	579.1355	$C_{26}H_{28}O_{15}$	-4.8	100	8.3	Luteolin 7-O-(2″-Oepentosyl)hexoside
34	25.26	577.1311	577.1351	$C_{30}H_{26}O_{12}$	-3.2	100	230.8	Procyanidin dimer B (isomer 10)
35	25.34	477.0649	477.0675	$C_{21}H_{18}O_{13}$	5.4	100	7.7	Quercetin glucuronide
36	26.02	575.1213	575.1195	$C_{30}H_{24}O_{12}$	-3.2	100	20.9	Proc. dimer A (isomer 2)
37	26.56	575.1201	575.1195	$C_{30}H_{24}O_{12}$	-1.1	100	44.5	Proc. dimer A (isomer 3)
38	26.86	609.1444	609.1461	$C_{27}H_{30}O_{16}$	2.8	100	29.8	Multinoside A
39	27.38	577.1302	577.1351	$C_{30}H_{26}O_{12}$	8.6	100	16.7	Procyanidin dimer B (isomer 11)
40	27.67	575.1203	575.1195	$C_{30}H_{24}O_{12}$	-1.3	100	17.2	Proc. dimer A (isomer 4) (A2)
41	28.23	433.0847	433.0776	$C_{20}H_{18}O_{11}$	-16.3	100	21.1	Quercetin-3-O-arabinoside
42	28.65	579.1377	579.1355	$C_{26}H_{28}O_{15}$	-3.7	100	8.2	Quercetin -xylosyl--rhamnoside
43	29.12	575.1203	575.1195	$C_{30}H_{24}O_{12}$	-1.4	100	27.5	Proc. dimer A (isomer 5)
44	29.40	575.1189	575.1195	$C_{30}H_{24}O_{12}$	1.0	100	30.5	Proc. dimer A (isomer 6)
45	29.82	593.1544	593.1512	$C_{27}H_{30}O_{15}$	-5.4	100	6.7	Kaempferol-O-glucosyl-rhamnoside
46	30.75	565.2280	565.2291	$C_{28}H_{38}O_{12}$	1.9	100	23.9	Quercetin derivative (Isomer 2)

Peak Number	Retention time (min)	*m/z* experimental [M-H]-	*m/z* calculated [M-H]-	Molecular formula	Error (ppm)	Score	mSigma	Proposed compound
47	30.96	575.1245	575.1195	$C_{30}H_{24}O_{12}$	-8.7	100	16.6	Proc. dimer A (isomer 7)
48	34.02	575.1195	575.1195	$C_{30}H_{24}O_{12}$	0.0	100	39.3	Proc. dimer A (isomer 8)
49	35.00	575.1217	575.1195	$C_{30}H_{24}O_{12}$	-3.7	100	69.9	Proc. dimer A (isomer 9)
50	37.06	301.0354	301.0354	$C_{15}H_{10}O_7$	-5.4	100	2.4	Quercetin
51	39.01	271.0618	271.0612	$C_{15}H_{12}O_5$	-2.1	100	35.5	Naringenin
52	39.23	285.0367	285.0405	$C_{15}H_{10}O_6$	13.3	100	35.7	Kaempferol
53	39.45	577.1377	577.1351	$C_{30}H_{26}O_{12}$	-4.5	100	32.3	Procyanidin dimer B (isomer 12)

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839 **Table 3.** Quantification of phenolic compounds in the optimum MAE peel extract of avocado by

840 HPLC-DAD-ESI-TOF-MS.

Phenolic compounds	Concentration (mg/100 g peel dm)			Concentration (µg/g extract)		
Phenolic acids						
4-hydroxybenzoic acid	15	±	0.2	450	±	5
Syringic acid	13	±	0.5	390	±	10
Tyrosol-glucosyl-pentoside	32	±	1.9	950	±	60
3-O-caffeoylquinic acid	18	±	0.3	535	±	8
4-O-caffeoylquinic acid	37	±	1.7	1,120	±	50
5-O-caffeoylquinic acid	32	±	1.4	950	±	40
Subtotal	147			4,395		
Flavonoids						
Naringenin	0.9	±	0.1	28	±	1
Kaempferol	1.9	±	0.1	58	±	2
Quercetin	2.2	±	0.1	67	±	3
Quercetin-3-O-arabinoside	0.9	±	0.1	28	±	1
Quercetin-3-β-glucoside	4.8	±	0.1	143	±	2
Quercetin glucuronide	3.2	±	0.2	97	±	5
Quercetin derivative (isomer 1)	7.5	±	0.3	226	±	8
Quercetin derivative (Isomer 2)	2.1	±	0.1	63	±	2
Quercetin-xylosyl-rhamnoside	7.1	±	0.3	213	±	8
Quercetin-O-arabinosyl-glucoside	13	±	1	400	±	4
Rutin	7.0	±	0.4	210	±	10
Quercetin-diglucoside (isomer 1)	18	±	1	530	±	20
Quercetin-diglucoside (isomer 2)	4.7	±	0.1	141	±	1
Kaempferol-O-glucosyl-rhamnoside	8.7	±	0.3	263	±	8
Luteolin 7-O-(2″-O-pentosyl)hexoside	3.7	±	0.1	110	±	2
Multinoside A	40	±	1	1,210	±	30
Subtotal	126			3,787		
Catechins						
(+)-Catechin	237	±	2	7,120	±	60
(-)-Epicatechin	59	±	1	1,760	±	10
Subtotal	296			8,880		
Condensed tannins						
Dimer A	75	±	5	2,300	±	100
Dimer B	530	±	7	15,900	±	200
Trimer A	38	±	2	1,130	±	50
Trimer B	150	±	4	4,600	±	100
Subtotal	793			23,930		
Total	1,362			40,992		

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842 **Table 4.** *In vitro* biological activities of avocado peel extract obtained using MAE optimum extraction

843 conditions.

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Biological activity	Units	MAE	Inhibitory Control
Antimicrobial activity [a]	Zone inhibition diameter		
Staphylococcus epidermidis (Gram +, pathogenic)	(mm)	7.2 ± 1.1	19.3 ± 0.5
Enterococcus faecalis (Gram +, opportunistic)	(mm)	6.3 ± 0.8	22.0 ± 1.5
Escherichia coli (Gram -, pathogenic)	(mm)	–	20.2 ± 1.2
Enterobacter hormaechei (Gram -, opportunistic)	(mm)	–	16.0 ± 1.3
Kluyveromyces marxianus (Yeast, spoilage)	(mm)	–	–
Galactomyces candidus (Mold, spoilage)	(mm)	–	9.4 ± 0.9
Antioxidant activity	Total antioxidant capacity (TAC)		
ABTS	mmol Eq T / g DE	1.34 ± 0.01	
FRAP	mmol Eq Fe(II) / g DE	2.66 ± 0.13	
ORAC	mmol Eq T / g DE	3.02 ± 0.05	
Inhibition of enzymes implicated in extracellular matrix degradation [b]	Remaining MMP activity (C = 100%)		
MMP1 (collagenase-1 or fibroblastic collagenase)	(%)	43.7 ± 3.8	0.9 ± 0.3
MMP2 (gelatinase A)	(%)	14.6 ± 2.5	0.6 ± 0.1
MMP3 (stromelysin-1)	(%)	59.6 ± 5.0	0.5 ± 0.3
MMP7 (matrilysine)	(%)	40.0 ± 1.5	49.5 ± 1.1

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846 a) The concentration of the MAE extract in the antimicrobial activity assays (disk diffusion method)

847 was 100 mg dry extract (DE)/mL using 20 µL extract/disc. Chloramphenicol (0.025 mg/mL) was

848 used as positive inhibitory control for bacteria strains; Cycloheximide (1 mg/mL) was used as positive

849 inhibitory control for the yeast and mold strains. b) The concentration of the MAE extract in the

850 assays to assess the matrix metalloproteinases (MMPs) inhibiting activity was 150 µg DE/mL. N-

851 Isobutyl-N-(4–239 methoxyphenylsulfonyl)-glycyl hydroxamic acid (NNGH, 0.5 µg/mL) was used

852 as positive inhibitory control of MMPs.

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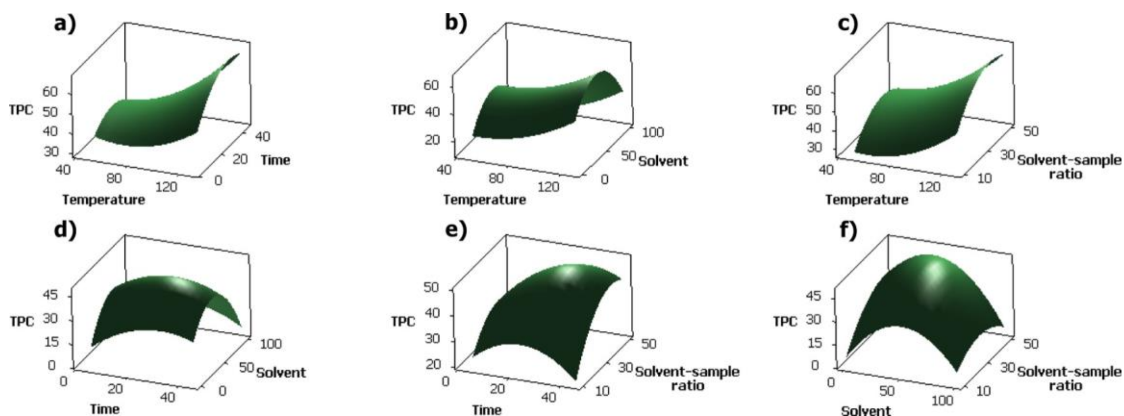

854 **Figure 1.** Response surface plots showing combined effects of process variables on TPC: a)
855 temperature vs time; b) temperature vs percentage ethanol in the solvent miXture, c) temperature vs
856 solvent-sample ratio; d) time vs percentage ethanol in the solvent miXture; e) time vs solvent-sample
857 ratio; f) percentage ethanol in the solvent miXture vs solvent-sample ratio.

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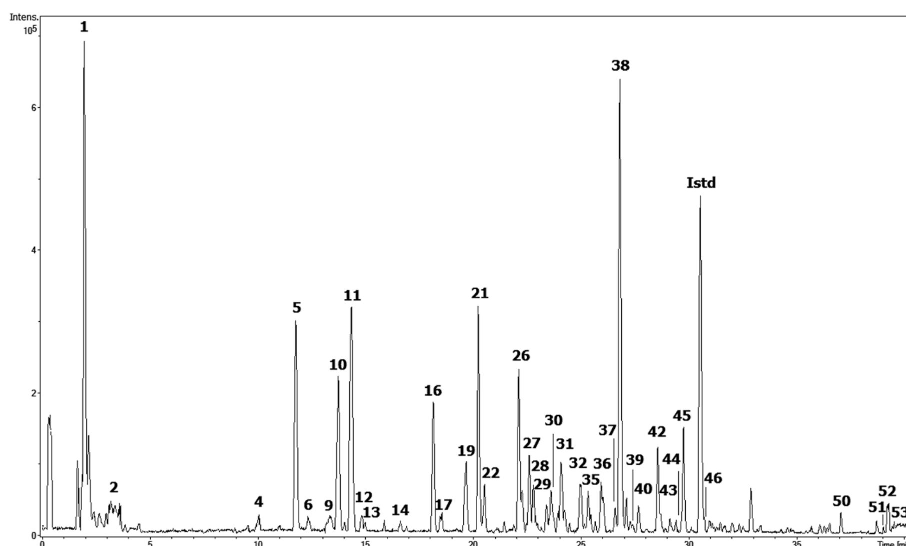

859 **Figure 2.** Base peak chromatogram of optimum MAE avocado peel extract obtained by HPLC-ESI-
860 TOF-MS.

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864 **Table 1S.** Analysis of variance (ANOVA) of the regression models.

Source	**SC Sec.**	**Df**	**MS**	**F-Ratio**	**P value**
x_1: Temperature	760.44	1	45.89	5.47	0.033
x_2: Time	47.43	1	8.43	1.01	0.331
x_3: Ethanol-water mixtures	227.00	1	309.83	36.96	0.000
x_4: Solvent-sample ratio	245.62	1	84.54	10.09	0.006
x_1^2	186.11	1	68.54	8.18	0.011
x_2^2	65.15	1	155.21	18.52	0.001
x_3^2	1029.38	1	1109.19	132.33	0.000
x_4^2	173.83	1	173.83	20.74	0.000
$x_1 x_2$	36.83	1	36.83	4.39	0.052
$x_1 x_3$	0.00	1	0	0	0.996
$x_1 x_4$	4.39	1	4.39	0.52	0.479
$x_2 x_3$	15.94	1	15.94	1.9	0.187
$x_2 x_4$	18.19	1	18.19	2.17	0.160
$x_3 x_4$	48.91	1	48.91	5.83	0.028

Lack-of-fit	109.55	10	109.55	2.68	0.120
Pure error	24.57	6	24.57		
Total (coor.)	2993.32	30			
R^2	0.955				
CV	2.89				

865 x_1: Temperature; x_2: Time; x_3: Ethanol-water mixtures; x_4: Solvent-sample ratio

866 **Table 2S.** Analytical parameters of the proposed method.

Analyte	**LOD (µg/mL)**	**LOQ (µg/mL)**	**Calibration range (µg/mL)**		**Calibration equations**	**R^2**
Catechin	0.95	3.17	LOQ -	120	y = 0.1231 x - 0.0399	0.997
Chlorogenic acid	0.19	0.63	LOQ -	100	y = 0.0055 x - 0.0075	0.991
Epicatechin	0.57	1.89	LOQ -	120	y = 0.2333 x - 0.0413	0.997
Procyanidin A2	0.07	0.22	LOQ -	120	y = 0.2981 x - 0.0440	0.994
Procyanidin B2	0.32	1.08	LOQ -	120	y = 0.1095 x - 0.0062	0.993
Quercetin-3-β-glucoside	0.07	0.22	LOQ -	120	y = 0.0634 x + 0.0167	0.998
Rutin	0.02	0.06	LOQ -	120	y = 0.1155 x - 0.0273	0.996

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