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Isolation of proteins from spent coffee grounds. Polyphenol removal and peptide identification in the protein hydrolysates by RP-HPLC-ESI-Q-TOF

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Abstract

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

Keywords

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

1. Introduction

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

also be incorporated into melanoidins, mainly by transglycosylation reactions (Moreira et al., 2017). Melanoidins are the high molecular weight nitrogenous and brown-coloured compounds formed during the roasting process of coffee (Bekedam, Roos, Schols, Van Boekel, & Smit, 2008; Moreira, Nunes, Domingues, & Coimbra, 2012), and the incorporation of phenolic compounds into the melanoidins is a significant pathway of CGA degradation during roasting (Coelho et al., 2014). Moreover, it has also been shown that the coffee preparation affects the concentration of these compounds (Bravo et al., 2012).

Apart from the mentioned compounds, the mean protein content of SCG is 13.6% (Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011; Silva, Nebra, Machado-Silva, & Sanchez, 1998), but this content might be overestimated due to the presence of other nitrogen-containing substances such as caffeine, trigonelline, free amines and/or amino acids (Delgado, Vignoli, Siika-aho, & Franco, 2008). The protein content also varies depending on the brewing and roasting processes, when the proteins can be fragmented, polymerized, and/or integrated into melanoidins (Bravo, et al., 2012; Cruz et al., 2012; Tokimoto, Kawasaki, Nakamura, Akutagawa, & Tanada, 2005). However, no works have focused on the identification of bioactive peptides from SCG proteins hydrolysates. Bioactive peptides are mainly found in a latent state as part of a protein from which they can be released by hydrolysis using different enzymes (Sarmadi & Ismail, 2010). In order to exploit SCG as a source of bioactive peptides, SCG proteins have to be extracted avoiding the extraction of other interfering compounds (such as polyphenols), and then hydrolysed. In the present work, a commonly used Tris-HCl based method for protein extraction from food by-products has been compared to a new urea-based method to isolate SCG proteins, and the best method has been applied to evaluate the protein content in SCG from different coffee-brewing preparations. In addition, a polyphenol extraction step to remove interferences has been studied, as well as the hydrolysis of the resultant proteins into peptides using alcalase and thermolysin enzymes. Furthermore, the effect of roasting degree in the presence of peptides with antioxidant and/or *in vitro* angiotensin-converting enzyme (ACE)-inhibitory activity was evaluated. Finally, the resulting peptides

have been identified by reversed-phase high-performance liquid chromatography coupled via electrospray ionization to a quadrupole time-of-flight mass spectrometer (RP-HPLC-ESI-Q-TOF).

2. Materials and Methods

2.1. Chemicals

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

2.2. Coffee samples

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

2.3. Spent coffee ground preparation

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

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

2.4. Polyphenol extraction

When specified, and before protein extraction, polyphenols were extracted from defatted SCG samples (in triplicate). For this aim, 5 mL of methanol:H_2O:acetic acid (70:28:2, v/v/v) were added to 100 mg of defatted SCG, and a high intensity focused ultrasound (HIFU) probe (model VCX130, Sonics Vibra-Cell, Hartford, CT, USA) was used for 5 min with an amplitude of 30% (HIFU standard conditions if not specified elsewhere). Thereafter, samples were vortexed in a mechanical stirrer (Selecta, Barcelona, Spain) at room temperature for 30 min, centrifuged at 4,000g for 10 min and the supernatants were transferred to a different vial. This procedure was repeated three times and the pellets were dried at 40 °C overnight. The collected supernatants after each extraction cycle were pooled together and directly analysed by HPLC-UV/HPLC-FLD.

2.5. Protein extraction and quantification

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

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

2.6. Protein analysis by SDS-PAGE

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

2.7. Protein hydrolysis

For protein hydrolysis, 0.8 mg of SCG proteins extracted with UB (for the comparison between the different hydrolytic enzymes and for the evaluation of the polyphenol extraction step) or 4 mL of UB (for the evaluation of the effect of the roasting degree) were transferred to 3 kDa molecular weight cut-off filters (Amicon Ultra 0.5 mL - 3 kDa, Merck Millipore, Burlington, MA, USA), previously washed once with 250 µL of a mixture of ACN:H_2O (20:80, v/v) at 14,000g for 10 min, and later on with 500 µL of Milli-Q water at 14,000g for 15 min. The samples were then centrifuged at 14,000g until the whole volume was loaded, and consecutive washes were performed with 300 µL of 50 mM AmBi in ACN:H_2O (20:80, v/v) (once) and then with 300 µL of 50 mM AmBi in Milli-Q water (twice) to remove urea excess. Between each washing step, the filter units were centrifuged at 14,000g for 15 min. Finally, protein hydrolysis was carried out inside the filter units with 300 µL of 50 mM AmBi buffer (pH 8.0) using two different enzymes: alcalase (0.15 AU enzyme/g protein) and thermolysin (0.1 g enzyme/g protein), and in similar conditions as in Hernández-Corroto, Marina, & García (2019) and Pérez-Míguez, Marina, & Castro-Puyana (2019). Hydrolysis was performed at 50 °C for 4 h by slight mixing (750 rpm) in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany), and the reactions were stopped by heating to 100 °C for 10 min. Resulting peptides were then centrifuged at 14,000g for 10 min, and 200 µL of 50 mM AmBi were added (twice) and centrifuged again to recover all peptides. Finally, samples were transferred to new tubes, dried in SpeedVac (Eppendorf AG, Hamburg, Germany) and dissolved in Milli-Q water to a final concentration of 2.5 mg/mL (considering the starting amount of loaded protein).

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

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

determined as previously described (Hernández-Corroto, Marina, & García, 2018; Hernández-Corroto, Marina, & García, 2019). See **Supplementary Material and Methods** for the full description.

2.9. HPLC-UV and HPLC-FLD analyses

Separation of polyphenol extracts (dissolved in methanol:H_2O:acetic acid (70:28:2, v/v/v)) and SCG protein hydrolysates (dissolved in Milli-Q water) were performed in a 1100 series LC system (Agilent Technologies, Germany). LC control, data acquisition, and data analysis were carried out using the Agilent LC/MSD ChemStation software (B.04.03). Injection volume was set to 5 µL and all samples were analyzed in duplicate. A porous-shell fused-core Ascentis Express C18 analytical column (100 mm × 2.1 mm, particle size 2.7 µm) protected by a C18 guard column (0.5 cm × 2.1 mm, particle size 2.7 µm), both from Supelco (Bellefonte, PA, USA) were used. The flow-rate was set to 0.25 mL/min and the column 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 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 with 0.1% (v/v) FA. Detection was carried out using an UV detector at 280 and 325 nm, and using a fluorescent detector (FLD) at λ_{exc}= 280 and λ_{em}= 304 and 348 nm.

2.10. RP-HPLC-ESI-Q-TOF peptide and polyphenol identification

Identification of peptides and polyphenols in SCG protein hydrolysates (dissolved in Milli-Q water) were performed by RP-HPLC-ESI-Q-TOF as previously described (Pérez-Míguez, Marina, & Castro-Puyana, 2019). Briefly, MS analysis was performed in a quadrupole Q-TOF series 6530 coupled to a HPLC (model 1100) both from Agilent Technologies (Germany), equipped with a Jet Stream thermal orthogonal ESI source. MS control, data acquisition, and data analysis were carried out using the Agilent Mass Hunter Qualitative Analysis software (B.07.00). Injection volume was set to 10 µL, and three replicates were injected in triplicates. For the chromatographic separation, an Ascentis Express Peptide ES-C18 analytical column (100 × 2.1 mm, particle size 2.7 µm) with a C18 guard column (0.5 cm × 2.1 mm, particle size

2.7 µm), both from Supelco (Bellefonte, Pa, USA) were employed. The column temperature was held at 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 and mobile phase B consisted of ACN with 0.3% (v/v) acetic acid. The gradient was 5% B for 3 min, 5–40% 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 conditions. ESI in positive ion mode (for peptides) or in negative ion mode (for polyphenols) were used at a capillary voltage of 3500 V and with a *m/z* range from 100 to 1700. UV signals were also recorded at 280 and 325 nm. MS analyses were performed employing the auto MS/MS mode using 3 precursor per cycle, dynamic exclusion after two spectra (released after 1 min), and collision energy of 5 V for every 100 Da. For proper mass accuracy, spectra were corrected using ions *m/z* 121.0509 ($C_5H_4N_4$) and 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$) in ESI positive mode, and *m/z* 119.0363 ($C_5H_4N_4$) and 980.0164 ($C_{18}H_{18}O_6N_3P_3F_{24}$ + formate) in ESI negative mode, simultaneously pumped into the ionization source at a 15 µL/min flow rate. Peaks software (Bioinformatics Solutions Inc.; Waterloo, ON, Canada) was employed for database search against Uniprot *Coffea arabica* reviewed proteome (downloaded on July 25th 2019 and containing 92 entries). Oxidation of methionine (+15.99 Da) was included as variable modification and peptide results were refined using a − 10lgP threshold of 15. Moreover, *de novo* peptide sequencing was performed using an error tolerance of 10 ppm for the precursor and 0.5 kDa for the fragment, and including oxidation of methionine as variable modification. *De novo* peptide sequences were accepted if the average local confidence (ALC, expected percentage of correct amino acids in the peptide sequence) was ≥ 80% in at least 5 from 9 injections. Moreover, since the MS system is not able to distinguish between isoleucine (I) and leucine (L) amino acids due to their equal molecular masses, only isoforms with L are presented.

2.11. Statistical analysis

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

differences are observed between the French Press SCG (2.40 mg of protein/100 mg of SCG) and the Filter SCG (2.26 mg of protein/100 mg of SCG), and the lowest protein content is obtained from mocha SCG (1.68 mg of protein/100 mg of SCG). The total solid and the protein content of coffee brews have been directly related with the extraction time used for the coffee preparation (Zanoni, Pagliarini, & Peri, 1992), but it is also known that the coffee weight-to-water volume ratio and the coffee grinding degree have a great impact (Cruz et al., 2012). It has been demonstrated that coffee brews from mocha machines contain more total solids per gram of roasted and ground coffee than coffee brews prepared with other procedures (Gloess, et al., 2013). In the present work we kept fixed the coffee weight-to-water volume ratio, the type of coffee, the roasting degree and the grinding degree, and we varied the pressure, temperature and extraction times, which are intrinsic parameters to the different coffee preparations used. The highest protein content in espresso SCG suggests that even thought a higher pressure is used for the espresso coffee preparation, the sorter time has a greater effect on the extracted proteins.

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

The used UB has been previously applied in different proteomics studies to extract proteins from complex tissues, and it has been combined with Amicon devices (3 kDa molecular cut-off filters) to remove contaminants before HPLC and/or MS analysis, and to hydrolyse proteins into peptides prior to HPLC-MS analyses (Holfeld, Valdés, Malmström, Segersten, & Lind, 2018; Wiśniewski, 2017). In the present study, the use of 3 kDa filters would allow to retain coffee proteins larger than 3 kDa, to discard the UB, and to remove free and small compounds from the samples, such as polyphenols. The use of these filters would also help removing big polyphenols and melanoidins that might remain on top of the filter after hydrolysis. This procedure has some drawbacks as very small proteins can be lost during sample loading and washing steps, and peptides bigger than 3 kDa can remain on top of the filter after

the hydrolysis. Considering these aspects, the peptide content and the antioxidant activity of alcalase and thermolysin protein hydrolysates from espresso SCG were measured (**Table 1**). The upper part shows that the peptide content and the ABTS antioxidant activity are similar in both hydrolytic extracts, but the antioxidant activity based on the hydroxyl radical assay is higher in alcalase hydrolysates. These protein hydrolysates were then characterized by HPLC-UV at 325 nm (maximum UV absorbance for CGA) (**Figure 1A**, upper part) and at 280 nm (maximum UV absorbance for caffeine) (**Figure 1B**, upper part). It can be observed that caffeine (t_R 8.75 min) and other phenolic compounds remain in the samples, but no differences were observed between thermolysin or alcalase hydrolysates profiles. These hydrolysates were also characterized by HPLC-FLD at λ_{exc}= 280 nm and λ_{em}= 304 and 348 nm (maximum fluorescence excitation and emission wavelengths of the two amino acid residues that are primarily responsible for the fluorescence of peptides/proteins, tryptophan and tyrosine) (**Figures 1C and 1D**, upper part). These results demonstrate that thermolysin hydrolysates are more complex than alcalase hydrolysates in terms of peptide content.

To try to reduce the quantity of phenolic compounds, a polyphenol extraction step was included before the protein extraction. The characterization of the polyphenolic extracts allowed the identification of caffeine, caffeic acid, 4-O-caffeoylquinic acid, 1,3-dicaffeoylquinic acid, 3-hydroxycoumarin and 1,5-dicaffeoylquinic acid using pure standards (**Figure 2**). In addition, a decrease of approximately 9 times after each extraction cycle was observed. Thereafter, the proteins were extracted and the protein concentration was measured, being 0.91 mg of protein/100 mg of SCG (a 69% reduction compared to the non-polyphenol extraction, 2.90 mg of protein/100 mg of SCG). This reduction can be explained by the composition of the solvent used (methanol:H_2O:acetic acid (70:28:2, v/v/v)), which has been previously used to extract polyphenols from coffee (Mussatto, Ballesteros, Martins, & Teixeira, 2011) or cocoa beans (D'Souza et al., 2017), but also to extract peptides from cocoa beans (D'Souza et al., 2018). The peptide content and the antioxidant activity of these extracts was also evaluated (**Table 1**, lower part). A

decrease of 47% of the peptide content (between the two alcalase hydrolysates) was observed when the polyphenol extraction step was included, but this effect was not observed when thermolysin was used. Moreover, the polyphenol extraction step increased the antioxidant activity of thermolysin hydrolysates, being this effect slightly higher but not significant with respect to the alcalase hydrolysates. These results are unexpected since it is commonly known that phenolic compounds have higher antioxidant activity than peptides. However, it has been widely discussed that polyphenols can also have inhibitory effects on enzymes involved in the hydrolysis of proteins (Cirkovic Velickovic & Stanic-Vucinic, 2018), therefore the polyphenol removal prior to the hydrolysis step could increase the release of peptides with antioxidant activity.

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

3.3. Effect of roasting degree on thermolysin SCG hydrolysates

The next step was the evaluation of the roasting degree effect on the protein/peptides extracts obtained from different SCG (LSCG, light spent coffee grounds; MSCG, medium spent coffee grounds; DSCG, dark spent coffee grounds). Three independent espresso coffees were prepared and polyphenols were extracted before proteins were isolated using UB. The characterization of the polyphenolic extracts

indicates that the area of caffeic acid, 4-O-caffeoylquinic acid, 1,3-dicaffeoylquinic acid and 1,5-dicaffeoylquinic acid decreased with the roasting process; the area of caffeine remained unchanged; and the area of 3-hydroxycoumarin is increased (**Supplementary Material, Figure S3**). These results agree well with previous reports where CGA such as 5-CQA, 4-CQA, 3-CQA, 3,5-diCQA, 4,5-diCQA, 5-FQA, 3,4-diCQA and 4-FQ, are degraded during roasting, whereas the formation of CGA lactones takes place (Farah, de Paulis, Trugo, & Martin, 2005; Moon, Yoo, & Shibamoto, 2009). The levels of these compounds may also be decreased by their incorporation into Maillard reaction products during the roasting process (Coelho et al., 2014; Delgado-Andrade & Morales, 2005; Bekedam, Roos, Schols, Van Boekel, & Smit, 2008). It has been suggested that melanoidins are derived from cross-linking of Maillard reaction products to proteins via reactive side chains of amino acids, and more recently it has been demonstrated that transglycosylation reactions to form new polysaccharides is the main mechanism for this incorporation (Moreira et al., 2017). On the other hand, the caffeine levels are not affected (Oestreich-Janzen, 2010).

Thereafter, the protein concentration was evaluated by Bradford assay, indicating that DSCG had higher amount of proteins (1.49 mg of protein/100 mg of SCG) than MSCG (1.08 mg of protein/100 mg of SCG), and three times more than LSCG (0.49 mg of protein/100 mg of SCG) (**Supplementary Material, Figure S4**). The SDS-PAGE analysis also shows a slightly more intense protein bands of ≈150 kDa in DSCG (**Supplementary Material, Figure S5**). The higher protein concentration of DSCG could be partially explained by the loss coffee weight during the roasting process (13% for light, 15% for medium, and 17% for dark roasted beans), which can enrich the material that is not degraded or loss. However, darker roasting has shown to produce more total soluble solids in the brew (Petracco 2005), and therefore there should be fewer proteins in their SCG. Moreover, it is also known that coffee proteins can be fragmented, polymerized or integrated into melanoidins through the Maillard reaction during the roasting process, but it is not well known how this reaction affects the functional properties (such as the

solubility) of proteins (Oliver, Melton, & Stanley, 2006). After protein quantification, the peptide content, the antioxidant activity and the *in vitro* ACE-inhibitory activity of thermolysin protein hydrolysates from LSCG, MSCG and DSCG were measured (**Table 2**). For comparison purposes, DSCG protein isolates were also hydrolysed with alcalase, and a control sample incubated without enzyme was included. As expected due to the higher amount of proteins, the highest peptide concentration among the three thermolysin hydrolysates was obtained in DSCG, followed by MSCG and LSCG (**Table 2**). There were not significant differences on the ABTS or hydroxyl scavenging activity between the samples, but the DSCG and the MSCG hydrolysates had higher *in vitro* ACE-inhibitory activity. The protein hydrolysates were then characterized by HPLC-UV and HPLC-FLD (**Supplementary Material, Figures S6-S9**). The acquisition at 280 nm shows that the area of some peaks are slightly increased (peaks at t_R 5.5 min and 14.7 min); some of them remained unchanged (peaks at t_R 23.0 min, 25.2 min, 25.5 min and 26.0 min); and others are decreased (peaks at t_R 19.0 min and 27.0 min) with the roasting process (**Supplementary Material, Figures S6**); and the chromatograms acquired at 325 nm indicate that most of the peaks were significantly increased with the roasting process (**Supplementary Material, Figures S7**). There were not significant differences in the FLD-chromatograms acquired at λ_{exc} = 280 nm and λ_{em} = 304 nm, but when λ_{em} was set to 348 nm (**Supplementary Material, Figures S8**), the general trend was a decrease in the area when increasing the roasting degree (**Supplementary Material, Figures S9**). As previously commented, the signals observed at 325 nm might be due to the presence of phenolic compounds that can be released during the incubation step at 50 °C. It has been observed that the incorporation of phenolic compounds into the melanoidins is a significant pathway of CGA degradation during roasting (Coelho et al., 2014), being transglycosylation reactions the main mechanism for this incorporation (Moreira et al., 2017). Different techniques such as alkaline hydrolysis, acid hydrolysis, the increase of the medium ionic strength, or the alkaline fusion method have been applied to release them (Bekedam, Roos, Schols, Van Boekel, & Smit, 2008; Monente, Ludwig, Irigoyen, De Peña, & Cid, 2015; Perrone,

Farah, & Donangelo, 2012; Delgado-Andrade & Morales, 2005; Coelho et al., 2014). For instance, covalently bound caffeic and ferulic acids decrease with roasting, while the content of dihydrocaffeic acid increases (Perrone, Farah, & Donangelo, 2012). And by using the alkaline fusion method, it has been suggested that the incorporation of phenolic compounds in coffee melanoidins is also related to the amount of proteins (Coelho et al., 2014). Complementary, the antioxidant activity of melanoidins from coffee brews obtained using roasted coffees is higher than those obtained from green coffees (Delgado-Andrade, Rufián-Henares, & Morales, 2005; Perrone, Farah, & Donangelo, 2012). Based on these results, different CGA acids could have been released from melanoidins, or some melanoidins could still be present in the SCG protein hydrolysates, but further studies are needed to confirm these hypotheses.

3.4. Evaluation of the enzymatic incubation on SCG hydrolysates

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

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 were 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.

Different peptides with a number of amino acids ranging from 4 to 10 were identified, and none of these peptides belong to the enzymes employed for hydrolysis. In the case of DSCG alcalase hydrolysates, 5 peptides (ALM(+15.99)APH, M(+15.99)EGL, CCVLLP, NVLAR and NLM(+15.99)APH, **Supplementary Material, Table S1**) were identified, and only one peptide (SHWH) was identified when no enzyme was used. In overall, thermolysin hydrolysates present a higher number of peptides (12 in LSCG, 14 in MSCG and 10 in DSCG) and, when combining the results with the database search, 37, 48 and 26 peptides were identified in LSCG, MSCG and DSCG thermolysin hydrolysates, respectively; 30 peptides in DSCG alcalase hydrolysates; and only 15 peptides when no enzyme was used. The possible antioxidant or antihypertensive/ACE-inhibitory activity of these peptides was searched in the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), but none of them have been reported. In overall, these results indicate that the peptide complexity of thermolysin hydrolysates is higher than alcalase hydrolysates, and some of the thermolysin released peptides (or a combination of them) would be responsible for the higher ACE-inhibitory activity observed. Since LSCG presents some antioxidant and ACE inhibitory activities, the common peptides identified in the three thermolysin hydrolysates using the *de novo* sequencing tool (CSDAVGVK and RCPQGGTHYG), and the common peptides identified as coming from LSCG, MSCG and DSCG proteins (YKPPYS and CVIPSN) would be the first candidates to be evaluated for their biological activity. The tentative identification of CSDAVGVK peptide in DSCG is shown as example in **Supplementary Material, Figure S12**. Moreover, the higher ACE-inhibitory activity observed for MSCG and DSCG thermolysin hydrolysates would be explained by the higher number of identified peptides (48 in the case of MSCG), or by the specific sequence of the identified peptides in the MSCG/DSCG thermolysin hydrolysates, but not in the LSCG or the alcalase hydrolysates. The following peptides meet this requirement: DPGDKKN, CASDPAQ and RLNQ, identified in the MSCG and the DSCG using the *de novo* sequencing tool; GGSMG, GVMDFQ, LDPIEF, GGGDL, YEAWL, DAHIPPG, DPHFGQPAVE, FGM(+15.99)NSLS, FPCDGP, GPVNIAY, GMAST and LGMAST, identified as coming from MSCG proteins;

and FLSRSD, IPNIH, LSDM(+15.99)NLS, M(+15.99)VDSFH and NRGGY, identified as coming from DSCG proteins.

Additionally, RP-HPLC with UV (at 325 nm) and MS (in ESI negative mode) detectors were used to tentatively identify the possible remaining polyphenols in SCG protein hydrolysates (**Supplementary Material, Figures S10B and S10D**). Among the different observed peaks in **Figure 3A**, peaks **1–5** showed 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** 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 of these polyphenols was performed by analysing the MS and MS/MS spectra (as exemplified in **Figure 4** for 3-FQA), and comparing them with the fragmentation patterns already reported (Kuhnert, Jaiswal, Matei, Sovdat, & Deshpande, 2010; Clifford, Johnston, Knight, & Kuhnert, 2003). Based on these analyses, peak **1** was tentatively assigned as 3-FQA, peak **2** as 1-FQA, peaks **3** and **5** as 5-FQA, and peak **4** as 4-FQA. However, peak **6** could not be identified. These phenolic compounds have already been identified in SCGs (Panusa, Zuorro, Lavecchia, Marrosu, & Petrucci, 2013; Bravo et al., 2012), and they might be partly responsible for the antioxidant activity exerted by the extracts.

4. Conclusion

In conclusion, the urea-based extraction buffer allows the extraction of more proteins than the conventional Tris-HCl buffers, and its application demonstrates that the highest protein content is obtained from espresso SCG. Moreover, the use of 3 kDa molecular cut-off filters allows the removal of the buffer and the hydrolysis of the proteins using thermolysin and alcalase enzymes, but not the complete removal of polyphenols. Moreover, these protein hydrolysates possess antioxidant and ACE-inhibitory activities, the latter being highest when SCG samples are obtained from dark/medium roasted coffees, and after the inclusion of a polyphenol step removal. Finally, several peptides that might be responsible for the ACE-inhibitory activity observed have been identified. However, further experiments

using synthetic peptides are needed to confirm which of the released peptides (or a combination of them) are the most bioactive.

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Conflict of Interest

The authors declare no conflict of interest

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Figure Captions

Figure 1. HPLC chromatograms of SCG proteins hydrolysed with alcalase and thermolysin (including the polyphenol extraction step). Chromatographic conditions were: Ascentis Express C18 analytical column (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 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-rate, 0.25 mL/min; temperature, 30 °C; injected volume, 5 µL; absorbance detection was performed at λ 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 348 nm (**D**).

Figure 2. HPLC chromatograms obtained from methanol:H_2O:acetic acid (70:28:2, v/v/v) extracts after one, two or three extraction cycles before SCG protein extraction. Separation conditions were the same as in **Figure 1**, and UV absorbance was detected at λ of 325 nm.

Figure 3. HPLC chromatograms of DSCG protein hydrolysed with thermolysin, alcalase or "no enzyme" with polyphenol extraction. Separation conditions were the same as in **Figure 1**, absorbance detection was performed at λ 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 348 nm (**D**). Peaks were lately identified by RP-HPLC-ESI-Q-TOF-MS/MS as: **1**, 3-FQA; **2**, 1-FQA; **3**, 5-FQA; **4**, 4-FQA; **5**, 5-FQA; **6**, not identified.

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

Tables

Table 1. Peptide content and antioxidant activity (ABTS and hydroxyl assays) of SCG (after espresso preparation of commercial medium roasted 100% Arabica coffee) proteins hydrolysed with alcalase and thermolysin (and including the polyphenol extraction step).

Sample	Peptide content (mg/mL)	ABTS assay (%)[1]	Hydroxyl radical assay (%)
Alcalase	$0.43^{a} \pm 0.08$	$22.1^{b} \pm 5.20$	$88.4^{a} \pm 11.6$
Thermolysin	$0.42^{a} \pm 0.05$	$19.8^{b} \pm 11.3$	$73.5^{b} \pm 3.55$
Alcalase (with polyphenol extraction)	$0.23^{b} \pm 0.02$	$30.7^{a,b} \pm 0.52$	$84.3^{a,b} \pm 4.85$
Thermolysin (with polyphenol extraction)	$0.39^{a} \pm 0.05$	$36.1^{a} \pm 2.39$	$95.4^{a} \pm 2.70$

*Different letters indicate significant differences between samples after ANOVA with LSD Post-hoc, p-value < 0.05.
[1] For ABTS antioxidant activity, samples were diluted to 1:150.

Table 2. Peptide content, antioxidant activity (ABTS and hydroxyl assays) and *in vitro* ACE-inhibitory activity of thermolysin hydrolysates from light, medium and dark espresso SCG (LSCG, MSCG, DSCG), alcalase hydrolysates from DSCG, and incubation of DSCG without enzyme.

Sample	Peptide content (mg/mL)	ABTS assay (%)[1]	Hydroxyl radical assay (%)	*In vitro* ACE-inhibitory activity (%)
LSCG – Thermolysin	$0.22^{b} \pm 0.05$	$30.5^{a,b} \pm 4.26$	$85.7^{a,b} \pm 7.84$	$61.7^{b} \pm 4.98$
MSCG– Thermolysin	$0.31^{a,b} \pm 0.09$	$28.7^{a,b} \pm 9.47$	$81.6^{b} \pm 10.2$	$83.0^{a} \pm 2.72$
DSCG – Thermolysin	$0.41^{a} \pm 0.08$	$32.9^{a,b} \pm 4.28$	$92.2^{a} \pm 11.3$	$81.5^{a} \pm 1.51$
DSCG – Alcalase	$0.21^{b} \pm 0.07$	$39.8^{a} \pm 5.49$	$96.1^{a} \pm 1.89$	$61.5^{b} \pm 3.84$
DSCG – No enzyme	$0.25^{b} \pm 0.03$	$24.8^{b} \pm 8.31$	$83.4^{a,b} \pm 6.27$	$37.7^{c} \pm 9.71$

*Different letters indicate significant differences between samples after ANOVA with LSD Post-hoc, p-value < 0.05.
[1] For ABTS antioxidant activity, samples were diluted to 1:150.

Table 3. Peptide sequence, belonging gene and protein name, molecular mass (Da) and −10lgP 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.

				−10lgP				
				Thermolysin			Alcalase	No Enzyme
Peptide sequence	Gene name	Protein name	Molecular mass (Da)	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		

YKSQNM	NU5C		769.3429		16.1			
HNYYGEPA	PETD	Cytochrome b6-f complex	949.3929		15.2	23.9		
GGGDL	PSAA	Photosystem I P700 chlorophyll a apoprotein A1	417.1859		16.3			
YEAWL	PSAA		680.3170		28.4			
DAHIPPG	PSAB	Photosystem I P700 chlorophyll a apoprotein A2	705.3445		15.3			
DPHFGQPAVE	PSAB		1095.4985		16.9			
FGM(+15.99)NSLS	PSAB		770.3269		17.7			
FPCDGP	PSAB		634.2421		18.6			
GPVNIAY	PSAB		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	DNA-directed RNA polymerase subunit beta''	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		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			
EEAAQ	SPDE	Spermidine synthase	546.2285				15.6	
EIDKM	SPDE		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 methyltransferase 1	518.2159			22.0		
NDLFP	XMT1		604.2856				17.4	
FLSRSD	YCF2	Protein Ycf2	723.3551			18.4		
GNM(+15.99)LGPA	YCF2		674.3057					16.8
IPNIH	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 assembly protein Ycf4	646.3762					18.0
VGSVG	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.

				LRC		MRC		DRC	
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**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

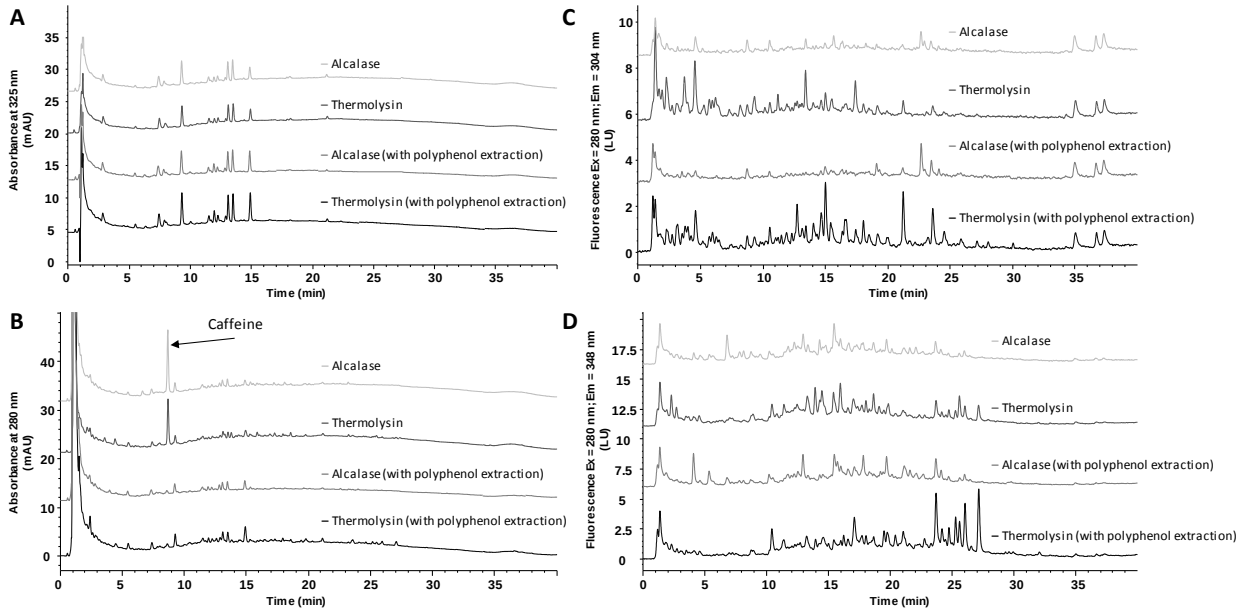
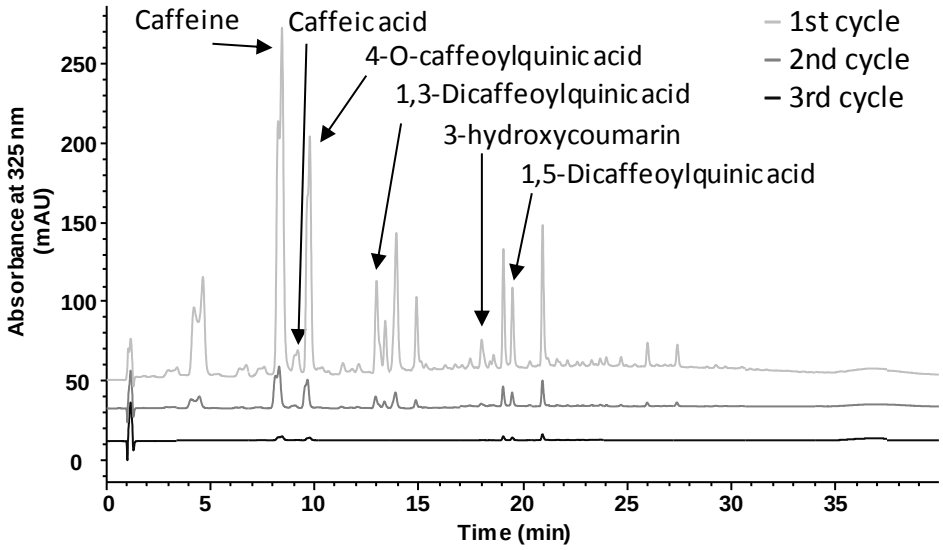
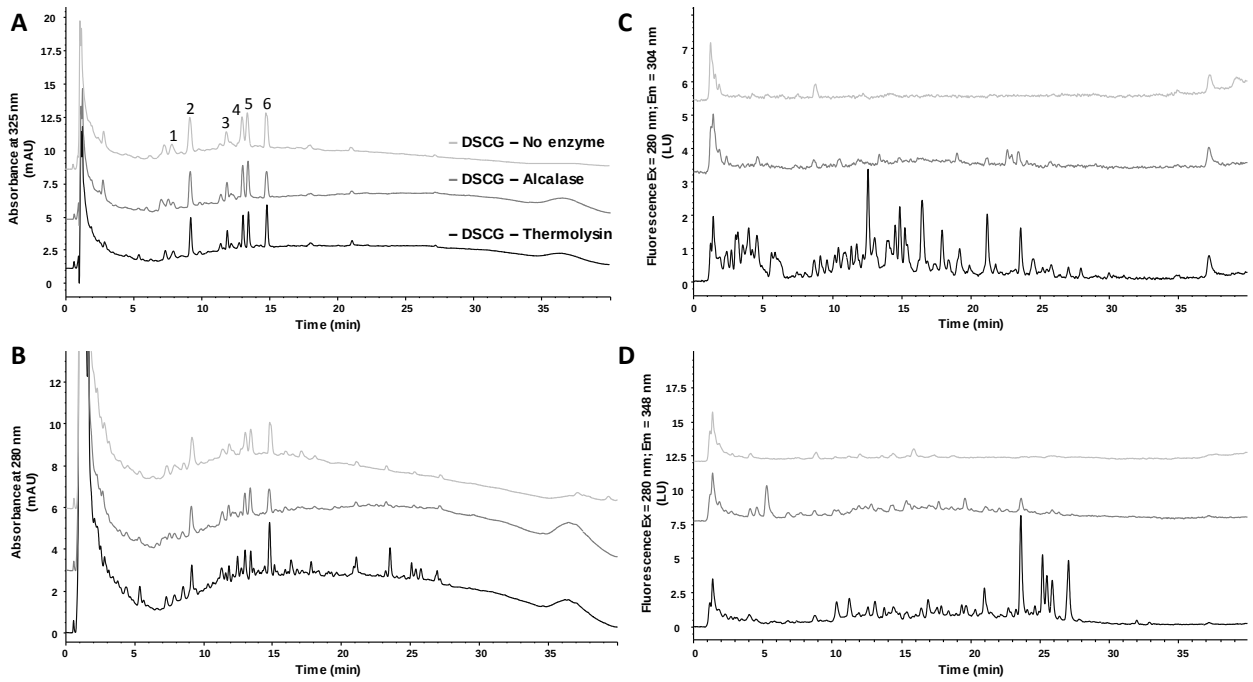


Figure 1



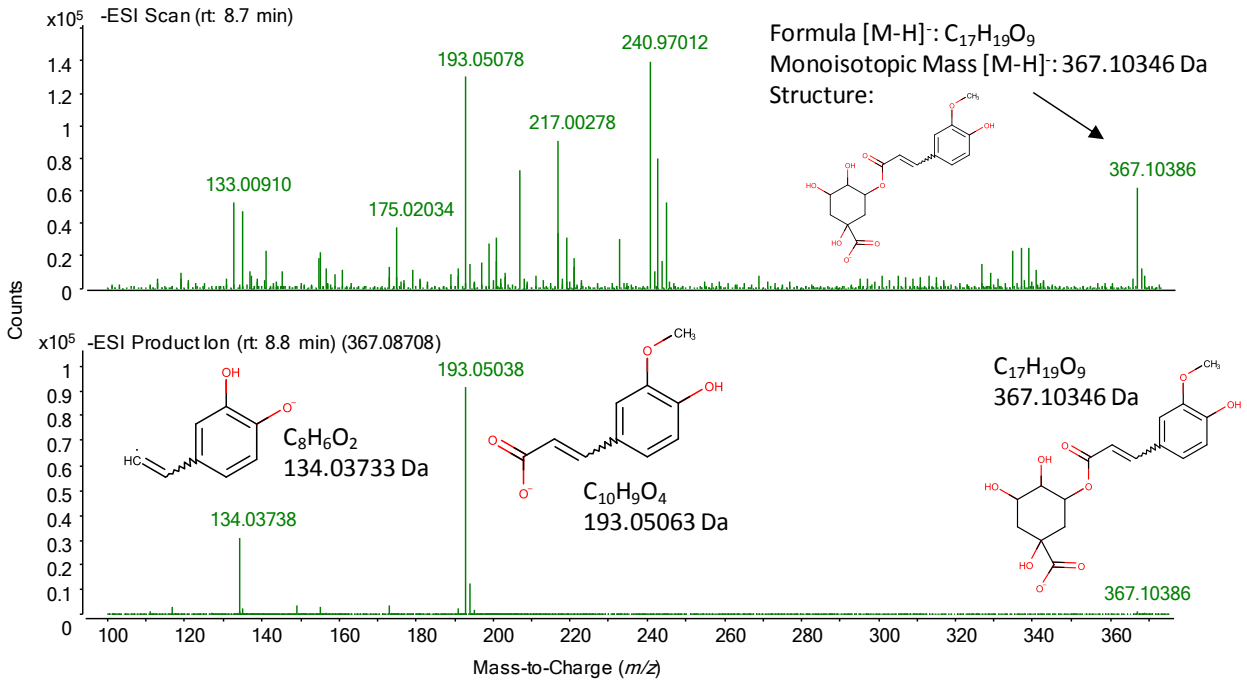
668

669 **Figure 2**



670

671 **Figure 3**


x10^5 -ESI Scan (rt: 8.7 min)
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Monoisotopic Mass [M-H]⁻: 367.10346 Da
Structure:
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193.05078
217.00278
133.00910
175.02034
367.10386
Counts
x10^5 -ESI Product Ion (rt: 8.8 min) (367.08708)
193.05038
C8H6O2
134.03733 Da
C10H9O4
193.05063 Da
C17H19O9
367.10346 Da
134.03738
367.10386
Mass-to-Charge (m/z)

Figure 4

Isolation of proteins from spent coffee grounds. Polyphenol removal and peptide identification in the protein hydrolysates by RP-HPLC-ESI-Q-TOF

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Abstract

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

Keywords

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

1. Introduction

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

also be incorporated into melanoidins, mainly by transglycosylation reactions (Moreira et al., 2017). Melanoidins are the high molecular weight nitrogenous and brown-coloured compounds formed during the roasting process of coffee (Bekedam, Roos, Schols, Van Boekel, & Smit, 2008; Moreira, Nunes, Domingues, & Coimbra, 2012), and the incorporation of phenolic compounds into the melanoidins is a significant pathway of CGA degradation during roasting (Coelho et al., 2014). Moreover, it has also been shown that the coffee preparation affects the concentration of these compounds (Bravo et al., 2012).

Apart from the mentioned compounds, the mean protein content of SCG is 13.6% (Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011; Silva, Nebra, Machado-Silva, & Sanchez, 1998), but this content might be overestimated due to the presence of other nitrogen-containing substances such as caffeine, trigonelline, free amines and/or amino acids (Delgado, Vignoli, Siika-aho, & Franco, 2008). The protein content also varies depending on the brewing and roasting processes, when the proteins can be fragmented, polymerized, and/or integrated into melanoidins (Bravo, et al., 2012; Cruz et al., 2012; Tokimoto, Kawasaki, Nakamura, Akutagawa, & Tanada, 2005). However, no works have focused on the identification of bioactive peptides from SCG proteins hydrolysates. Bioactive peptides are mainly found in a latent state as part of a protein from which they can be released by hydrolysis using different enzymes (Sarmadi & Ismail, 2010). In order to exploit SCG as a source of bioactive peptides, SCG proteins have to be extracted avoiding the extraction of other interfering compounds (such as polyphenols), and then hydrolysed. In the present work, a commonly used Tris-HCl based method for protein extraction from food by-products has been compared to a new urea-based method to isolate SCG proteins, and the best method has been applied to evaluate the protein content in SCG from different coffee-brewing preparations. In addition, a polyphenol extraction step to remove interferences has been studied, as well as the hydrolysis of the resultant proteins into peptides using alcalase and thermolysin enzymes. Furthermore, the effect of roasting degree in the presence of peptides with antioxidant and/or *in vitro* angiotensin-converting enzyme (ACE)-inhibitory activity was evaluated. Finally, the resulting peptides

have been identified by reversed-phase high-performance liquid chromatography coupled via electrospray ionization to a quadrupole time-of-flight mass spectrometer (RP-HPLC-ESI-Q-TOF) ~~technology~~.

2. Materials and Methods

2.1. Chemicals

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

2.2. Coffee samples

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

2.3. Spent coffee ground preparation

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

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

2.4. Polyphenol extraction

When specified, and before protein extraction, polyphenols were extracted from defatted SCG samples (in triplicate). For this aim, 5 mL of methanol:H_2O:acetic acid (70:28:2, v/v/v) were added to 100 mg of defatted SCG, and a high intensity focused ultrasound (HIFU) probe (model VCX130, Sonics Vibra-Cell, Hartford, CT, USA) was used for 5 min with an amplitude of 30% (HIFU standard conditions if not specified elsewhere). Thereafter, samples were vortexed in a mechanical stirrer (Selecta, Barcelona, Spain) at room temperature for 30 min, centrifuged at 4,000g for 10 min and the supernatants were transferred to a different vial. This procedure was repeated three times and the pellets were dried at 40 °C overnight. The collected supernatants after each extraction cycle were pooled together and directly analysed by HPLC-UV/HPLC-FLD.

2.5. Protein extraction and quantification

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

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

2.6. Protein analysis by SDS-PAGE

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

2.7. Protein hydrolysis

For protein hydrolysis, 0.8 mg of SCG proteins extracted with UB (for the comparison between the different hydrolytic enzymes and for the evaluation of the polyphenol extraction step) or 4 mL of UB (for the evaluation of the effect of the roasting degree) were transferred to 3 kDa molecular weight cut-off filters (Amicon Ultra 0.5 mL - 3 kDa, Merck Millipore, Burlington, MA, USA), previously washed once with 250 µL of a mixture of ACN:H_2O (20:80, v/v) at 14,000g for 10 min, and later on with 500 µL of Milli-Q water at 14,000g for 15 min. The samples were then centrifuged at 14,000g until the whole volume was loaded, and consecutive washes were performed with 300 µL of 50 mM AmBi in ACN:H_2O (20:80, v/v) (once) and then with 300 µL of 50 mM AmBi in Milli-Q water (twice) to remove urea excess. Between each washing step, the filter units were centrifuged at 14,000g for 15 min. Finally, protein hydrolysis was carried out inside the filter units with 300 µL of 50 mM AmBi buffer (pH 8.0) using two different enzymes: alcalase (0.15 AU enzyme/g protein) and thermolysin (0.1 g enzyme/g protein), and in similar conditions as in Hernández-Corroto, Marina, & García (2019) and Pérez-Míguez, Marina, & Castro-Puyana (2019). Hydrolysis was performed at 50 °C for 4 h by slight mixing (750 rpm) in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany), and the reactions were stopped by heating to 100 °C for 10 min. Resulting peptides were then centrifuged at 14,000g for 10 min, and 200 µL of 50 mM AmBi were added (twice) and centrifuged again to recover all peptides. Finally, samples were transferred to new tubes, dried in SpeedVac (Eppendorf AG, Hamburg, Germany) and dissolved in Milli-Q water to a final concentration of 2.5 mg/mL (considering the starting amount of loaded protein).

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

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

determined as previously described (Hernández-Corroto, Marina, & García, 2018; Hernández-Corroto, Marina, & García, 2019). See **Supplementary Material and Methods** for the full description.

2.9. HPLC-UV and HPLC-FLD analyses

Separation of polyphenol extracts (dissolved in methanol:H_2O:acetic acid (70:28:2, v/v/v)) and SCG protein hydrolysates (dissolved in Milli-Q water) were performed in a 1100 series LC system (Agilent Technologies, Germany). LC control, data acquisition, and data analysis were carried out using the Agilent LC/MSD ChemStation software (B.04.03). Injection volume was set to 5 µL and all samples were analyzed in duplicate. A porous-shell fused-core Ascentis Express C18 analytical column (100 mm × 2.1 mm, particle size 2.7 µm) protected by a C18 guard column (0.5 cm × 2.1 mm, particle size 2.7 µm), both from Supelco (Bellefonte, PA, USA) were used. The flow-rate was set to 0.25 mL/min and the column 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 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 with 0.1% (v/v) FA. Detection was carried out using an UV detector at 280 and 325 nm, and using a fluorescent detector (FLD) at λ_{exc}= 280 and λ_{em}= 304 and 348 nm.

2.10. RP-HPLC-ESI-Q-TOF peptide and polyphenol identification

Identification of peptides and polyphenols in SCG protein hydrolysates (dissolved in Milli-Q water) were performed by RP-HPLC-ESI-Q-TOF as previously described (Pérez-Míguez, Marina, & Castro-Puyana, 2019). Briefly, MS analysis was performed in a quadrupole Q-TOF series 6530 coupled to a HPLC (model 1100) both from Agilent Technologies (Germany), equipped with a Jet Stream thermal orthogonal ESI source. MS control, data acquisition, and data analysis were carried out using the Agilent Mass Hunter Qualitative Analysis software (B.07.00). Injection volume was set to 10 µL, and three replicates were injected in triplicates. For the chromatographic separation, an Ascentis Express Peptide ES-C18 analytical column (100 × 2.1 mm, particle size 2.7 µm) with a C18 guard column (0.5 cm × 2.1 mm, particle size

2.7 µm), both from Supelco (Bellefonte, Pa, USA) were employed. The column temperature was held at 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 and mobile phase B consisted of ACN with 0.3% (v/v) acetic acid. The gradient was 5% B for 3 min, 5–40% 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 conditions. ESI in positive ion mode (for peptides) or in negative ion mode (for polyphenols) were used at a capillary voltage of 3500 V and with a *m/z* range from 100 to 1700. UV signals were also recorded at 280 and 325 nm. MS analyses were performed employing the auto MS/MS mode using 3 precursor per cycle, dynamic exclusion after two spectra (released after 1 min), and collision energy of 5 V for every 100 Da. For proper mass accuracy, spectra were corrected using ions *m/z* 121.0509 ($C_5H_4N_4$) and 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$) in ESI positive mode, and *m/z* 119.0363 ($C_5H_4N_4$) and 980.0164 ($C_{18}H_{18}O_6N_3P_3F_{24}$ + formate) in ESI negative mode, simultaneously pumped into the ionization source at a 15 µL/min flow rate. Peaks software (Bioinformatics Solutions Inc.; Waterloo, ON, Canada) was employed for database search against Uniprot *Coffea arabica* reviewed proteome (downloaded on July 25th 2019 and containing 92 entries). Oxidation of methionine (+15.99 Da) was included as variable modification and peptide results were refined using a − 10lgP threshold of 15. Moreover, *de novo* peptide sequencing was performed using an error tolerance of 10 ppm for the precursor and 0.5 kDa for the fragment, and including oxidation of methionine as variable modification. *De novo* peptide sequences were accepted if the average local confidence (ALC, expected percentage of correct amino acids in the peptide sequence) was ≥ 80% in at least 5 from 9 injections. Moreover, since the MS system is not able to distinguish between isoleucine (I) and leucine (L) amino acids due to their equal molecular masses, only isoforms with L are presented.

2.11. Statistical analysis

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

differences are observed between the French Press SCG (2.40 mg of protein/100 mg of SCG) and the Filter SCG (2.26 mg of protein/100 mg of SCG), and the lowest protein content is obtained from mocha SCG (1.68 mg of protein/100 mg of SCG). The total solid and the protein content of coffee brews have been directly related with the extraction time used for the coffee preparation (Zanoni, Pagliarini, & Peri, 1992), but it is also known that the coffee weight-to-water volume ratio and the coffee grinding degree have a great impact (Cruz et al., 2012). It has been demonstrated that coffee brews from mocha machines contain more total solids per gram of roasted and ground coffee than coffee brews prepared with other procedures (Gloess, et al., 2013). In the present work we kept fixed the coffee weight-to-water volume ratio, the type of coffee, the roasting degree and the grinding degree, and we varied the pressure, temperature and extraction times, which are intrinsic parameters to the different coffee preparations used. The highest protein content in espresso SCG suggests that even thought a higher pressure is used for the espresso coffee preparation, the sorter time has a greater effect on the extracted proteins.

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

The used UB has been previously applied in different proteomics studies to extract proteins from complex tissues, and it has been combined with Amicon devices (3 kDa molecular cut-off filters) to remove contaminants before HPLC and/or MS analysis, and to hydrolyse proteins into peptides prior to HPLC-MS analyses (Holfeld, Valdés, Malmström, Segersten, & Lind, 2018; Wiśniewski, 2017). In the present study, the use of 3 kDa filters would allow to retain coffee proteins larger than 3 kDa, to discard the UB, and to remove free and small compounds from the samples, such as polyphenols. The use of these filters would also help removing big polyphenols and melanoidins that might remain on top of the filter after hydrolysis. This procedure has some drawbacks as very small proteins can be lost during sample loading and washing steps, and peptides bigger than 3 kDa can remain on top of the filter after

the hydrolysis. Considering these aspects, the peptide content and the antioxidant activity of alcalase and thermolysin protein hydrolysates from espresso SCG were measured (**Table 1**). The upper part shows that the peptide content and the ABTS antioxidant activity are similar in both hydrolytic extracts, but the antioxidant activity based on the hydroxyl radical assay is higher in alcalase hydrolysates. These protein hydrolysates were then characterized by HPLC-UV at 325 nm (maximum UV absorbance for CGA) (**Figure 1A**, upper part) and at 280 nm (maximum UV absorbance for caffeine) (**Figure 1B**, upper part). It can be observed that caffeine (t_R 8.75 min) and other phenolic compounds remain in the samples, but no differences were observed between thermolysin or alcalase hydrolysates profiles. These hydrolysates were also characterized by HPLC-FLD at λ_{exc}= 280 nm and λ_{em}= 304 and 348 nm (maximum fluorescence excitation and emission wavelengths of the two amino acid residues that are primarily responsible for the fluorescence of peptides/proteins, tryptophan and tyrosine) (**Figures 1C and 1D**, upper part). These results demonstrate that thermolysin hydrolysates are more complex than alcalase hydrolysates in terms of peptide content.

To try to reduce the quantity of phenolic compounds, a polyphenol extraction step was included before the protein extraction. The characterization of the polyphenolic extracts allowed the identification of caffeine, caffeic acid, 4-O-caffeoylquinic acid, 1,3-dicaffeoylquinic acid, 3-hydroxycoumarin and 1,5-dicaffeoylquinic acid using pure standards (**Figure 2**). In addition, a decrease of approximately 9 times after each extraction cycle was observed. Thereafter, the proteins were extracted and the protein concentration was measured, being 0.91 mg of protein/100 mg of SCG (a 69% reduction compared to the non-polyphenol extraction, 2.90 mg of protein/100 mg of SCG). This reduction can be explained by the composition of the solvent used (methanol:H_2O:acetic acid (70:28:2, v/v/v)), which has been previously used to extract polyphenols from coffee (Mussatto, Ballesteros, Martins, & Teixeira, 2011) or cocoa beans (D'Souza et al., 2017), but also to extract peptides from cocoa beans (D'Souza et al., 2018). The peptide content and the antioxidant activity of these extracts was also evaluated (**Table 1**, lower part). A

decrease of 47% of the peptide content (between the two alcalase hydrolysates) was observed when the polyphenol extraction step was included, but this effect was not observed when thermolysin was used. Moreover, the polyphenol extraction step increased the antioxidant activity of thermolysin hydrolysates, being this effect slightly higher but not significant with respect to the alcalase hydrolysates. These results are unexpected since it is commonly known that phenolic compounds have higher antioxidant activity than peptides. However, it has been widely discussed that polyphenols can also have inhibitory effects on enzymes involved in the hydrolysis of proteins (Cirkovic Velickovic & Stanic-Vucinic, 2018), therefore the polyphenol removal prior to the hydrolysis step could increase the release of peptides with antioxidant activity.

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

3.3. Effect of roasting degree on thermolysin SCG hydrolysates

The next step was the evaluation of the roasting degree effect on the protein/peptides extracts obtained from different SCG (LSCG, light spent coffee grounds; MSCG, medium spent coffee grounds; DSCG, dark spent coffee grounds). Three independent espresso coffees were prepared and polyphenols were extracted before proteins were isolated using UB. The characterization of the polyphenolic extracts

indicates that the area of caffeic acid, 4-O-caffeoylquinic acid, 1,3-dicaffeoylquinic acid and 1,5-dicaffeoylquinic acid decreased with the roasting process; the area of caffeine remained unchanged; and the area of 3-hydroxycoumarin is increased (**Supplementary Material, Figure S3**). These results agree well with previous reports where CGA such as 5-CQA, 4-CQA, 3-CQA, 3,5-diCQA, 4,5-diCQA, 5-FQA, 3,4-diCQA and 4-FQ, are degraded during roasting, whereas the formation of CGA lactones takes place (Farah, de Paulis, Trugo, & Martin, 2005; Moon, Yoo, & Shibamoto, 2009). The levels of these compounds may also be decreased by their incorporation into Maillard reaction products during the roasting process (Coelho et al., 2014; Delgado-Andrade & Morales, 2005; Bekedam, Roos, Schols, Van Boekel, & Smit, 2008). It has been suggested that melanoidins are derived from cross-linking of Maillard reaction products to proteins via reactive side chains of amino acids, and more recently it has been demonstrated that transglycosylation reactions to form new polysaccharides is the main mechanism for this incorporation (Moreira et al., 2017). On the other hand, the caffeine levels are not affected (Oestreich-Janzen, 2010).

Thereafter, the protein concentration was evaluated by Bradford assay, indicating that DSCG had higher amount of proteins (1.49 mg of protein/100 mg of SCG) than MSCG (1.08 mg of protein/100 mg of SCG), and three times more than LSCG (0.49 mg of protein/100 mg of SCG) (**Supplementary Material, Figure S4**). The SDS-PAGE analysis also shows a slightly more intense protein bands of ≈150 kDa in DSCG (**Supplementary Material, Figure S5**). The higher protein concentration of DSCG could be partially explained by the loss coffee weight during the roasting process (13% for light, 15% for medium, and 17% for dark roasted beans), which can enrich the material that is not degraded or loss. However, darker roasting has shown to produce more total soluble solids in the brew (Petracco 2005), and therefore there should be fewer proteins in their SCG. Moreover, it is also known that coffee proteins can be fragmented, polymerized or integrated into melanoidins through the Maillard reaction during the roasting process, but it is not well known how this reaction affects the functional properties (such as the

solubility) of proteins (Oliver, Melton, & Stanley, 2006). After protein quantification, the peptide content, the antioxidant activity and the *in vitro* ACE-inhibitory activity of thermolysin protein hydrolysates from LSCG, MSCG and DSCG were measured (**Table 2**). For comparison purposes, DSCG protein isolates were also hydrolysed with alcalase, and a control sample incubated without enzyme was included. As expected due to the higher amount of proteins, the highest peptide concentration among the three thermolysin hydrolysates was obtained in DSCG, followed by MSCG and LSCG (**Table 2**). There were not significant differences on the ABTS or hydroxyl scavenging activity between the samples, but the DSCG and the MSCG hydrolysates had higher *in vitro* ACE-inhibitory activity. The protein hydrolysates were then characterized by HPLC-UV and HPLC-FLD (**Supplementary Material, Figures S6-S9**). The acquisition at 280 nm shows that the area of some peaks are slightly increased (peaks at t_R 5.5 min and 14.7 min); some of them remained unchanged (peaks at t_R 23.0 min, 25.2 min, 25.5 min and 26.0 min); and others are decreased (peaks at t_R 19.0 min and 27.0 min) with the roasting process (**Supplementary Material, Figures S6**); and the chromatograms acquired at 325 nm indicate that most of the peaks were significantly increased with the roasting process (**Supplementary Material, Figures S7**). There were not significant differences in the FLD-chromatograms acquired at λ_{exc} = 280 nm and λ_{em} = 304 nm, but when λ_{em} was set to 348 nm (**Supplementary Material, Figures S8**), the general trend was a decrease in the area when increasing the roasting degree (**Supplementary Material, Figures S9**). As previously commented, the signals observed at 325 nm might be due to the presence of phenolic compounds that can be released during the incubation step at 50 °C. It has been observed that the incorporation of phenolic compounds into the melanoidins is a significant pathway of CGA degradation during roasting (Coelho et al., 2014), being transglycosylation reactions the main mechanism for this incorporation (Moreira et al., 2017). Different techniques such as alkaline hydrolysis, acid hydrolysis, the increase of the medium ionic strength, or the alkaline fusion method have been applied to release them (Bekedam, Roos, Schols, Van Boekel, & Smit, 2008; Monente, Ludwig, Irigoyen, De Peña, & Cid, 2015; Perrone,

Farah, & Donangelo, 2012; Delgado-Andrade & Morales, 2005; Coelho et al., 2014). For instance, covalently bound caffeic and ferulic acids decrease with roasting, while the content of dihydrocaffeic acid increases (Perrone, Farah, & Donangelo, 2012). And by using the alkaline fusion method, it has been suggested that the incorporation of phenolic compounds in coffee melanoidins is also related to the amount of proteins (Coelho et al., 2014). Complementary, the antioxidant activity of melanoidins from coffee brews obtained using roasted coffees is higher than those obtained from green coffees (Delgado-Andrade, Rufián-Henares, & Morales, 2005; Perrone, Farah, & Donangelo, 2012). Based on these results, different CGA acids could have been released from melanoidins, or some melanoidins could still be present in the SCG protein hydrolysates, but further studies are needed to confirm these hypotheses.

3.4. Evaluation of the enzymatic incubation on SCG hydrolysates

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

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 were 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.

Different peptides with a number of amino acids ranging from 4 to 10 were identified, and none of these peptides belong to the enzymes employed for hydrolysis. In the case of DSCG alcalase hydrolysates, 5 peptides (ALM(+15.99)APH, M(+15.99)EGL, CCVLLP, NVLAR and NLM(+15.99)APH, **Supplementary Material, Table S1**) were identified, and only one peptide (SHWH) was identified when no enzyme was used. In overall, thermolysin hydrolysates present a higher number of peptides (12 in LSCG, 14 in MSCG and 10 in DSCG) and, when combining the results with the database search, 37, 48 and 26 peptides were identified in LSCG, MSCG and DSCG thermolysin hydrolysates, respectively; 30 peptides in DSCG alcalase hydrolysates; and only 15 peptides when no enzyme was used. The possible antioxidant or antihypertensive/ACE-inhibitory activity of these peptides was searched in the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), but none of them have been reported. In overall, these results indicate that the peptide complexity of thermolysin hydrolysates is higher than alcalase hydrolysates, and some of the thermolysin released peptides (or a combination of them) would be responsible for the higher ACE-inhibitory activity observed. Since LSCG presents some antioxidant and ACE inhibitory activities, the common peptides identified in the three thermolysin hydrolysates using the *de novo* sequencing tool (CSDAVGVK and RCPQGGTHYG), and the common peptides identified as coming from LSCG, MSCG and DSCG proteins (YKPPYS and CVIPSN) would be the first candidates to be evaluated for their biological activity. The tentative identification of CSDAVGVK peptide in DSCG is shown as example in **Supplementary Material, Figure S12**. Moreover, the higher ACE-inhibitory activity observed for MSCG and DSCG thermolysin hydrolysates would be explained by the higher number of identified peptides (48 in the case of MSCG), or by the specific sequence of the identified peptides in the MSCG/DSCG thermolysin hydrolysates, but not in the LSCG or the alcalase hydrolysates. The following peptides meet this requirement: DPGDKKN, CASDPAQ and RLNQ, identified in the MSCG and the DSCG using the *de novo* sequencing tool; GGSMG, GVMDFQ, LDPIEF, GGGDL, YEAWL, DAHIPPG, DPHFGQPAVE, FGM(+15.99)NSLS, FPCDGP, GPVNIAY, GMAST and LGMAST, identified as coming from MSCG proteins;

and FLSRSD, IPNIH, LSDM(+15.99)NLS, M(+15.99)VDSFH and NRGGY, identified as coming from DSCG proteins.

Additionally, RP-HPLC with UV (at 325 nm) and MS (in ESI negative mode) detectors were used to tentatively identify the possible remaining polyphenols in SCG protein hydrolysates (**Supplementary Material, Figures S10B and S10D**). Among the different observed peaks in **Figure 3A**, peaks **1–5** showed 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** 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 of these polyphenols was performed by analysing the MS and MS/MS spectra (as exemplified in **Figure 4** for 3-FQA), and comparing them with the fragmentation patterns already reported (Kuhnert, Jaiswal, Matei, Sovdat, & Deshpande, 2010; Clifford, Johnston, Knight, & Kuhnert, 2003). Based on these analyses, peak **1** was tentatively assigned as 3-FQA, peak **2** as 1-FQA, peaks **3** and **5** as 5-FQA, and peak **4** as 4-FQA. However, peak **6** could not be identified. These phenolic compounds have already been identified in SCGs (Panusa, Zuorro, Lavecchia, Marrosu, & Petrucci, 2013; Bravo et al., 2012), and they might be partly responsible for the antioxidant activity exerted by the extracts.

4. Conclusion

In conclusion, the urea-based extraction buffer allows the extraction of more proteins than the conventional Tris-HCl buffers, and its application demonstrates that the highest protein content is obtained from espresso SCG. Moreover, the use of 3 kDa molecular cut-off filters allows the removal of the buffer and the hydrolysis of the proteins using thermolysin and alcalase enzymes, but not the complete removal of polyphenols. Moreover, these protein hydrolysates possess antioxidant and ACE-inhibitory activities, the latter being highest when SCG samples are obtained from dark/medium roasted coffees, and after the inclusion of a polyphenol step removal. Finally, several peptides that might be responsible for the ACE-inhibitory activity observed have been identified. However, further experiments

using synthetic peptides are needed to confirm which of the released peptides (or a combination of them) are the most bioactive.

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Conflict of Interest

The authors declare no conflict of interest

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Figure Captions

Figure 1. HPLC chromatograms of SCG proteins hydrolysed with alcalase and thermolysin (including the polyphenol extraction step). Chromatographic conditions were: Ascentis Express C18 analytical column (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 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-rate, 0.25 mL/min; temperature, 30 °C; injected volume, 5 µL; absorbance detection was performed at λ 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 348 nm (**D**).

Figure 2. HPLC chromatograms obtained from methanol:H_2O:acetic acid (70:28:2, v/v/v) extracts after one, two or three extraction cycles before SCG protein extraction. Separation conditions were the same as in **Figure 1**, and UV absorbance was detected at λ of 325 nm.

Figure 3. HPLC chromatograms of DSCG protein hydrolysed with thermolysin, alcalase or "no enzyme" with polyphenol extraction. Separation conditions were the same as in **Figure 1**, absorbance detection was performed at λ 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 348 nm (**D**). Peaks were lately identified by RP-HPLC-ESI-Q-TOF-MS/MS as: **1**, 3-FQA; **2**, 1-FQA; **3**, 5-FQA; **4**, 4-FQA; **5**, 5-FQA; **6**, not identified.

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

Tables

Table 1. Peptide content and antioxidant activity (ABTS and hydroxyl assays) of SCG (after espresso preparation of commercial medium roasted 100% Arabica coffee) proteins hydrolysed with alcalase and thermolysin (and including the polyphenol extraction step).

Sample	Peptide content (mg/mL)	ABTS assay (%)[1]	Hydroxyl radical assay (%)
Alcalase	$0.43^{a} \pm 0.08$	$22.1^{b} \pm 5.20$	$88.4^{a} \pm 11.6$
Thermolysin	$0.42^{a} \pm 0.05$	$19.8^{b} \pm 11.3$	$73.5^{b} \pm 3.55$
Alcalase (with polyphenol extraction)	$0.23^{b} \pm 0.02$	$30.7^{a,b} \pm 0.52$	$84.3^{a,b} \pm 4.85$
Thermolysin (with polyphenol extraction)	$0.39^{a} \pm 0.05$	$36.1^{a} \pm 2.39$	$95.4^{a} \pm 2.70$

*Different letters indicate significant differences between samples after ANOVA with LSD Post-hoc, p-value < 0.05.
[1] For ABTS antioxidant activity, samples were diluted to 1:150.

Table 2. Peptide content, antioxidant activity (ABTS and hydroxyl assays) and *in vitro* ACE-inhibitory activity of thermolysin hydrolysates from light, medium and dark espresso SCG (LSCG, MSCG, DSCG), alcalase hydrolysates from DSCG, and incubation of DSCG without enzyme.

Sample	Peptide content (mg/mL)	ABTS assay (%)[1]	Hydroxyl radical assay (%)	*In vitro* ACE-inhibitory activity (%)
LSCG – Thermolysin	$0.22^{b} \pm 0.05$	$30.5^{a,b} \pm 4.26$	$85.7^{a,b} \pm 7.84$	$61.7^{b} \pm 4.98$
MSCG– Thermolysin	$0.31^{a,b} \pm 0.09$	$28.7^{a,b} \pm 9.47$	$81.6^{b} \pm 10.2$	$83.0^{a} \pm 2.72$
DSCG – Thermolysin	$0.41^{a} \pm 0.08$	$32.9^{a,b} \pm 4.28$	$92.2^{a} \pm 11.3$	$81.5^{a} \pm 1.51$
DSCG – Alcalase	$0.21^{b} \pm 0.07$	$39.8^{a} \pm 5.49$	$96.1^{a} \pm 1.89$	$61.5^{b} \pm 3.84$
DSCG – No enzyme	$0.25^{b} \pm 0.03$	$24.8^{b} \pm 8.31$	$83.4^{a,b} \pm 6.27$	$37.7^{c} \pm 9.71$

*Different letters indicate significant differences between samples after ANOVA with LSD Post-hoc, p-value < 0.05.
[1] For ABTS antioxidant activity, samples were diluted to 1:150.

Table 3. Peptide sequence, belonging gene and protein name, molecular mass (Da) and −10lgP 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.

				−10lgP				
				Thermolysin			Alcalase	No Enzyme
Peptide sequence	Gene name	Protein name	Molecular mass (Da)	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		

YKSQNM	NU5C		769.3429		16.1			
HNYYGEPA	PETD	Cytochrome b6-f complex	949.3929		15.2	23.9		
GGGDL	PSAA	Photosystem I P700 chlorophyll a apoprotein A1	417.1859		16.3			
YEAWL	PSAA		680.3170		28.4			
DAHIPPG	PSAB	Photosystem I P700 chlorophyll a apoprotein A2	705.3445		15.3			
DPHFGQPAVE	PSAB		1095.4985		16.9			
FGM(+15.99)NSLS	PSAB		770.3269		17.7			
FPCDGP	PSAB		634.2421		18.6			
GPVNIAY	PSAB		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	DNA-directed RNA polymerase subunit beta''	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		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			
EEAAQ	SPDE	Spermidine synthase	546.2285				15.6	
EIDKM	SPDE		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 methyltransferase 1	518.2159	22.0		
NDLFP	XMT1		604.2856		17.4	
FLSRSD	YCF2	Protein Ycf2	723.3551	18.4		
GNM(+15.99)LGPA	YCF2		674.3057			16.8
IPNIH	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 assembly protein Ycf4	646.3762			18.0
VGSVG	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.

				LRC		MRC		DRC	
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**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

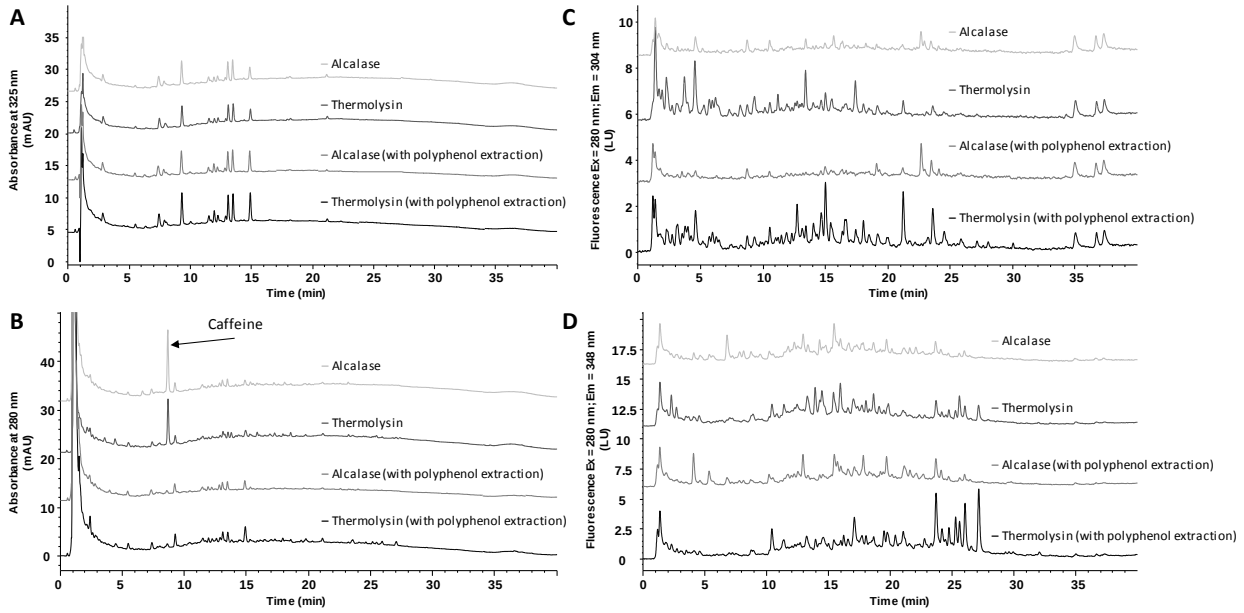
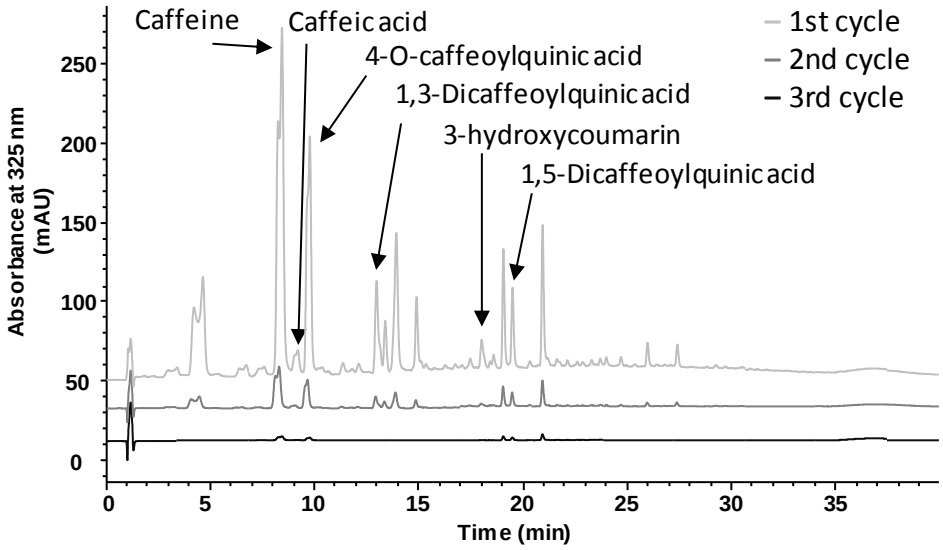
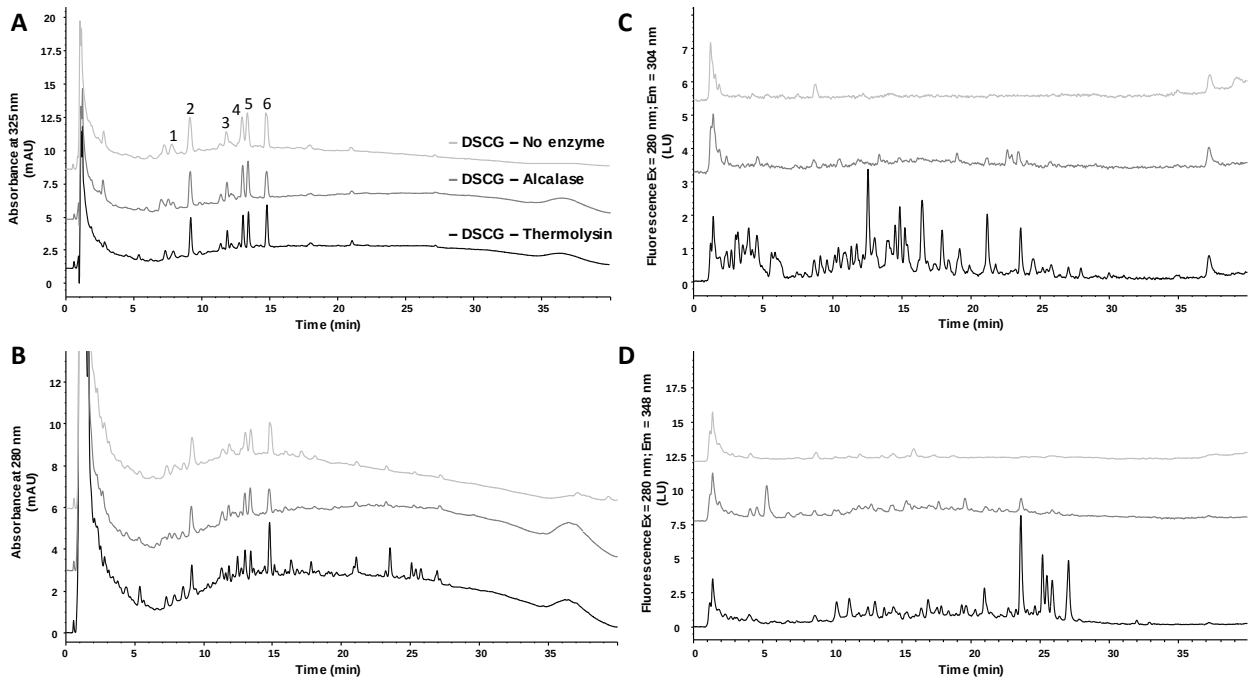


Figure 1



669

670 **Figure 2**



671

672 **Figure 3**

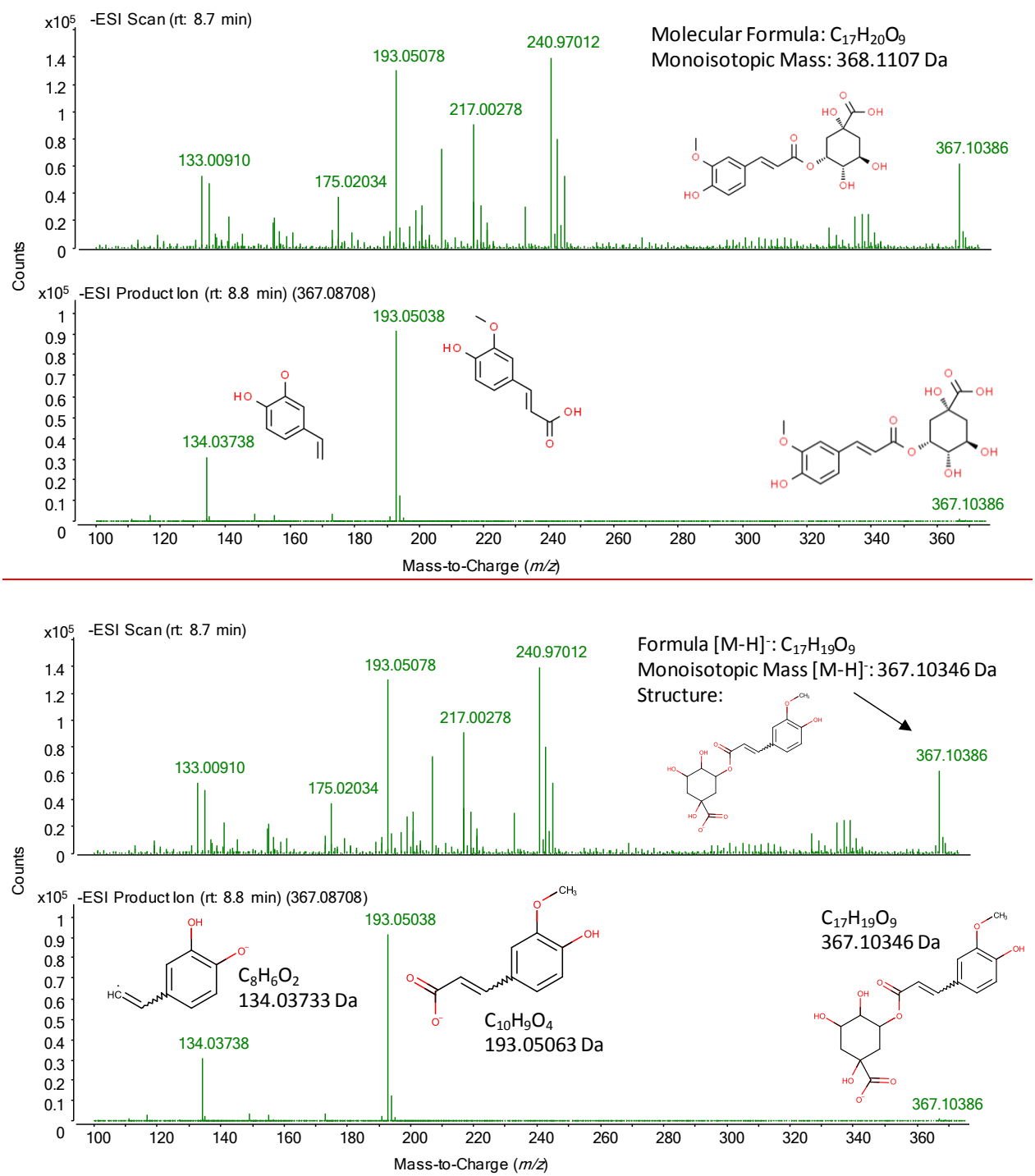

x10^5 -ESI Scan (rt: 8.7 min)
Molecular Formula: C17H20O9
Monoisotopic Mass: 368.1107 Da
193.05078
240.97012
217.00278
133.00910
175.02034
367.10386
Counts
x10^5 -ESI Product Ion (rt: 8.8 min) (367.08708)
193.05038
134.03738
367.10386
Mass-to-Charge (m/z)
x10^5 -ESI Scan (rt: 8.7 min)
Formula [M-H]-: C17H19O9
Monoisotopic Mass [M-H]-: 367.10346 Da
Structure:
193.05078
240.97012
217.00278
133.00910
175.02034
367.10386
Counts
x10^5 -ESI Product Ion (rt: 8.8 min) (367.08708)
C8H6O2
134.03733 Da
193.05038
C10H9O4
193.05063 Da
C17H19O9
367.10346 Da
134.03738
367.10386
Mass-to-Charge (m/z)

Figure 4

Supplementary material for online publication only

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*Graphical Abstract
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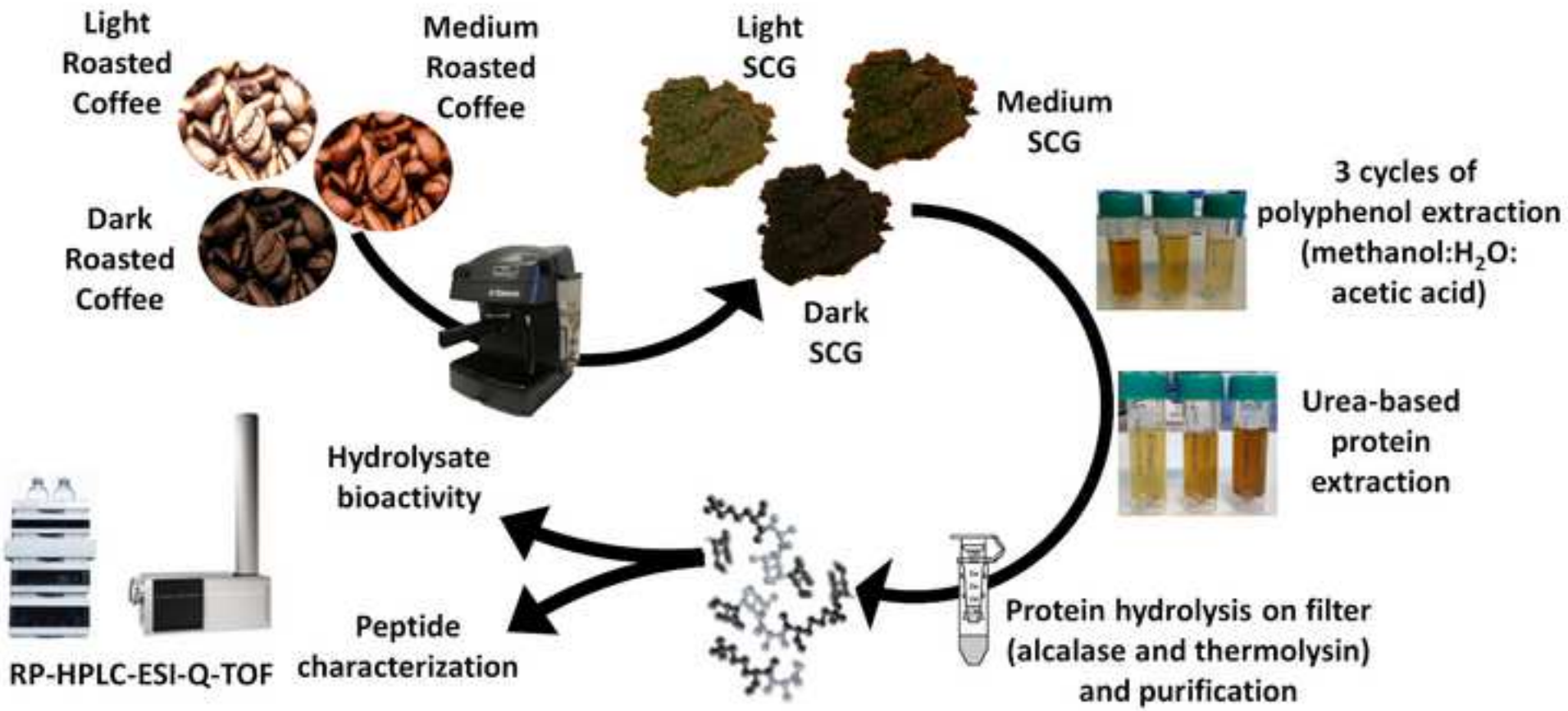

Graphical Abstract3.tif

Credit Author Statement

A.V.: investigation, formal analysis, validation, data curation, visualization, writing-original draft. **M.C.P.:** conceptualization, supervision, resources, writing-review & editing, project administration, funding acquisition. **M.L.M.:** conceptualization, supervision, resources, writing-review & editing, project administration, funding acquisition.

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