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Pérez-Míguez, Raquel, Marina, María Luisa & Castro-Puyana, María, 2019. High resolution liquid chromatography tandem mass spectrometry for the separation and identification of peptides in coffee silverskin protein hydrolysates. Microchemical journal, 149, p.103951.

Available at https://doi.org/10.1016/j.microc.2019.05.051

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(Article begins on next page)

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HIGH RESOLUTION LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR THE SEPARATION AND IDENTIFICATION OF PEPTIDES IN COFFEE SILVERSKIN PROTEIN HYDROLYSATES.

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ABSTRACT

An analytical methodology was developed for the first time in this work to investigate the peptide composition of coffee silverskin protein hydrolysates. Coffee silverskin is the only by-product produced in the coffee roasting process and it contains a relatively high amount of proteins (16.2-19.0%). Different extraction procedures were tested to obtain protein extracts from coffee silverskin samples which were subsequently submitted to enzymatic digestion using different enzymes. Protein hydrolysates from *Arabica* coffee silverskin obtained using three roasting degrees (light, medium and dark) were considered in order to evaluate the influence of this process on peptide composition. Antioxidant and hypocholesterolemic activities were investigated for these hydrolysates. A method based on the use of liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometer was developed enabling the separation and identification of different short chain peptides in the coffee silverskin hydrolysates using *de novo* sequencing tool. Different peptides, with a number of amino acids ranging from 4 to 12, were identified in the coffee silverskin analyzed. Peptides obtained were different depending on the enzymatic hydrolysis employed. As general trend, the results obtained showed that peptide composition in coffee silverskin protein hydrolysates was not significantly affected by the coffee roasting process.

Keywords: Peptides; liquid chromatography-tandem mass spectrometry; coffee silverskin; roasting process; bioactivity.

1. INTRODUCTION

Coffee has an important cultural and economic impact worldwide since it is one of the most consumed beverages in the world. The production of coffee beverage comprises many stages being the roasting of green coffee beans one of the most relevant. After this process, the coffee silverskin (CS) (a thin tegument of the outer layer of the beans which represents about 4.2 % (w/w) of coffee beans) is obtained [1]. This is the only by-product produced during roasting process. The CS chemical composition includes high levels of dietary fiber (50-60%), being mainly soluble dietary fiber (>85%), carbohydrates (glucose, xylose, galactose, mannose and arabinose), proteins (16.2-19.0%), fat (1.56-3.28%) and ash (7%)[2-8]. Moreover, CS also contains other bioactive compounds such as phenolic compounds (e.g. chlorogenic acids) that together with melanoidins (that are formed as products in the Maillard reaction) are responsible of its antioxidant capacity [1]. Because of this chemical composition, CS can be considered as a natural source of bioactive compounds with beneficial properties for human health [1, 8, 9-13]. Despite the most common application of CS has been as direct fuel, for composting and soil fertilization [1, 14], nowadays, it has been proposed as a new potential functional ingredient for food. In fact, CS has been incorporated to the formulation of flakes, breads, biscuits and snakes [9], to prepare an antioxidant beverage for body weight control [10], or used in cosmetics as active ingredient to improve skin hydration and firmness [15]. Although CS has been chemically characterized in different works [1, 4], no studies were aimed to investigate its endogenous peptides or the peptide composition of protein hydrolysates in spite of its relatively high content in proteins, as mentioned above.

The bioactivity of many peptides present in different foodstuff and food residues or byproducts includes antimicrobial, ACE inhibitory effect, cholesterol-lowering activity, and antioxidant properties, among others [16-18]. These bioactive capacities may be

directly linked with the presence of peptides in these samples or related to peptides originated during enzymatic hydrolysis, food processing or ripening [19]. Usually, the most common bioactive peptides are formed by short amino acid chains (around 2-30 amino acids) [20, 21].

Nowadays, liquid chromatography (LC) coupled to high resolution mass spectrometry (MS) is the preferred analytical technique to carry out the accurate analysis of peptides with a chain longer than 5 amino acids [22]. The detection of shorter peptides (2-4 amino acids) has, on the contrary, some analytical limitations since, for instance, their fragmentation makes their detection complicated [22-24]. Then, the separation and identification of short peptides is a challenge.

The main objectives of this work were: i) to study the peptide composition of protein hydrolysates of CS (Arabica variety) and their antioxidant and hypocholesterolemic activities, and ii) to evaluate the effects of submitting coffee beans to different degrees of roasting process on peptide composition. With this aim, a high intensity focused ultrasound probe was employed to extract soluble proteins, which were subsequently precipitated and submitted to enzymatic digestion using different enzymes. A method was developed for the separation and identification of peptides in the CS protein hydrolysates using liquid chromatography coupled to a quadrupole-time-of flight mass spectrometer (LC-(QTOF)MS) and de novo sequencing tool.

2. MATERIALS AND METHODS

2.1. Chemicals and samples.

All chemicals and reagents used in this work were of analytical grade. Sodium hydroxide**,** thermolysin enzyme, pepsin and pancreatin digestive enzymes, bovine serum albumin (BSA), oleic acid, phosphatidylcholine, taurocholate, cholesterol, sodium chloride (NaCl), L-glutathione (GSH), β-mercaptoethanol, o-phthalaldehyde (OPA), dithiothreitol

(DTT), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulphate, 1,10-phenantroline, ferrous sulphate, hydrogen peroxide, and potassium ferricyanide were purchased from Sigma-Aldrich (Steinheim, Germany). Alcalase 2.4 L FG and Flavourzyme 1000 L were generously donated by Novozymes Spain S.A (Madrid, Spain). Hydrochloric acid, acetone, hexane, methanol, ethanol and acetic acid were acquired in Scharlau (Barcelona, Spain). Tris-(hydroxymethyl)-aminomethane (Tris), sodium dihydrogen phosphate, disodium tetraborate, and sodium dodecyl sulfate (SDS) were purchased from Merck (Darmstadt, Germany). Cholesterol oxidase kit was from BioAssay Systems (Hayward, CA, USA). Miniprotean precast gels, Laemmli buffer, Tris/glycine/SDS running buffer, precision plus protein standards (recombinant proteins expressed by Escherichia coli with molecular weights of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa), silver stain kit, and Bradford reagent (Coomassie Blue G-250) were acquired from Bio-Rad (Hercules, CA, USA).

For the HPLC-MS/MS analysis, MS grade acetonitrile (ACN) and acetic acid from Sigma-Aldrich (Steinheim, Germany) were employed. The ultrapure water used was obtained from a Milli-Q (Millipore, Bedford, MA, USA) instrument.

Different coffee silverskin samples from *Arabica* coffee variety were provided by "Café Fortaleza" (Vitoria, Spain). These samples were obtained by roasting green coffee beans at three different roasting levels: light level (LCS) using a roasting temperature of 175 ºC during 12.36 min; medium level (MCS) employing 185 ºC during 14.11 min; dark level (DCS) by roasting the green coffee beans at 195 ºC during 17.06 min.

2.2. Total protein content.

The protein content of CS samples was determined by the Kjeldahl method [25]. Analyses were performed in triplicate. Nitrogen data were converted into protein values using a conversion factor of 5.3 and were expressed as g per 100 g of dried coffee silverskin.

2.3. Protein extraction from coffee silverskin.

Proteins from coffee silveskin were extracted following a procedure previously described in the literature with different modifications [26]. Briefly, 0.5 g of grounded coffee silverskin were defatted three times with 20 mL of hexane (to avoid interferences in the extraction). Then, 50 mg of proteins were extracted with 5 mL of 100 mM Tris-HCl buffer (pH 7.5) containing 0.25% (w/w) SDS and 0.25% (w/w) DTT using a high intensity focused ultrasound probe (HIFU) (model VCX130 from Sonics Vibra-Cell, Hartford, CT, USA) for 10 min at 50% of amplitude. After centrifugation, the proteins were precipitated using 10 mL of cold acetone and left at 4ºC overnight. Precipitated proteins were centrifuged (4000 × g, 10 min, 25ºC) and dissolved in the appropriate buffer for their digestion: 5 mM borate buffer (pH = 8.5) in the case of using alcalase, 5 mM phosphate buffer (pH = 8.0) for thermolysin or acid water solution (pH 2.0) for simulated gastrointestinal digestion, respectively.

2.4. SDS-PAGE.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis with a Bio-Rad Mini-protean system (Hercules, CA, USA) was performed to achieve the separation of coffee silverskin proteins. Proteins solutions were mixed with Laemmli buffer containing 5 % (v/v) β-mercaptoethanol, followed by heating at 100 °C during 5 min and loaded into commercial ready precast gels. Then, Tris/glycine/SDS was used as running buffer and protein separation was carried out by applying 80 V for 5 min and 150 V until performing the separation. Proteins standard solution with molecular weights from 10 to 100 kDa were also loaded into the gel. After separation, the gel was treated first with a fixing solution of water/MeOH/acetic acid (50/40/10 % (v/v)) by shaking for 30 min and then with water/EtOH/acetic acid (85/10/5 % (v/v)) two times (for 15 min each one). Then, an oxidizer solution was added to the gel during 5 min followed by the

addition of the silver reagent during 20 min. Finally, the gel was washed with water before adding the developer solution, and the reaction was stopped adding 5 % acetic acid solution.

2.5. Protein digestion.

Protein extracts obtained from CS were hydrolysed using different enzymes. CS proteins were dissolved in the corresponding digestion buffer at a final concentration of 5 mg/mL with the help of an ultrasonic probe for 5-10 min and with 30 % of wave amplitude. 5 mM borate buffer (pH = 8.5), 5 mM phosphate buffer (pH = 8.0) or acid water solution (pH 2.0) were employed as buffers for alcalase, thermolysin or simulated gastrointestinal digestion (using pepsin and pancreatin), respectively. After the addition of the appropriate amount of enzyme to the protein extract solution, it was incubated in a Thermomixer Compact (Eppendorf, Hamburg, Germany) at 50 °C (for alcalase, and thermolysin digestion) or 37 °C (for gastrointestinal digestion) with shaking at 700 rpm. The digestion was stopped by increasing the temperature to 100 °C for 10 min. After centrifugation (6000 g for 10 min, 24 ºC), the supernatant was collected for further analyses.

2.6. O-phthalaldehyde (OPA) assay.

Peptide content was measured using the OPA assay following the procedure described by Wang et al. (2008) with some modifications [27]. A 40 mg/mL solution of OPA reagent in MeOH was employed to prepare daily a mixture of 2.5 mL of disodium tetraborate (100 mM), 1.0 mL of SDS (5% (v/v)), 1.39 mL of water, 10 µL of β-mercaptoethanol, and 100 µL of OPA solution. This mixture was employed to incubate the CS protein hydrolysates during 8 min at room temperature. Then, the absorbance was measured at 340 nm using a spectrophotometer Cary 8454 from Agilent Technologies (Santa Clara, USA). All analyses were performed in triplicate.

2.7. *In vitro* bioactivity assays.

In vitro antioxidant capacity of CS protein hydrolysates was measured using two different assays evaluating the ability to scavenge free radicals: ABTS and hydroxyl radicals scavenging. Samples were prepared in triplicate and measured in duplicate. The assays were carried out following the procedures described by González-García et al [26]. The antioxidant capacity was expressed as percentage of inhibition.

In vitro hypolipidemic activity was evaluated measuring the peptides ability to reduce the absorption of dietary cholesterol (ability to reduce micellar cholesterol solubility). The assay was carried out following the procedure described by Prados et al [28]. The reduction in the micellar solubility of cholesterol was calculated using the following equation:

Cholesterol solubility reduction (%) = $((C0 - CS)/C0) * 100$

In this equation C_0 is referred to the initial concentration of cholesterol in micelles (without peptides) and Cs is referred to the concentration of cholesterol in micelles after adding peptides.

Both *in vitro* antioxidant capacity and *in vitro* hypolipidemic activity were evaluated preparing the CS protein hydrolysates in triplicate and measuring them in duplicate.

2.8. Peptide analysis by LC-(QTOF)MS.

A HPLC system 1100 from Agilent (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole-time-of flight mass spectrometer (QTOF/MS) Agilent 6530 equipped with an orthogonal electrospray ionization (ESI) source (Agilent Jet Stream, AJS) was employed to carry out peptide analysis. MS control, data acquisition, and data analysis were performed using Agilent Mass Hunter Workstation software B.07.00 from Agilent Technologies. An Ascentis Express Peptide ES-C18 analytical column (100 x 2.1 mm, particle size 2.7 μm) with an Ascentis Express Peptide ES-C18 guard column (0.5 cm × 2.1 mm, 2.7 μm particle size) from Supelco (Bellefonte, Pa, USA) were employed

to carry out the chromatographic separation. The column temperature was 25 ºC and the flow rate 0.3 mL/min. Ten µL of extract were injected. The mobile phases consisted of (A) water with 0.3 % acetic acid and (B) ACN with 0.3 % acetic acid in a gradient elution analysis programmed as follows: 0 min, 5 % (B); 0-3 min, 5 % (B); 5-40 min, 5-40 % (B); 40-43 min, 95 % (B), 43-45 min, 95 % (B) with 15 min of post-time.

The MS analyses were carried out using positive ionization mode (3500 V) and masses ranged from 100 to 1700 m/z. Nebulizer pressure was set at 50 psig and the drying gas flow rate was fixed to 12 L/min and 350 ºC. The sheath gas flow was 12 L/min at 400 ºC. 80 V was chosen for the fragmentor voltage (cone voltage after capillary), whereas the skimmer and octapole voltage were 60 V and 750 V, respectively. MS/MS analyses were performed employing the auto MS/MS mode using 1 precursor per cycle, dynamic exclusion after two spectra (released after 1 min), and collision energy of 5 V for every 100 Da. Internal mass calibration in positive ionization mode was performed using a reference compound solution from Agilent Technologies containing the ions *m/z* 121.0508 ($C_5H_4N_4$) and 922.0097 ($C_{18}H_{18}O_6N_3P_3F_{24}$). This solution was continuously pumped into the ionization source at a 15 µL/min flow rate using a 25 mL Gastight 1000 Series Hamilton syringe (Hamilton Robotics, Bonaduz, Switzerland) on a NE-3000 pump (New Era Pump Systems Inc., Farmingdale, NY, USA). The analyses were conducted in triplicate.

2.9. Peptide identification by *de novo* sequencing.

Three replicates of CS protein hydrolysates were injected in triplicate in the MS system. Blank samples containing the appropriate buffer solution instead of CS protein hydrolysates were also analyzed. MS/MS spectra were obtained for the most abundant ions of molecular species and analyzed using the PEAKS Studio Version 7 software (Bioinformatics Solutions Inc., Waterloo, Canada) in order to identify peptides from

coffee silverskin proteins. *De novo* sequencing tool was employed to carry out data analysis. The results were refined applying a certain average local confidence (ALC). Those peptides identified with an ALC (expected percentage of correct amino acids in the peptide sequence) above 80% and with a good precursor fragmentation pattern were considered for further interpretation. In addition, only those peptides appearing in at least 5 from 9 injections (three injections of each triplicate) from some of the analyzed CS samples were considered. Since in the MS system employed in this work was not possible to differentiate isoleucine (I) from leucine (L), only isoforms with L are presented in these results.

3. RESULTS AND DISCUSSION

3.1. Extraction of proteins

The protein contents of the three CS obtained after roasting green coffee beans at different levels were 12.0 ± 0.1 %, 11.9± 0.1 %, and 12.0 ± 0.4 % for light CS (LCS), medium CS (MCS), and dark CS (DCS), respectively (all percentages referred to sample dry weight). Considering that the protein content was very similar in all CS samples, and that MCS is expected to present intermediate properties between light and dark roasted degrees, it was chosen as model sample to evaluate different protein extraction procedures.

Then, to achieve the extraction of proteins from MCS, three different approaches were evaluated: (i) a solid-liquid extraction using as extracting solvent a mixture of water and an organic solvent, (ii) a subcritical water extraction, and (iii) a high intensity focused ultrasound (HIFU) using different buffer solutions containing different additives as extracting solvents.

In the first attempt, a solid-liquid extraction using ACN:water (20:80 v/v) as extracting solvent was performed using shaking (5 min) and sonication (3 min). Under these conditions, the percentage of proteins extracted from the MCS sample was lower than 1%

(protein content obtained by Bradford assay). The second approach consisted of the use of a subcritical water extraction following the procedure previously described by Yusaku Narita et al., [12] with some modifications. This protocol was based on the use subcritical water as extraction solvent at two different temperatures, 120ºC and 180ºC. In both cases, the protein content of the MSC extract was lower than 1%. Finally, the third approach investigated in this work was based on a method previously reported by our research group to extract proteins from olive seeds [29]. The methodology consisted of the extraction of proteins with 100 mM Tris-HCl buffer (pH 7.5) containing 0.5% (w/v) SDS and 0.5% (w/v) DTT using HIFU for 5 min at 30% amplitude and followed by the protein precipitation with cold acetone overnight. Under these conditions, it was possible to obtain an extract which protein content was 2.6 ± 0.3 %. From the three different protein extraction strategies investigated, the third one (based on the use of HIFU) was selected since it enabled to obtain the highest protein content. In order to increase the amount of proteins extracted from the MSC sample by this extraction procedure, an optimization of different extraction conditions, such as extracting buffer composition, HIFU probe conditions, and solvent/sample ratio was performed.

First, the composition of the extracting buffer was evaluated considering buffers of different nature (100 mM Tris-HCl and 100 mM phosphate) at three different pH values (6.5, 7.5 and 8.5) keeping constant the amount of SDS and DTT as well as the HIFU conditions described above. As it can be seen in **Table 1**, the Tris-HCl buffer enabled to achieve a protein content five times higher than those obtained using phosphate buffer. In addition, the intermediate pH value allowed obtaining a protein content slightly higher than the other pH values tested. Therefore, 100 mM Tris-HCl buffer at pH 7.5 was chosen for further experiments. Bearing in mind that protein denaturation is highly affected by the presence of SDS and DTT in the extraction solvent, the effect of the concentration of

both denaturing agents in the extraction media was studied by varying first the amount of SDS between 0.0 and 0.5% (w/v), and later the amount of DTT between 0.25 and 1.0% (w/v). Considering the percentage of proteins extracted under these conditions (see **Table 1**) and trying to use the lowest concentration of DTT and SDS to avoid interferences in the Bradford assay, percentages of 0.25% (w/v) of SDS and 0.25% (w/v) of DTT in 100 mM Tris-HCl buffer at pH 7.5 were chosen for further experiments. In this step, the possibility of adding also urea to the extraction solvent was also tested; however, it was discarded because the protein content was 1.8 ± 0.1 % when a concentration of 1 M urea was added to the Tris-HCl buffer and interferences in the Bradford assay were observed when a 4 M concentration of urea was employed.

The next step to increase the amount of proteins extracted from MSC was to investigate the influence of the extraction time and amplitude of the HIFU probe. Extraction time was tested in the range from 3 to 15 min whereas the amplitude was in the range from 20 to 50%. As **Table 1** shows, the estimation of the protein content showed better results when 50% amplitude during 10 min was employed. Under these optimized conditions, it was possible to extract 3.9 ± 0.5 % of proteins. The possibility to increase the amount of proteins extracted using two cycles of the extraction procedure was evaluated. However, it did not improve the value obtained using just one cycle.

Using the optimal extraction conditions, the effect of varying the ratio between extraction solvent and sample amount was also studied. Initially, 50 mg of sample were extracted with 5 mL of extracting solution (ratio 1:10). Then, the sample amount was increased up to 100 mg or 200 mg using the same volume of extracting solution (ratio 1:20 and 1:40, respectively). In both cases, a lower percentage of protein content (see **Table 1**) was observed compared to that obtained using a 1:10 ratio. An increase in the volume of the

extraction solvent from 5 mL to 10 mL was evaluated keeping ratios of 1:20 and 1:40, but this increase had no effect on the protein content.

Finally, trying to avoid interferences from other compounds, a cleaning procedure based on washing the MCS sample two times with methanol:water (80:20 % (v/v)) followed by washing with acetone:water (80:20 % (v/v)) was carried out before protein extraction. However, this clean up procedure did not allow to improve the protein content extracted from the MCS sample.

Once selected the best extraction conditions to obtain the highest protein content from CS samples, a SDS-PAGE analysis was carried out to evaluate if there were differences in the protein profile of the different CS samples (LCS, MCS, and DCS). **Figure S1** (see supplementary data) demonstrated that the electrophoretic profiles of the three different samples showed similar bands; an intense band between 15 and 20 kD and bands with molecular masses higher than 100 kD.

3.2. Evaluation of the bioactive capacity of CS protein hydrolysates

Peptides from the protein extracts of CS samples submitted to different roasting process were obtained by enzymatic digestion employing alcalase, themolysin and a simulated gastrointestinal digestion with pepsin and pancreatin enzymes. Then, CS protein hydrolysates were evaluated in terms of peptide content and bioactive capacity.

Figure 1A shows the hydrolysis degree obtained for LCS, MCS and DCS when alcalase, thermolysin and simulated gastrointestinal digestion were used to hydrolyse the proteins extracted from each sample. There were not significant differences in the hydrolysis degree obtained for CS samples when different enzymes were used in the protein digestion. Regarding the bioactivity of all CS protein hydrolysates, it was measured in terms of antioxidant activity (using ABTS and hydroxyl radical scavenging assays) and as the capacity of peptides to reduce micellar cholesterol solubility. In general, all protein

hydrolysates exhibited antioxidant capacity with percentages ranging from 9%, obtained for MCS submitted to simulated gastrointestinal digestion, to 35%, obtained for DCS submitted to enzymatic digestion with thermolysin (see **Figures 1B** and **1C**). As **Figures 1A** and 1**B** show, when hydroxyl radical scavenging assays were performed, thermolysin was the enzyme yielding the highest antioxidant capacity whereas ABTS assay showed similar antioxidant capacity results for the three enzymes studied. Regarding the capacity to reduce micellar cholesterol solubility, it ranged from 25 to 32% as it can be seen in **Figure 1D**, reaching the maximum cholesterol-lowering activities when thermolysin was employed to hydrolyse the proteins from the three CS samples.

3.3. Peptide analysis by LC-(QTOF)MS and *de novo* identification

A reversed phase LC-(QTOF)MS analytical methodology was developed enabling the separation and identification of peptides in CS protein hydrolysates. The most appropriate chromatographic and MS/MS parameters were chosen to ensure the detection of the largest number of peptides in each analysis. With this aim, protein hydrolysates of LCS, MCS and DCS obtained using thermolysin to carry out the enzymatic digestion were chosen as model samples.

Chromatographic parameters such as gradient program, column temperature, flow rate, and injection volume were evaluated in terms of chromatographic resolution and analysis time. Regarding the gradient program, it was optimized to achieve a good chromatographic separation using water with 0.3 % acetic acid as solvent A, and ACN with 0.3 % acetic acid as solvent B, at a flow rate of 0.2 mL/min, a column temperature of 25ºC and an injection volume of 5 µL. Among the different gradient programs studied, the following enabled to obtain an adequate chromatographic separation: 5 % (B) at 0 min, followed by 5 % (B) at 0-3 min, 5-40 % (B) from 5 to 40 min, then 95 % (B) from 40 to 43 min and it was kept during 2 min, and finally 15 min of post-time. Subsequently,

the effect of the column temperature (25, 35, 55 °C) was evaluated keeping constant the flow rate and the injection volume. The lowest value tested for this parameter (25 ºC) was chosen since it allowed to obtain the highest number of peptides. Then, the flow rate was varied from 0.1 mL/min to 0.3 mL/min showing that the best results were obtained for a value of 0.3 mL/min. Finally, the effect of the injection volume was also studied when injection volumes of 5, 10 and 15 µL were employed, being the injection of 10 µL which allowed to detect a higher number of peptides.

The MS/MS parameters were selected considering those previously employed by our research team [30] to carry out the identification of peptides from food by-products. Modifying the mass range from 100 to 1700 m/z instead to 1500 m/z, the collision energy was varied from 3 to 6V/100 Da, being 5V/100 Da the value that enabled to detect a high number of peptides.

Under the best LC-MS/MS conditions, protein hydrolysates from LCS, MCS, and DCS were analyzed. Considering the scarce number of databases providing information about peptides from vegetable and fruit sources, *de novo* sequencing tool from the PEAK Software was selected to carry out the tentative identification of the peptides present in each CS hydrolysate. **Figure 2** shows the total ion chromatogram corresponding to the protein hydrolysates from MCS extract obtained after thermolysin digestion and an example of the mass spectrum for LLYQ peptide present in this sample. **Tables 1, 2** and **3** summarize the peptides identified by MS/MS after the three enzymatic digestions employed (thermolysin, alcalase and simulated gastrointestinal digestion), along with their experimental molecular masses, ALC and accuracy. It should be mentioned that only isoforms with leucine (L) are presented in these results, although peptide sequences containing isoleucine (I) instead of L are also possible (it is not possible to differentiate I from L by the MS system used). As can be seen in these tables (see also **Figure S2**),

different peptides with a number of amino acids ranging from 4 to 12 were identified. After excluding those peptides that could correspond to the enzymes employed in the hydrolysis (marked in the tables), thermolysin hydrolysates were those presenting a higher number of peptides (33), followed by gastrointestinal hydrolysates (11) and alcalase hydrolysates (7). As **Figure 2S** shows, no common peptides were found for the three different hydrolysis procedures.

The results obtained for the bioactive capacity of each hydrolysate showed that thermolysin was the best option, and these results were supported with peptide identification. As general trend, the results obtained showed that the peptide composition in CS protein hydrolysates was not affected by the coffee roasting process since most of the peptides identified were present in the three CS samples. For instance, using thermolysin or alcalase to hydrolyse the proteins from LCS, MCS and DCS extracts, just two and three peptides for each enzyme (LSGGLD, TTLPGS, for thermolysin, and AVPLLK, VAPLLK, ALLL for alcalase) showed differences along the roasting process. In the case of using a simulated gastrointestinal digestion, the peptides seemed to be more influenced by the roasting since five peptides identified in these CS protein hydrolysates (ALVGGTN, QVGGL, LGGLDSS, LGTVV, MMDPLA) were not present in at least one of the roasting degrees.

The amino acid composition of the peptides identified in the different CS protein hydrolysates contained high percentage of leucine (L)/isoleucine (I) and valine (V) within their sequences. These amino acids are common among antioxidant peptides [31], exert radical scavenging and metal chelation capacity and allow hydrogen-transfer and lipid peroxyl radical trapping due to their high solubility in hydrophobic radical species [32]. Also, some peptides presented proline (P) in their sequences which is characteristic of antihypertensive peptides [33].

The potential bioactivity of the different peptides identified in LCS, MCS and DCS protein hydrolysates was found in the BIOPEP database [34]. Although most of them are currently not included in BIOPEP, some were found within longer peptide sequences with potential bioactivities (see **Tables 1-3**). For instance, several peptides have been previously reported to be part of longer peptides with antibacterial activity (namely, LLNK and TLNGV), ACE-inhibitory effect (peptides as AVGVK, FASY, LLYQ, FDAVGVK, and AFDAVGVK), or antioxidant capacity (APGAGVY).

4. CONCLUSIONS

The peptide composition of protein hydrolysates from *Arabica* CS obtained using three different roasting degrees (light, medium and dark) was studied for the first time in this work by LC-(QTOF)MS. Proteins from CS were extracted using a Tris-HCl buffer containing SDS and DTT using a high intensity focused ultrasound probe. Subsequently, protein extracts were submitted to enzymatic hydrolysis employing different enzymes. Then, antioxidant and cholesterol-lowering capacities of the protein hydrolysates were evaluated. Despite not many differences were found among the extracts, the highest activities were obtained using thermolysin in the protein hydrolysis. Using the developed LC-(QTOF)MS method and *de novo* sequencing tool, the peptide composition of all the CS protein hydrolysates was investigated. 51 peptides, containing between 4 and 12 amino acids, were identified in the CS hydrolysates, none of them being common to the three different protein hydrolysis employed. Moreover, the roasting process to which the CS samples were submitted was shown to have little influence on their peptide composition.

Acknowledgments

Authors thank financial support from the Comunidad of Madrid and European funding from FSE and FEDER programs (project S2018/BAA-4393, AVANSECAL-II-CM). R.P.M. thanks the University of Alcalá for her pre-doctoral contract. M.C.P. thanks the Spanish Ministry of Economy and Competitiveness (MINECO) for her "Ramón y Cajal" (RYC- 2013-12688) research contract. Authors gratefully acknowledge "Café Fortaleza" for providing coffee silverskin samples.

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FIGURE CAPTIONS

Figure 1. Hydrolysis degree (A), antioxidant capacity evaluated by two different antioxidant assays (B and C), and the capacity to reduce micellar cholesterol solubility (D) of the protein hydrolysates obtained using three different enzymatic digestions from LCS, MCS and DCS.

Figure 2. Total ion chromatogram from MCS extracts hydrolysed with thermolysin by LC-(QTOF)MS and an example of MS/MS spectrum of the peptide LLYQ observed at 14.5 min (molecular mass: 535.3060 Da).

Figure 1

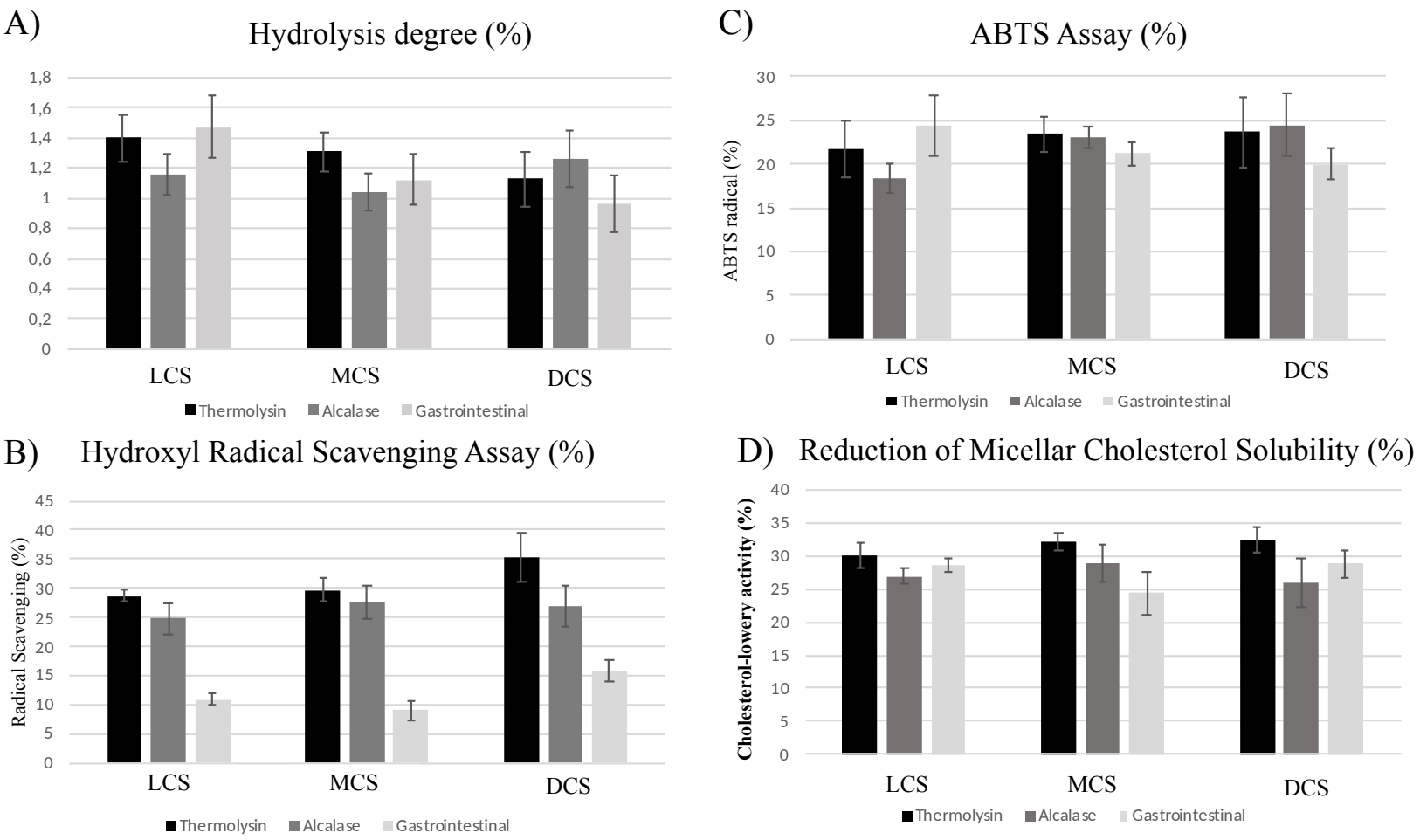
A)
Hydrolysis degree (%)
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
LCS
MCS
DCS
Thermolysin
Alcalase
Gastrointestinal
C)
ABTS Assay (%)
ABTS radical (%)
30
25
20
15
10
5
0
LCS
MCS
DCS
Thermolysin
Alcalase
Gastrointestinal
B)
Hydroxyl Radical Scavenging Assay (%)
Radical Scavenging (%)
45
40
35
30
25
20
15
10
5
0
LCS
MCS
DCS
Thermolysin
Alcalase
Gastrointestinal
D)
Reduction of Micellar Cholesterol Solubility (%)
Cholesterol-lowery activity (%)
40
35
30
25
20
15
10
5
0
LCS
MCS
DCS
Thermolysin
Alcalase
Gastrointestinal

Figure 2.

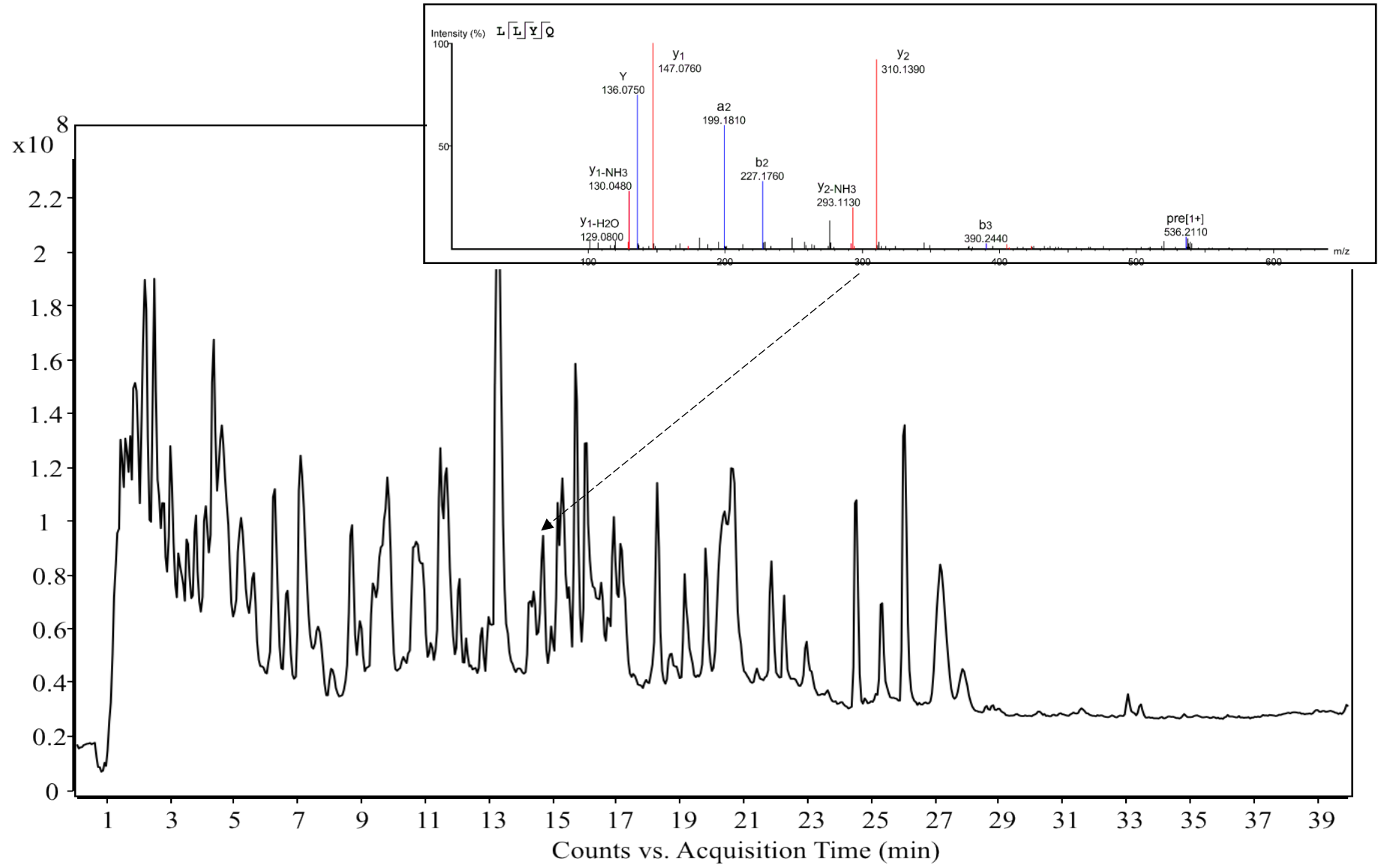
Intensity (%)
100
50
y1
147.0760
Y
136.0750
a2
199.1810
b2
227.1760
y2
310.1390
y1-NH3
130.0480
y2-NH3
293.1130
y1-H2O
129.0800
b3
390.2440
pre[1+]
536.2110
m/z
100
200
300
400
500
600
x10 8
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
Counts vs. Acquisition Time (min)

Table 1. Evaluation of different CS protein extraction conditions using HIFU.

	Extraction conditions	Protein extract (%) (Average ± SD)
	Extractant composition	
Buffer nature and pH	100 mM Tris-HCl pH 6.5	2.0 ± 0.4
	100 mM Tris-HCl pH 7.5	2.6 ± 0.3
	100 mM Tris-HCl pH 8.5	2.3 ± 0.6
	100 mM PB pH 6.5	0.4 ± 0.2
	100 mM PB pH 7.5	0.5 ± 0.4
	100 mM PB pH 8.5	0.5 ± 0.7
% SDS	0 % SDS	0.8 ± 0.5
	0.25 % SDS	2.8 ± 0.4
	0.5 % SDS	2.6 ± 0.3
% DTT	**0.25 % DTT**	2.9 ± 0.3
	0.5 % DTT	2.8 ± 0.4
	1 % DTT	2.3 ± 0.5
Urea	1 M Urea	1.8 ± 0.1
	4 M Urea	Interferences Bradford assay
	HIFU conditions	
Extraction time	3 min	3.3 ± 0.3
	5 min	2.9 ± 0.3
	10 min	3.4 ± 0.4
	15 min	3.2 ± 0.1
Amplitude	20%	2.9 ± 0.2
	30%	2.9 ± 0.3
	50%	3.9 ± 0.5
	Solvent/sample ratio	
5 mL extractant	**50 mg sample extracted (1:10)**	3.9 ± 0.5
	100 mg sample extracted (1:20)	3.0 ± 0.3
	200 mg sample extracted (1:40)	3.3 ± 0.3
10 mL extractant	200 mg sample extracted (1:20)	3.3 ± 0.4
	400 mg sample extracted (1:40)	3.3 ± 0.2

Table 2. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the thermolysin hydrolysates of LCS, MCS and DCS protein extracts using LC-MS/MS and *de novo* sequencing tool.

				LCS		MCS		DCS		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	LLNK[c]	1.6	486.3275	6 ± 2	93 ± 1	8 ± 1	95 ± 1	8 ± 2	96 ± 1	Antibacterial
2	AVGVK*[c]	1.71	472.3009	6 ± 4	91 ± 1	6 ± 3	91 ± 0	2 ± 4	83 ± 0	ACE-inhibitor
3	LLNAK	2.11	557.3537	4 ± 3	82 ± 1	7 ± 1	83 ± 1	8 ± 1	83 ± 1	-
4	VLGDQKN*	2.21	772.4079	3 ± 2	91 ± 0	5 ± 1	81 ± 30	6 ± 2	91 ± 0	-
5	LLSQ	3.7	459.2771	2 ± 2	94 ± 2	1 ± 1	94 ± 1	2 ± 1	95 ± 0	Haemolytic (ILSQ), Immunomodulating ((IISQ)
6	VTYD	3.75	496.2169	2 ± 2	83 ± 1	2 ± 1	83 ± 2	3 ± 2	83 ± 2	-
7	VYGDDGGQT	4.32	910.3668	2 ± 1	81 ± 1	1 ± 1	82 ± 1	2 ± 1	82 ± 1	-
8	VTDYT*	4.39	597.2646	2 ± 2	86 ± 1	1 ± 1	89 ± 1	2 ± 1	89 ± 1	-
9	LYGSTS*	5.02	626.2911	0.1 ± 3	88 ± 1	1 ± 1	87 ± 1	1 ± 1	86 ± 2	-
10	LSQGGTHYG	5.12	918.4196	2 ± 2	84 ± 1	1 ± 1	84 ± 2	1 ± 1	83 ± 2	-
11	YDAAP	5.3	535.2278	1 ± 3	82 ± 4	1 ± 1	82 ± 3	2 ± 1	82 ± 4	-
12	LYGST*	5.58	539.2591	2 ± 2	89 ± 1	1 ± 1	89 ± 1	2 ± 0	89 ± 1	-
13	YDAKTYR	5.68	915.4450	0.3 ± 3	85 ± 3	1 ± 1	85 ± 2	1 ± 1	84 ± 3	-
14	LTQY*[c]	6.96	523.2642	2 ± 2	82 ± 1	1 ± 1	82 ± 1	1 ± 2	82 ± 1	-
15	YSTY*[b]	7.31	532.2169	2 ± 2	88 ± 2	0 ± 1	85 ± 3	1 ± 2	88 ± 4	-
16	LYGSTSQE*	8.6	883.3923	2 ± 2	93 ± 2	1 ± 1	94 ± 1	2 ± 1	94 ± 1	-
17	LSGGLD[c]	8.7	561.2882	0.2 ± 2	88 ± 1	-	-	2 ± 3	87 ± 2	-
18	LSYDGNN*	9.73	781.3242	3 ± 2	91 ± 1	3 ± 1	90 ± 1	2 ± 1	91 ± 1	-
19	TTLPGS[a]	9.8	575.3036	1 ± 4	83 ± 2	-	-	1 ± 4	85 ± 2	-
20	SLGDSLSR	9.85	833.4243	2 ± 2	85 ± 2	2 ± 1	85 ± 1	1 ± 1	85 ± 1	-
21	LSGDSSLR (z=2)	9.96	833.4243	2 ± 2	87 ± 2	1 ± 1	87 ± 2	2 ± 1	86 ± 1	-
22	TYSTY*	10.14	633.2646	2 ± 3	89 ± 1	2 ± 2	88 ± 2	3 ± 2	88 ± 2	-
23	VHYSQGYNNA*	10.72	1151.4995	2 ± 3	82 ± 1	1 ± 1	83 ± 1	1 ± 1	82 ± 1	-
24	ANKNPDWE*[b]	10.8	973.4363	2 ± 3	89 ± 2	4 ± 2	89 ± 3	1 ± 1	89 ± 1	-

	(z=2)									
25	LNTTY	11.5	610.2963	2 ± 2	80 ± 0.5	2 ± 1	80 ± 1	1 ± 1	81 ± 1	-
26	MSDPAYK [c]	11.85	810.3582	3 ± 2	86 ± 2	3 ± 1	87 ± 3	3 ± 2	84 ± 3	-
27	LDVVAHE	12.89	718.3970	3 ± 2	93 ± 1	4 ± 1	93 ± 1	3 ± 2	94 ± 1	-
28	FASY*	12.97	486.2144	2 ± 2	96 ± 1	2 ± 1	96 ± 1	3 ± 2	96 ± 0	ACE-inhibitor
29	LLSQGGTHYG	13.25	1031.5037	3 ± 2	89 ± 1	3 ± 1	88 ± 2	3 ± 1	88 ± 2	-
30	LNEAL	14.24	558.3013	3 ± 2	87 ± 1	3 ± 1	88 ± 1	3 ± 2	88 ± 1	-
31	LLYQ [a]	14.5	535.3060	2 ± 2	89 ± 2	5 ± 2	88 ± 4	4 ± 2	89 ± 2	ACE-inhibitor, Immunomodulating
32	FDAVGVK*	14.67	734.3962	3 ± 2	95 ± 0.4	3 ± 1	95 ± 0.3	3 ± 1	95 ± 0	ACE-inhibitor
33	VTYDY*	15.17	659.2802	3 ± 2	93 ± 1	4 ± 1	92 ± 1	3 ± 2	92 ± 1	-
34	LNSGLLNKA [c]	15.6	928.5380	5 ± 2	85 ± 1	4 ± 0	86 ± 1	1 ± 3	86 ± 0	-
35	LNSGLLNAK [a, c]	15.6	928.5376	6 ± 1	87 ± 1	5 ± 1	87 ± 1	1 ± 3	86 ± 4	-
36	VTYDYYKN*(z=2)	15.8	1064.4814	3 ± 3	94 ± 0.4	4 ± 2	94 ± 1	3 ± 2	94 ± 0	-
37	LWAD*	15.95	503.2380	4 ± 3	82 ± 2	5 ± 1	82 ± 2	4 ± 2	83 ± 2	-
38	AFDAVGVK*	16.58	805.4333	4 ± 3	96 ± 0.5	5 ± 1	96 ± 0	4 ± 2	95 ± 1	ACE-inhibitor
39	VGPF [a]	18.6	418.2260	6 ± 2	82 ± 1	9 ± 1	84 ± 2	6 ± 2	86 ± 2	-
40	ELLPQ	18.75	598.3326	4 ± 3	85 ± 1	6 ± 3	87 ± 4	4 ± 3	86 ± 3	-
41	FASYDAPAVDAH [a,c]	18.8	631.2829	3 ± 3	83 ± 1	6 ± 2	83 ± 1	4 ± 2	84 ± 1	-
42	LFTYD	19.25	657.3010	3 ± 2	91 ± 1	4 ± 2	91 ± 1	3 ± 2	91 ± 1	-
43	VEFY* [b]	19.9	576.8228	3 ± 3	87 ± 1	-	-	3 ± 1	88 ± 0	-
44	LAPLP [c]	19.96	509.3213	5 ± 3	92 ± 1	6 ± 2	92 ± 1	6 ± 1	88 ± 6	-
45	LSGGLDVVAHE [a,c]	20.2	1045.7616	3 ± 5	89 ± 9	3 ± 1	93 ± 1	4 ± 2	92 ± 1	-
46	LSNLDVVAHE	20.37	1095.5559	3 ± 2	90 ± 3	3 ± 1	89 ± 3	3 ± 3	90 ± 3	-
47	LFTY	20.63	542.2740	3 ± 2	90 ± 1	3 ± 2	89 ± 1	2 ± 2	89 ± 0	-
48	LVEF*	21.84	506.2740	3 ± 3	90 ± 1	4 ± 2	90 ± 1	3 ± 2	90 ± 1	-
49	FWNGSQM* [a,c]	22.2	868.3576	3 ± 5	82 ± 1	4 ± 1	82 ± 2	-	-	-
50	FGLSDLT	24.5	751.3752	2 ± 4	83 ± 0	3 ± 2	83 ± 1	2 ± 1	83 ± 1	-
51	LFGTL	24.8	549.3163	3 ± 3	90 ± 1	4 ± 1	90 ± 1	2 ± 2	91 ± 1	-
52	LVEFY*	25.26	669.3373	0 ± 3	89 ± 1	2 ± 1	89 ± 1	0 ± 3	90 ± 1	-
53	VLEFY [b,c]	25.3	669.3432	2 ± 3	81 ± 1	2 ± 1	81 ± 1	2 ± 2	81 ± 1	-

54	EAPWL	25.52	614.3064	4 ± 3	87 ± 1	5 ± 2	88 ± 2	6 ± 2	86 ± 4	-
55	DVLPW	28.99	628.3220	4 ± 3	84 ± 2	4 ± 3	85 ± 5	3 ± 2	84 ± 2	-

* Peptides that could belong to the alcalase enzyme protein sequence.

a Peptides that were present in < 5 injections of LCS

b Peptides that were present in < 5 injections of MCS

c Peptides that were present in < 5 injections of DCS

Table 3. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the alcalase hydrolysates of LCS, MCS and DCS protein extracts using LC-MS/MS and *de novo* sequencing tool.

				LCS		MCS		DCS		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	VLVR	3.5	485.3326	0.1 ± 3	88 ± 2	0.3 ± 3	87 ± 2	4 ± 3	88 ± 2	-
2	LNVE	7.1	473.2485	0.4 ± 4	88 ± 3	0.3 ± 2	88 ± 4	5 ± 2	89 ± 3	-
3	APGAGVY*[a,b]	12.9	633.3122	3 ± 3	82 ± 2	2 ± 2	86 ± 1	4 ± 1	85 ± 2	Antioxidant
4	SVGAELE*[b,c]	14.2	703.3388	0.5 ± 2	87 ± 4	2 ± 0	89 ± 3	5 ± 1	83 ± 2	-
5	SFYYGK*[a,c]	15.6	763.3541	1 ± 2	85 ± 3	4 ± 2	88 ± 1	-	-	-
6	VVDL[a,b]	15.8	444.2584	1 ± 3	90 ± 3	2 ± 0	93 ± 2	7 ± 2	85 ± 3	-
7	VSLY*	17.7	480.2584	1 ± 3	92 ± 2	2 ± 2	93 ± 1	4 ± 3	91 ± 1	-
8	LVAL	19.1	414.2842	5 ± 3	90 ± 3	5 ± 3	90 ± 3	6 ± 2	90 ± 3	-
9	AVPLLK[b]	22.0	639.4319	0.4 ± 1	83 ± 4	1 ± 2	81 ± 2	-	-	-
10	VAPLLK[a,c]	22.1	639.4319	-	-	3 ± 2	83 ± 6	5 ± 2	82 ± 2	-
11	ALLL [a,b]	25.0	428.2999	-	-	4 ± 2	83 ± 3	6 ± 2	85 ± 6	celiac toxic (AIIL)

* Peptides that could belong to the alcalase enzyme protein sequence.

a Peptides that were present in < 5 injections of LCS

b Peptides that were present in < 5 injections of MCS

c Peptides that were present in < 5 injections of DCS

Table 4. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the gastrointestinal hydrolysate of LCS, MCS and DCS protein extracts using LC-MS/MS and *de novo* sequencing tool.

				LCS		MCS		DCS		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	WDAN [a]	3.6	505.2012	5 ± 4	81 ± 1	6 ± 1	81 ± 1	6 ± 2	82 ± 1	-
2	SSGEL*[a]	3.7	492.2265	-	-	7 ± 1	88 ± 4	7 ± 1	89 ± 3	-
3	LTGPT*[a]	4.3	488.2682	5 ± 3	86 ± 5	7 ± 1	87 ± 2	8 ± 2	86 ± 3	-
4	LLTHPN[a]	4.9	694.3833	4 ± 4	90 ± 1	9± 1	92 ± 1	8 ± 2	92 ± 1	-
5	ALVGGTN[a,b]	5.5	631.3355	-	-	9 ± 0	92 ± 2	9 ± 2	89 ± 6	-
6	TLNGV[a]	8.8	503.2786	6 ± 3	84 ± 1	7 ± 1	83 ± 2	8 ± 1	84 ± 2	Antibacterial (TIDGV)
7	TLDGV[a,b]	9.1	504.2627	5 ± 1	88 ± 1	9 ± 1	91 ± 1	7 ± 1	90 ± 1	-
8	PFAHP[a]	9.5	568.2846	5 ± 1	83 ± 2	5 ± 1	81 ± 1	6 ± 2	82 ± 1	-
9	QVGGL[a]	10.6	473.2682	-	-	8 ± 1	87 ± 2	7 ± 2	89 ± 2	-
10	LGGLDSS[a,b]	10.6	648.3151	-	-	7 ± 0	81 ± 0	-	-	-
11	NQFNHSSCST[a]	12.4	1124.4398	4 ± 4	89 ± 3	2 ± 1	87 ± 3	2 ± 2	88 ± 2	-
12	LGTVV[a,b]	14.8	488.3035	-	-	9 ± 1	84 ± 1	9 ± 1	81 ± 0	-
13	MMDPLA[a,b]	20.4	677.3035	-	-	5 ± 0	84 ± 3	6 ± 2	82 ± 1	-

* Peptides that could belong to the alcalase enzyme protein sequence.

a Peptides that were present in < 5 injections of LCS

b Peptides that were present in < 5 injections of DCS

Supplementary data

HIGH RESOLUTION LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR THE SEPARATION AND IDENTIFICATION OF PEPTIDES IN COFFEE SILVERSKIN PROTEIN HYDROLYSATES.

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Figure S1. SDS-PAGE gels corresponding to the protein extracts obtained for LCS, MCS and DCS.

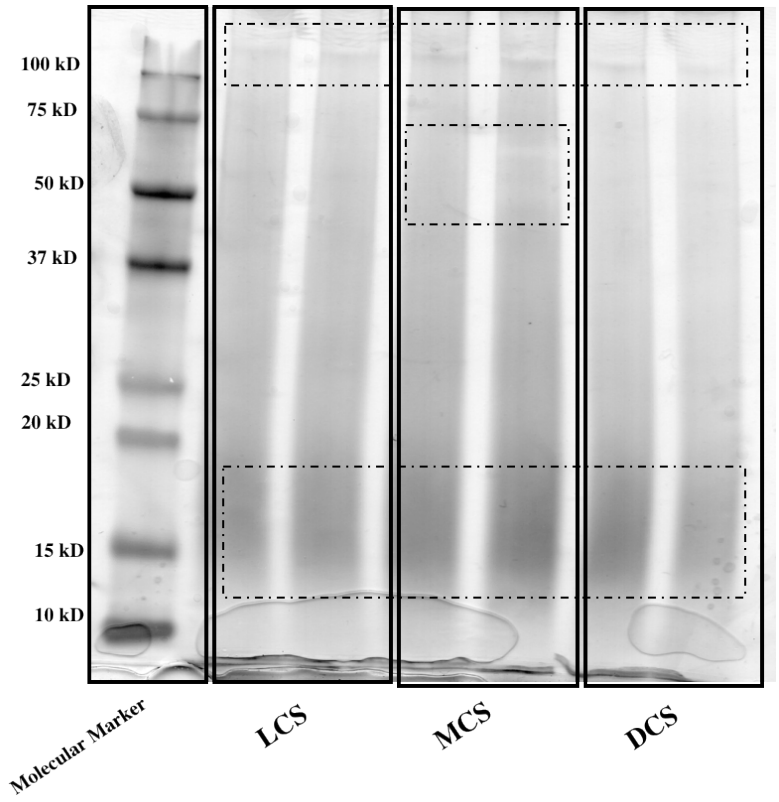

Figure S2. Venn diagrams for the total number of peptides obtained for LCS, MCS and DCS using three different enzymes.