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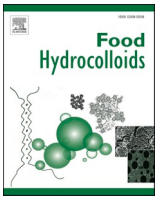

Production of soy protein concentrate with the recovery of bioactive compounds: From destruction to valorization

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ABSTRACT

This work aimed to develop a novel methodology based on aqueous micellar systems (AMS), for producing soy protein concentrates (SPC) from soybean flour and recovering high-valuable bioactive compounds as by-products. Ethoxylated aliphatic alcohols Tergitol 15-S-7 and Tergitol 15-S-9, non-toxic and biodegradable surfactants, were selected to form the AMS. The methodology consisted of an extractive stage of soybean flour with AMS, which rendered both a pellet, i.e., the SPC, and a supernatant containing the extracted bioactive compounds. The latter was further heated above the cloud point temperature, thus resulting in a biphasic system formed by a micelle-rich phase (MP) and an aqueous phase (AP). Obtained SPC showed a noticeable loss (~90%) of trypsin inhibitor activity, a total protein content close to 60%, soluble protein amounts varying from 19% to 34%, and remarkable released (by simulated digestion) antioxidant and antihypertensive activities. Those indicators are similar to or even better than those corresponding to SPC from the classical acid-extraction method. The AMS also exhibited an enhanced efficiency for extracting antinutrients such as non-digestible oligosaccharides, trypsin inhibitors, and lectins mostly recovered at the AP and separated from isoflavones, which were concentrated and isolated at the MP. The recovery of all the mentioned bioactive compounds, whether beneficial or undesirable, broadens their uses in research, food, and pharmacological fields.

This successful performance, simplicity, scalability, and sustainability make the proposed AMS-based extraction a powerful tool for processing plant derivatives and valorizing their by-products.

1. Introduction

Soybean [*Glycine* max (L.) Merr.] is the most important legume crop produced and consumed globally (Day, 2013; Jia et al., 2020). This legume and its by-products are considered one of the primary alternative protein sources for animal and human consumption (35–40%). Soybean has not only become an increasingly popular food. However, it has also attracted much interest because of the positive effect that its high intake produces on health, in particular in Asian populations (Kulling et al., 2001). Soy-based foods also contain a wide range of biologically active secondary metabolites, i.e., bioactive compounds, which can confer either beneficial or undesirable effects. Among the former, antioxidant and antihypertensive bioactivities have been widely reported (Balisteiro et al., 2013; Coscueta et al., 2016; González-Montoya et al., 2016). Consumption of this legume may reduce the risk of chronic diseases, such as cardiovascular diseases and cancer, as well as reduce the risk of osteoporosis and relieve the symptoms of menopause (Messina, 2014; Xiao et al., 2012). Phytochemicals responsible for such protective activities include saponins, phytates, protease inhibitors, phenolic acids, isoflavones, lecithin, lectin, and bioactive peptides (de Mejia et al., 2003; C. C. Lee et al., 2017; Lule et al., 2015; Xu et al., 2015). However, some declared beneficial activities, at specific doses and conditions, also have their dark side. Soybean contains various antinutritional bioactive compounds that exhibit undesirable physiological effects, such as preventing the absorption of nutrients. The main antinutritional compounds comprise lipoxygenase, trypsin inhibitors, lectin, and others in

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minor quantities, such as tannins, non-digestible oligosaccharides, saponins, alkaloids, phenolic compounds, and phytates. Trypsin inhibitors are the most critical components of antinutritional factors responsible for growth retardation and digestive and metabolic diseases (Boisen & Eggum, 1991; Gatel, 1994). Therefore, the inactivation of trypsin inhibitors becomes a requirement to improve the absorption of soy proteins in the digestive tract, thus representing a challenge for the food industry and research development (Liu, 1997). At present, "detoxification," i.e., the inactivation of soybean antinutrients, is carried out through various processes (Akande & Fabiyi, 2010; Newkirk, 2010, pp. 1–48). Denaturing the thermolabile antinutrients (trypsin inhibitors and lectins) by heat is the most widespread classical inactivation process. It causes the loss of their activity and results in improving protein digestibility. However, this process also leads to the indiscriminate destruction of other essential nutrients and bioactive molecules in the legume (Agrahar-Murugkar & Jha, 2010; Y. Chen, 2015; Jasti et al., 2015; Murugkar, 2015). Roasting in a rotary drum dryer or conventional grain dryer (temperatures vary between 110 and 170 °C) can reduce trypsin inhibitors up to 85%, while conventional drying with hot air at 100 °C for 2 h reduces trypsin inhibitors activity by 80% (Agrahar-Murugkar & Jha, 2010; Carvalho et al., 2013; Stewart et al., 2003). Other detoxification methodologies include extrusion, micronization, sterilization by autoclaving, dielectric thermal treatment technology, infrared, and enzymatic chemical treatment. However, they are not applicable on a macro scale due to their high cost (Vagadia et al., 2017). Besides, all the processes mentioned are destructive; this deprives the opportunity to valorize those antinutrients that present beneficial properties under certain conditions (Dang & Van Damme, 2015; Gomes et al., 2011). As an initial step, extracting and isolating those compounds is necessary. That would allow their toxicological and clinical evaluation application and their subsequent commercialization as supplemental ingredients.

Soybean flour, which is obtained from grinding dehulled soybeans, is one of the most widely marketed protein-rich food ingredients. Subjecting soybean flour to a washing process with appropriate extractive liquids allows for obtaining a soy protein concentrate (SPC). That is an ingredient with higher protein content/quality and lower content of bioactive compounds, e.g., antinutrients, which leach out in the washing liquids. This non-destructive detoxification process allows for recovering bioactive compounds; however, it is not yet applied for that purpose due to the lack of technologies capable of separating the different phytochemicals remaining in the extracts in a viable and sustainable way. Traditionally, SPC is obtained from defatted soybean flour by precipitating proteins and discarding soluble sugar and minor constituents with an alcohol-water mixture or a diluted acid solution in the pH range of 4.0–4.8 (Erickson, 1995). The yields of SPC for these conventional processes have been reported to vary between 60 and 70% concerning the flour protein content (Erickson, 1995). Thus, approximately two-thirds of the protein content of soy flour is recovered as insoluble residues after the extraction. In contrast, the remaining one-third, mainly containing protein antinutrients, is lost (Alibhai et al., 2006).

Recently, scientists have evaluated a wide range of new non-toxic, non-flammable, and biodegradable solvents to develop sustainable and environmentally friendly extraction methods (Bajkacz & Adamek, 2017). Certain surfactants exhibit the mentioned properties and represent an economical alternative to expensive and dangerous organic solvents. In aqueous media, they form aggregates, i.e., micelles, capable of interacting with hydrophilic or lipophilic molecules through hydrophobic, dipolar, and hydrogen bonding interactions; thus, these AMS become useful for extractive purposes (Sharma et al., 2015). Besides, they can separate into two phases, a micelle-poor one and a micelle-rich one, when heated above a critical temperature (cloud point), thus acquiring separating properties. AMS successfully recovered soybean phytochemicals, such as isoflavones. A Genapol X-080 AMS performed outstandingly in extracting daidzein from *Puerariae radix* (He et al., 2005). Furthermore, previous works carried out by our team demonstrated that Triton X-114, Genapol X-080, Tergitol 15-S-7 (Tg7), and Tergitol 15-S-9 (Tg9) AMS were suitable for extraction of total soy isoflavones (Cordisco et al., 2016; Coscueta et al., 2018). Genapol X-080 systems were suitable for extracting antinutrients from soybean flour; however, a complete characterization (protein content, antioxidant and antihypertensive activities) of the obtained SPC was not performed (Haidar et al., 2018).

In this context, this work aimed to develop a soybean flour detoxifying methodology, AMS-based, capable of producing protein concentrates with similar or improved quality to those obtained by classical methods and recovering high-valuable bioactive compounds as by-products. Thus, we proposed AMS formed by the biodegradable surfactants Tg7 and Tg9 as extractive systems since they represent a sustainable alternative industrially applicable. We considered antioxidant and antihypertensive activities, digestibility, and trypsin inhibitory activity as key parameters, other than protein content, to define the final SPC quality. Regarding the leached bioactive compounds, we have considered the molecular distribution pattern of the extracted proteins as one of the extraction efficiency indicators, together with the recovery and distribution coefficients of non-protein compounds (isoflavones and raffinose family oligosaccharides). Finally, we compared the traditional methodology and the one proposed here based on their advantages and disadvantages and provided future perspectives.

2. Materials and methods

2.1. Materials

The surfactants Tergitol 15-S-7 (Tg7) and Tergitol 15-S-9 (Tg9) were supplied from Sigma-Aldrich (St. Louis, MO, USA). White soybean flour (i.e., non-thermal treated soybean flour) was supplied by the food processing company Molinos Río de la Plata SA (San Lorenzo, Argentina). Crystallized salt-free bovine trypsin, porcine pepsin (800–1000 U mg^{-1} protein), pancreatin (4xUSP), angiotensin-I converting enzyme (peptidyl-dipeptidase A, EC 3.4.15.1, 5.1 U mg^{-1}), crystalline α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA), bile salts and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.) and used without further purification. Fluorescein [3′,6′-dihydroxyspiro (isobenzofuran-1[3H],9'[9H]-xanten)-3-one] was purchased from Fisher Scientific (Hanover Park, IL). AAPH [2,2′-azobis (2-amidi-nopropane) dihydrochloride] was purchased from Aldrich (Milwaukee, WI). The tripeptide Abz-Gly-Phe (NO_2)-Pro was obtained from Bachem Feinchemikailen (Bubendorf, Switzerland). Tris [tris (hydroxymethyl) aminomethane] was obtained from Fluka (Gmbh, Germany). Isoflavone standards (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in pure methanol until obtaining the following concentrations: daidzin 1.5–80.0 μg mL^{-1}; daidzein 0.3–17.0 μg mL^{-1}; genistin 0.7–40.0 μg mL^{-1} and genistein 0.2–13.0 μg mL^{-1}. All the other reagents were of analytical grade and used without further purification. Deionized water was used to prepare all the solutions.

2.2. Methods

White soybean flour (henceforth called soybean flour) was treated following traditional and AMS-based extraction processes. Samples from the resulting supernatants, treated solids, permeates, and extracts were subjected to different analytical procedures to determine performance indicators. Simultaneously, non-treated soybean flour was also evaluated for comparison purposes. Fig. 1, provided for a more precise understanding, shows a schematic diagram of all the developed processes and techniques whose descriptions are given below.

2.2.1. Production of soy protein concentrates

Soy protein concentrates (SPC) were prepared according to the classical acid-washing method (Sair, 1959). Briefly, a suspension of 20.00% m/V soybean flour and 25.00 mM citrate buffer pH 4.5 (3.00 g

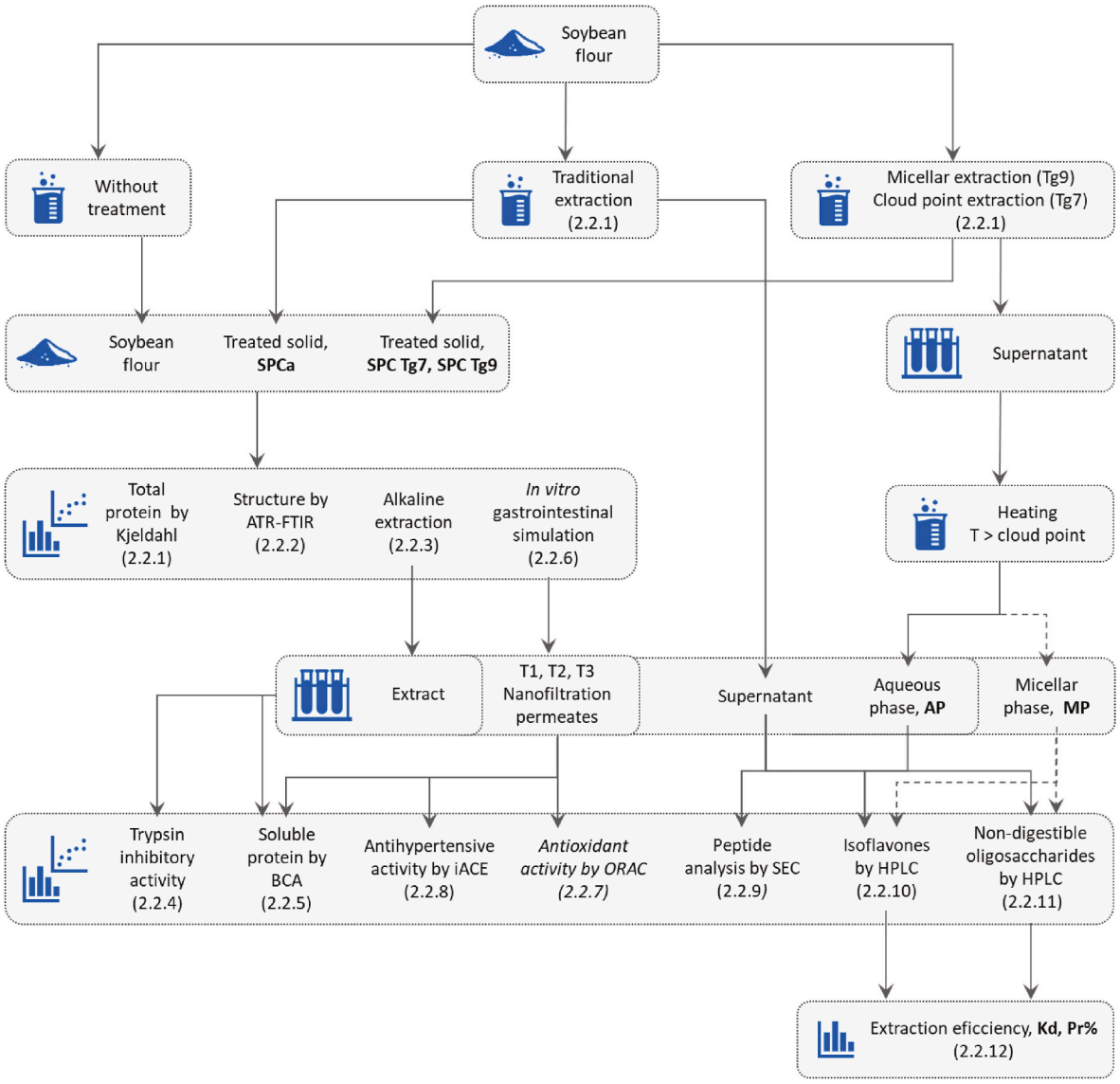

Fig. 1. Methodology outline. Flow diagram of the complete methodology, including the raw material (white soybean flour), the processes applied, the products and by-products obtained, and the analyses performed. For the processes and analyses, the subsection number corresponding to their description in the Materials and Methods section was also indicated.

of flour in 60.00 mL of buffer) was incubated in a bath at 40 °C and continuously stirred (150 rpm) for 45 min. Then, the system was centrifuged (4000 rpm) at 15 °C for 15 min, thus obtaining two fractions: a supernatant and an insoluble fraction, i.e., treated solid (SPCa).

SPC were also obtained by applying AMS as extractive solvents. AMS formed by two non-ionic surfactants, Tg7 and Tg9, were evaluated. Appropriate amounts of surfactants were dissolved in 50.00 mM sodium citrate (NaCit) at pH 4.5 until a final concentration of 5% m/m. This surfactant concentration was selected according to previous optimization tests (Coscueta et al., 2022). Higher concentrations were discarded to avoid high viscosity conditions representing a technical disadvantage. Each AMS was prepared by mixing 3.00 g of a given surfactant (Tg7 or Tg9) with 50.00 mM NaCit buffer pH 4.5 until a final system volume of e 60.00 mL. This mixture and a sample of soybean flour (3.00 g) were incubated separately in a thermostatic bath for 20 min to reach the extraction temperature (45 °C). After the incubation, both components (flour and surfactant solution) were placed into an Erlenmeyer, mixed, and stirred for 45 min inside the thermostatic bath, maintaining the temperature at 45 °C. Then, the system was centrifuged (4000 rpm) at room temperature for 10 min, thus obtaining a pellet (SPC) and a supernatant, separated by decantation. The supernatant was incubated again in a thermostatic bath and allowed to stand until total phase separation (approximately 15 min). A phase of low micelle concentration, i.e., aqueous phase (AP) and a phase rich in micelles (MP), were obtained by decanting. The phase separation temperatures, 45 °C for Tg7 and 60 °C for Tg9, were selected for each surfactant according to the phase diagram previously determined. These temperatures were a few degrees above the respective cloud point to obtain similar volumes of MP. Experiments were performed in triplicate for each surfactant.

All the resulting SPC (from traditional and AMS-based extractions) were neutralized (final pH 6.5) by adding 20.00 mL of 25.00 mM phosphate buffer solution at pH 7.0 and 0.50 mL of 1.00 M NaOH solution and then lyophilized. Finally, the Kjeldahl reference method analyzed the total protein content of each lyophilized SPC (SPCa, SPC Tg7, SPC Tg9).

2.2.2. *FTIR spectrometry*

Fourier transform infrared (FTIR) spectrometry characterized the soybean flour and the SPC. The spectra were acquired by averaging 30 measurements at wavenumbers from 500 to 4000 cm^{-1} with a

resolution of 4 cm^{-1}. The spectra were normalized for the maximum absorbance. The amide-I band's second derivative was used to identify the different spectral components of soybean flour and SPC. The subsequent spectral deconvolution was performed by applying a Gaussian fitting (Arrondo et al., 1993; Zana et al., 1998). Measurements were carried out in an infrared spectrometer, model ABB MB3000 (ABB, Switzerland), equipped with a deuterated triglycine sulfate detector and provided with a horizontal reflection accessory MIRacle™ (PIKE Technologies, USA), for attenuated total reflectance, with a diamond crystal plate/Se.

2.2.3. Extraction of trypsin inhibitors and soluble protein

Reference methods globally accepted for estimating trypsin inhibitory activity in soybean-derived products share the same principle: trypsin inhibitors are extracted from a weighed sample under the alkaline condition at which soy protein solubility is enhanced (Liu, 2021). In this work, the extraction/lixiviation was carried out by mixing 1.00 g of soybean flour/SPC with 50.00 mL (V_E) of 10.00 mM NaOH and stirring at room temperature for 3 h, according to the standard method proposed by Kakade et al. (1974) and later improved (AOCS, 2009; Kakade, 1974). A final centrifugation step (3500 rpm) for 10 min was carried out to recover the supernatant, which was conveniently diluted (with a dilution factor F_D) to determine the trypsin inhibitory activity (TIA) and soluble protein content.

2.2.4. Trypsin inhibitory activity (TIA)

TIA was measured according to the modified methodology proposed by Coscueta et al. (2017). The procedure was adapted to reduce the working volumes and develop the assay in a 96-well microplate, as shown in Table 1. The enzymatic reaction progress was monitored by absorbance measurements (400 nm) for 2 min. All the determinations were performed on the Multiskan GO (Thermo Fisher Scientific Corporation) spectrophotometer operated by the SkanIt 3.2 software (Thermo Fisher Scientific Corporation). The TIA was calculated as follows:

$$TIA' = 100\frac{0.350\ \left(m_{control} - m_{sample}\right)}{0.040}\ F_D V_E \quad (1)$$

where 100 is a conversion factor (to convert 0.01 u. Abs in trypsin inhibition units); $m_{control}$-m_{sample}, the difference between the slopes corresponding to the absorbance vs. time curves, in the absence (control) and presence of trypsin inhibitors (sample); F_D, the dilution factor of supernatant from extraction; V_E is the extraction volume of 0.01 M NaOH solution (50 mL, see section 2.2.3), used for 1 g of soybean flour; 0.040, the aliquot (mL) of supernatant dilution used in the continuous assay; and 0.350, the final reaction volume (mL) in the microplate well. Finally, the TIA of a given sample was expressed as a percentage of the total TIA present in soybean flour (TIA = $TIA'_{sample}/TIA'_{soybean\ flour}$ x 100) to facilitate the comparison of extractive efficiencies exhibited in the different treatments.

2.2.5. Determination of soluble protein content

We determined the soluble protein content by the bicinchoninic acid method (Smith et al., 1985), adapted for use in a 96-well microplate.

Table 1
Continuous method to determine trypsin inhibitory activity adapted for use in a microplate reader.

Reagents	Control[a]	Sample[a]
Tris buffer 0.050 M, pH 8.20	140	100
Trypsin working solution	70	70
Diluted supernatant	–	40
Incubate at 37 °C inside the microplate reader for 2 min, then add		
BAPNA working solution (pre-heated at 37 °C)	140	140

[a] Volumes expressed in μL.

This method measured the protein content in extracts from alkaline lixiviation of SPC and permeates from *in vitro* gastrointestinal simulation (see section 2.2.6). A stock bicinchoninic acid (BCA) solution was prepared with this composition: BCA 1.00% (m/V), sodium tartrate 0.16% (m/V), Na_2CO_3 2.00% (m/V), NaOH 0.40% (m/V), and $NaHCO_3$ 0.95% (m/V), the final pH being 11.2. A stock $CuSO_4$ solution of 4.00% (m/V) was also prepared. The working BCA reagent was prepared by mixing the BCA stock solution (diluted 1:10) with the stock $CuSO_4$ solution to a 50:1 ratio. A calibration curve made with bovine serum albumin (BSA) with concentrations of 50–1000 μg mL^{-1} was used. The protocol was carried out by placing 25 μL of sample in each well and then adding 200 μL of working BCA reagent simultaneously in all the wells. The microplate was incubated at 37 °C for 30 min before obtaining the absorbance values at 562 nm. Incubation and readings were performed on the Multiskan GO (Thermo Fisher Scientific Corporation) spectrophotometer operated by the SkanIt 3.2 software (Thermo Fisher Scientific Corporation).

2.2.6. In vitro simulated gastrointestinal digestion

The *in vitro* simulated gastrointestinal digestion (SGI) was carried out for all SPC and soybean flour. The digestive process in the mouth and esophagus, where carbohydrates are mainly affected, was not simulated since this work focused on the digestion of proteins and phenolic compounds. Replicates (300 mg) from each applied methodology were adequately pooled and homogenized into one sample. A given amount of the pooled sample (280 mg) was mixed with 6.00 mL of acidified water (pH 2.0). The pH was adjusted to 2.0 with 1.00 N HCl, and the final volume was completed to 7.00 mL with the same acidified water. The mixture was incubated at 37 °C and shaken at 130 rpm for 20 min to temper the digestive process. Before starting the SGI, 1.00 mL of supernatant, representing the sample at the initial time (T1), was removed from each experiment. The initial stage of the SGI began with the stomach digestion step, the gastric juice being simulated with pepsin 25 mg mL^{-1}, prepared in 0.10 N HCl (Aura, 2005). 0.3 mL of this "gastric juice" was added, left at 37 °C, and shaken at 130 rpm for 60 min. Then, the gastric stage was terminated by increasing the pH to 6.5 with 100.00 mM $NaHCO_3$ solution. An aliquot of 1.00 mL was then withdrawn, thus representing the sample corresponding to the end of the mentioned stage (T2). For the intestinal step, pancreatic juices were simulated with a solution of pancreatin 2 mg mL^{-1} and bile salts 12 mg mL^{-1} diluted in a solution of 100.00 mM $NaHCO_3$ (Laurent et al., 2007). 1.50 mL of pancreatic solution was added to the system. The temperature was returned to 37 °C and stirring decreased to 45 rpm. This stage was extended for 90 min (T3) and then stopped by freezing at −30 °C. Three independent SGI experiments were performed for each pool. Finally, all the samples from the SGI process were nano-filtered in 3 kDa pore membranes (Amicon® Ultra-4, Millipore), the resulting permeates (T1, T2, and T3) being subsequently analyzed (bioactive peptides and phenolic compounds). Enzymatic solutions were freshly prepared and sterilized by filtration with 0.22 μm membrane filters (Millipore, Billerica, MA, USA). After being sterilized, the solutions were kept in an ice bath to avoid enzymatic self-degradation. A thermostatic water bath at 37 °C was used to simulate the physiological temperature of the human body. Mechanical agitation (parallel peristaltic movements) was implemented, with intensities emulating those reached in each digestive compartment.

2.2.7. Antioxidant activity

The oxygen radical scavenging capacity (ORAC) was determined by the method proposed by Coscueta et al. (2021). The antioxidant activity of each sample (permeates T1, T2, and T3) was evaluated in triplicate and expressed as μmol Trolox Equivalent per gram of initial digested sample (SPC or soybean flour) on a dry basis.

2.2.8. ACE inhibitory activity

The ACE inhibitory activity was carried out using the fluorometric

assay described by Coscueta et al. (2021). The inhibitory activity on ACE (iACE) of each sample (permeates T1, T2, and T3) was evaluated in triplicate and expressed as the concentration capable of inhibiting 50% of the enzymatic activity (IC_{50}). A non-linear model calculated the IC_{50} values. The results were expressed as μL mL^{-1} to inhibit 50% of the enzymatic activity.

2.2.9. Size exclusion chromatography

The molecular mass distribution of the protein components in the supernatant from traditional extraction and the aqueous phase (AP) from AMS-based extraction was analyzed by size exclusion chromatography. The chromatographic runs were performed at a flow of 0.5 mL min^{-1} with 25 mM phosphate buffer (pH 7) containing 150 mM NaCl and 0.2 g L^{-1} NaN_3. Standard proteins with known molecular masses (Thyroglobulin, 669 kDa; Ferritin, 440 kDa; Aldolase, 158 kDa; Conalbumin, 75 kDa; Ovalbumin, 43 kDa; Carbonic anhydrase, 29 kDa; Ribonuclease A, 13.7 kDa; Aprotin, 6, 5 kDa) were used to calibrate the system. The AKTA pure 25 L system (GE Healthcare Life Sciences, Freiburg, Germany) was used in a configuration consisting of two high-performance piston pump systems, a pressure monitoring system for column protection, a mixing chamber, a V9-IA injection valve, a Superdex® 200 10/300 GL column connected in series to a Superdex Peptide 10/300 GL column (GE Healthcare Life Sciences, Freiburg, Germany), and a length U9-L UV detector fixed wave at 280 nm. The system was controlled by UNICORN software.

2.2.10. Determination of isoflavones by HPLC

The identification and quantification of extracted isoflavones on supernatant from traditional extraction and both phases (AP and MP) of micellar systems were carried out using high-performance liquid chromatography (HPLC). Initially, each analyzed sample was conveniently diluted (1:3 with pure MetOH) and filtered on a 0.45 μm filter. Chromatography was carried out on a reversed-phase column (COSMOSIL 5C18-AR-II Packed Column - 4.6 mm D.I. x 250 mm) with two mobile phases. Mobile phase A consisted of ultra-pure water acidified with glacial acetic acid (0.1% V/V), while phase B was constituted by acetonitrile acidified with glacial acetic acid (0.1% V/V). The operation involved injecting 20 μL of sample and eluting it with a linear gradient from 80 to 0% of phase A over 25 min, at a constant flow of 0.8 mL min^{-1} and a column temperature of 25 °C. Each isoflavone was identified by considering the absorbance spectrum and the retention time of standards. The concentration of each isoflavone was determined by measuring the area under the peak and interpolating it on the corresponding calibration curve. The results were expressed in μg of isoflavone per gram of soybean flour on a dry basis. The Waters e2695 modular separation system was used, with a UV/Vis photodiode array detector (PDA 190–600 nm). The acquisition of the data and the analysis were carried out using the Empower 3 software.

2.2.11. Determination of non-digestible oligosaccharides by HPLC

The content of raffinose family oligosaccharides in the supernatant from traditional extraction and both phases (AP and MP) of micellar systems was determined by HPLC. Aliquots (30 μL) of the undiluted liquid samples were analyzed by chromatography. The mobile phase was a 13.00 mM H_2SO_4 solution, with a flow of 0.8 mL min^{-1}, at isocratic elution. HPLC quality standards of raffinose, stachyose, glucose, and sucrose were used to identify the mentioned compounds in the samples by considering their retention time. The peaks for raffinose and stachyose were so close together that we had to measure them as one, which we called raffinose family oligosaccharides. The values were expressed in relative units (RU), given by the product between the integrated area and the mass of liquid obtained for each fraction, referred to as 1 g of soybean flour (RU g^{-1}). An HPLC system prepared for the identification and quantification of sugars was used, consisting of a Knauer WellChrom Pump K-1001 module (Knauer GmbH, Germany) and a differential refractive index (RI) detector K-2301 (Knauer GmbH, Germany). The separation was carried out on an Aminex HPX-87H 300 × 7.8 mm column (Bio-Rad, Hercules, USA) coupled to a Micro-Guard Cation H^+ precolumn (Bio-Rad, Hercules, USA), maintained at a temperature of 42 °C by an Eldex CH-150 column oven (Eldex Laboratories, Napa, CA). The data were acquired and analyzed using Clarity v.5.0.5.98 software (DataApex Ltd, Prague, Czech Republic).

2.2.12. Extraction efficiency parameters

The distribution coefficients (Kd) and phase recovery performance (Pr) in the different AMS were determined for both isoflavones and raffinose family oligosaccharides. The Kd was estimated as:

$$Kd = \frac{C_{MP}}{C_{AP}} \quad (2)$$

where C_{MP} and C_{AP} are the concentrations of the analyte (isoflavones or raffinose family oligosaccharides) in the MP (micellar phase) and AP (aqueous phase), respectively. On the other hand, the Pr was determined:

$$Pr(\%) = \frac{C_{MP/AP}V_{MP/AP}}{(C_{MP}V_{MP} + C_{AP}V_{AP})}\ 100 \quad (3)$$

where $C_{MP/AP}$ is the concentration of the analyte in either the MP or AP, selected according to the phase of the highest recovery, these $V_{MP/AP}$ is the volume of the corresponding phase. Equation (3) is only valid when analytes distribute entirely between the two phases without precipitating at the interphase.

2.2.13. Statistical analysis

All the experiments were carried out in triplicate, except for specific cases where something different was indicated, and the results were expressed as the mean value with their standard deviation (SD). Before any comparative statistical analysis, an exploratory study was carried out for all the data sets to determine the compliance of normality and homoscedasticity. Then the means were analyzed statistically by analysis of variance (ANOVA) followed by a posthoc test using Tukey (Tukey, 1949). A significance level of 5% was considered. All statistical analysis was carried out with the aid of RStudio V 1.0.143.

3. Results and discussion

According to previous results (Coscueta et al., 2022), we designed a new AMS-based process applied to soybean flour for producing SPC and recovering valuable bioactive compounds as by-products. A graphical scheme and technical details of the proposed methodology are presented in Fig. 2 to facilitate reading this article and interpreting the results.

Fig. 2 shows an initial micellar extraction performed by applying AMS on soybean flour. Then, a phase separation produced a MP and an AP by heating the extract (supernatant of the first extraction) to a temperature higher than the cloud point. It is important to note that the micellar extraction for Tg7 is considered a “cloud point” extraction since the extraction temperature (45 °C) is above the Tg7 CP (39 °C). This is not so for Tg9 since its cloud point (61 °C) is higher than the working temperature.

3.1. Producing soy protein concentrates: characterization of the product

3.1.1. FTIR spectroscopy

Although the literature has reported the non-ionic Tg7 and Tg9 as mild surfactants, it was necessary to evaluate their effect on soy protein structure since it might affect its functional properties (Nadar et al., 2017; Vicente et al., 2017). The spectroscopic analysis of polymeric molecules is complex due to the molecular vibrations that arise from numerous atoms. FTIR is an advantageous technique for the study of protein systems. There are several easily identifiable regions in the middle infrared spectrum, having nine characteristic bands called

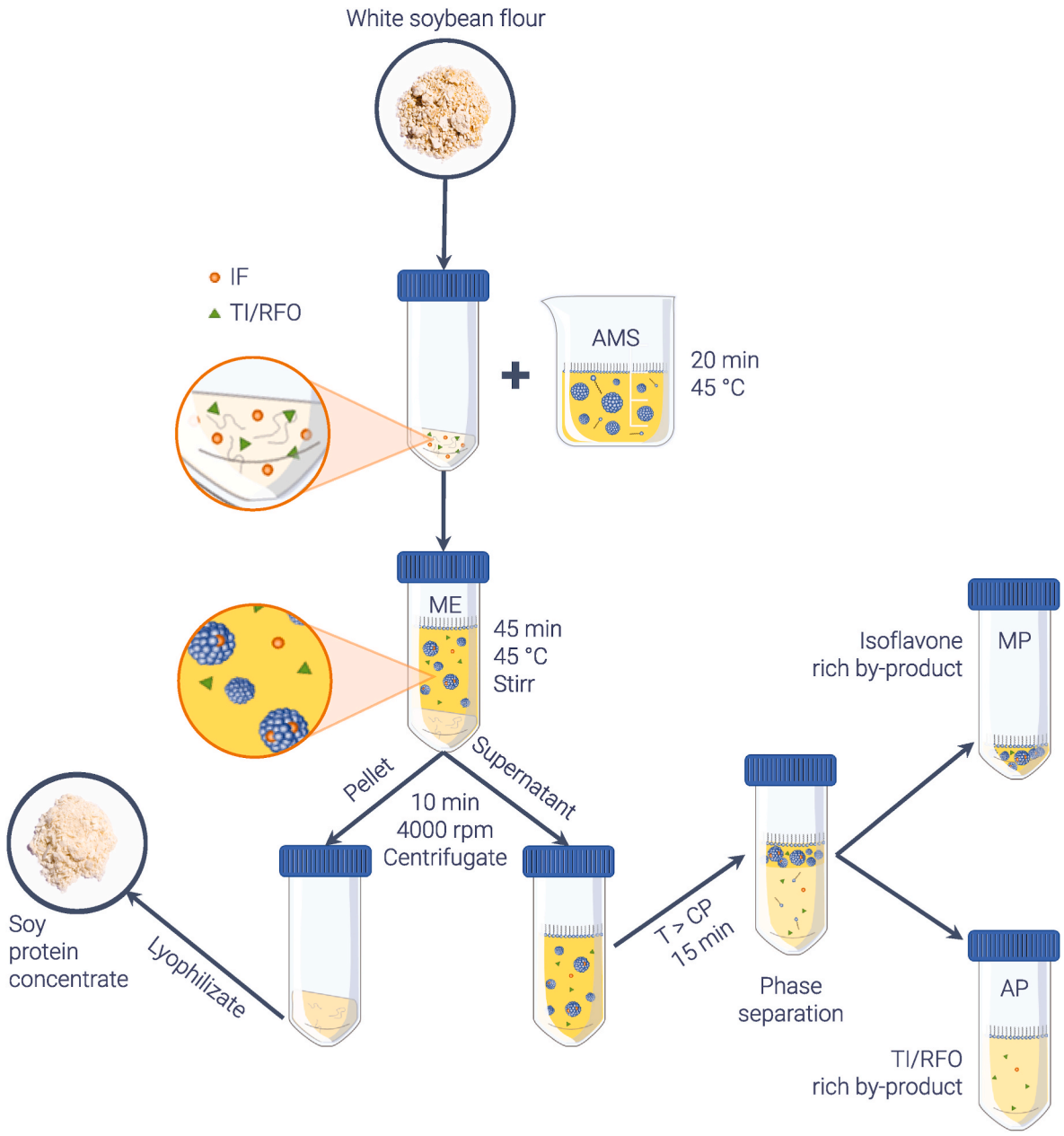

Fig. 2. Process outline. Scheme of the proposed methodology to produce SPC with SMA. ME: micellar extraction; MP: micellar phase; AP: aqueous phase.

amide-A, B, I, II ... VII; the amide-I, amide-II, and amide-III being the most widespread ones used in structural studies of protein.

Fig. 3A shows the FTIR spectra for flour (starting material) and SPC (products). The amide-A (3500-3200 cm^{-1}) and amide-B (3100-2500 cm^{-1}) bands come from a Fermi resonance between the first harmonic of amide-II and the vibration of the N–H stretch. Notably, the spectral band of amide-B showed an intense alteration after the process, which indicates a conformational change in the secondary structure of the matrix of the SPC (Chang & Tanaka, 2002). This band's intensity increased drastically for the SPCa and the SPC Tg9 compared to the soybean flour. However, only slight changes were noticeable for the SPC corresponding to Tg7. The amide-I and II bands are the two central regions of the infrared spectrum for protein. The amide-I (between 1700 and 1600 cm^{-1}) is mainly associated with the stretching vibration C═O (70–85%) and is directly related to the conformation of the main peptide chain (Chang & Tanaka, 2002). Amide-II (1600-1500 cm^{-1}) results from the N–H bending vibration (40–60%) and the C–N stretch vibration (18–40%). When structural alterations such as denaturation or aggregation occur, these two bands significantly decrease in intensity (Nishinari et al., 2014). Fig. 3B shows the highest and lowest amide-I and II signals for the flour and SPCa, respectively. In contrast, it shows intermediate intensities for the SPCs obtained by the micellar systems. The SPC Tg7 spectrum was like the flour, while the SPC Tg9 spectrum was higher than the SPCa. Amide-III (between 1300 and 1200 cm^{-1}) is a very complex band that results from a mixture of several coordinate shifts; it does not exhibit noticeable differences between SPCs and flour (Fig. 3B).

Even if the primary protein structures were the same, the secondary and the tertiary structures sometimes are not. The amide-B allowed us to analyze what happened to the secondary structures of the proteins. The most interesting regions in the FTIR spectra are the amide-I and II regions since changes in these regions were observed for other proteins (Arrondo et al., 1993; Susi & Byler, 1986). To analyze the secondary structures, we applied a deconvolution procedure of the amide-I band (Fig. 3C–F) (Fabian et al., 1993). The second derivative curve allowed the identification of the different spectral components used in a

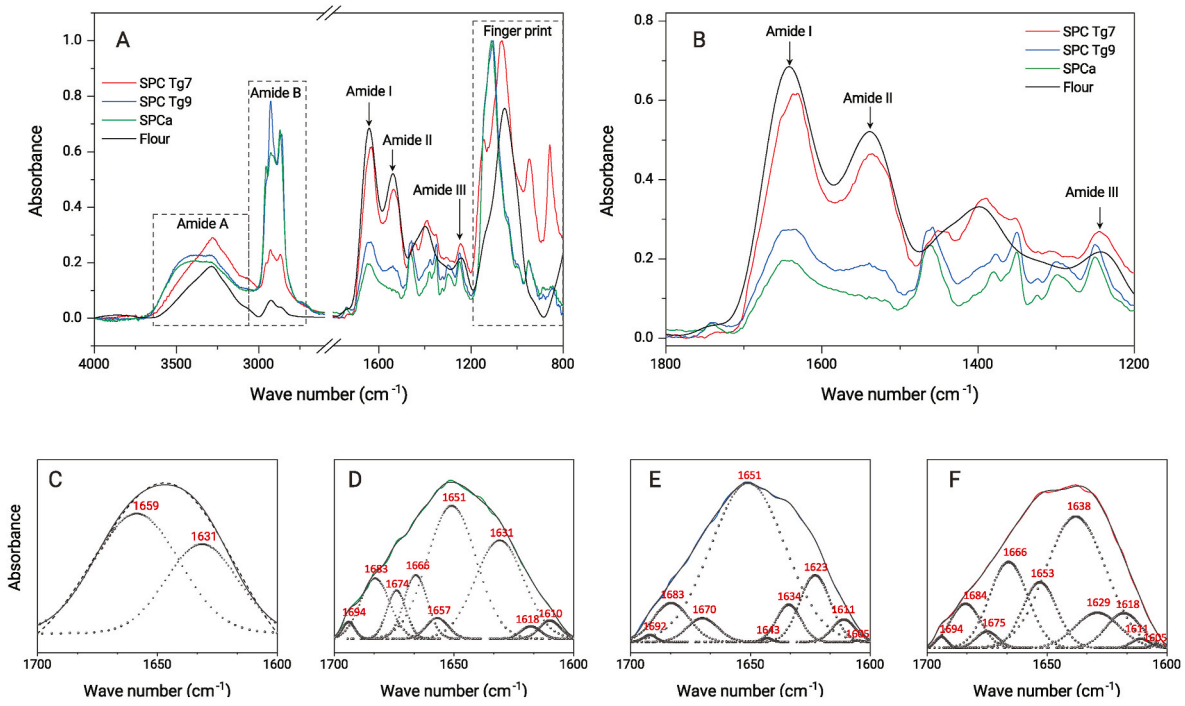

Fig. 3. FTIR. Standardized ATR-FTIR absorbance spectra of soybean flour and protein concentrates (SPCs). **A)** Full spectrum from 4000 to 800 cm^{-1}. **B)** Most important characteristic amide regions (I, II and III). Comparison of the amide-I region of the deconvoluted ATR-FTIR absorbance spectra for: **C)** soybean flour; **D)** SPCa; **E)** SPC Tg7; **F)** SPC Tg9. The curve fit was performed with Gaussian lines. The peak position of each component corresponding to the amide band was deduced from the second derivative spectra. The sum of the fitted curves is shown as a solid line, closely overlaid the original spectrum, shown as a dashed line (C) or colored line (D, E, and F).

deconvolution procedure with Gaussian curve fitting. Table 2 presents the contribution percentages of the various secondary structure types, calculated as the ratios between the areas of the curves corresponding to the different component bands and the total area below the spectral curve.

The data revealed that amide-I for all SPCs consists of nine or ten main components, unlike flour, which exhibits only two. We found the main band of the soybean flour and SPCa FTIR spectra in the amide-I region between 1660 and 1650 cm^{-1} (62% and 42% of the total area, respectively, Table 2). This corresponds to the vibrational movements of the amide residues of the main chain in a helical conformation. The soybean flour also presented another large band between 1650 and 1600 cm-1 corresponding to the β-sheet structure, which is in the minority (38%). Meanwhile, the SPCa also had smaller bands at 1637-1600 cm^{-1} and 1700-1682 cm^{-1}, related to β-sheet structures (44%), which slightly exceeded the content of α-helix (42%). Besides, it presented two bands corresponding to β-turn structures (1674 cm^{-1} and 1666 cm^{-1}). Both β-sheet and β-turn structures indicate a greater union of the amide protons by forming hydrogen bonds (FAO & Berk, 1992). We did not observe a common pattern in the FTIR spectra of the Tg7 and Tg9 SPCs (Table 2). The SPC Tg7 showed the principal peak in the region corresponding to the unordered structure (1639-1638 cm^{-1}), thus representing the highest structural percentage (43%). The second main structure was the β-sheet type, contributing about 24%. Finally, the SPC Tg9 had a structural distribution like the flour, with 67% of α-helix and 28% of β-sheet.

The structural alterations suffered by the SPC protein are related to the process conditions, such as temperature, surfactant concentration, and the medium's pH. It should be noticed that those conditions may affect inter/intramolecular interactions (formation/rupture of hydrogen bonds, hydrophobic effects) of protein molecules, thus resulting in conformational changes. Notably, the applied pH (pH 4.5) corresponds to the isoelectric point of most of the matrix proteins. This pH facilitates aggregation by non-electrostatic forces, thus decreasing protein solubility in the final concentrate (Derringer & Suich, 1980). We must not ignore that although the applied methodologies allow concentrate proteins, they cause the loss of soluble carbohydrates and a considerable amount of soluble protein. That enriches the final product in proteins already insoluble in the starting soybean flour, either by extracting the oil with hexane or the solvent removal step in the previous soybean processing. It should be noticed that changes in the secondary structure of soy proteins caused by a given treatment might affect not only their conformation but also functional properties such as gel transparency, solubility, surface hydrophobicity, and emulsifying capacity (X. Chen et al., 2013; Zhao, Chen, Chen, et al., 2008). Therefore, the information introduced here could be critical for further understanding and correlating the SPC applications.

Table 2
Protein secondary structure in soybean flour and SPC by FTIR analysis.

Product	Secondary structure (%) [a]			
	α-helix	β-sheet	Random coil	β-turns
Soybean flour	62	38	0	0
SPCa	42	44	0	14
SPC Tg7	12	24	43	20
SPC Tg9	67	28	0	4

[a] Structural composition of the amide-I, obtained from the area under each deconvoluted band and expressed as a percentage of the total area.

3.1.2. Protein content and trypsin inhibitory activity

The SPC produced by the different methodologies were analyzed and compared with each other and the starting soybean flour. Initially, we analyzed the total protein content by the Kjeldahl reference method (section 2.2.1). Then, we performed aqueous extractions to evaluate the trypsin inhibitory activity (TIA) and the amount of soluble protein by the bicinchoninic acid method (section 2.2.5). The results reported in Table 3 indicated that the total protein varied according to the type of

Table 3
Total protein content, soluble protein, and trypsin inhibitory activity analysis for SPC and soybean flour.

Product	Pt [a, b]	TIA [a, c]	Ps [a, d]
Soybean flour	54.6 ± 2.1 [a]	100 ± 7	84 ± 4
SPCa	64.0 ± 1.5 [b]	11 ± 1 [a]	29 ± 4 [a, b]
SPC Tg7	57.4 ± 0.7 [a, c]	10 ± 2 [a]	34 ± 7 [b]
SPC Tg9	60.7 ± 2.1 [b, c]	12 ± 3 [a]	19 ± 4 [a]

[a-d] Values from the same column that share a superscript do not present statistical differences, given the analysis of their variances (ANOVA) with Tukey's posthoc test for a significance level of 0.05.
[a] Values are expressed as mean ± SD on a dry basis.
[b] Total protein content (Pt) expressed as g proteins (100 g product) $^{-1}$.
[c] Trypsin inhibitory activity (TIA) expressed as a percentage from the starting soybean flour.
[d] Soluble protein content (Ps) expressed as g BSA (100 g total proteins) $^{-1}$.

surfactant used in the proposed technique. SPC Tg9 presented a total protein value comparable to SPCa, while SPC Tg7 showed a total protein like the starting soybean flour. When comparing the surfactants, Tg7 was demonstrated to be less efficient in concentrating proteins than Tg9, thus evidencing a higher protein extractive power.

As shown in Table 3, the inhibitory activity of trypsin was considerably reduced, around 90%, in all the SPC, thus reaching satisfactory levels that agreed with those predicted in a previous optimization work (Coscueta et al., 2022). In this regard, none of the methodologies differed significantly. However, this drastic reduction in the TIA resulted in a significant decrease in the protein solubility in 10 mM NaOH. That is a well-known disadvantage of protein concentration methods, i.e., the resulting SPC present low protein solubility after rehydration and poor functional properties. Protein exposure to extreme conditions may cause this low solubility (Alibhai et al., 2006; Fisher et al., 1986). We mean alcohol or acid extraction, heat treatment, precipitation, or centrifugation by extreme conditions. Besides, the reduction of protein solubility could also be the result of the enrichment of the final product is already insoluble proteins before the concentrating process, as we mentioned previously. Different treatments that allow for significant solubility recovery can mitigate this disadvantage's impact (Johnson, 1999). Also, particularly for the AMS-based extractions, the Tg9 led to the most significant protein solubility loss.

3.1.3. Digestibility and bioactivity

To analyze the performance of different SPC at *in vitro* simulated gastrointestinal digestion (SGI), we measured soluble protein, antioxidant activity, and antihypertensive activity at each stage (T1, T2, and T3). We measured soluble protein considering protein compounds with MW less than 3 kDa (enriched peptide fraction), the antioxidant activity by ORAC, and the antihypertensive activity from the inhibitory capacity on ACE (iACE). The response variable was recorded three times on the same experimental unit (pooled sample); therefore, we considered the collected data "repeated measures." So, we analyzed through the "Generalized Linear Models" procedure. Table A1 (see Annex) reports the results of fitting the general linear statistical models. These models relate the response variables soluble protein, antioxidant activity, and antihypertensive activity with three categorical predictive factors: experimental units (I), substrate (M: starting soybean flour or SPC from a given extractive methodology), and SGI's stage (T). Relationships between the observed responses and the predictor variables were statistically significant since, for each model, the P-value was less than 0.05. Besides, the determination coefficients (R^2) indicated that the adjusted models explained between 99.6% and 99.7% of the respective variability.

For the general digestion process (Table A2 in Annex), irrespective of the sample considered, the three responses adopted significantly different values according to the stage of the SGI. Interestingly, T2 showed higher soluble protein than T3. This finding is attributable to the analytical technique used for protein quantification, the bicinchoninic acid method. That assay depends on the ability of proteins to reduce Cu^{+2} to Cu^{+1} in an alkaline solution (biuret reaction), thus resulting in a purple product. Cysteine, tyrosine, and tryptophan residues are mainly responsible for reducing copper. However, unlike Coomassie dye-binding methods, the peptide skeletons also contribute to color formation, helping to minimize the variability derived from differences in protein composition. The peptide skeleton loss resulting from the release of peptides during stomach digestion (T2) may cause the decreasing signal. These peptides can be further degraded to tripeptides, dipeptides, and free amino acids in the intestinal stage (T3). On the other hand, the ORAC values increased during the gastric phase and practically kept constant in the intestinal phase. About iACE, the IC_{50} decreased significantly at each stage of the process, thus indicating a sustained increase in the inhibitory activity.

When considering the samples separately (Table A3 in Annex), we identified three homogeneous groups concerning ORAC and two for soluble protein and iACE. The mean soluble protein released during the digestion of the SPC Tg7 was the lowest one, while the remaining samples did not show differences. Concerning ORAC, the SPC Tg9 and the soybean flour did not differ significantly and surpassed the other samples, the SPC Tg7 showing the lowest level of this bioactivity. On the other hand, the maximum iACE was for the SPC Tg7 and the minimum for SPCa and SPC Tg9, while the soybean flour did not differ from the mentioned groups.

Fig. 4 shows the soluble protein content, ORAC, and iACE profiles as a function of each digestive stage. The lines represent each I and the color, each level of M. For soluble protein (Fig. 4A), the SPCa presented a different behavior than the others. As explained above, it showed a marked peptide release during the gastric phase and a later decrease in the signal during the intestinal phase. That could be indicative of more significant peptide degradation. The SPC Tg9 exhibited an increase in the peptide content during the first stage, keeping it constant until the end of digestion. About the soybean flour and SPC Tg7, the peptide release was almost continuous throughout the digestive process. The results for ORAC (Fig. 4B) differed, with SPC Tg7 and SPC Tg9 being markedly different from the other samples. SPC Tg7 showed constant antioxidant activity during the gastric phase, which increased during intestinal digestion. At the same time, SPC Tg9 evidenced a drastic increase and a later reduction in the gastric and intestinal stages, respectively. Unlike the other two responses, the iACE pattern (Fig. 4C) was similar for all samples throughout the SGI, with a greater increase in inhibitory activity during the gastric phase.

At the end of the digestion, the soluble protein was similar (Table 4) for all the products. At the same time, the antioxidant activity of both SPCa and SPC Tg7 was practically 30% lower than that corresponding to the soybean flour and SPC Tg9. Concerning iACE, all the products achieved high bioactivities.

It is expectable that soybean flour with higher protein solubility will release a greater number of peptides from its digestion and even higher antioxidant and antihypertensive activities. However, we did not observe this behavior in this work, probably due to its content of protease inhibitors. Protease inhibitors reduce proteolysis, releasing a larger proportion of peptides with more than 20 amino acids during flour digestion (Capriotti et al., 2015). These peptides probably play a nutritional role since their size belongs to the range (3–51 amino acids) compatible with the absorption through the intestinal epithelium. However, it should be noted that peptides that exceed 20 amino acids are known to exhibit minimal bioactivity properties (Roberts et al., 1999). For this reason, we previously filtrated with 3 kDa pore membranes samples used to determine ORAC and iACE bioactivities. Consequently, the retention of most peptides released during the filtration step could cause the reduced bioactivity values found for samples from soybean flour digestion.

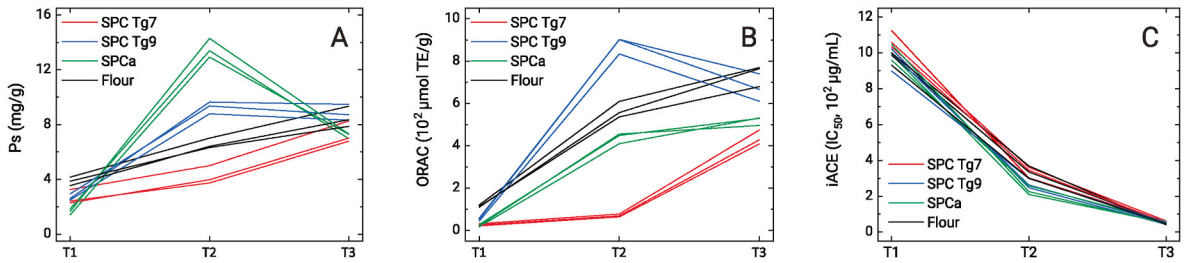

Fig. 4. SGI. Levels of each response depending on the stage of the SGI. The lines display the observations for each experimental unit for: **A**) soluble protein (Ps); **B**) ORAC; **C**) iACE. Stages of the process: T1, beginning of the gastric stage; T2, end of gastric stage; T3, end of the intestinal stage.

Table 4
Multiple comparisons for each response by product.

Substrate	Means observed at the end of the SGI [a]		
	Ps [b]	ORAC [c]	iACE [d]
Soybean flour	8.5 ± 0.8 [a]	737 ± 51 [a]	47 ± 6 [a]
SPCa	7.2 ± 0.2 [a]	518 ± 20 [b]	53 ± 6 [a]
SPC Tg7	7.4 ± 0.8 [a]	437 ± 34 [b]	58 ± 4 [a]
SPC Tg9	8.8 ± 0.6 [a]	672 ± 65 [a]	49 ± 6 [a]

[a, b] Values from the same column that share a superscript do not present statistical differences, given the analysis of their variances (ANOVA) with Tukey's posthoc test for a significance level of 0.05.

[a] Values expressed mean ± SD on a dry basis.

[b] Soluble protein content (Ps) in mg BSA (g of substrate)$^{-1}$.

[c] ORAC in µmol TE (g of substrate)$^{-1}$.

[d] iACE as IC_{50}, µg mL^{-1}.

3.2. Recovering bioactive compounds: characterization of by-products

3.2.1. Protein characterization by size

After applying both methodologies to prepare protein concentrates, we characterized the protein size profile of the supernatant from the classical extraction (acid extraction) and the AP from AMS-based extraction, presumably rich in antinutritional factors. For that, we used size exclusion chromatography (SEC).

We identified three main regions when analyzing the chromatograms (Fig. 5). The first region (I) corresponds to large proteins and polypeptides, the second one (II) to medium/small peptides, while the third (III) contains free amino acids and small molecules capable of absorbing at 280 nm. We focused our principal interest on the region I since it corresponds to the protein antinutritional factors in soybean flour. Even though they are unwanted compounds for nutritional purposes, they have biological capacities in the health area, making it interesting to recover them intact.

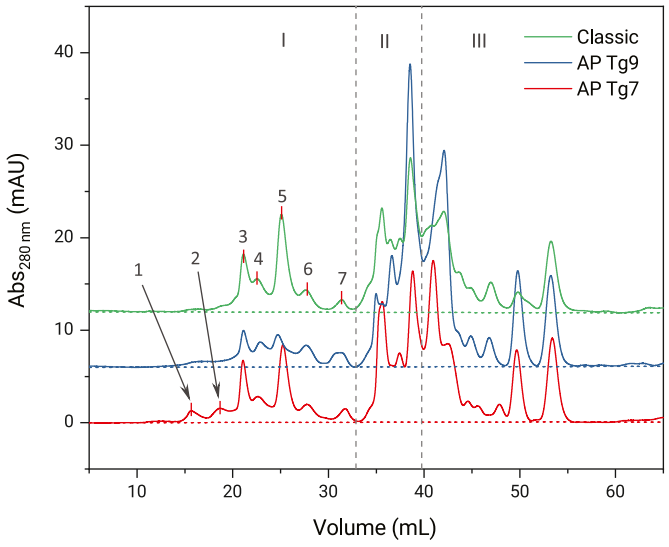

Fig. 5. Size exclusion chromatography. Chromatograms (solid lines) of molecular size distribution of the APs obtained by the classical method and the AMSs of Tg7 and Tg9. The horizontal dotted lines represent the baseline of each chromatogram, and the vertical dashed ones divide regions: I, II and III. The numbers represent the peaks corresponding to region I.

Within region I, peaks 1 and 2, with approximate average MW of ~690 kDa and ~296 kDa, respectively, may include protein aggregates and the native quaternary structure of glycinin. Peak 3 (~120 kDa MW) may correspond to β-conglycinin (native and denatured) and denatured glycinin. Peak 4 (~72 kDa MW) may include subunits of β-conglycinin (α, α′, and β) and acid subunits of glycinin. On the other hand, peak 5 (~30 kDa MW) may comprise the basic subunits of glycinin, lectin, and the Kunitz-type trypsin inhibitor. The Bowman-Birk type trypsin inhibitor was in peak 6 (~13 kDa MW). The soybean's principal antinutrients are lectin, the Kunitz, and Bowman-Birk trypsin inhibitors, all being the extraction's target molecules. Finally, the peak 7 (~4 kDa MW) may contain a peptide of high commercial value and currently growing interest, lunasin (Seber et al., 2012; Serra et al., 2016; Singh et al., 2017). We fractionated both peaks 5 and 6 prior to analysis by mass spectrometry (MALDI-TOF/TOF), thus confirming the presence of the lectin and the trypsin inhibitors.

Table A4 (see Annex) shows the areas calculated for each peak in region I and the total area for regions II and III. Neither the micellar extraction with Tg9 nor the classical method extracted protein aggregates or glycinin, while the Tg7 extracted them. For peak 3, the AP Tg9 differed from the others showing the lowest value, while for peak 4, it only differed from the AP Tg7. Notably, the Tg7 system exhibited the highest leaching ability when the first four peaks were analyzed globally. Concerning antinutritional factors, both the classic method and the AP Tg7 showed to be the best ones to extract Kunitz trypsin inhibitors and lectins. At the same time, the AP Tg9 exhibited the best performance in removing the Bowman-Birk trypsin inhibitor.

Additionally, it is worth mentioning that Tg9 evidenced a marked extractive capability for peak 7, which could contain the lunasin. This is a remarkable feature given the high value that this peptide has acquired for its proven beneficial properties for health, namely antioxidant, anti-inflammatory, and chemopreventive (de Mejia et al., 2021; Dong et al., 1995; French et al., 2004; Hao et al., 2020; Pivato et al., 2012; Zhao, Chen, Xue, & Lee, 2008). Finally, from inspection of regions II and III, the AP Tg9 presented the highest content of small peptides, free amino acids, and small molecules that absorb at 280 nm relative to the classic and Tg7 AP.

3.2.2. Extraction and purification of isoflavones

The potential ability to extract and isolate isoflavones of high commercial value is one of the proposed methodology's attractive properties. Table 5 reports isoflavone amounts leached by each system, determined by HPLC. The AMS differed markedly from the control (classical process), exhibiting a higher extractive ability of total isoflavone content. A more detailed analysis showed that the AMS Tg7 stood out from the classical method when extracting the glycosylated forms

Table 5
Comparison of the amount of isoflavones extracted by the different methodologies.

Isoflavone			Methodology		
			Micellar extraction		Classic extraction
			Tg7	Tg9	
Aglycones [a]	Daidzein	Amount [b]	62.0 ± 3.6 [a, b]	61.1 ± 1,4 [a]	70.0 ± 3.1 [b]
		Kd [c]	23.8 ± 2.5	6.1 ± 0,6 [a]	NA
		Pr [d]	85.8 ± 3.1	57.6 ± 2.4	NA
	Genistein	Amount [b]	40.2 ± 2.0 [a]	30.3 ± 1.8 [a]	35.0 ± 2.4 [a]
		Kd [c]	14.3 ± 2.7 [a]	14.2 ± 2.8 [a]	NA
		Pr [d]	78.7 ± 6.5 [a]	78.2 ± 7.0 [a]	NA
Glycones [a]	Daidzin	Amount [b]	490.1 ± 20.3 [a]	513.9 ± 22.6 [a]	183.0 ± 15.8
		Kd [c]	4.2 ± 0.2	6.0 ± 0.3	NA
		Pr [d]	49.3 ± 1.1	57.2 ± 1.3	NA
	Genistin	Amount [b]	770.4 ± 21.4	461.2 ± 4.3 [a]	418.7 ± 15.3 [a]
		Kd [c]	7.4 ± 0.4	11.2 ± 1.1	NA
		Pr [d]	63.0 ± 1.3	71.4 ± 2.0	NA
Total [a]		Amount [b]	1362.8 ± 47.2	1066.5 ± 30.1	706.7 ± 36.7
		Kd [c]	6.4 ± 0.4	8.0 ± 0.7	NA
		Pr [d]	59.5 ± 1.5 [a]	64.0 ± 1.9 [a]	NA

[a, b] Values from the same row that share a superscript do not present statistical differences, given the analysis of their variances (ANOVA) with Tukey's posthoc test for a significance level of 0.05.
[a] Values are expressed as mean ± SD.
[b] Amount in μg (g of soybean flour)$^{-1}$.
[c] Distribution coefficient (Kd).
[d] Phase rich in micelles recovery performance (Pr) expressed in %.

(daidzin and genistin). However, for the aglycones, those two systems were not different. On the other hand, Tg9 highly exceeded the control at extracting daidzin. However, it led to achieving similar or even lower genistein and daidzein extraction, respectively, probably due to its more hydrophilic nature. Table 5 also reveals that all the extracts have different compositions of isoflavones, with the glycosylated forms being the predominant ones in all cases. These results agree with the higher content of β-glycosides concerning the aglycones in soybean and derivatives. For example, it is well known that the aglycones of soybean flour are approximately 2–3% of the total isoflavone content, while the unconjugated β-glycosides represent 34% of the total isoflavone (Andrade et al., 2016). Table 5 also includes the distribution coefficients (Kd) of isoflavones between the micelle-rich/-poor phases (MP and AP). All the Kd measured were ≫1, thus confirming a marked isoflavones preference for the MP. Table 5 also shows the phase recovery performance at the micellar phase (Pr). The calculation of this efficiency parameter needed the phase volumes in each AMS. The mean MP volumes of the Tg7 and Tg9 systems were 10.3 and 9.1 mL, respectively, while their corresponding mean AP volumes were 44.7 and 40.9 mL. The differences in the total volumes recovered were due to the liquid retained by the solid in each case. Note that the reduced volume of MP regarding that of AP (four times lower) and Kd values higher than 1 results in increased isoflavone concentration in the MP. On the other hand, the high found Pr values demonstrate the remarkable effectiveness of AMS at recovering the isoflavones in the MP. Considering the extractive and concentrating properties, the AMS Tg7 was the best system to recover all the isoflavones. It allowed not only to extract more isoflavones than the traditional method but also to achieve four to five times higher concentrations. This finding is technologically promising since it involves a considerable reduction of volumes to be handled, thus leading to industrially advantageous processes.

When analyzing our results and those corresponding to other methodologies (Table 6), we found that the amounts of aglycones recovered with the proposed processes are similar or superior to most of them. The extraction with 70% ethanol achieved the highest amount of genistein and daidzein, but the extracted matrix was another soy derivative, such as okara (Jankowiak et al., 2014). When comparing the yields from ethanol and pure water extractions, water alone cannot extract the less hydrophilic isoflavones. However, adding surfactants can revert that (Cao et al., 2012). It is also important to note that direct micelles improve extractive performance. AMS here proposed (Tg7/Tg9) and Triton X-114 AMS (Cordisco et al., 2016) extracted more isoflavones (aglycones and β-glycosides) than those obtained by reverse micelles (Zhao et al., 2010). That suggests that the use of direct micelles improves extractive performance. When MP concentrates the target compounds, the surfactant presence may compromise the applicability of the final product. To solve this problem, in previous work, our research group applied a second extractive step, i.e., a back extraction, to make isoflavones displace from the AM to the AP (Cordisco et al., 2016). It is worth mentioning that the decision to include this step in the process will depend on the final use of isoflavones, i.e., as cosmetics or food ingredients.

3.2.3. *Extraction of raffinose family oligosaccharides*

Soy carbohydrates are largely undesirable due to their low digestibility. In this regard, the process evaluated here could extract oligosaccharides belonging to the raffinose family. Table 7 shows that the amount of leached oligosaccharide by the AMS was 48–53% higher than that of the traditional methodology. So, both AMS Tg7 and AMS Tg9 had a better extracting performance, i.e., higher detoxifying capacity. According to the Kd values lower than 1, those oligosaccharides slightly prefer the AP, probably due to their hydrophilic nature. This finding, together with the larger volumes of the AP (3.3–4.5 times the MP volume), are responsible for the high obtained Pr, close to 85% for both surfactants.

Considering that it is also possible to separate most of the raffinose family oligosaccharides from the isoflavones by simply decanting the phases, the proposed methodology constitutes a better alternative than those currently used. Besides, soy galactooligosaccharides represent an alternative and economical carbon source for microbial conversion to value-added products such as succinic acid and ethanol (Thakker et al., 2014). Consequently, the applied AMS-based extraction allows obtaining a by-product for further uses in bio-conversion processes.

4. Conclusions

This study, aimed to perform a novel AMS-based extracting methodology, was driven by the following research questions: Are SPC from the traditional and proposed methodology of comparable quality? -What about their nutritional and bioactive properties? -Compared to the classical methodology, how is the AMS performing in extracting soluble proteins, antinutrients, and isoflavones? -Is it possible to recover these components separately from the extracts to valorize them? In light of all presented results, it is possible to answer all the questions and conclude:

-The AMS-based extractive methodology produced SPC noticeably depleted in trypsin inhibitor content (TIA loss of 90%) with total protein close to 60% and variable protein soluble amounts ranging from 19% to 34%, these parameters being similar to those corresponding to SPC from classical methods. The secondary structure content, determined as a fingerprint of the obtained product, showed either alpha helix (SPC Tg7) or random coils (SPC Tg9) increases concerning SPC control (SPCa). This finding might affect their functional properties (emulsifying, solubility, etc.) and open potential new applications. Additionally, remarkable antioxidant and antihypertensive activities, other than high soluble protein content, were detected in peptides released during *in vitro* digestibility assays, thus confirming the high quality of the obtained product.

-The AMS-based methodology also exhibited a markedly enhanced

Table 6
Soy isoflavones extracted with different methodologies.

Methodology	Source	Amount [a]				Reference
		Daidzein	Genistein	Daidzin	Genistin	
AMS Tg7	Flour	62.00 ± 3.60 [a]	40.20 ± 2.00 [a]	490 ± 20 [a]	770.40 ± 21.40 [a]	**
AMS Tg9	Flour	61.10 ± 1.40 [a]	30.30 ± 1.80 [b, c]	514 ± 23 [a]	461.20 ± 4.30 [b]	**
AMS Triton X-114	Flour	142.60 ± 2.60	69.60 ± 0.20	350 ± 8 [b]	736.50 ± 3.30 [a]	Cordisco et al. (2016)
Water/acetone/ethanol	Flour	NA	38.00 ± 0.01 [a]	382 ± 12 [b]	467.00 ± 8.00 [b]	Andrade et al. (2016)
Acetonitrile 80%	Bean	36.60 ± 2.00 [b]	36.30 ± 0.40 [a, b]	670 ± 18 [c]	644.70 ± 22.40	Lee et al. (2015)
Methanol 80%	Bean	22.10 ± 2.50 [c]	26.40 ± 1.30 [c]	631 ± 15 [c]	587.60 ± 2.20	Lee et al. (2015)
Water	Okara	22.00 ± 10.00 [c]	3.00 ± 4.00 [d]	108 ± 22	71.00 ± 16.00 [c]	Jankowiak et al. (2014)
Ethanol 70%	Okara	173.00 ± 8.00	194.00 ± 14.00 *	160 ± 7 [d]	171.00 ± 7.00	Jankowiak et al. (2014)
Reverse AMS Triton X-100	Flour	NA	79.03 ± 4.31	168 ± 5 [d]	118.24 ± 4.98	Zhao et al. (2010)
Supercritical CO_2	Flour	30.93 ± 0.04 [b, c]	1.71 ± 0.01 [d]	–	53.64 ± 0.06 [c]	Rostagno et al. (2002)

[a-d] Values from the same column that share a superscript do not present statistical differences, given the analysis of their variances (ANOVA) with Tukey's posthoc test for a significance level of 0.05.
* Values not included in the analysis due to being out of homoscedasticity (Bartlett's test).
** Values corresponding to the present work.
[a] Amount of isoflavones expressed as mean ± SD, in μg (g of soybean flour) $^{-1}$.

Table 7
Comparison of the amount of raffinose family oligosaccharides extracted by the different methodologies.

Methodology		Raffinose family oligosaccharides [a]		
		Amount [b]	Kd [c]	Pr [d]
Micellar extraction	Tg7	17.9 ± 1.0 [a]	0.76 ± 0.05 [a]	85.1 ± 1.3 [a]
	Tg9	18.5 ± 1.3 [a]	0.75 ± 0.02 [a]	85.7 ± 0.9 [a]
Classic extraction		12.1 ± 0.8	NA	NA

[a] Values from the same column that share a superscript do not present statistical differences among themselves, given the analysis of their variances (ANOVA) with Tukey's posthoc test for a significance level of 0.05.
[a] Values are expressed as mean ± SD.
[b] Amount in RU (g of soybean flour) $^{-1}$.
[c] Distribution coefficient (Kd).
[d] Aqueous phase recovery performance (Pr) expressed in %.

extractive performance of raffinose family oligosaccharides (~50% higher than the traditional one), thus resulting in a successful soybean flour detoxifying strategy. Due to its non-destructive character, it allowed recovering raffinose family oligosaccharides and other valuable by-products, such as Kunitz and Bowman-Birk trypsin inhibitors, lectins, and lunasin, potentially applicable in research and fermentation fields. Additionally, these compounds, primarily recovered in the aqueous phase of AMS, were separated from isoflavones (isolated and concentrated in the micelle phase). Regarding this issue, AMS demonstrated improved efficiency in extracting isoflavones, thus exhibiting both selectivity for aglycone/glycone isoflavone forms and high yields, e.g., almost twice as large as that of the traditional method and even higher than those obtained with organic solvents such as methanol. These advantages make AMS a powerful tool for the food and cosmetic industries.

Finally, it is possible to affirm that the proposed AMS-based extraction constitutes a sustainable, simple, and scalable alternative that deserves consideration among the new technologies for processing plant derivatives and obtaining more valuable products and by-products.

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CRediT author statement

Ezequiel R. Coscueta: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Luciana Pellegrini Malpiedi:** Methodology, Formal analysis, Writing - Review & Editing. **Maria Manuela Pintado:** Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Bibiana B. Nerli:** Conceptualization, Resources, Writing - Review & Editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Annex.

Table A1
Analysis of variance for fitted general linear models.

Response	Source	SS[a]	DF[b]	MS[c]	F	P	R^2
Ps	Model	687	24	28.604	228	0.000	0.996
	Residue	2510	20	0.126			
	Total	689	44				

(*continued on next page*)

Table A1 (*continued*)

Response	Source	SS[a]	DF[b]	MS[c]	F	P	R^2
ORAC	Model	3557270	24	148219	276	0.000	0.997
	Residue	10759	20	538			
	Total	3568030	44				
iACE	Model	7580240	24	315843	251	0.000	0.997
	Residue	25209	20	1260			
	Total	7605440	44				

P < 0,05 is considered significant.
[a] SS, sum of squares.
[b] DF, degrees of freedom.
[c] MS, mean square.

Table A2
Multiple comparisons for each response by T.

T	Least squares mean [a]		
	Ps [b]	ORAC [c]	iACE [d]
T1	2.59 ± 0.09	51 ± 6	1015 ± 9
T2	9.63 ± 0.09	460 ± 6	283 ± 9
T3	8.37 ± 0.09	577 ± 6	57 ± 9

[a] Values are expressed on dry basis, as mean ± SEM calculated by least squares.
[b] Ps in mg (g of product) $^{-1}$.
[c] ORAC in μmol TE (g of product) $^{-1}$.
[d] iACE as IC_{50}, μg mL^{-1}. All the values in the same column present statistical differences among themselves, given the analysis of their variances (ANOVA) with Tukey's posthoc test for a significance level of 0.05.

Table A3
Multiple comparisons for each response by product.

Product	Least squares mean [a]		
	Ps [b]	ORAC [c]	iACE [d]
Flour	6.3 ± 0.3 [a]	473 ± 13 [a]	452 ± 12 [a, b]
SPCa	7.5 ± 0.3 [a]	326 ± 13	428 ± 12 [a]
SPC Tg7	4.8 ± 0.3	178 ± 13	489 ± 12 [b]
SPC Tg9	6.9 ± 0.3 [a]	534 ± 13 [a]	431 ± 12 [a]

[a, b] Values from the same column that share a superscript do not present statistical differences among themselves, given the analysis of their variances (ANOVA) with Tukey's posthoc test for a significance level of 0.05.
[a] Values are expressed on a dry basis, as mean ± SEM calculated by least squares.
[b] Ps in mg (g of product) $^{-1}$.
[c] ORAC in μmol TE (g of product) $^{-1}$.
[d] iACE as IC_{50}, μg mL^{-1}.

Table A4
Peak areas belonging to the region I and total areas of regions II and III.

Region	Peak	Area (mL*mAU) [a]		
		Tg7 AP	Tg9 AP	Classic AP
I	1	1.6 ± 0.1	NA	NA
	2	2.5 ± 0.2	NA	NA
	3	7.3 ± 0.5 [a]	5.4 ± 0.5	7.3 ± 0.6 [a]
	4	4.7 ± 0.3 [a]	4.5 ± 0.3 [a]	4.8 ± 0.4 [a]
	5	11.2 ± 0.8 [a]	7.3 ± 0.5	14.4 ± 1.0 [a]
	6	4.0 ± 0.2	5.8 ± 0.5	3.1 ± 0.2
	7	2.4 ± 0.1	5.1 ± 0.3	1.1 ± 0.1
II		49.6 ± 4.2 [a]	77.1 ± 3.9	49.2 ± 3.2 [a]
III		66.3 ± 4.0	94.3 ± 7.1	51.3 ± 2.3

[a, b] Values from the same row that share a superscript do not present statistical differences, given the analysis of their variances (ANOVA) with Tukey's posthoc test for a significance level of 0.05.
[a] Values are expressed as mean ± SD.

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