1 **A multi-analytical platform based on pressurized-liquid extraction, in vitro assays and**
2 **liquid chromatography/gas chromatography coupled to high resolution mass**
3 **spectrometry for food by-products valorisation. Part 1: Withanolides-rich extracts**
4 **optimization from goldenberry (*Physalis peruviana L.*) calyces as case study**

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6 Diego Ballesteros-Vivas[1,2a], Gerardo Alvarez-Rivera[2a], Andrea del Pilar Sánchez-Camargo[1],

7 Elena Ibáñez[2], Fabián Parada-Alfonso[1], Alejandro Cifuentes[2]*

8

9 [1] High Pressure Laboratory, Department of Chemistry, Faculty of Science, Universidad

10 Nacional de Colombia, Carrera 30 #45-03, Bogotá D.C., 111321, Colombia.

11 [2] Laboratory of Foodomics, Institute of Food Science Research, CIAL, CSIC, Nicolás

12 Cabrera 9, 28049 Madrid, Spain.

13 [a] These two authors have contributed equally to this work.

14

15 *Corresponding author:

16 Prof. Dr. Alejandro Cifuentes, Laboratory of Foodomics, Institute of Food Science Research,

17 CIAL (CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain, e-mail: a.cifuentes@csic.es, Tel.: +34

18 910017955; fax: +34 910017905.

19

20 **Keywords:**

21

22 Goldenberry calyx; Valorization; Pressurized-liquid extraction; LC-Q-TOF; GC-Q-TOF;

23 High-resolution mass spectrometry; Withanolides; Phenolic antioxidants;

24

25

26

27 **Abbreviations:**

28

29 4βHWE: 4β-hydroxywithanolide E

30 ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

31 ACN: acetonitrile

32 ANOVA: analysis of variance

33 Db: dry weight basis

34 DPPH: 2,2-diphenyl-1-picrylhydrazyl

35 EIC: extracted ion chromatogram

36 EtOAc: ethyl acetate

37 EtOH: ethanol

38 GAE: gallic acid equivalent

39 GC: gas chromatography

40 HSP: Hansen solubility parameters

41 LC: liquid chromatography

42 PLE: pressurized liquid extraction

43 q-TOF-MS/MS: quadrupole time-of-flight tandem mass spectrometry

44 TEAC: trolox equivalent antioxidant capacity

45 TFC: total flavonoid content

46 TPC: total phenolic content

47 WE: withanolide E

48 **ABSTRACT**

49 In this work, a multi-analytical platform that allows obtaining and characterizing high-added
50 value compounds from natural sources is presented, with a huge potential in traditional
51 medicine, natural products characterization, functional foods, etc. Namely, the proposed multi-
52 analytical platform is based on the combination of pressurized liquid extraction (PLE), liquid
53 chromatography (LC) and gas chromatography quadrupole time-of-flight mass spectrometry
54 GC-q-TOF-MS(/MS), *in vitro* assays and modelling tools for guiding extraction optimization.
55 As case study, goldenberry or cape gooseberry fruit (*Physalys peruviana L.*) was selected. In
56 particular, the potential of *P. peruviana* calyces, an important by-product of goldenberry
57 processing, as promising source of bioactive compounds was evaluated. Selection of the most
58 suitable solvent for PLE was based on the Hansen solubility parameters (HSP) approach using
59 4β-hydroxywithanolide E (4βHWE) and withanolide E (WE) as target compounds due to their
60 bioactive potential. A surface response methodology was further applied for the optimization
61 of the PLE parameters: temperature (50, 100 and 150 ºC) and solvent composition (% EtOH in
62 the mixture EtOH/EtOAc). The effects of the independent variables on extraction yield,
63 withanolides content (4βHWE and WE), total phenolic content (TPC), total flavonoids content
64 (TFC) and antioxidant activity (EC_{50} and TEAC) were evaluated in order to obtain withanolide-
65 rich extracts from *P. peruviana* calyces. The extract obtained under optimal conditions (at 125
66 °C and 75% EtOH v/v) exhibited satisfactory extraction yield (14.7 %) and moderate
67 antioxidant activity (with an EC_{50} value of 77.18 μg mL^{-1} and 1.08 mM trolox g^{-1}), with
68 4βHWE and WE concentrations of 8.8 and 2.3 mg g^{-1}, respectively. LC-q-TOF-MS/MS
69 analysis of the extract allowed the quantitation of 4βHWE and WE and the tentative
70 identification of several other withanolides structures. The obtained results demonstrate the
71 great potential of this multi-analytical approach for developing valorization strategies of food

72 by-products under sustainable conditions, to obtain bioactive-enriched extracts with potential
73 medicinal or health-promoting properties.

74 **1. INTRODUCTION**

75 Scientific disciplines which study traditional medicine include herbalism, ethnomedicine,
76 ethnobotany, and medical anthropology. In the search of different sources of bioactive
77 compounds that can have a positive effect on our health, valorization of food by-products is
78 one of the main challenges that administrations, scientists and agro-food industries have to face
79 nowadays. *Physalis peruviana* L. is an herbaceous and perennial plant, belonging to the family
80 *Solanaceae* [1]. *P. peruviana* produces an exotic fruit into a bladder-like calyx, which is known
81 as goldenberry or cape gooseberry, being Colombia the largest producer of goldenberry
82 worldwide [2]. This fruit has gained importance and popularity due to its organoleptic
83 properties, nutritional quality values, and mainly due to the content of health-promoting
84 compounds [1].

85 Goldenberry fruit can be transformed in derived products such as juice, sauces, syrups,
86 marmalades, and snacks, although it is mainly commercialized as fresh product [3]. Industrial
87 processing of goldenberries generates two by-products: goldenberry pomace, including seeds
88 and skin, and the calyx. According to Ramadan [4], during juice processing, goldenberry
89 pomace represents approx. 27.4% of fruit weight, whereas the calyx represents 5% of the raw
90 fruit [5]. The calyx is an inedible waste, weakly ribbed and pubescent straw-coloured
91 parchment-like husk, which protects the fruit against insects, birds, diseases, and climatic
92 situations during its development [1,6]. Previous research works have described goldenberry
93 calyces as a potential source of bioactive compounds, particularly withanolides [7,8]. These
94 compounds are a family of C_{28} ergostane-type steroids with diverse oxygenation in the steroid
95 skeleton in which C-26 and C-22, or C-26 and C-23, are oxidized in order to form a δ- or γ-
96 lactone. According to the difference in the substituted groups of C-17 side chain, withanolides

97 can be divided into type A (δ-lactone or δ-lactol) and type B (γ-lactone or γ-lactol) [9] (see
98 Figure 1). Some interesting biological and pharmacological activities of this group of naturally
99 steroids, include anti-inflammatory, antitumor, cytotoxic, hepatotoxic and antimicrobial
100 activities [10][7][5][11][12] [13].
101 The valorisation of food industry by-products is an emerging trend in food science which has
102 shown to be a valuable alternative to extract natural compounds with demonstrated bioactivity
103 from food processing generated wastes, usually discarded or employed to produce animal feed.
104 In this line, advanced extraction techniques such as pressurized liquid extraction (PLE) are
105 considered powerful tools for the efficient extraction of bioactive compounds, providing high
106 extraction yields in short extraction times using low volumes of extraction solvent [14]. Several
107 review works [15–20] have reported the advantages of this extraction technique with especial
108 emphasis in Foodomics applications. The selection of a suitable solvent for the extraction of
109 the target bioactive, in agreement with green chemistry principles, is also a critical issue for
110 the development of a sustainable valorisation process. In this regard, Hansen solubility
111 parameters (HSP) have been recently employed as a useful tool to selectively extract valuable
112 compounds from natural sources, employing PLE as extraction technique [21–26]. This
113 theoretical approach is based on the estimation of HSP of the target compound in order to
114 choose the most appropriate extraction solvent, thus reducing the number of experiments and
115 solvent consumption [21,27].
116 Most of reported studies in literature about *P. peruviana* are focused on the fruit itself [28–30],
117 whereas the identification of bioactive substances of the calyx has not been comprehensively
118 described yet [7,31]. Considering the potential of goldenberries calyx as promising source of
119 bioactive phytochemicals, a complete valorization strategy based on PLE (along with HSP
120 approach as theoretical prediction tool to select the most suitable solvent), advanced analytical
121 techniques (based on LC and GC coupled to Q-TOF mass spectrometry), *in vitro* assays and

122 experimental design to optimize extraction conditions is proposed in this work to obtain
123 withanolides-enriched extracts from goldenberry calyces.
124
125 **2. MATERIAL AND METHODS**

126 *2.1 Samples of Physalis peruviana*

127 The calyces of the goldenberry fruit (*Physalis peruviana*) were collected in the central supply
128 marketplace (www.corabastos.com.co) in Bogotá D.C., Colombia in June, 2017. Previously,
129 calyces had been separated from the mature fruit and discarded. The calyces were washed with
130 distilled water and dried at room temperature for 48 h in the darkness. The dried sample was
131 ground in knife mill (Grindomix GM200-Retsch GmbH, Haan, Germany) using dry ice and
132 then vacuum-packed and stored at -20 °C until its use.
133
134 *2.2 Reagents and materials*

135 Gallic acid, quercetin, trolox, withanolide A, 2,2'-azino-bis(3-ethylbenzothiazoline-6-
136 sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium acetate,
137 ammonium acetate, sodium carbonate, formic acid, potassium persulfate, aluminum chloride,
138 were purchased from Sigma-Aldrich (Madrid, Spain). Merck (Darmstadt, Germany) provided
139 the Folin-Ciocalteu phenol reagent. The solvents employed were HPLC-grade. Acetonitrile,
140 ethanol and methanol were acquired from VWR Chemicals (Barcelona, Spain), whereas
141 dichloromethane was provided from Fluka AG (Buchs, Switzerland) and ethyl acetate from
142 Scharlau (Barcelona, Spain). Ultrapure water was obtained from a Millipore system (Billerica,
143 MA, USA). For the UPLC-q-TOF-MS analyses, MS grade ACN and water from LabScan
144 (Dublin, Ireland) were employed.
145
146 *2.3 Estimation of Hansen Solubility Parameters at normal conditions using HSPiP® software*

147 Based on the neural network technique of Yamamoto-Molecular Break method (Y-MB)[32],
148 HSPiP® software (Version 5.0m UK) was employed for estimating the Hansen solubility
149 parameters (HSP) for Withanolide E and 4β-hydroxywithanolide E. The individual Hansen
150 parameters, which include dispersion δ_D (related to the van der Waals forces), dipole moment
151 δ_P, and hydrogen bond interactions δ_H, were calculated. For this purpose, the methodology
152 described by Sánchez-Camargo et al. [24] was followed. Briefly, by means of their canonical
153 SMILES (Simplified molecular input line syntax) notations (Withanolide E,
154 CC1=C(C(=O)OC(C1)C(C)(C2(CCC3(C2(CCC4C3CC5C6(C4(C(=O)C=CC6)C)O5)C)O)O)
155 O)C and 4βHWE, CC1=C(C(=O)OC(C1)C(C)(C
156 2(CCC3 (C2(CCC4C3CC5C6(C4(C(=O)C=CC6O)C)O5)C)O)O)O)C) and the "Do It
157 Yourself" tool available in the software menu, the HSPs were calculated. The SMILES
158 notations were obtained from PubChem website. After the individual parameters calculations,
159 the most suitable bio-solvents for a selective extraction were carefully chosen from a list of
160 101 items from the solvent optimizer menu, taking into account the *Ra* term as criteria. This
161 last term refers to the affinity between solute and solvent, and it measures "the distance" from
162 each other, as given by Equation 1. Thus, the smaller *Ra* corresponds to the greater affinity
163 between solvent and solute. In equation 1, subscript *i* refers to the solute and *j* refers to the
164 solvent.

165 $$R_a = \sqrt{4\left(\delta_{Di-}\delta_{Dj}\right)^2 + \left(\delta_{Pi-}\delta_{Pj}\right)^2 + \left(\delta_{Hi-}\delta_{Hj}\right)^2} \quad \text{Eq. (1)}$$

166 In order to consider possible modifications of solubility parameters under pressurized liquid
167 conditions, Marrero & Gani group contribution method was used for estimation of critical data
168 [33]. With those results, Jayasri and Yaseen [34] method was employed to assess the
169 temperature dependence of the solubility parameter for the target compounds. The physical
170 properties of the sub-critical bio-solvents were calculated following the Gunn-Yamada method
171 [35]. On the other hand, the effect of sub-critical conditions (pressure and temperature) of the

172 bio-based solvents was evaluated by Williams and co-workers' method [27]. As it will be
173 described later, pure ethanol and ethyl acetate, and their mixtures were used for the
174 experimental design for pressurized liquid extraction. The solubility parameters of the mixed
175 fluid were calculated by the Eq. (2), as follows:

176 $$\delta_{Mix-D,P,H} = \Phi_{Ethanol} \times \delta_{Ethanol} + \Phi_{Ethyl\ acetate} \times \delta_{Ethyl\ acetate} \quad \text{Eq. (2)}$$

177 where Φ is the volume fraction and δ is the solubility parameter (*D, P* and *H*) of ethanol or
178 ethyl acetate.

179

180 *2.4 Pressurized liquid extraction (PLE)*

181 Extractions were performed using an accelerated solvent extractor (ASE 200, Dionex,
182 Sunnyvale, CA, USA) equipped with a solvent controller unit. For each extraction, dried
183 sample of goldenberry calyces (~1 g) was mixed with sea sand (~2 g). The mixture was loaded
184 into an 11 mL stainless steel extraction cell. PLE experiments were developed at static
185 extraction mode for 20 min and 100 bar. After the extraction, the solvent was evaporated under
186 a stream of nitrogen at 25 °C (TurboVap® LV Biotage, Uppsala, Sweden). Extraction yield
187 was expressed as g of extract/100 g dry weight basis of sample.

188

189 *2.4.1 Experimental design*

190 PLE was optimized using a central composite design (CCD). The extraction temperature (50,
191 100 and 150 ºC) and percentage of EtOH in the mixture solvent EtOH/EtOAc (0, 50 and 100%
192 v/v) were considered as independent variables. Extraction yield (g of extract/100 g dry
193 weight basis of sample), relative content of 4βHWE and WE (mg/g dry weight basis of
194 sample), total phenolic content (mg Gallic acid equivalents/g dry weight basis of sample), total
195 flavonoid content (mg Quercetin equivalents/g dry weight basis of sample) and antioxidant
196 capacity (EC50 µg/mL extract and TEAC mM Trolox/g extract) were selected as responses

197 variables. The experimental design results and data analysis were performed using a response
198 surface methodology with STATISTICA 12 (Stat Soft, Inc., Tulsa, OK 74104, USA). The
199 effects of the independent variables on the response variables in the extraction process were
200 assessed using the pure error, considering a level of confidence of 95% for all the variables and
201 the quadratic model proposed for each response variable $\boldsymbol{Y_i}$ was [36]:

202 $$Y_i = \beta_0 + \beta_1 T + \beta_2 S + \beta_{1,1} T^2 + (\beta_{1,2} T \times S) + \beta_{2,2} S^2 + error \qquad \text{Eq. (3)}$$

203 where T is the temperature, S is the solvent composition (percentage of ethanol in the mixture),
204 β_0 is the intercept, β_1, β_2 are the linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$ are the quadratic coefficients,
205 $\beta_{1,2}$ is the linear-by-linear interaction coefficient, and *error* is the error variable. ANOVA was
206 used for evaluation of the significance of independent variables' influence and interactions (the
207 differences were statistically significant at $p < 0.05$). Standardized Pareto charts were applied
208 to obtain the significance of impact of tested variables on responses. The adequacy of the model
209 was evaluated by the coefficient of determination (R^2), and p values for the model and lack-of-
210 fit testing [37]. A multiple response optimization was carried out by the combination of
211 experimental factors, looking for maximizing the desirability function.
212
213 *2.4.2 Dynamic maceration (DM) procedure*

214 For comparison, DM was performed using EtOH, EtOAc and EtOH/EtOAc (50:50 v/v) as
215 solvents. Samples of goldenberry calyces (~1 g) were placed in conical centrifuge tube (Falcon
216 15 mL) with 5 mL of solvent and agitated at 750 rpm, 20 ºC for 24 h (Thermomixer R
217 Eppendorf AG, Hamburg, Germany). Subsequently, the extract was separated from the sample
218 by centrifugation (Eppendorf Centrifuge 5810 R, Hamburg, Germany) at 5000 rpm, 20 °C for
219 10 min. After the separation, the solvent was evaporated under a stream of nitrogen at 25 °C
220 (TurboVap® LV Biotage, Uppsala, Sweden). Extraction yield was expressed as g of
221 extract/100 g dry weight basis of sample.

222

223 *2.5 Determination of total phenolic content (TPC)*

224 The total phenolic content was determined according to the method of Hosu et al. [38] with
225 some modifications. Briefly, 50 µL of Folin-Ciocalteu reagent (0.2 M) and 600 µL of distilled
226 water were added to 10 µL of extract solution (4 mg mL^{-1} EtOH). The reaction mixture was
227 allowed to react 5 min and then, 150 µL of Na_2CO_3 (20% w/v) and 190 µL of distilled water
228 were added. Reaction mixtures were incubated at room temperature, in dark place for 120 min,
229 and then 300 µL were transferred to a 96-well microplate. The absorbance was measured at
230 760 nm in a microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski,
231 VT, USA). A calibration curve was obtained using 0–100 µg gallic acid/mL EtOH and was
232 used to calculate the total phenolic content of extracts. The phenolic content was expressed as
233 mg of gallic acid (GAE) per g of dry weight basis (Db) of sample. All analyses were done by
234 triplicate.

235

236 *2.6 Determination of total flavonoid content (TFC)*

237 The total flavonoid content of extracts was determined according to the method of Hosu et al..
238 [38] with some modifications. Briefly, 30 µL of $AlCl_3$ (10% w/v) were added to 100 µL of
239 extract solution (4 mg mL^{-1} EtOH). The reaction mixture was allowed to react 5 min and then,
240 30 µL of potassium acetate (1 M), 300 µL of absolute EtOH and 540 µL of distilled water
241 were added. Reaction mixtures were incubated at room temperature for 30 min and then 300
242 µL were transferred to a 96-well microplate. The absorbance was measured at 415 nm in a
243 microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski, VT, USA).
244 A calibration curve was obtained using 0–100 µg quercetin mL^{-1} EtOH and was used to
245 calculate the total flavonoid content of extracts. The flavonoid content was expressed as mg of
246 quercetin (QE) per g of dry weight basis (Db) of sample. All analyses were done by triplicate.

247

248 *2.7* Antioxidant capacity assays

249 *DPPH assay*

250 Free radical scavenging capacity was evaluated using the procedure described by Brand-
251 Williams, Cuvelier and Berset [39]. The extracts were diluted in MeOH at concentrations
252 between 0.3 and 5.0 mg mL^{-1}. An aliquot (25 µL) of the different concentrations of the extracts
253 was added to 975 µL of DPPH solution (6×10^{-5} M in MeOH). After vortexing for 1 min, the
254 reaction mixture was allowed to stand in the dark for 4 h min at room temperature.
255 Subsequently, 300 µL of the reaction mixture were transferred into a 96-well microplate, and
256 the absorbance was measured at 516 nm in a microplate spectrophotometer reader (Synergy
257 HT, BioTek Instruments, Winooski, VT, USA). A DPPH methanol solution was used as the
258 reference sample. The DPPH concentration remaining in the reaction medium at the end of the
259 reaction was calculated using a calibration curve. The percentage of remaining DPPH was then
260 plotted against the extract concentration to obtain the EC_{50}, the amount of antioxidant necessary
261 to decrease the initial DPPH concentration by 50%. Therefore, the lower the EC_{50} value, the
262 higher the antioxidant capacity. Measurements were done in at least triplicate.

263

264 *TEAC assay*

265 The total antioxidant capacity was determined by the TEAC method, which is based on the
266 capacity of antioxidants to capture the radical 2,20-azino-bis-(3 ethylbenzothiazoline-6-
267 sulfonic acid) ($ABTS^{\bullet+}$) [40]. The $ABTS^{\bullet+}$ radical cation was prepared by mixing an ABTS
268 stock solution (7.00 mM) with potassium persulfate solution (2.45 mM). The reaction was kept
269 in the dark at room temperature for 16 h. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5mM
270 phosphate buffer (pH 7.4) to an absorbance of 0.7 at 734 nm. The extracts were diluted with
271 5mM phosphate buffer at concentrations between 0.3 and 5.0 mg mL^{-1}. An aliquot (10 µL) of

272 the different concentrations of the extracts was added to 990 μL of $ABTS^{•+}$ solution. Afterward,
273 300 μL of the mixture were transferred into a 96-well microplate and the absorbance was
274 measured at 734 nm every 5 min for 45 min in a microplate spectrophotometer reader (Synergy
275 HT, BioTek Instruments, Winooski, VT, USA). Trolox (0.25-2.0 mM) was used for calibration
276 and results expressed as TEAC values (mM trolox g^{-1} extract). These values were obtained
277 from five different concentrations of each extract that were tested in the assay and gave a linear
278 response between 20 and 80% of the blank absorbance [36]. All analyses were performed in
279 triplicate.

280

281 *2.8 Analysis of withanolides by liquid chromatography-mass spectrometry (UPLC-q-TOF-*
282 *MS)*

283 Liquid chromatography coupled to a high-resolution mass spectrometer was employed to
284 analyze and quantify WE and 4βHWE. These analyses were performed using an ultrahigh
285 performance liquid chromatography (UPLC) system 1290 from Agilent (Agilent Technologies,
286 Santa Clara, CA, USA) coupled to a quadrupole-time-of-flight mass spectrometer (q-TOF MS)
287 Agilent 6540 that was equipped with an orthogonal ESI source (Agilent Jet Stream, AJS, Santa
288 Clara, CA, USA), and controlled by a PC running the Mass Hunter Workstation software 4.0
289 (MH) from Agilent. The chromatographic method was carried out using a Zorbax Eclipse Plus
290 C18 column (2.1 × 100mm, 1.8 μm particle diameter, Agilent Technologies, Santa Clara, CA)
291 at 30 °C. The mobile phase composition was water with ammonium acetate (5 mM at pH 3.0
292 adjusted with formic acid, A) and acetonitrile (+0.1% formic acid, B). The gradient program
293 was as follows: 0 min, 0% B; 12 min, 80% B; 14 min, 100% B; 16 min, 100% B; 17 min, 0%
294 B. A flow rate of 0.5 mL/min and an injection volume of 20 μL were employed. The analyses
295 were performed in positive ion mode (ESI+). The mass spectrometer was used in MS and
296 MS/MS modes for the structural analysis of all compounds. MS parameters were the following:

297 capillary voltage, 4000 V; nebulizer pressure, 40 psi; drying gas flow rate, 10 L/min; gas
298 temperature, 350 ºC; skimmer voltage, 45 V; fragmentor voltage, 110 V. The MS and Auto
299 MS/MS modes were set to acquire m/z ranging between 50-1100 and 50-800 amu, respectively,
300 at a scan rate of 5 spectra per second. WE and 4βHWE were analyzed and relatively quantified
301 by structural analogy respect to Withanolide A, since there no commercial standards were
302 available from WE and 4βHWE. A calibration curve was obtained using 0.2–20 μg
303 Withanolide A mL^{-1} in MeOH and was used to calculate WE and 4βHWE relative content in
304 the extracts.

305

306 **3. RESULTS AND DISCUSSION**

307 *3.1 Theoretical selection of the bio-based solvents for whitanolides extraction by Hansen*
308 *Solubility Parameters estimation*

309 The theoretical approach of HSP has been used since the first half of the last century [32].
310 However, it has recently received special attention for its usefulness as a predictive tool for the
311 dissolution of an analyte in a particular solvent. Some recent applications for the extraction of
312 compounds from natural sources include the prediction of the best cosolvents to solubilise fatty
313 acids in supercritical CO_2 [25], the selection of bio-based solvents for the selective extraction
314 of tannins from algae [23], carotenoids from microalgae [24], betulin from birch bark [21] and
315 the study of β-carotene and lutein solubility in supercritical CO_2 [22]. HSP estimation is based
316 on the principle of "like dissolves like" and it can be very favourable to obtain a first
317 approximation for selective extraction processes, indicating the most suitable solvent(s) for a
318 given application, avoiding the selection of impractical experimental conditions [24]. The
319 results of the prediction of HSP by Yamamoto-molecular break method (HSPiP®) are given in
320 Table 1 at ambient conditions. As can be seen, similar results for the estimation of the solubility
321 parameters for WE and 4βHWE structures were obtained. The dispersion (δ_D) forces showed

322 a higher influence on the solubility parameters, due to the presence of steroid skeleton.
323 Moreover, calculating *Ra* value it is possible to get a better idea about the optimal solvents to
324 be chosen for the selective extraction. In descending order, d-limonene, ethyl acetate, ethyl
325 lactate, ethanol and water were the calculated as the preferred bio-solvents from the software
326 database. Despite d-limonene was calculated as the preferred solvent and it has already been
327 employed as solvent for green extraction processes [24,26], the evaporation of d-limonene to
328 obtaining dry extracts is a quite hard task considering the physico-chemical properties (boiling
329 point: 178 °C at 1.01 bar) of this solvent, thus limiting its application. For this reason, ethyl
330 acetate, ethanol, and their mixtures were preferred for PLE as selective extraction solvents. The
331 results of *Ra* for different solvent mixtures at 50, 100 and 150 °C are presented in Table 2.
332 From the theoretical estimation, low temperature (50 °C) and pure ethyl acetate provide the
333 lowest *Ra* values (*Ra* = 9.01 and 8.84, for WE and 4βHWE, respectively). This can be easily
334 explained by the similarity of the individual solubility parameters corresponding to the target
335 compounds and ethyl acetate, mainly δ_H and δ_P parameters (See Table 1). In contrast,
336 increasing temperature and proportion of ethanol increases the *Ra* value, thus showing a lower
337 selectivity. In this sense, it is important to highlight that HSP is an approximated model mainly
338 based on thermodynamics data and that transport phenomena are not considered that can be
339 influenced by temperature, as we already demonstrated in a previous work done in our
340 laboratory [23]. On the other hand, other aspects related with molecule conformation and
341 structure, and distribution of polar groups within the molecule can affect the solubilisation of
342 the target compounds into the solvent. Therefore, further experimental optimization is
343 recommended in order to overcome limitations imposed by the theoretical models.

344

345 <**Table 1**. Hansen Parameter Solubility for withanolides and solvents calculated by HSPiP
346 Software®>

347 <**Table 2**. Ra values for WE and 4βHWE at different subcritical mixtures solvents>

348

349 *3.2 Monitorization of withanolides enrichment of PLE extracts by LC-q-TOF*

350 WE and 4βHWE were considered as target analytes to monitor the withanolides enrichment
351 during PLE optimization, since they have been described as relevant compounds in *P.*
352 *peruviana* extracts from a pharmacological point of view [12,41,42]. It is well reported in
353 literature that withanolide compounds can be determined by generating the protonated
354 molecular ion $[M+H]^+$ as well as the adducts $[M+NH_4]^+$ or $[M+Na]^+$ [43,44]. Thus, operating
355 in positive ionization mode and using ammonium acetate (5 mM) in the mobile phase to
356 improve detectability, a targeted analysis of m/z ions 504.2956 and 520.2905, corresponding
357 to $[M+NH4]^+$ adducts of WE and 4βHWE, respectively, was carried out. As shown in the EIC
358 (extracted ion chromatogram) obtained in MS full scan acquisition mode (Figure 2A), two
359 major peaks at 6.1 and 7.6 min. were detected and assigned to 4βHWE and WE, respectively.
360 Other minor peaks with the same masses were also identified as isobaric forms of both target
361 withanolides (see tentative structural elucidation in [45]).
362 As shown in Figure 2B, MS^2 product ion spectra of target withanolides exhibit characteristic
363 fragmentation patterns in which the breakdown of precursor ion $[M+NH4]^+$ led to diagnostic
364 ions m/z 485.2545, 299.1642 and 169.0856 for 4βHWE and m/z 469.2591, 283.1694 and
365 169.0856 for WE. These product ions are mainly originated by the loss of a water molecule
366 $[M+H-H_2O]^+$ from the precursor molecular ion, and by the removal of the lactone part after
367 cleavage in C_{17}–C_{20} positions, subsequent rearrangement (-2H) and loss of a water molecule,
368 leading to the protonated ergostane moiety $[M+H-Lac]^+$ and the lactone part $[Lac]^+$. The
369 observed fragmentation pattern is consistent with the mass fragmentation of withanolides
370 reported in literature [44]. As mentioned in Section 2.8., relative quantitation of withanolides
371 4βHWE and WE was performed based on commercially available congener withanolide A.

372 Accurate mass values of diagnostic ions and performance parameters for the quantitation of the
373 target withanolides are summarized in Table 3.
374
375 <**Table 3**. Accurate m/z values of [M+NH4]$^+$ precursor ions, confirmation product ions and
376 performance parameters for the quantitation of the target withanolides.>
377
378 *3.3 Optimization of the PLE procedure*
379 In order to optimize the extraction of withanolides, particularly 4βHWE and WE, from
380 goldenberry calyces using PLE, a central composite design was applied. For this purpose,
381 temperature (50, 100 and 150 ºC) and solvent composition (percentage of EtOH in the mixture
382 EtOH/EtOAc: 0, 50 and 100% v/v) were studied on 4βHWE and WE contents. In addition, the
383 effects of the independent variables on extraction yield, TPC, TFC and antioxidant activity
384 (EC_{50} and TEAC) were also evaluated. Results are summarized in Table 4. As can be seen,
385 different behaviours in the response variables can be observed; in order to determine the factors
386 that influence each of the response variables, Pareto charts and surface responses were
387 constructed (see Figure 3). As can be seen, the highest extraction yield (16.02 %) was obtained
388 at the intermediate temperature evaluated (100 ºC) and 50% EtOH as extraction solvent; Pareto
389 chart for extraction yield (Figure 3 A) shows that both, solvent composition and temperature
390 significantly contribute to the final yield, being the negative quadratic effect of solvent
391 composition the most important. Therefore, a maximum can be reached considering
392 intermediate conditions of temperature and solvent composition.
393 As for 4βHWE, the highest content was 9.26 mg g^{-1} Db of sample obtained at 150 ºC-100%
394 EtOH v/v and this response was highly influenced by the linear and quadratic effect of the
395 temperature (Figure 3B). Regarding WE content, this variable was affected by temperature
396 (quadratic and negative) and solvent composition (linear and positive) (Figure 3C) and its

397 highest value was 2.37 mg WE g^{-1} Db of sample (100 ºC-50% EtOH v/v). Calderón, Ruiz &
398 Castellanos (2012) [7] purified 4βHWE from goldenberry calyx methanolic extract (Soxhlet
399 extraction at 60 ºC for 3h), getting 3.2 mg 4βHWE g^{-1} Db of sample. Henrich et al. [46]
400 extracted and isolated 4βHWE (2.9 mg) and WE (6.9 mg) from aerial part of *P. peruviana*
401 employing MeOH:CH_2Cl_2 (1:1); being the obtained 4βHWE results close to the ones achieved
402 by dynamic maceration extraction (2.83-3.36 mg g^{-1} Db of sample) in the present study.
403 However, the content of WE obtained is lower than the value reported previously [46].
404 Nevertheless, the extraction and solvent employed in this work are "green" in contrast to those
405 used by the mentioned authors.
406 Phenolic compounds are broadly distributed in the plant kingdom and they are the most
407 abundant secondary metabolites of plants. Phenolic compounds have been considered powerful
408 antioxidants by their capacity for scavenging and suppressing radical species [47]. Likewise,
409 these compounds may play a role in the inhibition of carcinogenesis by affecting the molecular
410 events in the initiation, promotion, and progression stages, especially, thanks to their
411 antioxidant capacity [48]. TPC and TFC are initial approximations to phenolic content. In this
412 work, the highest TPC value (37.83 mg GAE g^{-1} Db) was observed at 150 ºC and 100% EtOH
413 v/v and this response was principally influenced by the linear effect of the temperature and by
414 the interaction of the independent variables (Figure 3D). This behavior may be explained by
415 the mass transfer, which is favored by temperature, as well as, by the thermal stability and
416 medium polarity of the phenolic compounds extracted at the evaluated conditions. Medina-
417 Medrano et al. [49], studied the phenolic content in different organs of some species (*P.*
418 *subulate*, *P. solanacea*, *P. patula*, *P. hederifolia* var. *hederifolia* and *P. angulata*) of the genus
419 *Physalis* including the calyces. The TPC values reported by those authors (54.15-176.58 mg
420 GAE g^{-1} dry tissue) were higher than those found in this work. On the other hand, TFC results
421 were not statistically different among the different PLE conditions assayed and were similar to

422 those obtained under DM extraction. In PLE extraction, TFC response was only influenced by
423 quadratic and negative effect of solvent composition (Figure 3E). The highest TFC value was
424 1.61 mg QE g^{-1} Db and it was significantly lower that values reported previously in calyces for
425 some species of the genus *Physalis* (16.48-39.63 mg QE g^{-1} dry tissue) [49]; these differences
426 may be due, among other factors, to the solvent employed for extraction (80% methanol in
427 [49]), species and location. It is important to emphasize that significant tissue and species-
428 dependent variations were already observed by Medina-Medrano et al. [49] for TPC and TFC;
429 since no previous reports on *P. peruviana* calyces TPC and TFC could be found in the literature,
430 it is difficult to compare with results obtained in the present study.
431 Antioxidant activity was evaluated by DPPH and TEAC assays. Although these assays are
432 usually classified as *Single Electron Transfer* (SET) reactions, these two indicator radicals in
433 fact may be neutralized either by direct reduction via electron transfers or by radical quenching
434 via H atom transfer [50]. For this reason, DPPH and TEAC assays are some of the most used
435 methods to evaluate antioxidant activity *in vitro*. Antioxidant capacity evaluated by DPPH was
436 expressed in methanol extract concentration in μg mL^{-1} required to inhibit 50% of radical
437 (EC_{50}). Therefore, the lower EC_{50} value, the higher the antioxidant capacity. The lower EC_{50}
438 value obtained was 42.19 μg mL^{-1} at 150 ºC and 100% EtOH v/v and this response was
439 principally affected by linear and negative effect of both, temperature and solvent composition
440 (Figure 3F). As for TEAC, the best value obtained was 1.35 mM trolox g^{-1} under the best
441 conditions for DPPH. TEAC response was influenced by solvent composition and temperature
442 (Figure 3G). The best results of EC_{50} and TEAC are in agreement with the highest values of
443 TPC and TFC under the same extraction conditions; therefore, in this case, the antioxidant
444 capacity can be related to the phenolic content. Comparatively, the *P. peruviana* calyces'
445 extracts had a moderate antioxidant capacity with respect to the one reported previously for
446 other species of the genus *Physalis* (0.95-60.29 μg mL^{-1} extract) [49]. Due to low TPC and

447 TFC contents, as well as moderate antioxidant activity of *P. peruviana* calyces' extracts, PLE
448 extraction was optimized respect to 4βHWE and WE responses. To achieve this purpose,
449 desirability function combining 4βHWE and WE responses was calculated in the range from 0
450 to 1 (Figure 4A), where 1 represents the most desirable response. Profiles for predicted values
451 estimated by desirability function are shown in Figure 4B. Optimal conditions were 125 °C and
452 75% EtOH v/v at 0.8 desirability value. Predicted response values obtained by desirability
453 approach under optimum conditions were checked experimentally using same extraction
454 conditions. Extraction yield, TPC, TFC and antioxidant activity variable responses were also
455 predicted and evaluated. Table 5 presents experimental and predicted results for responses
456 evaluated at optimum condition estimated. At these conditions, 4βHWE and WE content were
457 simultaneously improved, whereas that extraction yield, TFC and TEAC results were close to
458 the maximum values obtained by experimental design. However, TPC and EC_{50} values
459 decreased after applying the desirability function, since they were not included in the final
460 desirability optimization function. Results obtained during validation of optimized conditions
461 were close to predicted values and they were within the confidence intervals. This confirms
462 that selected RSM model was successfully applied for PLE of *P. peruviana* calyces in order to
463 obtain extracts with maximal 4βHWE and WE contents.
464 Although results obtained using RSM for optimizing 4βHWE and WE contents seemed
465 contradictory with data presented in Table 2 in terms of Ra distances, it is important to consider
466 that other effects more associated to the dielectric constant of the solvent (or relative static
467 permittivity) should be also taken into account, including the possibility of having oxygen
468 atoms (in whitanolides molecules) acting as hydrogen bond acceptor that could improve its
469 solubility in more polar solvents; moreover, other aspects related with molecule conformation,
470 structure, and distribution of polar groups within the molecule can affect the solubilisation of

471 the target compounds into the solvent, aspects that are not easy explained from a theoretical
472 point of view.
473
474 <**Table 4.** Experimental design conditions (experiments 1 to 10) and results for each response
475 variable studied for the optimization of the PLE and macerations (experiments I, II and III) of
476 the goldenberries calyces. Values presented are mean ± sd.>
477
478 <**Table 5.** Experimental and predicted values for all response variables at 75% EtOH and 125
479 ºC.>
480

481 **4. CONCLUSIONS**

482 A sustainable valorisation strategy was successfully developed in this work, demonstrating the
483 great potential of the proposed multi-analytical platform based on pressurized-liquid
484 extraction, green solvents, in vitro assays and liquid chromatography coupled to q-TOF mass
485 spectrometry to obtain and characterize bioactive extracts with huge potential as functional
486 foods and in traditional medicine.
487 PLE was implemented optimizing withanolides 4βHWE and WE recoveries from *P. peruviana*
488 calyces, which are reported as highly valuable health-promoting compounds. Optimization also
489 allowed maximizing the extraction yield, total phenolic and total flavonoids content. By using
490 a RSM and considering temperature and solvent composition (mixtures of EtOH/EtOAc) as
491 factors, optimized conditions obtained were 125 °C and 75% EtOH in the EtOH/EtOAc solvent
492 mixture. Results provided satisfactory extraction yield (14.7 %) and moderate antioxidant
493 activity (EC_{50} 77.18 µg mL^{-1}; 1.08 mM trolox g^{-1}), with 4βHWE and WE concentration levels
494 at 8.8 and 2.3 mg g^{-1}, respectively.
495 Further research work will be carried out to evaluate the biological activity of the obtained PLE
496 calyces extracts to better understand the promising benefits on human health under a foodomics

497 approach. In addition, the potential implementation of the developed PLE procedure at
498 industrial level might also be considered after appropriate scale-up study and economic
499 evaluation, taking into account the potential applications of the obtained bioactives-rich extract
500 in nutraceutical application.

501

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503

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684

685 **Figure captions**

686 **Figure 1.** Chemical structures of Withanolides Type A and Type B.

687

688 **Figure 2.** Detection of target withanolides 4β-WE and WE in PLE extracts of *P. peruviana* by
689 LC- ESI(+)-q-TOF analysis. (A) Overlapped extracted ion chromatograms of PLE extracts
690 obtained in optimal (black) and non-optimal (orange) conditions; (B) MS/MS fragmentation
691 spectra of 4β-WE and WE compounds with annotated product ions.

692

693 **Figure 3**. Standardized Pareto charts for the response variables studied and their corresponding
694 response surfaces.

695

696 **Figure 4.** A) Desirability surface to maximize 4βHWE and WE response variables and B)
697 Profiles for predicted values at estimated optimal point 75% EtOH v/v and 125 ºC.

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699