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14 **Abstract**

15 Several works have been focused on the extraction of polysaccharides, polyphenols and caffeine from
16 spent coffee grounds (SCG) and their application in food formulations, but the peptide bioactivity from
17 SCG protein hydrolysates has never been addressed. In the present work and for the first time, two
18 different methods to isolate proteins from SCG have been compared, demonstrating that a urea-based
19 extraction buffer provides a higher yield. This extraction method was then applied to compare the
20 protein content in SCG from different coffee-brewing preparations, showing a higher protein content in
21 SCG from espresso coffee machines. In addition, a polyphenol extraction step to remove interferences
22 has been evaluated and the hydrolysis of the extracted proteins using alcalase and thermolysin enzymes
23 has been compared. The effect of roasting degree on the antioxidant and *in vitro* angiotensin-converting
24 enzyme (ACE)-inhibitory activity has been evaluated. The results show that the ACE-inhibitory activity is
25 higher when SCG proteins are obtained from medium and dark roasted coffees and then hydrolyzed with
26 thermolysin. Finally, the peptides contained in these hydrolysates have been identified by reversed-
27 phase high-performance liquid chromatography coupled via electrospray ionization to a quadrupole
28 time-of-flight mass spectrometer (RP-HPLC-ESI-Q-TOF).

31 **Keywords**

32 Bioactivity; Liquid chromatography–tandem mass spectrometry; peptide; polyphenol; spent coffee
33 grounds.

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34 **1. Introduction**

35 Coffee industry produces a large amount of residues that can be >50% of the fruit mass in the coffee
36 producing countries (Tsai, Liu & Hsieh, 2012). Among them, the solid residues obtained during the
37 brewing process, the so called spent coffee grounds (SCG) (Cruz et al., 2012), are usually incinerated or
38 disposed of in landfills with the subsequent air pollution and soil contamination. Therefore, strategies for
39 the proper management of these residues are needed. Due to the large amounts of organic compounds
40 in SCG (Campos-Vega, Loarca-Piña, Vergara-Castañeda, & Oomah, 2015), these residues have been used
41 as a source of value-added products, such as for biodiesel production (Karmee, Swanepoel, & Marx,
42 2018), as a precursor for activated carbon production (Kante, Nieto-Delgado, Rangel-Mendez, &
43 Bandosz, 2012), to formulate products for animal feeding (Givens & Barber, 1986; Xu, Cai, Zhang, &
44 Ogawa, 2006), or to extract polysaccharides as a first step to exploit SCG in fermentative processes
45 (Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011). SCG are also rich in antioxidant compounds such
46 as phenols and other non-protein nitrogenous compounds such as caffeine, which have been associated
47 with health benefits (Campos-Vega, Oomah, Loarca-Piña, & Vergara-Castañeda, 2013; Campos-Vega,
48 Loarca-Piña, Vergara-Castañeda, & Oomah, 2015). The main phenolic compounds in SCG residues are
49 similar to those obtained in coffee brews, being chlorogenic acids (CGA) such as caffeoylquinic acids
50 (CQAs), dicaffeoylquinic acids (diCQAs), feruloylquinic acids (FQAs), caffeoylquinic acid lactones (CQLs),
51 feruloylquinic acid lactones (FQLs) and p-coumaroylquinic acids (pCoQAs) the most abundant (Bravo,
52 Arbillaga, de Peña, & Cid, 2013; Farah, de Paulis, Trugo, & Martin, 2005; Panusa, Zuorro, Lavecchia,
53 Marrosu, & Petrucci, 2013). Furthermore, several works have demonstrated that the concentration of
54 these compounds depends on the brewing and roasting procedures (Gloess et al., 2013; Ludwig et al.,
55 2012; Bravo, et al., 2012; Cruz et al., 2012). Consequently, the extraction of phenolic compounds from
56 SCG has been extensively studied using different solvents, solvent-to-solid ratios, or extraction times
57 (Yen, Wang, Chang, & Duh, 2005; Mussatto, Ballesteros, Martins, & Teixeira, 2011). Moreover, CGA can

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4 58 also be incorporated into melanoidins, mainly by transglycosylation reactions (Moreira et al., 2017).

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6 59 Melanoidins are the high molecular weight nitrogenous and brown-coloured compounds formed during
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8 60 the roasting process of coffee (Bekedam, Roos, Schols, Van Boekel, & Smit, 2008; Moreira, Nunes,
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10 61 Domingues, & Coimbra, 2012), and the incorporation of phenolic compounds into the melanoidins is a
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12 62 significant pathway of CGA degradation during roasting (Coelho et al., 2014). Moreover, it has also been
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14 63 shown that the coffee preparation affects the concentration of these compounds (Bravo et al., 2012).
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19 64 Apart from the mentioned compounds, the mean protein content of SCG is 13.6% (Mussatto, Carneiro,
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21 65 Silva, Roberto, & Teixeira, 2011; Silva, Nebra, Machado-Silva, & Sanchez, 1998), but this content might
22
23 66 be overestimated due to the presence of other nitrogen-containing substances such as caffeine,
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25 67 trigonelline, free amines and/or amino acids (Delgado, Vignoli, Siika-aho, & Franco, 2008). The protein
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27 68 content also varies depending on the brewing and roasting processes, when the proteins can be
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29 69 fragmented, polymerized, and/or integrated into melanoidins (Bravo, et al., 2012; Cruz et al., 2012;
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31 70 Tokimoto, Kawasaki, Nakamura, Akutagawa, & Tanada, 2005). However, no works have focused on the
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33 71 identification of bioactive peptides from SCG proteins hydrolysates. Bioactive peptides are mainly found
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35 72 in a latent state as part of a protein from which they can be released by hydrolysis using different
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37 73 enzymes (Sarmadi & Ismail, 2010). In order to exploit SCG as a source of bioactive peptides, SCG proteins
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39 74 have to be extracted avoiding the extraction of other interfering compounds (such as polyphenols), and
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41 75 then hydrolysed. In the present work, a commonly used Tris-HCl based method for protein extraction
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43 76 from food by-products has been compared to a new urea-based method to isolate SCG proteins, and the
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45 77 best method has been applied to evaluate the protein content in SCG from different coffee-brewing
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47 78 preparations. In addition, a polyphenol extraction step to remove interferences has been studied, as well
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49 79 as the hydrolysis of the resultant proteins into peptides using alcalase and thermolysin enzymes.
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51 80 Furthermore, the effect of roasting degree in the presence of peptides with antioxidant and/or *in vitro*
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53 81 angiotensin-converting enzyme (ACE)-inhibitory activity was evaluated. Finally, the resulting peptides
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4 82 have been identified by reversed-phase high-performance liquid chromatography coupled via
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6 83 electrospray ionization to a quadrupole time-of-flight mass spectrometer (RP-HPLC-ESI-Q-TOF).
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10 84 **2. Materials and Methods**

11 12 13 85 **2.1. Chemicals**

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16 86 All chemicals and reagents were of analytical grade. Water was daily obtained from a Milli-Q system
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18 87 from Millipore (Bedford, MA, USA). Sodium dodecylsulfate (SDS), tris(hydroxymethyl)aminomethane
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20 88 (TRIS), sodium chloride, hydrochloric acid, sodium dihydrogen phosphate and sodium hydroxide were
21
22 89 obtained from Merck (Darmstadt, Germany). Acetic acid, acetone, methanol, n-hexane (96%), and
23
24 90 acetonitrile (ACN) were purchased from Scharlab (Barcelona, Spain). Formic acid (FA) was from Fisher
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26 91 Scientific (Geel, Belgium). Ammonium bicarbonate (AmBi), dithiothreitol (DTT), sodium tetraborate,
27
28 92 thermolysin enzyme, angiotensin converting enzyme (ACE), 2-[4-(2-hydroxyethyl)-1-piperazinyl]-
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30 93 ethanesulfonic acid (HEPES), hippuryl-histidyl-leucine (HHL), 1,10-phenanthroline, 2-mercaptoethanol,
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32 94 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), albumin from bovine serum (BSA),
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34 95 hydrogen peroxide, ferrous sulfate, L-gluthathion (GSH), ortho-phthalaldehyde (OPA), potassium
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36 96 persulfate, trigonelline hydrochloride, caffeine, caffeic acid, 4-O-caffeoylquinic acid, and 3-
37
38 97 hydroxycoumarin were purchased from Sigma (St. Louis, MO, USA). 1,3-dicaffeoylquinic acid, and 1,5-
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40 98 dicaffeoylquinic acid were purchased from Plantachem (Pinnow, Germany). Tris/glycine/SDS running
41
42 99 buffer, Laemmli buffer, Bio-Safe Coomassie G250 stain, Mini Protean precast gels, Precision Plus Protein
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44 100 Standards (molecular masses of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa) and Quick Start
45
46 101 Bradford – 1xDye reagent were obtained from Bio-Rad-Laboratories (Hercules, CA, USA). Alcalase 2.4 L
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48 102 FG enzyme was kindly donated by Novozymes Spain S.A. (Madrid, Spain).
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57 103 **2.2. Coffee samples**

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104 Commercial medium roasted 100% Arabica coffee (CMRC) already grounded (fine grind) from “Tchibo”
105 (Hamburg, Germany) was used as standard coffee for the optimization of the protein extraction method,
106 and for the comparison of the different coffee-brewing preparations. Additionally, three differently
107 roasted 100% Arabica coffee beans (light, medium and dark) were grounded (fine grind) and provided by
108 “Café Fortaleza” (Vitoria, Spain) for the evaluation of the roasting process in the presence of peptides
109 with antioxidant and potential *in vitro* ACE-inhibitory activities. Coffee beans were roasted at 175 °C
110 during 12.36 min (light roasted), at 185 °C during 14.11 min (medium roasted), and at 195 °C during
111 17.06 min (dark roasted). The weight loss of each sample was evaluated in order to control the roasting
112 process being 13% for light, 15% for medium, and 17% for dark roasted beans.

113 **2.3. Spent coffee ground preparation**

114 An espresso machine, a mocha coffeemaker, a plunger coffeemaker, and filter paper were used to
115 generate different SCG, trying to keep a coffee weight-to-water volume ratio at 1g/8.75mL. All
116 experiments were performed in triplicate. Espresso coffee brews were prepared from 8 g of ground-
117 roasted coffee, using an espresso coffee machine (Saeco Via Venneto, Italy) with a 15 bar pressure
118 pump, and dispensing water during 10 s at 90 °C. Mocha, plunger and filter coffee brews were prepared
119 from 20 g of ground-roasted coffee. For mocha coffee, a mocha coffeemaker (Vitro-Fulgor, Valira, Spain)
120 was used, and the heating temperature and extraction time were approximately 10 min at 93 °C. For the
121 plunger coffee brew, hot water (98 °C) was added to the coffee powder in the plunger coffeemaker (0.5 L
122 capacity), and the water was kept in contact with the coffee for 5 min before the plunger was pushed
123 down. For the filter coffee brew, coffee powder was placed in a filter paper, hot water (98 °C) was added
124 slowly, and extraction took place in 5 min. All generated SCG were dried in an oven at 103 ± 2 °C until
125 constant weight. Thereafter, 1 g was defatted three times with 25 mL of hexane maintaining the solution

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126 under stirring for 30 min. After each cycle, samples were centrifuged at 4,000g for 10 min, and hexane
127 was discarded. Samples were kept overnight at 40 °C to completely remove the hexane.

128 **2.4. Polyphenol extraction**

129 When specified, and before protein extraction, polyphenols were extracted from defatted SCG samples
130 (in triplicate). For this aim, 5 mL of methanol:H₂O:acetic acid (70:28:2, v/v/v) were added to 100 mg of
131 defatted SCG, and a high intensity focused ultrasound (HIFU) probe (model VCX130, Sonics Vibra-Cell,
132 Hartford, CT, USA) was used for 5 min with an amplitude of 30% (HIFU standard conditions if not
133 specified elsewhere). Thereafter, samples were vortexed in a mechanical stirrer (Selecta, Barcelona,
134 Spain) at room temperature for 30 min, centrifuged at 4,000g for 10 min and the supernatants were
135 transferred to a different vial. This procedure was repeated three times and the pellets were dried at 40
136 °C overnight. The collected supernatants after each extraction cycle were pooled together and directly
137 analysed by HPLC-UV/HPLC-FLD.

138 **2.5. Protein extraction and quantification**

139 Two different extraction methods were compared. In the first one, 100 mg of defatted SCG were mixed
140 with 5 mL of Tris Buffer (TB, consisting on 100 mM Tris-HCl (pH 8.5), 0.5% (w/v) SDS and 0.75% (w/v)
141 DTT) and HIFU was applied. Thereafter, samples were vortexed in a mechanical stirrer for 60 min,
142 centrifuged at 4,000g for 10 min, and 4 mL of TB supernatant was taken. Then, the proteins in the
143 supernatant were precipitated with 10 mL of cold acetone at -20 °C overnight. Samples were then
144 centrifuged again at 4,000g for 10 min and the precipitated proteins were dried at 40 °C overnight.
145 Pellets were weighed and ≈ 1 mg was dissolved in TB to a final concentration of 10 mg/mL for SDS-PAGE
146 analysis. In addition, another ≈ 1 mg was dissolved to a final concentration of 10 mg/mL in a Bradford
147 compatibility Quantification Buffer (QB, 100 mM Tris-HCl (pH 8.5) containing 0.025% (w/v) SDS and
148 without DTT) for SDS-PAGE analysis and protein quantification. These samples were then diluted 1:3 to

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4 149 fit in the BSA standard calibration curve, and the protein content was estimated (Bradford, 1976), using
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6 150 the same extraction buffer as blank. In the second method, 100 mg of defatted SCG (with or without
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9 151 polyphenols, depending on the experiment) were mixed with 5 mL of Urea Buffer (UB, consisting on 7 M
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11 152 urea, 2 M thiourea and 1 M AmBi in water), and HIFU was applied. Samples were then vortexed for 60
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14 153 min, centrifuged at 4,000g for 10 min, and supernatant was centrifuged again at 14,000g for 10 min to
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16 154 remove the remaining debris. Thereafter, protein concentration was determined in the supernatants by
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18 155 Bradford assay after checking the compatibility of urea and thiourea, and using this buffer as blank. For
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21 156 both protein extraction methods, the absorbance corresponding to a mixture of 12.3 μ L of sample with 1
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23 157 mL of Bradford solution was measured at 595 nm using a spectrophotometer Cary 8454 UV-Vis (Agilent
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25 158 Technologies, Germany). The protein concentration was then calculated by interpolation in a calibration
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28 159 curve prepared using a BSA standard at concentrations ranging from 0 to 0.3 mg/mL. Every sample was
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30 160 measured by triplicate.

31 32 33 161 **2.6. Protein analysis by SDS-PAGE**

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36 162 SDS-PAGE separation was carried out in a Mini-Protean from Bio Rad. Samples were prepared in
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39 163 triplicate by mixing the same volume of SCG protein isolate and Laemmli buffer, containing 5% (v/v) β -
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41 164 mercaptoethanol and by heating for 5 min at 100 °C. Electrophoresis was carried out on commercial
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44 165 Ready Gel Precast Gels using Tris/glycine/SDS as running buffer and applying 80 V for 5 min and 200 V for
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46 166 30 min. For the estimation of molecular weights, protein standards (10, 15, 20, 25, 37, 50, 75, 100, 150,
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49 167 and 250 kDa) were used as ladder. After separation, proteins were fixed with 50 mL of 10% (v/v) glacial
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51 168 acetic acid/40% (v/v) methanol gently shaking for 30 min and stained with 50 mL of Bio-Safe Coomassie
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53 169 stain by slightly shaking for 1 h. At the end, the gel was washed with Milli-Q water for 2 h and images
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56 170 were acquired using a scanner (Epson Perfection V39, Sowa, Japan).

57 58 59 171 **2.7. Protein hydrolysis**

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4 172 For protein hydrolysis, 0.8 mg of SCG proteins extracted with UB (for the comparison between the
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6 173 different hydrolytic enzymes and for the evaluation of the polyphenol extraction step) or 4 mL of UB (for
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9 174 the evaluation of the effect of the roasting degree) were transferred to 3 kDa molecular weight cut-off
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11 175 filters (Amicon Ultra 0.5 mL - 3 kDa, Merck Millipore, Burlington, MA, USA), previously washed once with
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13 176 250 μ L of a mixture of ACN:H₂O (20:80, v/v) at 14,000g for 10 min, and later on with 500 μ L of Milli-Q
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16 177 water at 14,000g for 15 min. The samples were then centrifuged at 14,000g until the whole volume was
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18 178 loaded, and consecutive washes were performed with 300 μ L of 50 mM AmBi in ACN:H₂O (20:80, v/v)
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21 179 (once) and then with 300 μ L of 50 mM AmBi in Milli-Q water (twice) to remove urea excess. Between
22
23 180 each washing step, the filter units were centrifuged at 14,000g for 15 min. Finally, protein hydrolysis was
24
25 181 carried out inside the filter units with 300 μ L of 50 mM AmBi buffer (pH 8.0) using two different
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27 182 enzymes: alcalase (0.15 AU enzyme/g protein) and thermolysin (0.1 g enzyme/g protein), and in similar
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30 183 conditions as in Hernández-Corroto, Marina, & García (2019) and Pérez-Míguez, Marina, & Castro-
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32 184 Puyana (2019). Hydrolysis was performed at 50 °C for 4 h by slight mixing (750 rpm) in a Thermomixer
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35 185 Compact (Eppendorf AG, Hamburg, Germany), and the reactions were stopped by heating to 100 °C for
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37 186 10 min. Resulting peptides were then centrifuged at 14,000g for 10 min, and 200 μ L of 50 mM AmBi
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40 187 were added (twice) and centrifuged again to recover all peptides. Finally, samples were transferred to
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42 188 new tubes, dried in SpeedVac (Eppendorf AG, Hamburg, Germany) and dissolved in Milli-Q water to a
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44 189 final concentration of 2.5 mg/mL (considering the starting amount of loaded protein).

47 48 190 **2.8. Determination of peptide content, antioxidant and *in vitro* ACE-inhibitory activities**

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51 191 The peptide content (based on the OPA assay), the antioxidant activity (based on the ABTS and hydroxyl
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53 192 radical scavenging assays), and the *in vitro* ACE-inhibitory activity (based on the hydrolysis of the
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56 193 tripeptide HHL into hippuric acid (HA) by the action of ACE) of SCG protein hydrolysates were

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4 194 determined as previously described (Hernández-Corroto, Marina, & García, 2018; Hernández-Corroto,
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6 195 Marina, & García, 2019). See **Supplementary Material and Methods** for the full description.
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10 196 **2.9. HPLC-UV and HPLC-FLD analyses**

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13 197 Separation of polyphenol extracts (dissolved in methanol:H₂O:acetic acid (70:28:2, v/v/v)) and SCG
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15 198 protein hydrolysates (dissolved in Milli-Q water) were performed in a 1100 series LC system (Agilent
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18 199 Technologies, Germany). LC control, data acquisition, and data analysis were carried out using the
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20 200 Agilent LC/MSD ChemStation software (B.04.03). Injection volume was set to 5 μ L and all samples were
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22 201 analyzed in duplicate. A porous-shell fused-core Ascentis Express C18 analytical column (100 mm \times 2.1
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25 202 mm, particle size 2.7 μ m) protected by a C18 guard column (0.5 cm \times 2.1 mm, particle size 2.7 μ m), both
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27 203 from Supelco (Bellefonte, PA, USA) were used. The flow-rate was set to 0.25 mL/min and the column
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30 204 temperature was 30 $^{\circ}$ C. The gradient was 5–35% B in 30 min, 35–50% B in 5 min, 50–95% B in 5 min, and
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32 205 95–5% B in 2 min. Mobile phase A was water with 0.1% (v/v) of FA and mobile phase B consisted of ACN
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34 206 with 0.1% (v/v) FA. Detection was carried out using an UV detector at 280 and 325 nm, and using a
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37 207 fluorescent detector (FLD) at λ_{exc} = 280 and λ_{em} = 304 and 348 nm.
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40 208 **2.10. RP-HPLC-ESI-Q-TOF peptide and polyphenol identification**

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43 209 Identification of peptides and polyphenols in SCG protein hydrolysates (dissolved in Milli-Q water) were
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46 210 performed by RP-HPLC-ESI-Q-TOF as previously described (Pérez-Míguez, Marina, & Castro-Puyana,
47
48 211 2019). Briefly, MS analysis was performed in a quadrupole Q-TOF series 6530 coupled to a HPLC (model
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50 212 1100) both from Agilent Technologies (Germany), equipped with a Jet Stream thermal orthogonal ESI
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53 213 source. MS control, data acquisition, and data analysis were carried out using the Agilent Mass Hunter
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55 214 Qualitative Analysis software (B.07.00). Injection volume was set to 10 μ L, and three replicates were
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58 215 injected in triplicates. For the chromatographic separation, an Ascentis Express Peptide ES-C18 analytical
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60 216 column (100 \times 2.1 mm, particle size 2.7 μ m) with a C18 guard column (0.5 cm \times 2.1 mm, particle size
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4 217 2.7 μm), both from Supelco (Bellefonte, Pa, USA) were employed. The column temperature was held at
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6 218 25 °C and the flow rate was set to 0.3 mL/min. Mobile phase A was water with 0.3% (v/v) of acetic acid
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9 219 and mobile phase B consisted of ACN with 0.3% (v/v) acetic acid. The gradient was 5% B for 3 min, 5–40%
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11 220 B in 37 min, 40–95% B in 3 min, 95% B in 2 min, with 15 min of post-time to come back to initial
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13 221 conditions. ESI in positive ion mode (for peptides) or in negative ion mode (for polyphenols) were used at
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16 222 a capillary voltage of 3500 V and with a m/z range from 100 to 1700. UV signals were also recorded at
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18 223 280 and 325 nm. MS analyses were performed employing the auto MS/MS mode using 3 precursor per
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21 224 cycle, dynamic exclusion after two spectra (released after 1 min), and collision energy of 5 V for every
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23 225 100 Da. For proper mass accuracy, spectra were corrected using ions m/z 121.0509 ($\text{C}_5\text{H}_4\text{N}_4$) and
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25 226 922.0098 ($\text{C}_{18}\text{H}_{18}\text{O}_6\text{N}_3\text{P}_3\text{F}_{24}$) in ESI positive mode, and m/z 119.0363 ($\text{C}_5\text{H}_4\text{N}_4$) and 980.0164
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27 227 ($\text{C}_{18}\text{H}_{18}\text{O}_6\text{N}_3\text{P}_3\text{F}_{24}$ + formate) in ESI negative mode, simultaneously pumped into the ionization source at a
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30 228 15 $\mu\text{L}/\text{min}$ flow rate. Peaks software (Bioinformatics Solutions Inc.; Waterloo, ON, Canada) was
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32 229 employed for database search against Uniprot *Coffea arabica* reviewed proteome (downloaded on July
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34 230 25th 2019 and containing 92 entries). Oxidation of methionine (+15.99 Da) was included as variable
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37 231 modification and peptide results were refined using a -10lgP threshold of 15. Moreover, *de novo*
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40 232 peptide sequencing was performed using an error tolerance of 10 ppm for the precursor and 0.5 kDa for
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42 233 the fragment, and including oxidation of methionine as variable modification. *De novo* peptide
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44 234 sequences were accepted if the average local confidence (ALC, expected percentage of correct amino
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47 235 acids in the peptide sequence) was $\geq 80\%$ in at least 5 from 9 injections. Moreover, since the MS system
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49 236 is not able to distinguish between isoleucine (I) and leucine (L) amino acids due to their equal molecular
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52 237 masses, only isoforms with L are presented.

53 54 55 238 **2.11. Statistical analysis**

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4 239 Statistical analysis was performed using Statistica software version 7.1 (StatSoft, Inc., USA). One-way
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6 240 ANOVA with LSD Post-hoc test was employed to determine any significant differences between mean
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9 241 values using $p < 0.05$.

12 242 **3. Results and Discussion**

15 243 **3.1. Protein extraction from SCG**

18 244 Different methods have been previously developed to extract proteins from food waste matrices, and
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21 245 the biological activity of the released bioactive peptides after hydrolysis has been evaluated (Vásquez-
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23 246 Villanueva, Marina, & García, 2016; Hernández-Corroto, Marina, & García, 2018; Hernández-Corroto,
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26 247 Marina, & García, 2019). In the present work, the pH (from 6.5 to 8.5) and the DTT content (from 0.25 to
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28 248 0.75% (w/v)) of a 0.5% (w/v) SDS and 100 mM Tris-HCl buffer has been optimized to maximize the
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31 249 extraction of proteins from SCG of an espresso machine. The best buffer composition is 100 mM Tris-HCl
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33 250 (pH 8.5), 0.5% (w/v) SDS and 0.75% (w/v) DTT, which yields less than 0.3 mg of protein/100 mg of SCG.
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35 251 This quantity is low compared to the protein content of 13.6% determined by the Kjeldahl method in SCG
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38 252 (Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011) and therefore a different buffer based on urea (UB)
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40 253 has been compared with the optimized TB. By using UB, the total protein quantity obtained is 2.89 mg of
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42 254 protein/100 mg of SCG. This result highlights an increase of more than 10 times in the protein content
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45 255 when UB is used instead of TB. Moreover, the UB, the TB and the QB protein extracts have been
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47 256 characterized by SDS-PAGE, and the protein profiles are shown in **Supplementary Material Figure S1**. A
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50 257 protein band around 150 kDa is observed with the three buffers, and this band is slightly more intense
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52 258 when UB is used. Another band below 10 kDa is also observed when UB or TB are used.

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55 259 Based on the higher protein content, the UB has been selected to evaluate the protein content in SCG
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58 260 from four different coffee preparations. As observed in **Supplementary Material, Figure S2**, the highest
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60 261 protein content is obtained from espresso SCG (2.89 mg of protein/100 mg of SCG). No significant

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4 262 differences are observed between the French Press SCG (2.40 mg of protein/100 mg of SCG) and the
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6 263 Filter SCG (2.26 mg of protein/100 mg of SCG), and the lowest protein content is obtained from mocha
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9 264 SCG (1.68 mg of protein/100 mg of SCG). The total solid and the protein content of coffee brews have
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11 265 been directly related with the extraction time used for the coffee preparation (Zanoni, Pagliarini, & Peri,
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13 266 1992), but it is also known that the coffee weight-to-water volume ratio and the coffee grinding degree
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16 267 have a great impact (Cruz et al., 2012). It has been demonstrated that coffee brews from mocha
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18 268 machines contain more total solids per gram of roasted and ground coffee than coffee brews prepared
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21 269 with other procedures (Gloess, et al., 2013). In the present work we kept fixed the coffee weight-to-
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23 270 water volume ratio, the type of coffee, the roasting degree and the grinding degree, and we varied the
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25 271 pressure, temperature and extraction times, which are intrinsic parameters to the different coffee
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28 272 preparations used. The highest protein content in espresso SCG suggests that even though a higher
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30 273 pressure is used for the espresso coffee preparation, the shorter time has a greater effect on the
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32 274 extracted proteins.

36 275 **3.2. Evaluation of the peptide content and the antioxidant activity in espresso SCG hydrolysates**

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39 276 The used UB has been previously applied in different proteomics studies to extract proteins from
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41 277 complex tissues, and it has been combined with Amicon devices (3 kDa molecular cut-off filters) to
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44 278 remove contaminants before HPLC and/or MS analysis, and to hydrolyse proteins into peptides prior to
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46 279 HPLC-MS analyses (Holfeld, Valdés, Malmström, Segersten, & Lind, 2018; Wiśniewski, 2017). In the
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49 280 present study, the use of 3 kDa filters would allow to retain coffee proteins larger than 3 kDa, to discard
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51 281 the UB, and to remove free and small compounds from the samples, such as polyphenols. The use of
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53 282 these filters would also help removing big polyphenols and melanoidins that might remain on top of the
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56 283 filter after hydrolysis. This procedure has some drawbacks as very small proteins can be lost during
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58 284 sample loading and washing steps, and peptides bigger than 3 kDa can remain on top of the filter after
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4 285 the hydrolysis. Considering these aspects, the peptide content and the antioxidant activity of alcalase
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6 286 and thermolysin protein hydrolysates from espresso SCG were measured (**Table 1**). The upper part
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8 287 shows that the peptide content and the ABTS antioxidant activity are similar in both hydrolytic extracts,
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11 288 but the antioxidant activity based on the hydroxyl radical assay is higher in alcalase hydrolysates. These
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13 289 protein hydrolysates were then characterized by HPLC-UV at 325 nm (maximum UV absorbance for CGA)
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16 290 (**Figure 1A**, upper part) and at 280 nm (maximum UV absorbance for caffeine) (**Figure 1B**, upper part). It
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18 291 can be observed that caffeine (t_R 8.75 min) and other phenolic compounds remain in the samples, but no
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21 292 differences were observed between thermolysin or alcalase hydrolysates profiles. These hydrolysates
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23 293 were also characterized by HPLC-FLD at λ_{exc} = 280 nm and λ_{em} = 304 and 348 nm (maximum fluorescence
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25 294 excitation and emission wavelengths of the two amino acid residues that are primarily responsible for
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27 295 the fluorescence of peptides/proteins, tryptophan and tyrosine) (**Figures 1C and 1D**, upper part). These
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30 296 results demonstrate that thermolysin hydrolysates are more complex than alcalase hydrolysates in terms
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32 297 of peptide content.

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36 298 To try to reduce the quantity of phenolic compounds, a polyphenol extraction step was included before
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38 299 the protein extraction. The characterization of the polyphenolic extracts allowed the identification of
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40 300 caffeine, caffeic acid, 4-O-caffeoylquinic acid, 1,3-dicaffeoylquinic acid, 3-hydroxycoumarin and 1,5-
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42 301 dicaffeoylquinic acid using pure standards (**Figure 2**). In addition, a decrease of approximately 9 times
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44 302 after each extraction cycle was observed. Thereafter, the proteins were extracted and the protein
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46 303 concentration was measured, being 0.91 mg of protein/100 mg of SCG (a 69% reduction compared to the
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48 304 non-polyphenol extraction, 2.90 mg of protein/100 mg of SCG). This reduction can be explained by the
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50 305 composition of the solvent used (methanol:H₂O:acetic acid (70:28:2, v/v/v)), which has been previously
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52 306 used to extract polyphenols from coffee (Mussatto, Ballesteros, Martins, & Teixeira, 2011) or cocoa
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54 307 beans (D'Souza et al., 2017), but also to extract peptides from cocoa beans (D'Souza et al., 2018). The
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56 308 peptide content and the antioxidant activity of these extracts was also evaluated (**Table 1**, lower part). A
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4 309 decrease of 47% of the peptide content (between the two alcalase hydrolysates) was observed when the
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6 310 polyphenol extraction step was included, but this effect was not observed when thermolysin was used.
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9 311 Moreover, the polyphenol extraction step increased the antioxidant activity of thermolysin hydrolysates,
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11 312 being this effect slightly higher but not significant with respect to the alcalase hydrolysates. These results
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13 313 are unexpected since it is commonly known that phenolic compounds have higher antioxidant activity
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15 314 than peptides. However, it has been widely discussed that polyphenols can also have inhibitory effects
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17 315 on enzymes involved in the hydrolysis of proteins (Cirkovic Velickovic & Stanic-Vucinic, 2018), therefore
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19 316 the polyphenol removal prior to the hydrolysis step could increase the release of peptides with
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21 317 antioxidant activity.
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26 318 The protein hydrolysates after the polyphenol extraction were also characterized by HPLC-UV (**Figures**
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28 319 **1A and 1B**, lower part) and by HPLC-FLD (**Figures 1C and 1D**, lower part). The chromatograms obtained
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30 320 at 325 nm show that alcalase and thermolysin hydrolysates are similar, but the area of some of the
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32 321 previous observed peaks increased when polyphenols were extracted. As it will be lately discussed, these
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34 322 compounds could be released from the protein isolates during the incubation at 50 °C. On the other
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36 323 hand, the chromatograms acquired at 280 nm clearly shows that caffeine (t_R 8.75 min) almost
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38 324 disappeared when the polyphenols extraction was included. In addition, more peaks were observed after
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40 325 min 20 when thermolysin in combination with the polyphenol extraction was used. This effect was also
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42 326 observed in the HPLC-FLD chromatograms (**Figures 1C and 1D**).
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48 327 **3.3. Effect of roasting degree on thermolysin SCG hydrolysates**

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52 328 The next step was the evaluation of the roasting degree effect on the protein/peptides extracts obtained
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54 329 from different SCG (LSCG, light spent coffee grounds; MSCG, medium spent coffee grounds; DSCG, dark
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56 330 spent coffee grounds). Three independent espresso coffees were prepared and polyphenols were
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58 331 extracted before proteins were isolated using UB. The characterization of the polyphenolic extracts
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4 332 indicates that the area of caffeic acid, 4-O-caffeoylquinic acid, 1,3-dicaffeoylquinic acid and 1,5-
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6 333 dicaffeoylquinic acid decreased with the roasting process; the area of caffeine remained unchanged; and
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9 334 the area of 3-hydroxycoumarin is increased (**Supplementary Material, Figure S3**). These results agree
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11 335 well with previous reports where CGA such as 5-CQA, 4-CQA, 3-CQA, 3,5-diCQA, 4,5-diCQA, 5-FQA, 3,4-
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13 336 diCQA and 4-FQ, are degraded during roasting, whereas the formation of CGA lactones takes place
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16 337 (Farah, de Paulis, Trugo, & Martin, 2005; Moon, Yoo, & Shibamoto, 2009). The levels of these
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18 338 compounds may also be decreased by their incorporation into Maillard reaction products during the
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21 339 roasting process (Coelho et al., 2014; Delgado-Andrade & Morales, 2005; Bekedam, Roos, Schols, Van
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23 340 Boekel, & Smit, 2008). It has been suggested that melanoidins are derived from cross-linking of Maillard
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25 341 reaction products to proteins via reactive side chains of amino acids, and more recently it has been
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28 342 demonstrated that transglycosylation reactions to form new polysaccharides is the main mechanism for
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30 343 this incorporation (Moreira et al., 2017). On the other hand, the caffeine levels are not affected
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32 344 (Oestreich-Janzen, 2010).

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36 345 Thereafter, the protein concentration was evaluated by Bradford assay, indicating that DSCG had higher
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38 346 amount of proteins (1.49 mg of protein/100 mg of SCG) than MSCG (1.08 mg of protein/100 mg of SCG),
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41 347 and three times more than LSCG (0.49 mg of protein/100 mg of SCG) (**Supplementary Material, Figure**
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43 348 **S4**). The SDS-PAGE analysis also shows a slightly more intense protein bands of \approx 150 kDa in DSCG
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45 349 (**Supplementary Material, Figure S5**). The higher protein concentration of DSCG could be partially
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48 350 explained by the loss coffee weight during the roasting process (13% for light, 15% for medium, and 17%
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50 351 for dark roasted beans), which can enrich the material that is not degraded or loss. However, darker
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52 352 roasting has shown to produce more total soluble solids in the brew (Petracco 2005), and therefore
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55 353 there should be fewer proteins in their SCG. Moreover, it is also known that coffee proteins can be
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57 354 fragmented, polymerized or integrated into melanoidins through the Maillard reaction during the
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59 355 roasting process, but it is not well known how this reaction affects the functional properties (such as the
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4 356 solubility) of proteins (Oliver, Melton, & Stanley, 2006). After protein quantification, the peptide content,
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6 357 the antioxidant activity and the *in vitro* ACE-inhibitory activity of thermolysin protein hydrolysates from
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9 358 LSCG, MSCG and DSCG were measured (**Table 2**). For comparison purposes, DSCG protein isolates were
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11 359 also hydrolysed with alcalase, and a control sample incubated without enzyme was included. As
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14 360 expected due to the higher amount of proteins, the highest peptide concentration among the three
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16 361 thermolysin hydrolysates was obtained in DSCG, followed by MSCG and LSCG (**Table 2**). There were not
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18 362 significant differences on the ABTS or hydroxyl scavenging activity between the samples, but the DSCG
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21 363 and the MSCG hydrolysates had higher *in vitro* ACE-inhibitory activity. The protein hydrolysates were
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23 364 then characterized by HPLC-UV and HPLC-FLD (**Supplementary Material, Figures S6-S9**). The acquisition
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25 365 at 280 nm shows that the area of some peaks are slightly increased (peaks at t_R 5.5 min and 14.7 min);
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28 366 some of them remained unchanged (peaks at t_R 23.0 min, 25.2 min, 25.5 min and 26.0 min); and others
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30 367 are decreased (peaks at t_R 19.0 min and 27.0 min) with the roasting process (**Supplementary Material,**
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33 368 **Figures S6**); and the chromatograms acquired at 325 nm indicate that most of the peaks were
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35 369 significantly increased with the roasting process (**Supplementary Material, Figures S7**). There were not
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37 370 significant differences in the FLD-chromatograms acquired at $\lambda_{exc} = 280$ nm and $\lambda_{em} = 304$ nm, but when
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40 371 λ_{em} was set to 348 nm (**Supplementary Material, Figures S8**), the general trend was a decrease in the
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42 372 area when increasing the roasting degree (**Supplementary Material, Figures S9**). As previously
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44 373 commented, the signals observed at 325 nm might be due to the presence of phenolic compounds that
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47 374 can be released during the incubation step at 50 °C. It has been observed that the incorporation of
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49 375 phenolic compounds into the melanoidins is a significant pathway of CGA degradation during roasting
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52 376 (Coelho et al., 2014), being transglycosylation reactions the main mechanism for this incorporation
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54 377 (Moreira et al., 2017). Different techniques such as alkaline hydrolysis, acid hydrolysis, the increase of
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56 378 the medium ionic strength, or the alkaline fusion method have been applied to release them (Bekedam,
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59 379 Roos, Schols, Van Boekel, & Smit, 2008; Monente, Ludwig, Irigoyen, De Peña, & Cid, 2015; Perrone,
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4 380 Farah, & Donangelo, 2012; Delgado-Andrade & Morales, 2005; Coelho et al., 2014). For instance,
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6 381 covalently bound caffeic and ferulic acids decrease with roasting, while the content of dihydrocaffeic
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8 382 acid increases (Perrone, Farah, & Donangelo, 2012). And by using the alkaline fusion method, it has been
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10 suggested that the incorporation of phenolic compounds in coffee melanoidins is also related to the
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12 amount of proteins (Coelho et al., 2014). Complementary, the antioxidant activity of melanoidins from
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14 coffee brews obtained using roasted coffees is higher than those obtained from green coffees (Delgado-
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16 Andrade, Rufián-Henares, & Morales, 2005; Perrone, Farah, & Donangelo, 2012). Based on these results,
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18 different CGA acids could have been released from melanoidins, or some melanoidins could still be
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20 present in the SCG protein hydrolysates, but further studies are needed to confirm these hypotheses.
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26 389 **3.4. Evaluation of the enzymatic incubation on SCG hydrolysates**

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29 390 To evaluate the effect of the enzymatic incubation, DSCG protein isolates were also hydrolysed with
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31 alcalase, and a control sample incubated without enzyme was included. As expected from our previous
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33 results (**Table 1**), lower amounts of peptides were obtained when alcalase was used (**Table 2**), and the
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35 antioxidant activity was similar when the different enzymes were used (a significantly lower ABTS activity
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37 was observed when the samples were incubated with no enzyme). However, the ACE-inhibitory activity
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39 of alcalase hydrolysates was lower than its thermolysin counterpart, and less than 50% of the capacity
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41 was found when no enzyme was added (**Table 2**). The HPLC-UV characterization demonstrated that
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43 there were not significant differences in the intensity of peaks obtained at 325 nm (**Figure 3A**), but a
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45 more complex profile was obtained at 280 nm when thermolysin was used (**Figure 3B**). These differences
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47 were greater when the characterization was performed using HPLC-FLD (**Figures 3C and 3D**). Altogether,
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49 these results suggest that the antioxidant activity of the protein hydrolysates is partly derived from the
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51 released polyphenols during the incubation step, but the ACE-inhibitory activity is mainly derived from
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53 the released peptides, being higher when thermolysin enzyme is used.
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3.5. Identification of peptides and polyphenols in SCG hydrolysates by RP-HPLC-ESI-Q-TOF-MS/MS

To identify the released peptides, thermolysin hydrolysates from LSCG, MSCG and DSCG, alcalase hydrolysates from DSCG, and DSCG proteins incubated without enzyme were analyzed by RP-HPLC-ESI-Q-TOF in positive mode (**Supplementary Material, Figures S10A and S10C**). The chromatographic and MS/MS parameters were similar to those applied in a recent work for the identification of peptides in coffee silverskin protein hydrolysates (Pérez-Míguez, Marina, & Castro-Puyana, 2019). As a first step, MS/MS data were searched against *Coffea arabica* proteome, and the peptides (and proteins containing those peptides) are reported in **Table 3**. In total, peptides belonging to 35 different proteins were identified based on the peptide sequence. The most overrepresented proteins in these peptides were PSAB and RPOC2 (11 peptides), followed by NU5C and TI214 (8 peptides), ATPB and YCF2 (7 peptides), and RPOB (4 peptides). The PSAB protein amino acid sequence and the identified peptides in MSCG is shown as example in **Supplementary Material, Figure S11**. The sample with the highest number of peptides was the MSCG thermolysin hydrolysate (34 peptides), followed by the LSCG thermolysin hydrolysate (25 peptides), and the DSCG alcalase hydrolysates (25 peptides). 16 peptides were also identified in DSCG thermolysin hydrolysate, 5 of them belonging to YCF2. In addition, 14 peptides could be identified in the sample where no enzyme was added, demonstrating that some peptides can also be released by the incubation processes (as observed for polyphenols). It is interesting to note that some proteins could only be identified in one specific sample, such NDHK in LSCG thermolysin hydrolysates, ACCD and PSBA in MSCG thermolysin hydrolysates, SPDE in DSCG alcalase hydrolysates, or YCF2, identified in all DSCG hydrolysates.

To complement the previous information, the *de novo* sequencing tool from the PEAK Software was used to carry out the tentative identification of more peptides. **Table 4** summarizes the peptides identified by MS/MS in thermolysin hydrolysates along with their experimental molecular masses, ALC and accuracy.

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4 426 Different peptides with a number of amino acids ranging from 4 to 10 were identified, and none of these
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6 427 peptides belong to the enzymes employed for hydrolysis. In the case of DSCG alcalase hydrolysates, 5
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8 428 peptides (ALM(+15.99)APH, M(+15.99)EGL, CCVLLP, NVLAR and NLM(+15.99)APH, **Supplementary**
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10 **Material, Table S1**) were identified, and only one peptide (SHWH) was identified when no enzyme was
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12 429 used. In overall, thermolysin hydrolysates present a higher number of peptides (12 in LSCG, 14 in MSCG
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14 430 and 10 in DSCG) and, when combining the results with the database search, 37, 48 and 26 peptides were
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16 431 identified in LSCG, MSCG and DSCG thermolysin hydrolysates, respectively; 30 peptides in DSCG alcalase
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18 432 hydrolysates; and only 15 peptides when no enzyme was used. The possible antioxidant or
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20 433 antihypertensive/ACE-inhibitory activity of these peptides was searched in the BIOPEP database
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22 434 (<http://www.uwm.edu.pl/biochemia/index.php/en/biopep>), but none of them have been reported. In
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24 435 overall, these results indicate that the peptide complexity of thermolysin hydrolysates is higher than
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26 436 alcalase hydrolysates, and some of the thermolysin released peptides (or a combination of them) would
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28 437 be responsible for the higher ACE-inhibitory activity observed. Since LSCG presents some antioxidant and
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30 438 ACE inhibitory activities, the common peptides identified in the three thermolysin hydrolysates using the
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32 439 *de novo* sequencing tool (CSDAVGVK and RCPQGGTHYG), and the common peptides identified as coming
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34 440 from LSCG, MSCG and DSCG proteins (YKPPYS and CVIPSN) would be the first candidates to be evaluated
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36 441 for their biological activity. The tentative identification of CSDAVGVK peptide in DSCG is shown as
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38 442 example in **Supplementary Material, Figure S12**. Moreover, the higher ACE-inhibitory activity observed
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40 443 for MSCG and DSCG thermolysin hydrolysates would be explained by the higher number of identified
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42 444 peptides (48 in the case of MSCG), or by the specific sequence of the identified peptides in the
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44 445 MSCG/DSCG thermolysin hydrolysates, but not in the LSCG or the alcalase hydrolysates. The following
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46 446 peptides meet this requirement: DPGDKKN, CASDPAQ and RLNQ, identified in the MSCG and the DSCG
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48 447 using the *de novo* sequencing tool; GGSMG, GVMDFQ, LDPIEF, GGGDL, YEAWL, DAHPPG, DPHFGQPAVE,
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50 448 FGM(+15.99)NSLS, FPCDGP, GPVNIAY, GMAST and LGMAST, identified as coming from MSCG proteins;
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4 450 and FLSRSD, IPNIH, LSDM(+15.99)NLS, M(+15.99)VDSFH and NRRGY, identified as coming from DSCG
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6 451 proteins.
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10 452 Additionally, RP-HPLC with UV (at 325 nm) and MS (in ESI negative mode) detectors were used to
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12 453 tentatively identify the possible remaining polyphenols in SCG protein hydrolysates (**Supplementary**
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14 454 **Material, Figures S10B and S10D**). Among the different observed peaks in **Figure 3A**, peaks **1–5** showed
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16 455 a $[M-H]^-$ ion at m/z 367.1, with different product ions at m/z 193.0, 191.0, 173.0 and 134.0, while peak **6**
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18 456 showed a $[M-H]^-$ ion at m/z 193.0 and MS/MS product ions at m/z 178 and m/z 134). The identification
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21 457 of these polyphenols was performed by analysing the MS and MS/MS spectra (as exemplified in **Figure 4**
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24 458 for 3-FQA), and comparing them with the fragmentation patterns already reported (Kuhnert, Jaiswal,
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26 459 Matei, Sovdat, & Deshpande, 2010; Clifford, Johnston, Knight, & Kuhnert, 2003). Based on these
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29 460 analyses, peak **1** was tentatively assigned as 3-FQA, peak **2** as 1-FQA, peaks **3** and **5** as 5-FQA, and peak **4**
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31 461 as 4-FQA. However, peak **6** could not be identified. These phenolic compounds have already been
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33 462 identified in SCGs (Panusa, Zuurro, Lavecchia, Marrosu, & Petrucci, 2013; Bravo et al., 2012), and they
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36 463 might be partly responsible for the antioxidant activity exerted by the extracts.
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39 464 **4. Conclusion**

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42 465 In conclusion, the urea-based extraction buffer allows the extraction of more proteins than the
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44 466 conventional Tris-HCl buffers, and its application demonstrates that the highest protein content is
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47 467 obtained from espresso SCG. Moreover, the use of 3 kDa molecular cut-off filters allows the removal of
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49 468 the buffer and the hydrolysis of the proteins using thermolysin and alcalase enzymes, but not the
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52 469 complete removal of polyphenols. Moreover, these protein hydrolysates possess antioxidant and ACE-
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54 470 inhibitory activities, the latter being highest when SCG samples are obtained from dark/medium roasted
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57 471 coffees, and after the inclusion of a polyphenol step removal. Finally, several peptides that might be
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59 472 responsible for the ACE-inhibitory activity observed have been identified. However, further experiments
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473 using synthetic peptides are needed to confirm which of the released peptides (or a combination of
474 them) are the most bioactive.

475
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481 **Conflict of Interest**

482 The authors declare no conflict of interest

483
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621 **Figure Captions**

622 **Figure 1.** HPLC chromatograms of SCG proteins hydrolysed with alcalase and thermolysin (including the
623 polyphenol extraction step). Chromatographic conditions were: Ascentis Express C18 analytical column
624 (100 mm × 2.1 mm, 2.7 μm); gradient: 5–35% B in 30 min, 35–50% B in 5 min, 50–95% B in 5 min, and
625 95–5% B in 2 min; mobile phases: water with 0.1% (v/v) of FA (A) and ACN with 0.1% (v/v) FA (B); flow-
626 rate, 0.25 mL/min; temperature, 30 °C; injected volume, 5 μL; absorbance detection was performed at λ
627 of 325 nm (A) and 280 nm (B), and fluorescence was acquired at λ_{exc} of 280 nm and λ_{em} of 304 nm (C) and
628 348 nm (D).

629 **Figure 2.** HPLC chromatograms obtained from methanol:H₂O:acetic acid (70:28:2, v/v/v) extracts after
630 one, two or three extraction cycles before SCG protein extraction. Separation conditions were the same
631 as in **Figure 1**, and UV absorbance was detected at λ of 325 nm.

632 **Figure 3.** HPLC chromatograms of DSCG protein hydrolysed with thermolysin, alcalase or “no enzyme”
633 with polyphenol extraction. Separation conditions were the same as in **Figure 1**, absorbance detection
634 was performed at λ of 325 nm (A) and 280 nm (B), and fluorescence was acquired at λ_{exc} of 280 nm and
635 λ_{em} of 304 nm (C) and 348 nm (D). Peaks were lately identified by RP-HPLC-ESI-Q-TOF-MS/MS as: **1**, 3-
636 FQA; **2**, 1-FQA; **3**, 5-FQA; **4**, 4-FQA; **5**, 5-FQA; **6**, not identified.

637 **Figure 4.** Tentatively identification of 3-FQA in SCG protein hydrolysates by RP-HPLC-ESI(-)-Q-TOF-
638 MS/MS analysis.

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4 **639 Tables**

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7 **640 Table 1.** Peptide content and antioxidant activity (ABTS and hydroxyl assays) of SCG (after espresso
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10 **641** preparation of commercial medium roasted 100% Arabica coffee) proteins hydrolysed with alcalase and
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12 **642** thermolysin (and including the polyphenol extraction step).
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Sample	Peptide content (mg/mL)	ABTS assay (%) ¹	Hydroxyl radical assay (%)
Alcalase	0.43 ^a ± 0.08	22.1 ^b ± 5.20	88.4 ^a ± 11.6
Thermolysin	0.42 ^a ± 0.05	19.8 ^b ± 11.3	73.5 ^b ± 3.55
Alcalase (with polyphenol extraction)	0.23 ^b ± 0.02	30.7 ^{a,b} ± 0.52	84.3 ^{a,b} ± 4.85
Thermolysin (with polyphenol extraction)	0.39 ^a ± 0.05	36.1 ^a ± 2.39	95.4 ^a ± 2.70

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25 **643** *Different letters indicate significant differences between samples after ANOVA with LSD Post-hoc, p-value < 0.05.
26 **644** ¹ For ABTS antioxidant activity, samples were diluted to 1:150.
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31 **646 Table 2.** Peptide content, antioxidant activity (ABTS and hydroxyl assays) and *in vitro* ACE-inhibitory
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33 **647** activity of thermolysin hydrolysates from light, medium and dark espresso SCG (LSCG, MSCG, DSCG),
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35 **648** alcalase hydrolysates from DSCG, and incubation of DSCG without enzyme.
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Sample	Peptide content (mg/mL)	ABTS assay (%) ¹	Hydroxyl radical assay (%)	<i>In vitro</i> inhibitory (%)	ACE-activity
LSCG – Thermolysin	0.22 ^b ± 0.05	30.5 ^{a,b} ± 4.26	85.7 ^{a,b} ± 7.84	61.7 ^b ± 4.98	
MSCG– Thermolysin	0.31 ^{a,b} ± 0.09	28.7 ^{a,b} ± 9.47	81.6 ^b ± 10.2	83.0 ^a ± 2.72	
DSCG – Thermolysin	0.41 ^a ± 0.08	32.9 ^{a,b} ± 4.28	92.2 ^a ± 11.3	81.5 ^a ± 1.51	
DSCG – Alcalase	0.21 ^b ± 0.07	39.8 ^a ± 5.49	96.1 ^a ± 1.89	61.5 ^b ± 3.84	
DSCG – No enzyme	0.25 ^b ± 0.03	24.8 ^b ± 8.31	83.4 ^{a,b} ± 6.27	37.7 ^c ± 9.71	

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51 **649** *Different letters indicate significant differences between samples after ANOVA with LSD Post-hoc, p-value < 0.05.
52 **650** ¹ For ABTS antioxidant activity, samples were diluted to 1:150.
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Table 3. Peptide sequence, belonging gene and protein name, molecular mass (Da) and $-10\lg P$ of the peptides identified in thermolysin hydrolysates from light, medium and dark SCG (LSCG, MSCG, DSCG), alcalase hydrolysates from DSCG, or DSCG incubated with no enzyme, using LC-MS/MS and database search.

Peptide sequence	Gene name	Protein name	Molecular mass (Da)	$-10\lg P$				
				Thermolysin			Alcalase	No Enzyme
				LSCG	MSCG	DSCG	DSCG	DSCG
GGSMG	ACCD	Acetyl-coenzyme A carboxylase carboxyl transferase	407.1475		15.2			
GVMDFQ	ACCD		695.2949		22.9			
LDPIEF	ACCD		732.3694		16.7			
EVIAVNQ	AGAL	Alpha-galactosidase	771.4127		20.1			
AVATDT	ATPA	ATP synthase subunit alpha	576.2755					18.1
GDGLMI	ATPA		604.2891				18.6	
GIARI	ATPA		528.3384				16.3	
AVAM(+15.99)SS	ATPB	ATP synthase subunit beta	580.2527					17.8
DTGAP	ATPB		459.1965		15.7			
GAVDT	ATPB		461.2122	20.1	23.0			
NLGAV	ATPB	ATP synthase subunit epsilon	472.2645	15.3				
PGARMR	ATPB		686.3646					27.8
TRGM(+15.99)E	ATPB		608.2588				15.7	
YM(+15.99)EM(+15.99)K	ATPB	ATP synthase epsilon	732.2822	20.0				
RIVWDS	ATPE		774.4024	40.4	39.4			
TFSTVRD	CCS1		Caffeine synthase 1	824.4028				15.0
TILHF	CEMA	Chloroplast envelope membrane protein	629.3537				30.4	
GPNTM(+15.99)	CS3	Probable caffeine synthase 3	534.2108				27.0	
LIAAM(+15.99)PGSF	CS4	Probable caffeine synthase 4	921.4630	23.6				
GAMPGS	DXMT1	3,7-dimethylxanthine N-methyltransferase	518.2159	24.3	27.4			
SRPPI	DXMT1		568.3333				22.7	
KIRPPG	NDHH	NAD(P)H-quinone oxidoreductase H	666.4177			17.8	15.5	
YDVAPGG	NDHJ	NAD(P)H-quinone oxidoreductase J	677.3020				17.1	
FDFDRYG	NDHK	NAD(P)H-quinone oxidoreductase K	918.3871	16.5				
TITGGM	NDHK		578.2734	20.6				
NSSST	NU1C	NAD(P)H-quinone oxidoreductase 1	494.1973			26.8		
FVMAIGM(+15.99)I	NU4C	NAD(P)H-quinone oxidoreductase 4	896.4500			19.1		
YFFDSG	NU4C		734.2911		18.4	16.7		
AFSTMSQ	NU5C	NAD(P)H-quinone oxidoreductase 5	770.3269	30.5				
KPPYS	NU5C		590.3064				22.3	
LAFSTMSQ	NU5C		883.4109	18.1				
LFPTATK	NU5C	NAD(P)H-quinone oxidoreductase 5	776.4432	22.5				
LWGRG	NU5C		587.3180	16.3				
WIINN	NU5C		658.3439	15.5				
YKPPYS	NU5C		753.3697	28.1	27.6	18.4		

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YKSQNM	NU5C		769.3429	16.1		
HNYYGEP	PETD	Cytochrome b6-f complex	949.3929	15.2	23.9	
GGGDL	PSAA	Photosystem I P700	417.1859	16.3		
YEAWL	PSAA	chlorophyll a apoprotein A1	680.3170	28.4		
DAHIPP	PSAB		705.3445	15.3		
DPHFGQPAVE	PSAB		1095.4985	16.9		
FGM(+15.99)NLS	PSAB		770.3269	17.7		
FPCDGP	PSAB		634.2421	18.6		
GPVNIAY	PSAB	Photosystem I P700 chlorophyll a apoprotein A2	732.3806	30.4		
NVLAR	PSAB		571.3442			26.4
RGGALG	PSAB		529.2972			15.8
RTNFGIGH	PSAB		900.4566	18.7		
SLAWT	PSAB		576.2908			15.1
SRGEY	PSAB		610.2711	18.1		
VLPHPQ	PSAB		689.3860	15.0		
DGMPLG	PSBA	Photosystem II protein D1	588.2578	20.4		
VMHERNAH	PSBA		992.4611	24.7		
GHASFA	PSBB	Photosystem II CP47 reaction center protein	588.2656			21.7
M(+15.99)GLPWY	PSBB		781.3469			15.4
SDPYGLT	PSBB		751.3388	21.1	18.9	
PVVFAS	PSBZ	Photosystem II reaction protein z	618.3377		20.6	
YKGRCYH	RBL	Ribulose bisphosphate carboxylase	925.4229	17.3		
NPVDH	RK2A	50S ribosomal protein L2-A	580.2605	17.8		
M(+15.99)AVPK	RK32	50S ribosomal protein L32	560.2992			48.5
AAATVGGE	RPOB	DNA-directed RNA polymerase subunit beta	674.3235	17.9		
AEELY	RPOB		623.2802			23.3
EGMATI	RPOB		620.2839			22.1
RSNKNTC	RPOB		821.3814	15.1		
NNTLT	RPOC1	DNA-directed RNA polymerase subunit beta'	561.2759		21.4	
ASFQET	RPOC2		681.2969	20.2		
EAVGI	RPOC2		487.2642	24.7		
GLMSDPQGQM(+15.99)	RPOC2		1078.4424	15.9		
GTIEM(+15.99)	RPOC2		565.2418			20.1
IDHFGM	RPOC2	DNA-directed RNA polymerase subunit beta''	718.3109			15.6
LGGPC	RPOC2		445.1995	24.2		
NQDIGIEL	RPOC2		900.4553		28.1	16.3
QEREN	RPOC2		674.2983	17.8		
SGARG	RPOC2		446.2237			16.7
SIDSISM	RPOC2		751.3422	18.4		
SSGIT	RPOC2		463.2278	15.1		
HFGHGT	RR2	30S ribosomal protein S2	654.2874		20.6	
GMAST	RR4	30S ribosomal protein S4	465.1893		32.0	
LGMAST	RR4		578.2734		22.1	
EAAAQ	SPDE		546.2285			15.6
EIDKM	SPDE	Spermidine synthase	634.2996			19.2
IAHLP	SPDE		549.3275			25.2
CVIPSN	TI214	Protein TIC 214	631.2999	33.2	27.1	25.7

EM(+15.99)KGT	TI214		580.2527			22.8
FGEMIK	TI214		723.3625		25.2	
NNIPF	TI214		603.3016		30.0	
RWVYT	TI214		723.3704	17.5		
TGQLM(+15.99)	TI214		564.2578			46.6
TVWGM(+15.99)	TI214		608.2628		21.8	
WGDALN	TI214		674.3024			15.9
GAMPGS	XMT1	Monomethylxanthine	518.2159		22.0	
NDLFP	XMT1	methyltransferase 1	604.2856		17.4	
FLSRSD	YCF2		723.3551		18.4	
GNM(+15.99)LGPA	YCF2		674.3057			16.8
IPNIH	YCF2	Protein Ycf2	592.3333		15.3	
KNTQEK	YCF2		746.3923		27.1	
LSDM(+15.99)NLS	YCF2		794.3480		16.5	
M(+15.99)VDSFH	YCF2		750.3007		19.6	16.3
NRGGY	YCF2		565.2609		16.3	
GSRKIS	YCF4	Photosystem I	646.3762			18.0
VGSVG	YCF4	assembly protein Ycf4	417.2224		17.4	
WNVGN	YCF4		588.2656			15.4

* Light, medium and dark shades correspond to light (LSCG), medium (MSCG) and dark (DSCG) thermolysin hydrolysates, respectively.

Table 4. Peptide sequence, retention time (RT), molecular mass, mass accuracy and average local confidence (ALC) of the peptides identified in thermolysin hydrolysates from light, medium and dark SCG (LSCG, MSCG, DSCG) using LC-MS/MS and the *de novo* sequencing tool.

ID	Peptide sequence	RT (min)	Molecular mass (Da)	LRC		MRC		DRC	
				Mass accuracy (ppm)	ALC (%)	Mass accuracy (ppm)	ALC (%)	Mass accuracy (ppm)	ALC (%)
1	DPGDKKN	2.4	772.3715	-	-	7 ± 3	88 ± 5	6 ± 2	87 ± 2
2	SSSDPAQ	6.1	690.282	5 ± 1	89 ± 2	8 ± 1	89 ± 1	-	-
3	CASDPAQ	6.4	690.2643	-	-	9 ± 0	88 ± 1	9 ± 1	86 ± 2
4	AWAH	8.5	483.223	10 ± 0	82 ± 0	3 ± 0	84 ± 0	1 ± 1	83 ± 1
5	M(+15.99)EGSTSSGL	9.2	883.3593	5 ± 2	81 ± 1	2 ± 2	82 ± 1	5 ± 2	81 ± 1
6	GWAEGR	9.9	674.3136	1 ± 1	84 ± 2	-	-	-	-
7	RLNQ	12.2	529.2972	-	-	6 ± 3	83 ± 0	4 ± 2	83 ± 1
8	M(+15.99)DAVGVK	14.2	734.3633	8 ± 2	95 ± 1	5 ± 2	93 ± 2	2 ± 2	94 ± 1
9	NAGHM(+15.99)PN	14.8	755.3021	9 ± 0	90 ± 1	4 ± 3	88 ± 2	-	-
10	VTYDYYQN	15.1	1064.4451	2 ± 0	91 ± 0	4 ± 3	90 ± 1	8 ± 0	90 ± 1
11	M(+15.99)APHWN	18.1	770.317	8 ± 1	90 ± 0	4 ± 2	90 ± 2	-	-
12	RNSGLLNQ	18.5	900.4777	8 ± 1	88 ± 2	10 ± 0	88 ± 0	-	-
13	EANLDVVAHE	18.7	1095.5195	2 ± 1	88 ± 5	4 ± 3	88 ± 3	-	-

14	AATYDYYNQ	18.8	1107.4509	1 ± 1	91 ± 1	4 ± 3	89 ± 1	8 ± 0	90 ± 1
15	CSDAVGVK	19.9	777.3691	8 ± 1	92 ± 2	4 ± 2	91 ± 1	2 ± 1	91 ± 2
16	RCPQGGTHYG	20.2	1074.4666	6 ± 1	84 ± 1	4 ± 2	83 ± 1	4 ± 1	84 ± 1
17	NFDAVGVQ	21.2	848.4028	6 ± 2	88 ± 1	3 ± 2	87 ± 3	5 ± 2	89 ± 2
18	M(+15.99)WDGSQM	21.6	869.3048	5 ± 1	88 ± 1	3 ± 5	85 ± 1	6 ± 1	87 ± 2
19	RM(+15.99)APH	21.8	626.2958	1 ± 1	92 ± 1	4 ± 2	91 ± 1	9 ± 0	91 ± 1
20	LM(+15.99)APHWN	22.4	883.4011	3 ± 2	89 ± 3	1 ± 2	86 ± 2	-	-
21	M(+15.99)GLSDLT	22.9	751.3422	7 ± 2	82 ± 1	4 ± 2	84 ± 2	1 ± 2	86 ± 3
22	CSAPHW	26.2	699.2798	9 ± 0	81 ± 0	4 ± 3	82 ± 1	1 ± 1	84 ± 2
23	WLPPF	27.4	658.3478	5 ± 2	92 ± 1	7 ± 1	93 ± 1	-	-

* Dark shade: peptides identified in < 5 injections. Only isoforms with leucine (L) are presented in these results although peptide sequences containing isoleucine (I) instead of L are also possible.

Figures

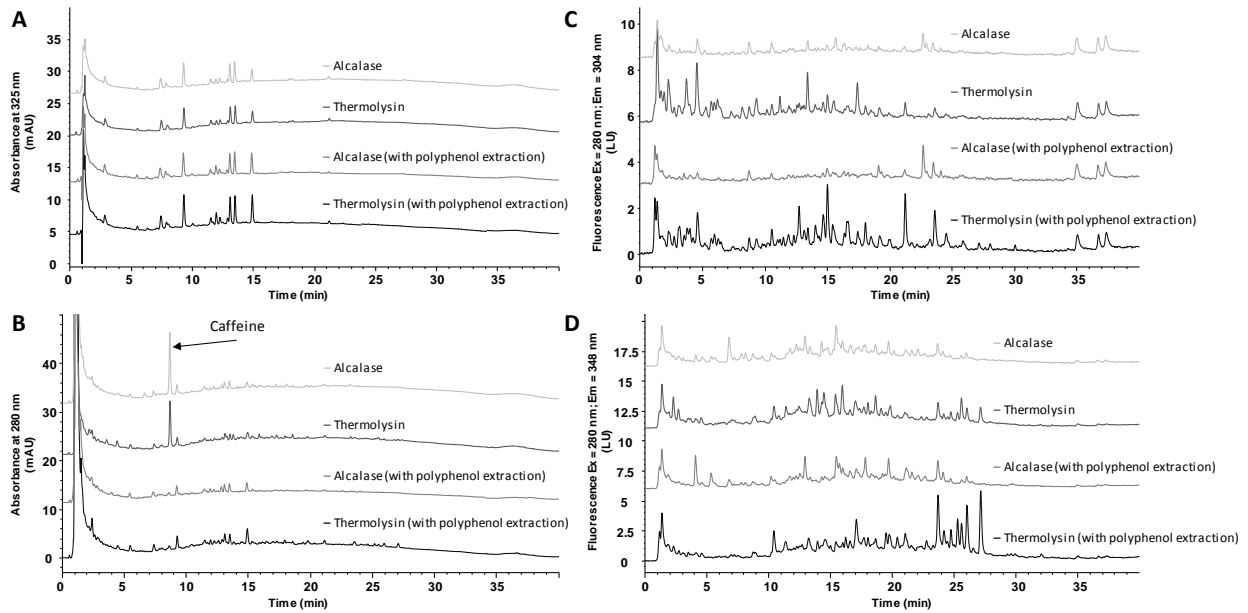
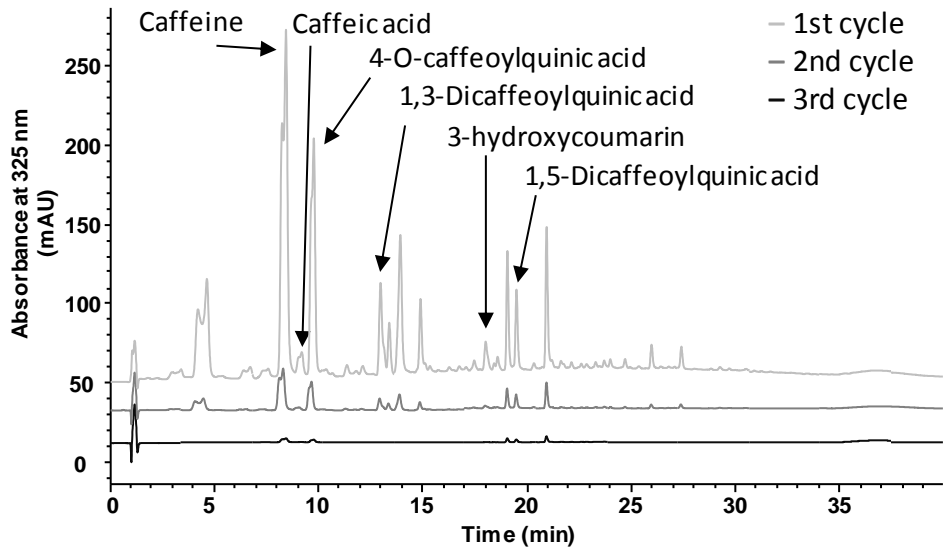


Figure 1

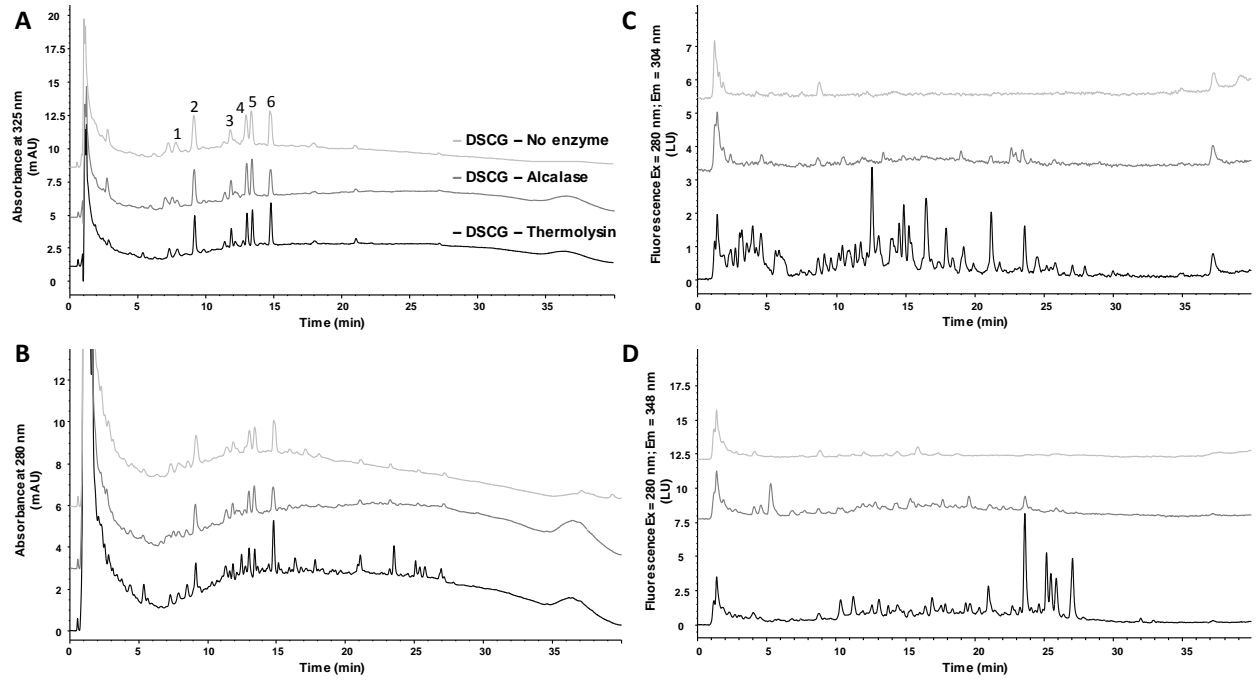
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669 **Figure 2**

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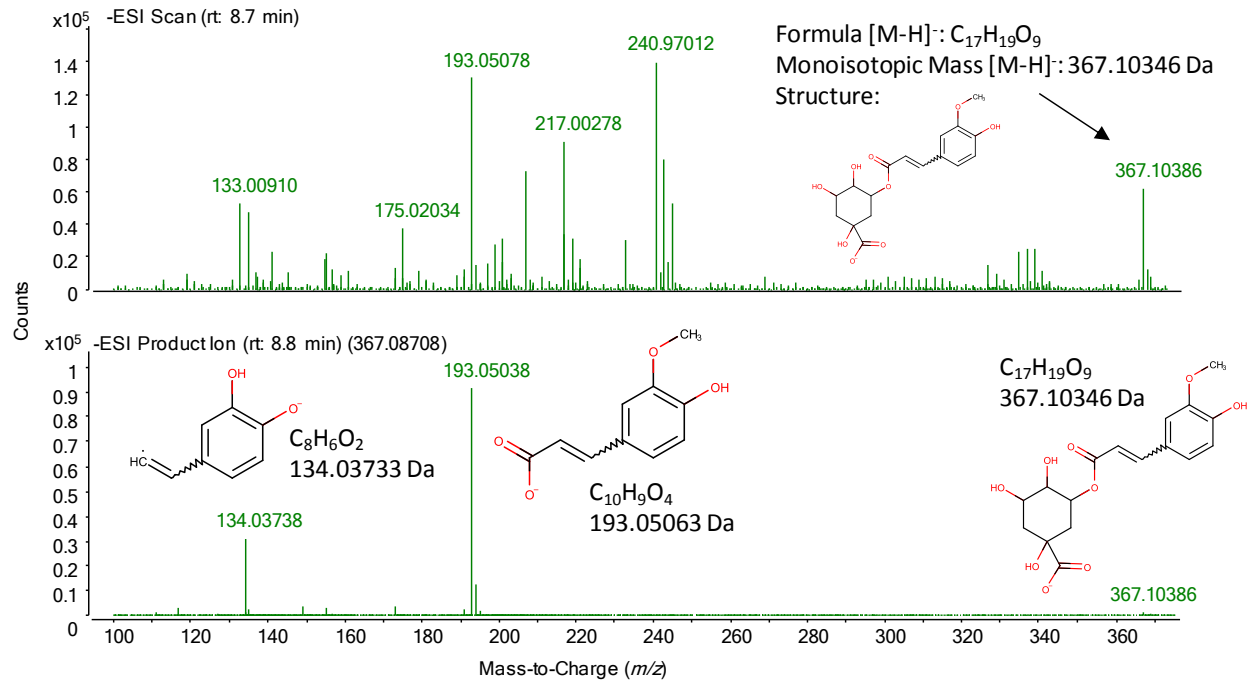


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671 **Figure 3**

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674 **Figure 4**

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14 **Abstract**

15 Several works have been focused on the extraction ~~and application~~ of polysaccharides, polyphenols and
16 caffeine from spent coffee grounds (SCG) and their application in food formulations, but the peptide
17 bioactivity from SCG protein hydrolysates has never been addressed. In the present work and for the
18 first time, two different methods to isolate proteins from SCG have been compared, demonstrating that
19 a urea-based extraction buffer provides a higher yield. This extraction method was then applied to
20 compare the protein content in SCG from different coffee-brewing preparations, showing a higher
21 protein content in SCG from espresso coffee machines. In addition, a polyphenol extraction step to
22 remove interferences has been evaluated and the hydrolysis of the extracted proteins using alcalase and
23 thermolysin enzymes has been compared. The effect of roasting degree on the antioxidant and *in vitro*
24 angiotensin-converting enzyme (ACE)-inhibitory activity has been evaluated. The results show that the
25 ACE-inhibitory activity is higher when SCG proteins are obtained from medium and dark roasted coffees
26 and then hydrolyzed with thermolysin. Finally, the peptides contained in these hydrolysates have been
27 identified by reversed-phase high-performance liquid chromatography coupled via electrospray
28 ionization to a quadrupole time-of-flight mass spectrometer (RP-HPLC-ESI-Q-TOF)-~~technology~~.

31 **Keywords**

32 Bioactivity; Liquid chromatography–tandem mass spectrometry; peptide; polyphenol; spent coffee
33 grounds.

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34 **1. Introduction**

35 Coffee industry produces a large amount of residues that can be >50% of the fruit mass in the coffee
36 producing countries (Tsai, Liu & Hsieh, 2012). Among them, the solid residues obtained during the
37 brewing process, the so called spent coffee grounds (SCG) (Cruz et al., 2012), are usually incinerated or
38 disposed of in landfills with the subsequent air pollution and soil contamination. Therefore, strategies for
39 the proper management of these residues are needed. Due to the large amounts of organic compounds
40 in SCG (Campos-Vega, Loarca-Piña, Vergara-Castañeda, & Oomah, 2015), these residues have been used
41 as a source of value-added products, such as for biodiesel production (Karmee, Swanepoel, & Marx,
42 2018), as a precursor for activated carbon production (Kante, Nieto-Delgado, Rangel-Mendez, &
43 Bandosz, 2012), to formulate products for animal feeding (Givens & Barber, 1986; Xu, Cai, Zhang, &
44 Ogawa, 2006), or to extract polysaccharides as a first step to exploit SCG in fermentative processes
45 (Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011). SCG are also rich in antioxidant compounds such
46 as phenols and other non-protein nitrogenous compounds such as caffeine, which have been associated
47 with health benefits (Campos-Vega, Oomah, Loarca-Piña, & Vergara-Castañeda, 2013; Campos-Vega,
48 Loarca-Piña, Vergara-Castañeda, & Oomah, 2015). The main phenolic compounds in SCG residues are
49 similar to those obtained in coffee brews, being chlorogenic acids (CGA) such as caffeoylquinic acids
50 (CQAs), dicaffeoylquinic acids (diCQAs), feruloylquinic acids (FQAs), caffeoylquinic acid lactones (CQLs),
51 feruloylquinic acid lactones (FQLs) and p-coumaroylquinic acids (pCoQAs) the most abundant (Bravo,
52 Arbillaga, de Peña, & Cid, 2013; Farah, de Paulis, Trugo, & Martin, 2005; Panusa, Zuorro, Lavecchia,
53 Marrosu, & Petrucci, 2013). Furthermore, several works have demonstrated that the concentration of
54 these compounds depends on the brewing and roasting procedures (Gloess et al., 2013; Ludwig et al.,
55 2012; Bravo, et al., 2012; Cruz et al., 2012). Consequently, the extraction of phenolic compounds from
56 SCG has been extensively studied using different solvents, solvent-to-solid ratios, or extraction times
57 (Yen, Wang, Chang, & Duh, 2005; Mussatto, Ballesteros, Martins, & Teixeira, 2011). Moreover, CGA can

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4 58 also be incorporated into melanoidins, mainly by transglycosylation reactions (Moreira et al., 2017).

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6 59 Melanoidins are the high molecular weight nitrogenous and brown-coloured compounds formed during
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9 60 the roasting process of coffee (Bekedam, Roos, Schols, Van Boekel, & Smit, 2008; Moreira, Nunes,
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11 61 Domingues, & Coimbra, 2012), and the incorporation of phenolic compounds into the melanoidins is a
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13 62 significant pathway of CGA degradation during roasting (Coelho et al., 2014). Moreover, it has also been
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15 63 shown that the coffee preparation affects the concentration of these compounds (Bravo et al., 2012).

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19 64 Apart from the mentioned compounds, the mean protein content of SCG is 13.6% (Mussatto, Carneiro,
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21 65 Silva, Roberto, & Teixeira, 2011; Silva, Nebra, Machado-Silva, & Sanchez, 1998), but this content might
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23 66 be overestimated due to the presence of other nitrogen-containing substances such as caffeine,
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25 67 trigonelline, free amines and/or amino acids (Delgado, Vignoli, Siika-aho, & Franco, 2008). The protein
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27 68 content also varies depending on the brewing and roasting processes, when the proteins can be
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29 69 fragmented, polymerized, and/or integrated into melanoidins (Bravo, et al., 2012; Cruz et al., 2012;
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31 70 Tokimoto, Kawasaki, Nakamura, Akutagawa, & Tanada, 2005). However, no works have focused on the
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33 71 identification of bioactive peptides from SCG proteins hydrolysates. Bioactive peptides are mainly found
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35 72 in a latent state as part of a protein from which they can be released by hydrolysis using different
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37 73 enzymes (Sarmadi & Ismail, 2010). In order to exploit SCG as a source of bioactive peptides, SCG proteins
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39 74 have to be extracted avoiding the extraction of other interfering compounds (such as polyphenols), and
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41 75 then hydrolysed. In the present work, a commonly used Tris-HCl based method for protein extraction
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43 76 from food by-products has been compared to a new urea-based method to isolate SCG proteins, and the
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45 77 best method has been applied to evaluate the protein content in SCG from different coffee-brewing
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47 78 preparations. In addition, a polyphenol extraction step to remove interferences has been studied, as well
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49 79 as the hydrolysis of the resultant proteins into peptides using alcalase and thermolysin enzymes.
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51 80 Furthermore, the effect of roasting degree in the presence of peptides with antioxidant and/or *in vitro*
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53 81 angiotensin-converting enzyme (ACE)-inhibitory activity was evaluated. Finally, the resulting peptides
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4 82 have been identified by reversed-phase high-performance liquid chromatography coupled via
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6 83 electrospray ionization to a quadrupole time-of-flight mass spectrometer (RP-HPLC-ESI-Q-TOF)
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9 84 technology.

10 11 12 85 **2. Materials and Methods**

13 14 15 86 **2.1. Chemicals**

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18 87 All chemicals and reagents were of analytical grade. Water was daily obtained from a Milli-Q system
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21 88 from Millipore (Bedford, MA, USA). Sodium dodecylsulfate (SDS), tris(hydroxymethyl)aminomethane
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23 89 (TRIS), sodium chloride, hydrochloric acid, sodium dihydrogen phosphate and sodium hydroxide were
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25
26 90 obtained from Merck (Darmstadt, Germany). Acetic acid, acetone, methanol, n-hexane (96%), and
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28 91 acetonitrile (ACN) were purchased from Scharlab (Barcelona, Spain). Formic acid (FA) was from Fisher
29
30 92 Scientific (Geel, Belgium). Ammonium bicarbonate (AmBi), dithiothreitol (DTT), sodium tetraborate,
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32 93 thermolysin enzyme, angiotensin converting enzyme (ACE), 2-[4-(2- hydroxyethyl)-1-piperazinyl]-
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34 94 ethanesulfonic acid (HEPES), hippuryl-histidyl-leucine (HHL), 1,10-phenanthroline, 2-mercaptoethanol,
35
36 95 2,2'-azino-bis(3-ethylbenzothiazoline- 6-sulphonic acid) (ABTS), albumin from bovine serum (BSA),
37
38 96 hydrogen peroxide, ferrous sulfate, L-gluthathion (GSH), ortho-phthalaldehyde (OPA), potassium
39
40 97 persulfate, trigonelline hydrochloride, caffeine, caffeic acid, 4-O-caffeoylquinic acid, and 3-
41
42 98 hydroxycoumarin were purchased from Sigma (St. Louis, MO, USA). 1,3-dicaffeoylquinic acid, and 1,5-
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44 99 dicaffeoylquinic acid were purchased from Plantachem (Pinnow, Germany). Tris/glycine/SDS running
45
46 100 buffer, Laemmli buffer, Bio-Safe Coomassie G250 stain, Mini Protean precast gels, Precision Plus Protein
47
48 101 Standards (molecular masses of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa) and Quick Start
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50 102 Bradford – 1xDye reagent were obtained from Bio-Rad-Laboratories (Hercules, CA, USA). Alcalase 2.4 L
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52 103 FG enzyme was kindly donated by Novozymes Spain S.A. (Madrid, Spain).

53 54 55 56 57 58 59 104 **2.2. Coffee samples**

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105 Commercial medium roasted 100% Arabica coffee (CMRC) already grounded (fine grind) from “Tchibo”
106 (Hamburg, Germany) was used as standard coffee for the optimization of the protein extraction method,
107 and for the comparison of the different coffee-brewing preparations. Additionally, three differently
108 roasted 100% Arabica coffee beans (light, medium and dark) were grounded (fine grind) and provided by
109 “Café Fortaleza” (Vitoria, Spain) for the evaluation of the roasting process in the presence of peptides
110 with antioxidant and potential *in vitro* ACE-inhibitory activities. Coffee beans were roasted at 175 °C
111 during 12.36 min (light roasted), at 185 °C during 14.11 min (medium roasted), and at 195 °C during
112 17.06 min (dark roasted). The weight loss of each sample was evaluated in order to control the roasting
113 process being 13% for light, 15% for medium, and 17% for dark roasted beans.

2.3. Spent coffee ground preparation

115 An espresso machine, a mocha coffeemaker, a plunger coffeemaker, and filter paper were used to
116 generate different SCG, trying to keep a coffee weight-to-water volume ratio at 1g/8.75mL. All
117 experiments were performed in triplicate. Espresso coffee brews were prepared from 8 g of ground-
118 roasted coffee, using an espresso coffee machine (Saeco Via Venneto, Italy) with a 15 bar pressure
119 pump, and dispensing water during 10 s at 90 °C. Mocha, plunger and filter coffee brews were prepared
120 from 20 g of ground-roasted coffee. For mocha coffee, a mocha coffeemaker (Vitro-Fulgor, Valira, Spain)
121 was used, and the heating temperature and extraction time were approximately 10 min at 93 °C. For the
122 plunger coffee brew, hot water (98 °C) was added to the coffee powder in the plunger coffeemaker (0.5 L
123 capacity), and the water was kept in contact with the coffee for 5 min before the plunger was pushed
124 down. For the filter coffee brew, coffee powder was placed in a filter paper, hot water (98 °C) was added
125 slowly, and extraction took place in 5 min. All generated SCG were dried in an oven at 103 ± 2 °C until
126 constant weight. Thereafter, 1 g was defatted three times with 25 mL of hexane maintaining the solution

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127 under stirring for 30 min. After each cycle, samples were centrifuged at 4,000g for 10 min, and hexane
128 was discarded. Samples were kept overnight at 40 °C to completely remove the hexane.

129 **2.4. Polyphenol extraction**

130 When specified, and before protein extraction, polyphenols were extracted from defatted SCG samples
131 (in triplicate). For this aim, 5 mL of methanol:H₂O:acetic acid (70:28:2, v/v/v) were added to 100 mg of
132 defatted SCG, and a high intensity focused ultrasound (HIFU) probe (model VCX130, Sonics Vibra-Cell,
133 Hartford, CT, USA) was used for 5 min with an amplitude of 30% (HIFU standard conditions if not
134 specified elsewhere). Thereafter, samples were vortexed in a mechanical stirrer (Selecta, Barcelona,
135 Spain) at room temperature for 30 min, centrifuged at 4,000g for 10 min and the supernatants were
136 transferred to a different vial. This procedure was repeated three times and the pellets were dried at 40
137 °C overnight. The collected supernatants after each extraction cycle were pooled together and directly
138 analysed by HPLC-UV/HPLC-FLD.

139 **2.5. Protein extraction and quantification**

140 Two different extraction methods were compared. In the first one, 100 mg of defatted SCG were mixed
141 with 5 mL of Tris Buffer (TB, consisting on 100 mM Tris-HCl (pH 8.5), 0.5% (w/v) SDS and 0.75% (w/v)
142 DTT) and HIFU was applied. Thereafter, samples were vortexed in a mechanical stirrer for 60 min,
143 centrifuged at 4,000g for 10 min, and 4 mL of TB supernatant was taken. Then, the proteins in the
144 supernatant were precipitated with 10 mL of cold acetone at -20 °C overnight. Samples were then
145 centrifuged again at 4,000g for 10 min and the precipitated proteins were dried at 40 °C overnight.
146 Pellets were weighed and ≈ 1 mg was dissolved in TB to a final concentration of 10 mg/mL for SDS-PAGE
147 analysis. In addition, another ≈ 1 mg was dissolved to a final concentration of 10 mg/mL in a Bradford
148 compatibility Quantification Buffer (QB, 100 mM Tris-HCl (pH 8.5) containing 0.025% (w/v) SDS and
149 without DTT) for SDS-PAGE analysis and protein quantification. These samples were then diluted 1:3 to

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4 150 fit in the BSA standard calibration curve, and the protein content was estimated (Bradford, 1976), using
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6 151 the same extraction buffer as blank. In the second method, 100 mg of defatted SCG (with or without
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8 152 polyphenols, depending on the experiment) were mixed with 5 mL of Urea Buffer (UB, consisting on 7 M
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10 153 urea, 2 M thiourea and 1 M AmBi in water), and HIFU was applied. Samples were then vortexed for 60
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12 154 min, centrifuged at 4,000g for 10 min, and supernatant was centrifuged again at 14,000g for 10 min to
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14 155 remove the remaining debris. Thereafter, protein concentration was determined in the supernatants by
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16 156 Bradford assay after checking the compatibility of urea and thiourea, and using this buffer as blank. For
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18 157 both protein extraction methods, the absorbance corresponding to a mixture of 12.3 μ L of sample with 1
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20 158 mL of Bradford solution was measured at 595 nm using a spectrophotometer Cary 8454 UV-Vis (Agilent
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22 159 Technologies, Germany). The protein concentration was then calculated by interpolation in a calibration
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24 160 curve prepared using a BSA standard at concentrations ranging from 0 to 0.3 mg/mL. Every sample was
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26 161 measured by triplicate.
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33 162 **2.6. Protein analysis by SDS-PAGE**

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36 163 SDS-PAGE separation was carried out in a Mini-Protean from Bio Rad. Samples were prepared in
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38 164 triplicate by mixing the same volume of SCG protein isolate and Laemmli buffer, containing 5% (v/v) β -
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40 165 mercaptoethanol and by heating for 5 min at 100 °C. Electrophoresis was carried out on commercial
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42 166 Ready Gel Precast Gels using Tris/glycine/SDS as running buffer and applying 80 V for 5 min and 200 V for
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44 167 30 min. For the estimation of molecular weights, protein standards (10, 15, 20, 25, 37, 50, 75, 100, 150,
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46 168 and 250 kDa) were used as ladder. After separation, proteins were fixed with 50 mL of 10% (v/v) glacial
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48 169 acetic acid/40% (v/v) methanol gently shaking for 30 min and stained with 50 mL of Bio-Safe Coomassie
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50 170 stain by slightly shaking for 1 h. At the end, the gel was washed with Milli-Q water for 2 h and images
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52 171 were acquired using a scanner (Epson Perfection V39, Sowa, Japan).
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59 172 **2.7. Protein hydrolysis**

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4 173 For protein hydrolysis, 0.8 mg of SCG proteins extracted with UB (for the comparison between the
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6 174 different hydrolytic enzymes and for the evaluation of the polyphenol extraction step) or 4 mL of UB (for
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9 175 the evaluation of the effect of the roasting degree) were transferred to 3 kDa molecular weight cut-off
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11 176 filters (Amicon Ultra 0.5 mL - 3 kDa, Merck Millipore, Burlington, MA, USA), previously washed once with
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13 177 250 μ L of a mixture of ACN:H₂O (20:80, v/v) at 14,000g for 10 min, and later on with 500 μ L of Milli-Q
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16 178 water at 14,000g for 15 min. The samples were then centrifuged at 14,000g until the whole volume was
17
18 179 loaded, and consecutive washes were performed with 300 μ L of 50 mM AmBi in ACN:H₂O (20:80, v/v)
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21 180 (once) and then with 300 μ L of 50 mM AmBi in Milli-Q water (twice) to remove urea excess. Between
22
23 181 each washing step, the filter units were centrifuged at 14,000g for 15 min. Finally, protein hydrolysis was
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25 182 carried out inside the filter units with 300 μ L of 50 mM AmBi buffer (pH 8.0) using two different
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28 183 enzymes: alcalase (0.15 AU enzyme/g protein) and thermolysin (0.1 g enzyme/g protein), and in similar
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30 184 conditions as in Hernández-Corroto, Marina, & García (2019) and Pérez-Míguez, Marina, & Castro-
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32 185 Puyana (2019). Hydrolysis was performed at 50 °C for 4 h by slight mixing (750 rpm) in a Thermomixer
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35 186 Compact (Eppendorf AG, Hamburg, Germany), and the reactions were stopped by heating to 100 °C for
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37 187 10 min. Resulting peptides were then centrifuged at 14,000g for 10 min, and 200 μ L of 50 mM AmBi
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40 188 were added (twice) and centrifuged again to recover all peptides. Finally, samples were transferred to
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42 189 new tubes, dried in SpeedVac (Eppendorf AG, Hamburg, Germany) and dissolved in Milli-Q water to a
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44 190 final concentration of 2.5 mg/mL (considering the starting amount of loaded protein).

47 191 **2.8. Determination of peptide content, antioxidant and *in vitro* ACE-inhibitory activities**

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51 192 The peptide content (based on the OPA assay), the antioxidant activity (based on the ABTS and hydroxyl
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53 193 radical scavenging assays), and the *in vitro* ACE-inhibitory activity (based on the hydrolysis of the
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56 194 tripeptide HHL into hippuric acid (HA) by the action of ACE) of SCG protein hydrolysates were

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4 195 determined as previously described (Hernández-Corroto, Marina, & García, 2018; Hernández-Corroto,
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6 196 Marina, & García, 2019). See **Supplementary Material and Methods** for the full description.
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10 197 **2.9. HPLC-UV and HPLC-FLD analyses**

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13 198 Separation of polyphenol extracts (dissolved in methanol:H₂O:acetic acid (70:28:2, v/v/v)) and SCG
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15 199 protein hydrolysates (dissolved in Milli-Q water) were performed in a 1100 series LC system (Agilent
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18 200 Technologies, Germany). LC control, data acquisition, and data analysis were carried out using the
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20 201 Agilent LC/MSD ChemStation software (B.04.03). Injection volume was set to 5 µL and all samples were
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22 202 analyzed in duplicate. A porous-shell fused-core Ascentis Express C18 analytical column (100 mm × 2.1
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25 203 mm, particle size 2.7 µm) protected by a C18 guard column (0.5 cm × 2.1 mm, particle size 2.7 µm), both
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27 204 from Supelco (Bellefonte, PA, USA) were used. The flow-rate was set to 0.25 mL/min and the column
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29
30 205 temperature was 30 °C. The gradient was 5–35% B in 30 min, 35–50% B in 5 min, 50–95% B in 5 min, and
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32 206 95–5% B in 2 min. Mobile phase A was water with 0.1% (v/v) of FA and mobile phase B consisted of ACN
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34 207 with 0.1% (v/v) FA. Detection was carried out using an UV detector at 280 and 325 nm, and using a
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37 208 fluorescent detector (FLD) at λ_{exc} = 280 and λ_{em} = 304 and 348 nm.
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40 209 **2.10. RP-HPLC-ESI-Q-TOF peptide and polyphenol identification**

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43 210 Identification of peptides and polyphenols in SCG protein hydrolysates (dissolved in Milli-Q water) were
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45 211 performed by RP-HPLC-ESI-Q-TOF as previously described (Pérez-Míguez, Marina, & Castro-Puyana,
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47
48 212 2019). Briefly, MS analysis was performed in a quadrupole Q-TOF series 6530 coupled to a HPLC (model
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50 213 1100) both from Agilent Technologies (Germany), equipped with a Jet Stream thermal orthogonal ESI
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53 214 source. MS control, data acquisition, and data analysis were carried out using the Agilent Mass Hunter
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55 215 Qualitative Analysis software (B.07.00). Injection volume was set to 10 µL, and three replicates were
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57 216 injected in triplicates. For the chromatographic separation, an Ascentis Express Peptide ES-C18 analytical
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60 217 column (100 × 2.1 mm, particle size 2.7 µm) with a C18 guard column (0.5 cm × 2.1 mm, particle size
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4 218 2.7 μm), both from Supelco (Bellefonte, Pa, USA) were employed. The column temperature was held at
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6 219 25 °C and the flow rate was set to 0.3 mL/min. Mobile phase A was water with 0.3% (v/v) of acetic acid
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9 220 and mobile phase B consisted of ACN with 0.3% (v/v) acetic acid. The gradient was 5% B for 3 min, 5–40%
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11 221 B in 37 min, 40–95% B in 3 min, 95% B in 2 min, with 15 min of post-time to come back to initial
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13 222 conditions. ESI in positive ion mode (for peptides) or in negative ion mode (for polyphenols) were used at
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16 223 a capillary voltage of 3500 V and with a m/z range from 100 to 1700. UV signals were also recorded at
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18 224 280 and 325 nm. MS analyses were performed employing the auto MS/MS mode using 3 precursor per
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21 225 cycle, dynamic exclusion after two spectra (released after 1 min), and collision energy of 5 V for every
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23 226 100 Da. For proper mass accuracy, spectra were corrected using ions m/z 121.0509 ($\text{C}_5\text{H}_4\text{N}_4$) and
24
25 227 922.0098 ($\text{C}_{18}\text{H}_{18}\text{O}_6\text{N}_3\text{P}_3\text{F}_{24}$) in ESI positive mode, and m/z 119.0363 ($\text{C}_5\text{H}_4\text{N}_4$) and 980.0164
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27 228 ($\text{C}_{18}\text{H}_{18}\text{O}_6\text{N}_3\text{P}_3\text{F}_{24}$ + formate) in ESI negative mode, simultaneously pumped into the ionization source at a
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30 229 15 $\mu\text{L}/\text{min}$ flow rate. Peaks software (Bioinformatics Solutions Inc.; Waterloo, ON, Canada) was
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32 230 employed for database search against Uniprot *Coffea arabica* reviewed proteome (downloaded on July
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34 231 25th 2019 and containing 92 entries). Oxidation of methionine (+15.99 Da) was included as variable
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36 232 modification and peptide results were refined using a – 10lgP threshold of 15. Moreover, *de novo*
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39 233 peptide sequencing was performed using an error tolerance of 10 ppm for the precursor and 0.5 kDa for
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41 234 the fragment, and including oxidation of methionine as variable modification. *De novo* peptide
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44 235 sequences were accepted if the average local confidence (ALC, expected percentage of correct amino
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46 236 acids in the peptide sequence) was $\geq 80\%$ in at least 5 from 9 injections. Moreover, since the MS system
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49 237 is not able to distinguish between isoleucine (I) and leucine (L) amino acids due to their equal molecular
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51 238 masses, only isoforms with L are presented.

55 239 **2.11. Statistical analysis**

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Statistical analysis was performed using Statistica software version 7.1 (StatSoft, Inc., USA). One-way ANOVA with LSD Post-hoc test was employed to determine any significant differences between mean values using $p < 0.05$.

3. Results and Discussion

3.1. Protein extraction from SCG

Different methods have been previously developed to extract proteins from food waste matrices, and the biological activity of the released bioactive peptides after hydrolysis has been evaluated (Vásquez-Villanueva, Marina, & García, 2016; Hernández-Corroto, Marina, & García, 2018; Hernández-Corroto, Marina, & García, 2019). In the present work, the pH (from 6.5 to 8.5) and the DTT content (from 0.25 to 0.75% (w/v)) of a 0.5% (w/v) SDS and 100 mM Tris-HCl buffer has been optimized to maximize the extraction of proteins from SCG of an espresso machine. The best buffer composition is 100 mM Tris-HCl (pH 8.5), 0.5% (w/v) SDS and 0.75% (w/v) DTT, which yields less than 0.3 mg of protein/100 mg of SCG. This quantity is low compared to the protein content of 13.6% determined by the Kjeldahl method in SCG (Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011) and therefore a different buffer based on urea (UB) has been compared with the optimized TB. By using UB, the total protein quantity obtained is 2.89 mg of protein/100 mg of SCG. This result highlights an increase of more than 10 times in the protein content when UB is used instead of TB. Moreover, the UB, the TB and the QB protein extracts have been characterized by SDS-PAGE, and the protein profiles are shown in **Supplementary Material Figure S1**. A protein band around 150 kDa is observed with the three buffers, and this band is slightly more intense when UB is used. Another band below 10 kDa is also observed when UB or TB are used.

Based on the higher protein content, the UB has been selected to evaluate the protein content in SCG from four different coffee preparations. As observed in **Supplementary Material, Figure S2**, the highest protein content is obtained from espresso SCG (2.89 mg of protein/100 mg of SCG). No significant

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4 263 differences are observed between the French Press SCG (2.40 mg of protein/100 mg of SCG) and the
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6 264 Filter SCG (2.26 mg of protein/100 mg of SCG), and the lowest protein content is obtained from mocha
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9 265 SCG (1.68 mg of protein/100 mg of SCG). The total solid and the protein content of coffee brews have
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11 266 been directly related with the extraction time used for the coffee preparation (Zanoni, Pagliarini, & Peri,
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14 267 1992), but it is also known that the coffee weight-to-water volume ratio and the coffee grinding degree
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16 268 have a great impact (Cruz et al., 2012). It has been demonstrated that coffee brews from mocha
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18 269 machines contain more total solids per gram of roasted and ground coffee than coffee brews prepared
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21 270 with other procedures (Gloess, et al., 2013). In the present work we kept fixed the coffee weight-to-
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23 271 water volume ratio, the type of coffee, the roasting degree and the grinding degree, and we varied the
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25 272 pressure, temperature and extraction times, which are intrinsic parameters to the different coffee
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28 273 preparations used. The highest protein content in espresso SCG suggests that even though a higher
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30 274 pressure is used for the espresso coffee preparation, the shorter time has a greater effect on the
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33 275 extracted proteins.

36 276 **3.2. Evaluation of the peptide content and the antioxidant activity in espresso SCG hydrolysates**

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39 277 The used UB has been previously applied in different proteomics studies to extract proteins from
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41 278 complex tissues, and it has been combined with Amicon devices (3 kDa molecular cut-off filters) to
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44 279 remove contaminants before HPLC and/or MS analysis, and to hydrolyse proteins into peptides prior to
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46 280 HPLC-MS analyses (Holfeld, Valdés, Malmström, Segersten, & Lind, 2018; Wiśniewski, 2017). In the
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49 281 present study, the use of 3 kDa filters would allow to retain coffee proteins larger than 3 kDa, to discard
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51 282 the UB, and to remove free and small compounds from the samples, such as polyphenols. The use of
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53 283 these filters would also help removing big polyphenols and melanoidins that might remain on top of the
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56 284 filter after hydrolysis. This procedure has some drawbacks as very small proteins can be lost during
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58 285 sample loading and washing steps, and peptides bigger than 3 kDa can remain on top of the filter after
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4 286 the hydrolysis. Considering these aspects, the peptide content and the antioxidant activity of alcalase
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6 287 and thermolysin protein hydrolysates from espresso SCG were measured (**Table 1**). The upper part
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8 288 shows that the peptide content and the ABTS antioxidant activity are similar in both hydrolytic extracts,
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11 289 but the antioxidant activity based on the hydroxyl radical assay is higher in alcalase hydrolysates. These
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13 290 protein hydrolysates were then characterized by HPLC-UV at 325 nm (maximum UV absorbance for CGA)
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16 291 (**Figure 1A**, upper part) and at 280 nm (maximum UV absorbance for caffeine) (**Figure 1B**, upper part). It
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18 292 can be observed that caffeine (t_R 8.75 min) and other phenolic compounds remain in the samples, but no
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21 293 differences were observed between thermolysin or alcalase hydrolysates profiles. These hydrolysates
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23 294 were also characterized by HPLC-FLD at λ_{exc} = 280 nm and λ_{em} = 304 and 348 nm (maximum fluorescence
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25 295 excitation and emission wavelengths of the two amino acid residues that are primarily responsible for
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27 296 the fluorescence of peptides/proteins, tryptophan and tyrosine) (**Figures 1C and 1D**, upper part). These
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30 297 results demonstrate that thermolysin hydrolysates are more complex than alcalase hydrolysates in terms
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32 298 of peptide content.

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36 299 To try to reduce the quantity of phenolic compounds, a polyphenol extraction step was included before
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38 300 the protein extraction. The characterization of the polyphenolic extracts allowed the identification of
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40 301 caffeine, caffeic acid, 4-O-caffeoylquinic acid, 1,3-dicaffeoylquinic acid, 3-hydroxycoumarin and 1,5-
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42 302 dicaffeoylquinic acid using pure standards (**Figure 2**). In addition, a decrease of approximately 9 times
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44 303 after each extraction cycle was observed. Thereafter, the proteins were extracted and the protein
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46 304 concentration was measured, being 0.91 mg of protein/100 mg of SCG (a 69% reduction compared to the
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48 305 non-polyphenol extraction, 2.90 mg of protein/100 mg of SCG). This reduction can be explained by the
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50 306 composition of the solvent used (methanol:H₂O:acetic acid (70:28:2, v/v/v)), which has been previously
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52 307 used to extract polyphenols from coffee (Mussatto, Ballesteros, Martins, & Teixeira, 2011) or cocoa
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54 308 beans (D'Souza et al., 2017), but also to extract peptides from cocoa beans (D'Souza et al., 2018). The
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56 309 peptide content and the antioxidant activity of these extracts was also evaluated (**Table 1**, lower part). A
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4 310 decrease of 47% of the peptide content (between the two alcalase hydrolysates) was observed when the
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6 311 polyphenol extraction step was included, but this effect was not observed when thermolysin was used.
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9 312 Moreover, the polyphenol extraction step increased the antioxidant activity of thermolysin hydrolysates,
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11 313 being this effect slightly higher but not significant with respect to the alcalase hydrolysates. These results
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13 314 are unexpected since it is commonly known that phenolic compounds have higher antioxidant activity
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15 315 than peptides. However, it has been widely discussed that polyphenols can also have inhibitory effects
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17 316 on enzymes involved in the hydrolysis of proteins (Cirkovic Velickovic & Stanic-Vucinic, 2018), therefore
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19 317 the polyphenol removal prior to the hydrolysis step could increase the release of peptides with
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21 318 antioxidant activity.

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26 319 The protein hydrolysates after the polyphenol extraction were also characterized by HPLC-UV (**Figures**
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28 320 **1A and 1B**, lower part) and by HPLC-FLD (**Figures 1C and 1D**, lower part). The chromatograms obtained
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30 321 at 325 nm show that alcalase and thermolysin hydrolysates are similar, but the area of some of the
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32 322 previous observed peaks increased when polyphenols were extracted. As it will be lately discussed, these
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34 323 compounds could be released from the protein isolates during the incubation at 50 °C. On the other
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36 324 hand, the chromatograms acquired at 280 nm clearly shows that caffeine (t_R 8.75 min) almost
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38 325 disappeared when the polyphenols extraction was included. In addition, more peaks were observed after
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40 326 min 20 when thermolysin in combination with the polyphenol extraction was used. This effect was also
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42 327 observed in the HPLC-FLD chromatograms (**Figures 1C and 1D**).

43 328 **3.3. Effect of roasting degree on thermolysin SCG hydrolysates**

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48 329 The next step was the evaluation of the roasting degree effect on the protein/peptides extracts obtained
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50 330 from different SCG (LSCG, light spent coffee grounds; MSCG, medium spent coffee grounds; DSCG, dark
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52 331 spent coffee grounds). Three independent espresso coffees were prepared and polyphenols were
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54 332 extracted before proteins were isolated using UB. The characterization of the polyphenolic extracts
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4 333 indicates that the area of caffeic acid, 4-O-caffeoylquinic acid, 1,3-dicaffeoylquinic acid and 1,5-
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6 334 dicaffeoylquinic acid decreased with the roasting process; the area of caffeine remained unchanged; and
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9 335 the area of 3-hydroxycoumarin is increased (**Supplementary Material, Figure S3**). These results agree
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11 336 well with previous reports where CGA such as 5-CQA, 4-CQA, 3-CQA, 3,5-diCQA, 4,5-diCQA, 5-FQA, 3,4-
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13 337 diCQA and 4-FQ, are degraded during roasting, whereas the formation of CGA lactones takes place
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16 338 (Farah, de Paulis, Trugo, & Martin, 2005; Moon, Yoo, & Shibamoto, 2009). The levels of these
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18 339 compounds may also be decreased by their incorporation into Maillard reaction products during the
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21 340 roasting process (Coelho et al., 2014; Delgado-Andrade & Morales, 2005; Bekedam, Roos, Schols, Van
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23 341 Boekel, & Smit, 2008). It has been suggested that melanoidins are derived from cross-linking of Maillard
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25 342 reaction products to proteins via reactive side chains of amino acids, and more recently it has been
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28 343 demonstrated that transglycosylation reactions to form new polysaccharides is the main mechanism for
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30 344 this incorporation (Moreira et al., 2017). On the other hand, the caffeine levels are not affected
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33 345 (Oestreich-Janzen, 2010).

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36 346 Thereafter, the protein concentration was evaluated by Bradford assay, indicating that DSCG had higher
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38 347 amount of proteins (1.49 mg of protein/100 mg of SCG) than MSCG (1.08 mg of protein/100 mg of SCG),
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41 348 and three times more than LSCG (0.49 mg of protein/100 mg of SCG) (**Supplementary Material, Figure**
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43 349 **S4**). The SDS-PAGE analysis also shows a slightly more intense protein bands of \approx 150 kDa in DSCG
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45 350 (**Supplementary Material, Figure S5**). The higher protein concentration of DSCG could be partially
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48 351 explained by the loss coffee weight during the roasting process (13% for light, 15% for medium, and 17%
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50 352 for dark roasted beans), which can enrich the material that is not degraded or loss. However, darker
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52 353 roasting has shown to produce more total soluble solids in the brew (Petracco 2005), and therefore
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55 354 there should be fewer proteins in their SCG. Moreover, it is also known that coffee proteins can be
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57 355 fragmented, polymerized or integrated into melanoidins through the Maillard reaction during the
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59 356 roasting process, but it is not well known how this reaction affects the functional properties (such as the
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4 357 solubility) of proteins (Oliver, Melton, & Stanley, 2006). After protein quantification, the peptide content,
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6 358 the antioxidant activity and the *in vitro* ACE-inhibitory activity of thermolysin protein hydrolysates from
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9 359 LSCG, MSCG and DSCG were measured (**Table 2**). For comparison purposes, DSCG protein isolates were
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11 360 also hydrolysed with alcalase, and a control sample incubated without enzyme was included. As
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14 361 expected due to the higher amount of proteins, the highest peptide concentration among the three
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16 362 thermolysin hydrolysates was obtained in DSCG, followed by MSCG and LSCG (**Table 2**). There were not
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18 363 significant differences on the ABTS or hydroxyl scavenging activity between the samples, but the DSCG
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21 364 and the MSCG hydrolysates had higher *in vitro* ACE-inhibitory activity. The protein hydrolysates were
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23 365 then characterized by HPLC-UV and HPLC-FLD (**Supplementary Material, Figures S6-S9**). The acquisition
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25 366 at 280 nm shows that the area of some peaks are slightly increased (peaks at t_R 5.5 min and 14.7 min);
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28 367 some of them remained unchanged (peaks at t_R 23.0 min, 25.2 min, 25.5 min and 26.0 min); and others
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30 368 are decreased (peaks at t_R 19.0 min and 27.0 min) with the roasting process (**Supplementary Material,**
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32 369 **Figures S6**); and the chromatograms acquired at 325 nm indicate that most of the peaks were
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35 370 significantly increased with the roasting process (**Supplementary Material, Figures S7**). There were not
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37 371 significant differences in the FLD-chromatograms acquired at $\lambda_{exc} = 280$ nm and $\lambda_{em} = 304$ nm, but when
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40 372 λ_{em} was set to 348 nm (**Supplementary Material, Figures S8**), the general trend was a decrease in the
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42 373 area when increasing the roasting degree (**Supplementary Material, Figures S9**). As previously
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44 374 commented, the signals observed at 325 nm might be due to the presence of phenolic compounds that
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47 375 can be released during the incubation step at 50 °C. It has been observed that the incorporation of
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49 376 phenolic compounds into the melanoidins is a significant pathway of CGA degradation during roasting
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51 377 (Coelho et al., 2014), being transglycosylation reactions the main mechanism for this incorporation
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54 378 (Moreira et al., 2017). Different techniques such as alkaline hydrolysis, acid hydrolysis, the increase of
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56 379 the medium ionic strength, or the alkaline fusion method have been applied to release them (Bekedam,
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59 380 Roos, Schols, Van Boekel, & Smit, 2008; Monente, Ludwig, Irigoyen, De Peña, & Cid, 2015; Perrone,
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4 381 Farah, & Donangelo, 2012; Delgado-Andrade & Morales, 2005; Coelho et al., 2014). For instance,
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6 382 covalently bound caffeic and ferulic acids decrease with roasting, while the content of dihydrocaffeic
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9 383 acid increases (Perrone, Farah, & Donangelo, 2012). And by using the alkaline fusion method, it has been
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11 384 suggested that the incorporation of phenolic compounds in coffee melanoidins is also related to the
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14 385 amount of proteins (Coelho et al., 2014). Complementary, the antioxidant activity of melanoidins from
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16 386 coffee brews obtained using roasted coffees is higher than those obtained from green coffees (Delgado-
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18 387 Andrade, Rufián-Henares, & Morales, 2005; Perrone, Farah, & Donangelo, 2012). Based on these results,
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21 388 different CGA acids could have been released from melanoidins, or some melanoidins could still be
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23 389 present in the SCG protein hydrolysates, but further studies are needed to confirm these hypotheses.

26 390 **3.4. Evaluation of the enzymatic incubation on SCG hydrolysates**

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29 391 To evaluate the effect of the enzymatic incubation, DSCG protein isolates were also hydrolysed with
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32 392 alcalase, and a control sample incubated without enzyme was included. As expected from our previous
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34 393 results (**Table 1**), lower amounts of peptides were obtained when alcalase was used (**Table 2**), and the
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37 394 antioxidant activity was similar when the different enzymes were used (a significantly lower ABTS activity
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39 395 was observed when the samples were incubated with no enzyme). However, the ACE-inhibitory activity
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41 396 of alcalase hydrolysates was lower than its thermolysin counterpart, and less than 50% of the capacity
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44 397 was found when no enzyme was added (**Table 2**). The HPLC-UV characterization demonstrated that
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46 398 there were not significant differences in the intensity of peaks obtained at 325 nm (**Figure 3A**), but a
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48 399 more complex profile was obtained at 280 nm when thermolysin was used (**Figure 3B**). These differences
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51 400 were greater when the characterization was performed using HPLC-FLD (**Figures 3C and 3D**). Altogether,
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53 401 these results suggest that the antioxidant activity of the protein hydrolysates is partly derived from the
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56 402 released polyphenols during the incubation step, but the ACE-inhibitory activity is mainly derived from
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58 403 the released peptides, being higher when thermolysin enzyme is used.

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3.5. Identification of peptides and polyphenols in SCG hydrolysates by RP-HPLC-ESI-Q-TOF-MS/MS

To identify the released peptides, thermolysin hydrolysates from LSCG, MSCG and DSCG, alcalase hydrolysates from DSCG, and DSCG proteins incubated without enzyme were analyzed by RP-HPLC-ESI-Q-TOF in positive mode (**Supplementary Material, Figures S10A and S10C**). The chromatographic and MS/MS parameters were similar to those applied in a recent work for the identification of peptides in coffee silverskin protein hydrolysates (Pérez-Míguez, Marina, & Castro-Puyana, 2019). As a first step, MS/MS data were searched against *Coffea arabica* proteome, and the peptides (and proteins containing those peptides) are reported in **Table 3**. In total, peptides belonging to 35 different proteins were identified based on the peptide sequence. The most overrepresented proteins in these peptides were PSAB and RPOC2 (11 peptides), followed by NU5C and TI214 (8 peptides), ATPB and YCF2 (7 peptides), and RPOB (4 peptides). The PSAB protein amino acid sequence and the identified peptides in MSCG is shown as example in Supplementary Material, Figure S11. The sample with the highest number of peptides was the MSCG thermolysin hydrolysate (34 peptides), followed by the LSCG thermolysin hydrolysate (25 peptides), and the DSCG alcalase hydrolysates (25 peptides). 16 peptides were also identified in DSCG thermolysin hydrolysate, 5 of them belonging to YCF2. In addition, 14 peptides could be identified in the sample where no enzyme was added, demonstrating that some peptides can also be released by the incubation processes (as observed for polyphenols). It is interesting to note that some proteins could only be identified in one specific sample, such NDHK in LSCG thermolysin hydrolysates, ACCD and PSBA in MSCG thermolysin hydrolysates, SPDE in DSCG alcalase hydrolysates, or YCF2, identified in all DSCG hydrolysates.

To complement the previous information, the *de novo* sequencing tool from the PEAK Software was used to carry out the tentative identification of more peptides. **Table 4** summarizes the peptides identified by MS/MS in thermolysin hydrolysates along with their experimental molecular masses, ALC and accuracy.

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4 427 Different peptides with a number of amino acids ranging from 4 to 10 were identified, and none of these
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6 428 peptides belong to the enzymes employed for hydrolysis. In the case of DSCG alcalase hydrolysates, 5
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9 429 peptides (ALM(+15.99)APH, M(+15.99)EGL, CCVLLP, NVLAR and NLM(+15.99)APH, **Supplementary**
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11 430 **Material, Table S1**) were identified, and only one peptide (SHWH) was identified when no enzyme was
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14 431 used. In overall, thermolysin hydrolysates present a higher number of peptides (12 in LSCG, 14 in MSCG
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16 432 and 10 in DSCG) and, when combining the results with the database search, 37, 48 and 26 peptides were
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18 433 identified in LSCG, MSCG and DSCG thermolysin hydrolysates, respectively; 30 peptides in DSCG alcalase
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20 434 hydrolysates; and only 15 peptides when no enzyme was used. The possible antioxidant or
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23 435 antihypertensive/ACE-inhibitory activity of these peptides was searched in the BIOPEP database
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25 436 (<http://www.uwm.edu.pl/biochemia/index.php/en/biopep>), but none of them have been reported. In
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28 437 overall, these results indicate that the peptide complexity of thermolysin hydrolysates is higher than
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30 438 alcalase hydrolysates, and some of the thermolysin released peptides (or a combination of them) would
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33 439 be responsible for the higher ACE-inhibitory activity observed. Since LSCG presents some antioxidant and
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35 440 ACE inhibitory activities, the common peptides identified in the three thermolysin hydrolysates using the
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37 441 *de novo* sequencing tool (CSDAVGVK and RCPQGGTHYG), and the common peptides identified as coming
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40 442 from LSCG, MSCG and DSCG proteins (YKPPYS and CVIPSN) would be the first candidates to be evaluated
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42 443 for their biological activity. The tentative identification of CSDAVGVK peptide in DSCG is shown as
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44 444 example in Supplementary Material, Figure S12. Moreover, the higher ACE-inhibitory activity observed
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47 445 for MSCG and DSCG thermolysin hydrolysates would be explained by the higher number of identified
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49 446 peptides (48 in the case of MSCG), or by the specific sequence of the identified peptides in the
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52 447 MSCG/DSCG thermolysin hydrolysates, but not in the LSCG or the alcalase hydrolysates. The following
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54 448 peptides meet this requirement: DPGDKKN, CASDPAQ and RLNQ, identified in the MSCG and the DSCG
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56 449 using the *de novo* sequencing tool; GGSMG, GVMDFQ, LDPIEF, GGGDL, YEAWL, DAHIPPG, DPHFGQPAVE,
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59 450 FGM(+15.99)NSLS, FPCDGP, GPVNIAY, GMAST and LGMAST, identified as coming from MSCG proteins;

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4 451 and FLSRSD, IPNIH, LSDM(+15.99)NLS, M(+15.99)VDSFH and NRRGY, identified as coming from DSCG
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6 452 proteins.
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10 453 Additionally, RP-HPLC with UV (at 325 nm) and MS (in ESI negative mode) detectors were used to
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12 454 tentatively identify the possible remaining polyphenols in SCG protein hydrolysates (**Supplementary**
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14 455 **Material, Figures S10B and S10D**). Among the different observed peaks in **Figure 3A**, peaks **1–5** showed
16 456 a $[M-H]^-$ ion at m/z 367.1, with different product ions at m/z 193.0, 191.0, 173.0 and 134.0, while peak **6**
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19 457 showed a $[M-H]^-$ ion at m/z 193.0 and MS/MS product ions at m/z 178 and m/z 134). The identification
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21 458 of these polyphenols was performed by analysing the MS and MS/MS spectra (as exemplified in **Figure 4**
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24 459 for 3-FQA), and comparing them with the fragmentation patterns already reported (Kuhnert, Jaiswal,
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26 460 Matei, Sovdat, & Deshpande, 2010; Clifford, Johnston, Knight, & Kuhnert, 2003). Based on these
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28 461 analyses, peak **1** was tentatively assigned as 3-FQA, peak **2** as 1-FQA, peaks **3** and **5** as 5-FQA, and peak **4**
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31 462 as 4-FQA. However, peak **6** could not be identified. These phenolic compounds have already been
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33 463 identified in SCGs (Panusa, Zuurro, Lavecchia, Marrosu, & Petrucci, 2013; Bravo et al., 2012), and they
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35 464 might be partly responsible for the antioxidant activity exerted by the extracts.
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39 465 **4. Conclusion**

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42 466 In conclusion, the urea-based extraction buffer allows the extraction of more proteins than the
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44 467 conventional Tris-HCl buffers, and its application demonstrates that the highest protein content is
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46 468 obtained from espresso SCG. Moreover, the use of 3 kDa molecular cut-off filters allows the removal of
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48 469 the buffer and the hydrolysis of the proteins using thermolysin and alcalase enzymes, but not the
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50 470 complete removal of polyphenols. Moreover, these protein hydrolysates possess antioxidant and ACE-
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52 471 inhibitory activities, the latter being highest when SCG samples are obtained from dark/medium roasted
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54 472 coffees, and after the inclusion of a polyphenol step removal. Finally, several peptides that might be
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56 473 responsible for the ACE-inhibitory activity observed have been identified. However, further experiments
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474 using synthetic peptides are needed to confirm which of the released peptides (or a combination of
475 them) are the most bioactive.

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482 **Conflict of Interest**

483 The authors declare no conflict of interest

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622 Figure Captions

623 Figure 1. HPLC chromatograms of SCG proteins hydrolysed with alcalase and thermolysin (including the
624 polyphenol extraction step). Chromatographic conditions were: Ascentis Express C18 analytical column
625 (100 mm × 2.1 mm, 2.7 μm); gradient: 5–35% B in 30 min, 35–50% B in 5 min, 50–95% B in 5 min, and
626 95–5% B in 2 min; mobile phases: water with 0.1% (v/v) of FA (A) and ACN with 0.1% (v/v) FA (B); flow-
627 rate, 0.25 mL/min; temperature, 30 °C; injected volume, 5 μL; absorbance detection was performed at λ
628 of 325 nm (A) and 280 nm (B), and fluorescence was acquired at λ_{exc} of 280 nm and λ_{em} of 304 nm (C) and
629 348 nm (D).

630 Figure 2. HPLC chromatograms obtained from methanol:H₂O:acetic acid (70:28:2, v/v/v) extracts after
631 one, two or three extraction cycles before SCG protein extraction. Separation conditions were the same
632 as in **Figure 1**, and UV absorbance was detected at λ of 325 nm.

633 Figure 3. HPLC chromatograms of DSCG protein hydrolysed with thermolysin, alcalase or “no enzyme”
634 with polyphenol extraction. Separation conditions were the same as in **Figure 1**, absorbance detection
635 was performed at λ of 325 nm (A) and 280 nm (B), and fluorescence was acquired at λ_{exc} of 280 nm and
636 λ_{em} of 304 nm (C) and 348 nm (D). Peaks were lately identified by RP-HPLC-ESI-Q-TOF-MS/MS as: **1**, 3-
637 FQA; **2**, 1-FQA; **3**, 5-FQA; **4**, 4-FQA; **5**, 5-FQA; **6**, not identified.

638 Figure 4. Tentatively identification of 3-FQA in SCG protein hydrolysates by RP-HPLC-ESI(-)-Q-TOF-
639 MS/MS analysis.

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4 **640 Tables**

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7 **641 Table 1.** Peptide content and antioxidant activity (ABTS and hydroxyl assays) of SCG (after espresso
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10 **642** preparation of commercial medium roasted 100% Arabica coffee) proteins hydrolysed with alcalase and
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12 **643** thermolysin (and including the polyphenol extraction step).
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Sample	Peptide content (mg/mL)	ABTS assay (%) ¹	Hydroxyl radical assay (%)
Alcalase	0.43 ^a ± 0.08	22.1 ^b ± 5.20	88.4 ^a ± 11.6
Thermolysin	0.42 ^a ± 0.05	19.8 ^b ± 11.3	73.5 ^b ± 3.55
Alcalase (with polyphenol extraction)	0.23 ^b ± 0.02	30.7 ^{a,b} ± 0.52	84.3 ^{a,b} ± 4.85
Thermolysin (with polyphenol extraction)	0.39 ^a ± 0.05	36.1 ^a ± 2.39	95.4 ^a ± 2.70

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25 **644** *Different letters indicate significant differences between samples after ANOVA with LSD Post-hoc, p-value < 0.05.
26 **645** ¹ For ABTS antioxidant activity, samples were diluted to 1:150.
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31 **647 Table 2.** Peptide content, antioxidant activity (ABTS and hydroxyl assays) and *in vitro* ACE-inhibitory
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33 **648** activity of thermolysin hydrolysates from light, medium and dark espresso SCG (LSCG, MSCG, DSCG),
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35 **649** alcalase hydrolysates from DSCG, and incubation of DSCG without enzyme.
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Sample	Peptide content (mg/mL)	ABTS assay (%) ¹	Hydroxyl radical assay (%)	<i>In vitro</i> inhibitory (%)	ACE-activity
LSCG – Thermolysin	0.22 ^b ± 0.05	30.5 ^{a,b} ± 4.26	85.7 ^{a,b} ± 7.84	61.7 ^b ± 4.98	
MSCG– Thermolysin	0.31 ^{a,b} ± 0.09	28.7 ^{a,b} ± 9.47	81.6 ^b ± 10.2	83.0 ^a ± 2.72	
DSCG – Thermolysin	0.41 ^a ± 0.08	32.9 ^{a,b} ± 4.28	92.2 ^a ± 11.3	81.5 ^a ± 1.51	
DSCG – Alcalase	0.21 ^b ± 0.07	39.8 ^a ± 5.49	96.1 ^a ± 1.89	61.5 ^b ± 3.84	
DSCG – No enzyme	0.25 ^b ± 0.03	24.8 ^b ± 8.31	83.4 ^{a,b} ± 6.27	37.7 ^c ± 9.71	

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51 **650** *Different letters indicate significant differences between samples after ANOVA with LSD Post-hoc, p-value < 0.05.
52 **651** ¹ For ABTS antioxidant activity, samples were diluted to 1:150.
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57 **653**
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4 **Table 3.** Peptide sequence, belonging gene and protein name, molecular mass (Da) and -10lgP of the
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654 **Table 3.** Peptide sequence, belonging gene and protein name, molecular mass (Da) and -10lgP of the
655 peptides identified in thermolysin hydrolysates from light, medium and dark SCG (LSCG, MSCG, DSCG),
656 alcalase hydrolysates from DSCG, or DSCG incubated with no enzyme, using LC-MS/MS and database
657 search.

Peptide sequence	Gene name	Protein name	Molecular mass (Da)	-10lgP				
				Thermolysin			Alcalase	No Enzyme
				LSCG	MSCG	DSCG	DSCG	DSCG
GGSMG	ACCD	Acetyl-coenzyme A carboxylase carboxyl transferase	407.1475		15.2			
GVMDFQ	ACCD		695.2949		22.9			
LDPIEF	ACCD		732.3694		16.7			
EVIAVNQ	AGAL	Alpha-galactosidase	771.4127		20.1			
AVATDT	ATPA	ATP synthase subunit alpha	576.2755					18.1
GDGLMI	ATPA		604.2891				18.6	
GIARI	ATPA		528.3384				16.3	
AVAM(+15.99)SS	ATPB	ATP synthase subunit beta	580.2527					17.8
DTGAP	ATPB		459.1965		15.7			
GAVDT	ATPB		461.2122	20.1	23.0			
NLGAV	ATPB	472.2645	15.3					
PGARMR	ATPB	686.3646						27.8
TRGM(+15.99)E	ATPB	608.2588					15.7	
YM(+15.99)EM(+15.99)K	ATPB	732.2822	20.0					
RIVWDS	ATPE	ATP synthase epsilon	774.4024	40.4	39.4			
TFSTVRD	CCS1	Caffeine synthase 1	824.4028				15.0	
TILHF	CEMA	Chloroplast envelope membrane protein	629.3537				30.4	
GPNTM(+15.99)	CS3	Probable caffeine synthase 3	534.2108				27.0	
LIAAM(+15.99)PGSF	CS4	Probable caffeine synthase 4	921.4630	23.6				
GAMPGS	DXMT1	3,7-dimethylxanthine N-methyltransferase	518.2159	24.3	27.4			
SRPPI	DXMT1		568.3333				22.7	
KIRPPG	NDHH	NAD(P)H-quinone oxidoreductase H	666.4177			17.8	15.5	
YDVAPGG	NDHJ	NAD(P)H-quinone oxidoreductase J	677.3020				17.1	
FDFDRYG	NDHK	NAD(P)H-quinone oxidoreductase K	918.3871	16.5				
TITGGM	NDHK		578.2734	20.6				
NSSST	NU1C	NAD(P)H-quinone oxidoreductase 1	494.1973			26.8		
FVMAIGM(+15.99)I	NU4C	NAD(P)H-quinone oxidoreductase 4	896.4500			19.1		
YFFDSG	NU4C		734.2911		18.4	16.7		
AFSTMSQ	NU5C	NAD(P)H-quinone oxidoreductase 5	770.3269	30.5				
KPPYS	NU5C		590.3064				22.3	
LAFSTMSQ	NU5C		883.4109	18.1				
LFPTATK	NU5C		776.4432	22.5				
LWGRG	NU5C		587.3180	16.3				
WIINN	NU5C		658.3439	15.5				
YKPPYS	NU5C	753.3697	28.1	27.6	18.4			

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YKSQNM	NU5C		769.3429	16.1		
HNYYGEP	PETD	Cytochrome b6-f complex	949.3929	15.2	23.9	
GGGDL	PSAA	Photosystem I P700	417.1859	16.3		
YEAWL	PSAA	chlorophyll a apoprotein A1	680.3170	28.4		
DAHIPPG	PSAB		705.3445	15.3		
DPHFGQPAVE	PSAB		1095.4985	16.9		
FGM(+15.99)NLS	PSAB		770.3269	17.7		
FPCDGP	PSAB		634.2421	18.6		
GPVNIAY	PSAB	Photosystem I P700 chlorophyll a apoprotein A2	732.3806	30.4		
NVLAR	PSAB		571.3442			26.4
RGGALG	PSAB		529.2972			15.8
RTNFGIGH	PSAB		900.4566	18.7		
SLAWT	PSAB		576.2908			15.1
SRGEY	PSAB		610.2711	18.1		
VLPHPQ	PSAB		689.3860	15.0		
DGMPLG	PSBA	Photosystem II protein D1	588.2578	20.4		
VMHERNAH	PSBA		992.4611	24.7		
GHASFA	PSBB	Photosystem II CP47 reaction center protein	588.2656			21.7
M(+15.99)GLPWY	PSBB		781.3469			15.4
SDPYGLT	PSBB		751.3388	21.1	18.9	
PVVFAS	PSBZ	Photosystem II reaction protein z	618.3377		20.6	
YKGRCYH	RBL	Ribulose bisphosphate carboxylase	925.4229	17.3		
NPVDH	RK2A	50S ribosomal protein L2-A	580.2605	17.8		
M(+15.99)AVPK	RK32	50S ribosomal protein L32	560.2992			48.5
AAATVGGE	RPOB	DNA-directed RNA polymerase subunit beta	674.3235	17.9		
AEELY	RPOB		623.2802			23.3
EGMATI	RPOB		620.2839			22.1
RSNKNTC	RPOB		821.3814	15.1		
NNTLT	RPOC1	DNA-directed RNA polymerase subunit beta'	561.2759		21.4	
ASFQET	RPOC2		681.2969	20.2		
EAVGI	RPOC2		487.2642	24.7		
GLMSDPQGQM(+15.99)	RPOC2		1078.4424	15.9		
GTIEM(+15.99)	RPOC2		565.2418			20.1
IDHFGM	RPOC2	DNA-directed RNA polymerase subunit beta''	718.3109			15.6
LGGPC	RPOC2		445.1995	24.2		
NQDIGIEL	RPOC2		900.4553		28.1	16.3
QEREN	RPOC2		674.2983	17.8		
SGARG	RPOC2		446.2237			16.7
SIDSISM	RPOC2		751.3422	18.4		
SSGIT	RPOC2		463.2278	15.1		
HFGHGT	RR2	30S ribosomal protein S2	654.2874		20.6	
GMAST	RR4	30S ribosomal protein S4	465.1893		32.0	
LGMAST	RR4		578.2734		22.1	
EAAAQ	SPDE		546.2285			15.6
EIDKM	SPDE	Spermidine synthase	634.2996			19.2
IAHLP	SPDE		549.3275			25.2
CVIPSN	TI214	Protein TIC 214	631.2999	33.2	27.1	25.7

EM(+15.99)KGT	TI214		580.2527			22.8
FGEMIK	TI214		723.3625		25.2	
NNIPF	TI214		603.3016		30.0	
RWVYT	TI214		723.3704	17.5		
TGQLM(+15.99)	TI214		564.2578			46.6
TVWGM(+15.99)	TI214		608.2628		21.8	
WGDALN	TI214		674.3024			15.9
GAMPGS	XMT1	Monomethylxanthine	518.2159		22.0	
NDLFP	XMT1	methyltransferase 1	604.2856		17.4	
FLSRSD	YCF2		723.3551		18.4	
GNM(+15.99)LGPA	YCF2		674.3057			16.8
IPNIH	YCF2	Protein Ycf2	592.3333		15.3	
KNTQEK	YCF2		746.3923		27.1	
LSDM(+15.99)NLS	YCF2		794.3480		16.5	
M(+15.99)VDSFH	YCF2		750.3007		19.6	16.3
NRGGY	YCF2		565.2609		16.3	
GSRKIS	YCF4	Photosystem I	646.3762			18.0
VGSVG	YCF4	assembly protein Ycf4	417.2224		17.4	
WNVGN	YCF4		588.2656			15.4

* Light, medium and dark shades correspond to light (LSCG), medium (MSCG) and dark (DSCG) thermolysin hydrolysates, respectively.

Table 4. Peptide sequence, retention time (RT), molecular mass, mass accuracy and average local confidence (ALC) of the peptides identified in thermolysin hydrolysates from light, medium and dark SCG (LSCG, MSCG, DSCG) using LC-MS/MS and the *de novo* sequencing tool.

ID	Peptide sequence	RT (min)	Molecular mass (Da)	LRC		MRC		DRC	
				Mass accuracy (ppm)	ALC (%)	Mass accuracy (ppm)	ALC (%)	Mass accuracy (ppm)	ALC (%)
1	DPGDKKN	2.4	772.3715	-	-	7 ± 3	88 ± 5	6 ± 2	87 ± 2
2	SSSDPAQ	6.1	690.282	5 ± 1	89 ± 2	8 ± 1	89 ± 1	-	-
3	CASDPAQ	6.4	690.2643	-	-	9 ± 0	88 ± 1	9 ± 1	86 ± 2
4	AWAH	8.5	483.223	10 ± 0	82 ± 0	3 ± 0	84 ± 0	1 ± 1	83 ± 1
5	M(+15.99)EGSTSSGL	9.2	883.3593	5 ± 2	81 ± 1	2 ± 2	82 ± 1	5 ± 2	81 ± 1
6	GWAEGR	9.9	674.3136	1 ± 1	84 ± 2	-	-	-	-
7	RLNQ	12.2	529.2972	-	-	6 ± 3	83 ± 0	4 ± 2	83 ± 1
8	M(+15.99)DAVGVK	14.2	734.3633	8 ± 2	95 ± 1	5 ± 2	93 ± 2	2 ± 2	94 ± 1
9	NAGHM(+15.99)PN	14.8	755.3021	9 ± 0	90 ± 1	4 ± 3	88 ± 2	-	-
10	VTYDYYQN	15.1	1064.4451	2 ± 0	91 ± 0	4 ± 3	90 ± 1	8 ± 0	90 ± 1
11	M(+15.99)APHWN	18.1	770.317	8 ± 1	90 ± 0	4 ± 2	90 ± 2	-	-
12	RNSGLLNQ	18.5	900.4777	8 ± 1	88 ± 2	10 ± 0	88 ± 0	-	-
13	EANLDVVAHE	18.7	1095.5195	2 ± 1	88 ± 5	4 ± 3	88 ± 3	-	-

14	AATYDYYNQ	18.8	1107.4509	1 ± 1	91 ± 1	4 ± 3	89 ± 1	8 ± 0	90 ± 1
15	CSDAVGVK	19.9	777.3691	8 ± 1	92 ± 2	4 ± 2	91 ± 1	2 ± 1	91 ± 2
16	RCPQGGTHYG	20.2	1074.4666	6 ± 1	84 ± 1	4 ± 2	83 ± 1	4 ± 1	84 ± 1
17	NFDAVGVQ	21.2	848.4028	6 ± 2	88 ± 1	3 ± 2	87 ± 3	5 ± 2	89 ± 2
18	M(+15.99)WDGSQM	21.6	869.3048	5 ± 1	88 ± 1	3 ± 5	85 ± 1	6 ± 1	87 ± 2
19	RM(+15.99)APH	21.8	626.2958	1 ± 1	92 ± 1	4 ± 2	91 ± 1	9 ± 0	91 ± 1
20	LM(+15.99)APHWN	22.4	883.4011	3 ± 2	89 ± 3	1 ± 2	86 ± 2	-	-
21	M(+15.99)GLSDLT	22.9	751.3422	7 ± 2	82 ± 1	4 ± 2	84 ± 2	1 ± 2	86 ± 3
22	CSAPHW	26.2	699.2798	9 ± 0	81 ± 0	4 ± 3	82 ± 1	1 ± 1	84 ± 2
23	WLPFP	27.4	658.3478	5 ± 2	92 ± 1	7 ± 1	93 ± 1	-	-

* Dark shade: peptides identified in < 5 injections. Only isoforms with leucine (L) are presented in these results although peptide sequences containing isoleucine (I) instead of L are also possible.

Figures

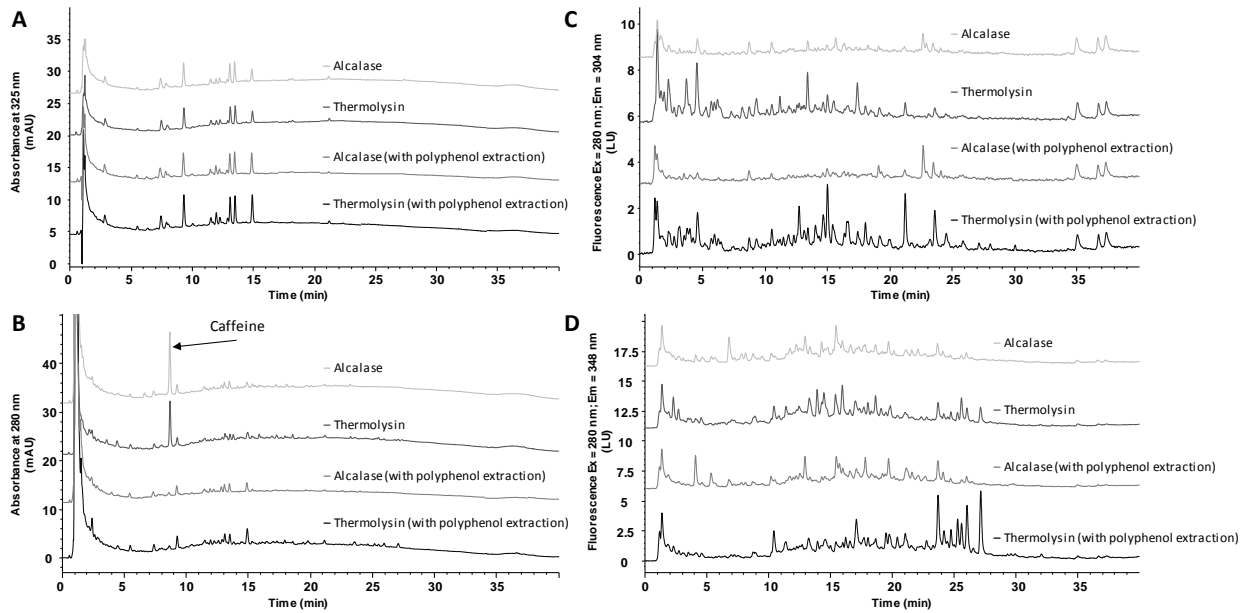
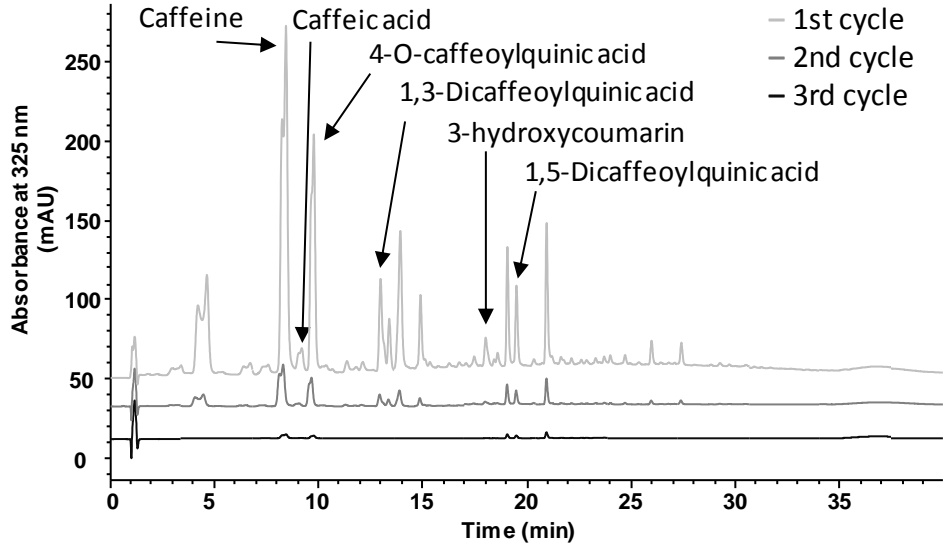


Figure 1

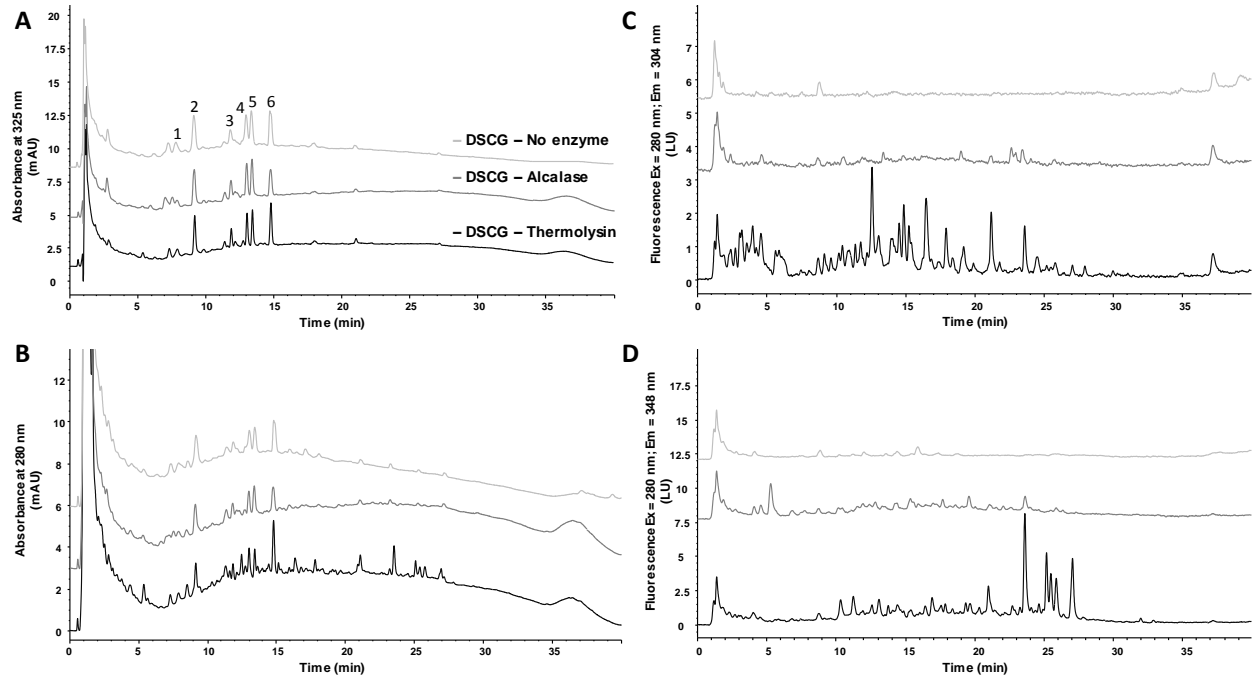
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670 **Figure 2**

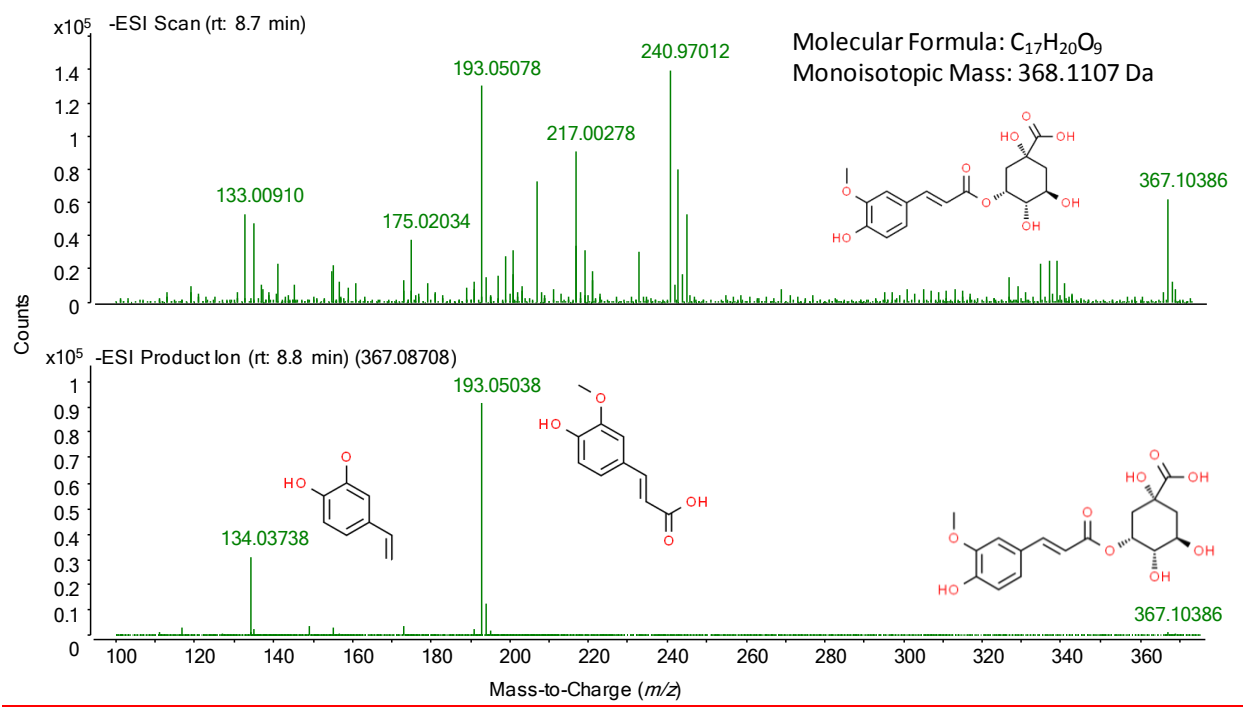
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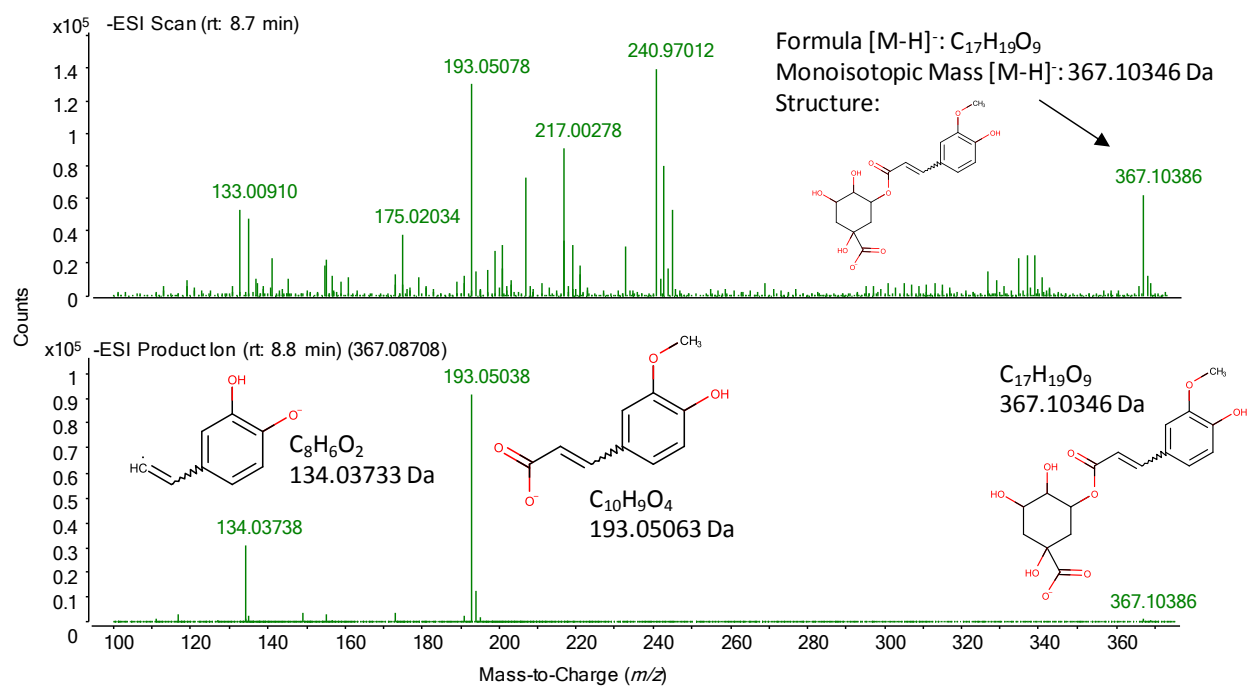
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672 **Figure 3**

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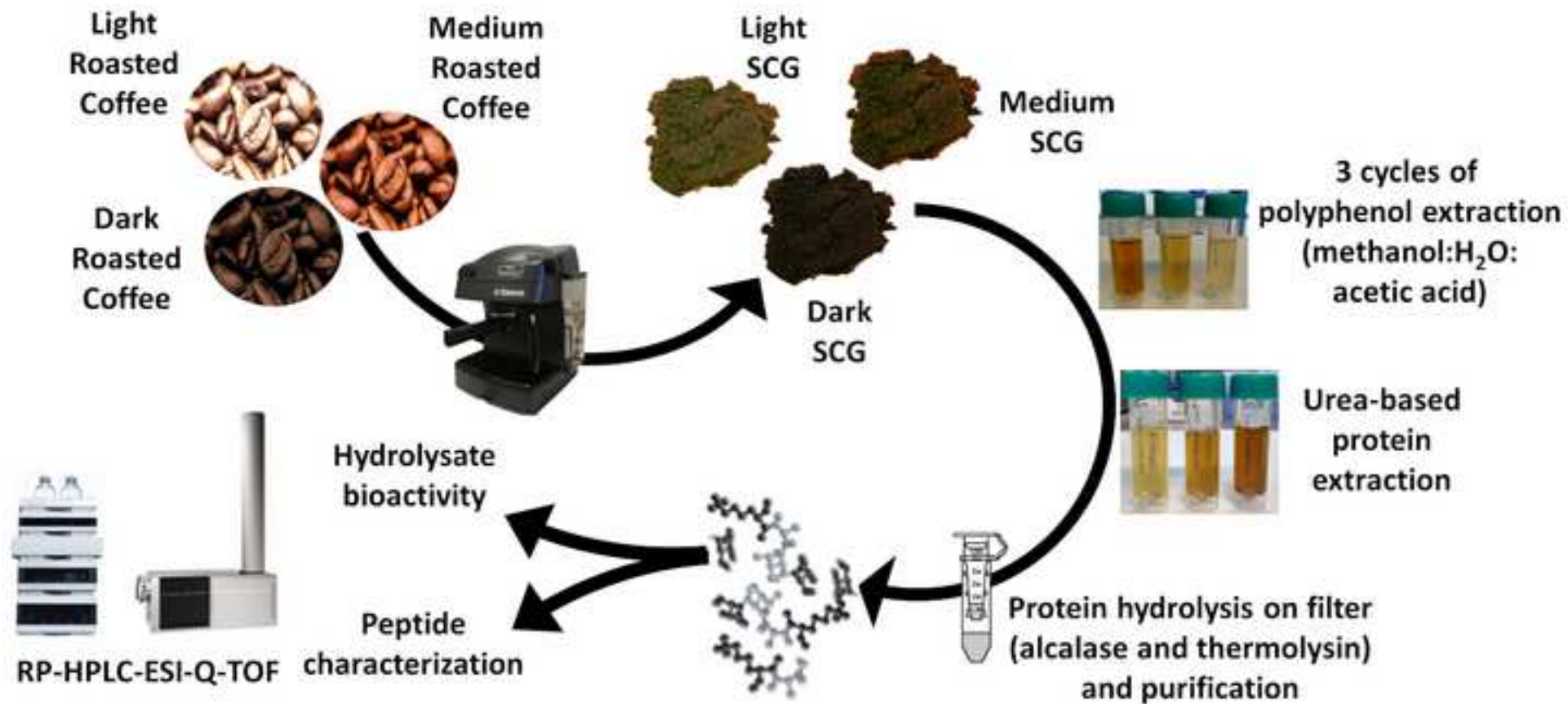


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675 **Figure 4**

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