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Aqueous micellar two-phase system as an alternative method to selectively remove soy antinutritional factors

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Abstract

In this work, different antinutritional factors (trypsin inhibitors, isoflavones and raffinose family oligosaccharides) were selectively removed from soy flour by using aqueous micellar two-phase systems (AMTPS). The effects of independent variables including temperature (30‑60 °C), time (10‑40 min) and solid to liquid ratio (0.025‑0.050 g/L) on the extraction of each antinutritional factor were analyzed using a full factorial design. As general tendency, temperature and time were the most significant parameters ($p < 0.05$). The best condition for the selective recovery (97% of isoflavones at top phase, and more than 50 % of the rest of ANFs at bottom phase) were 5 g/L of Genapol X-080, 0.2 mol/L of sodium citrate pH 5.00, 30 °C, 40 min and 0.050 g/L. Besides, *in vitro* gastrointestinal digestions assays demonstrated that the treated soy flour improved its protein digestibility. The findings of this work represent the introduction of a novel methodology to selectively remove soy antinutritional factors.

Keywords: trypsin inhibitor; isoflavone; surfactant; selective partitioning.

Abbreviations used: AMTPS, aqueous micellar two-phase system; ANFs, antinutritional factors; BAPNA, α-N-benzoyl-DL-arginine-p-nitroanilide; CI, confidence interval; D, deactivated soy flour; GX, Genapol X-080; IF, isoflavones; IGD, *in vitro* gastrointestinal digestion; K_r, partition coefficient; ND, non deactivated soy flour; RA, relative units; RFOs, raffinose family oligosaccharides; TI trypsin inhibitors; TIU, trypsin inhibitors units; S_T, selectivity at top phase.

1. Introduction

At present, soybeans (*Glycine max (L) Merril*) represent one of the most important sources of nutritional proteins (Yu, Yuan, Fu, & Zhu, 2016). It is estimated that 60% of total processed food includes ingredients derived from soy (Kumar & Mulimani, 2010). Nevertheless, a soy-based diet can present some disadvantages due to the presence of certain components known as antinutritional factors (ANFs) (Becker-Ritt, Mulinari, Vasconcelos, & Carlini, 2004). These compounds, such as oligosaccharides, phytoestrogens and protease inhibitors, can negatively affect animals and humans health when consumed frequently (Vagadia, Vanga, & Raghavan, 2017; Yu et al., 2016). Trypsin inhibitors (TI) are considered as the major soy ANFs (Sousa et al., 2015). High levels of these proteins could inhibit digestive proteases, thus affecting protein digestibility, and causing certain diseases, such as pancreatic hypertrophy (Vagadia et al., 2017). Examples of less harmful ANFs are raffinose and stachyose (RFOs), which have been associated with nutrient digestibility reduction, flatulence and abdominal discomfort (Dersjant-Li & Peisker, 2010; Kumar & Mulimani, 2010). Additionally, soy isoflavones (IF), also known as phytoestrogens, can exhibit undesirable physiological effects on human metabolism, principally at childhood (Portman, Navarro, Bruce, & Lampe, 2016).

A plethora of processing methods, such as soaking, cooking, toasting and chemical treatments, have already been explored in order to inactive/reduce soy ANFs (Akbarian et al., 2014; Dersjant-Li & Peisker, 2010). Heating seems to be the most suitable processing method to reduce TI activity. Trypsin inhibitory effect have been reduced up to 85% (i.e. remaing only 15% of initial TI activity) using different heating

protocols such as oven dry heat and salt-bed roasting (Coscueta et al., 2017). However, extreme working conditions such as high temperatures can compromise the availability of other components. Thus, alternative methodologies, such as radiation and oxidation, are being evaluated (Vagadia et al., 2017). With regard to RFOs, solvent extraction and enzymatic degradation are the most common means to eliminate them (Dersjant-Li & Peisker, 2010; Kumar & Mulimani, 2010). Respecting to IF reduction, solvent extractions in aqueous and organic media represent the most used methodologies (Jankowiak, Kantzas, Boom, & Van Der Goot, 2014; Sun, Li, & Wang, 2011).

Although most of the previously mentioned ANFs are known for their adverse effects, it is true that many of them also have beneficial effects on health (Thompson, 1993). For example, TI is known to be involved in many biological functions, such as blood coagulation, platelet aggregation, anti-carcinogenesis and granulo-cytopoietic activity (da Silva Bezerra et al., 2015). Besides, IF consumption has been associated with reduced menopause symptoms, reduced incidences of hyperglycemia and improved bone quality (Ahn & Park, 2017; Cordisco, Haidar, Coscueta, Nerli, & Malpiedi, 2016). Thereby, the development of strategies for the selective and non-destructive removal of soy ANFs represents a research area of great interest.

Aqueous micellar two-phase systems (AMTPS) represent an attractive tool to selectively extract soy ANFs. This methodology, which is based on solid-liquid and liquid-liquid extraction, depends on the ability of some surfactants to form two immiscible aqueous phases, a micelle-rich phase and a micelle-poor phase, over

certain temperature defined as cloud point (Gu & Galera-Gómez, 1995). Thereby, the physicochemical differences between both phases allow the separation of biomolecules present in a mixture (Bordier, 1981). At present, this technique has gained relevance as an eco-friendly methodology to purify a wide variety of molecules such as enzymes, antibodies, antibiotics and polyphenols (Sharma, Kori, & Parmar, 2015).

Preliminary works carried out by our research group have already demonstrated that IF can be successfully purified at the micelle-rich phase of AMTPSs of Triton X-114 and sodium tartrate (Cordisco et al., 2016). Under optimal working conditions, IF were purified with a recovery percentage of 93 and a purification factor of almost 10. However, other ANFs have not been analyzed.

Thus, in this context, the main aim of this work was to evaluate for the first time the feasibility of using AMTPS to selectively extract different antinutritional factors (raffinose, trypsin inhibitor and isoflavones) from soy flour. Genapol X-080 was selected as micelle-forming surfactant since its use was approved by the Food and Drug Administration (FDA). Protein availability of the treated soy flour was also evaluated.

2. Materials and Methods

2.1. Materials

Defatted soybean flour, both deactivated (D, treated with oven dry heat at 80 °C for 1 h) and non-deactivated (ND) samples, were obtained from the food processing

company Molinos Río de la Plata SA (San Lorenzo, Argentina). Trypsin (bovine), pepsin (from porcine gastric mucosa), pancreatin (from porcine pancreas), bile (from bovine bile), α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and Tris buffer were purchased from Sigma-Aldrich (St. Louis, USA) and used without further purification. The non-ionic surfactant polyethylene glycol monoalkyl ether (Genapol) X-080 (GX), citric acid and bicinchoninic acid (BCA) were supplied by Sigma–Aldrich (St. Louis, USA) and used as received. All the other reagents were of analytical grade and used without further purification.

2.2. Experimental design

The extraction of soy ANFs was performed with the aid of a 2^3 –full factorial design with three repetitions at the central point (Table 1). Temperature (X_1, °C), time (X_2, min) and solid to liquid ratio (X_3, g/L) were the independent variables. The recovered amount of each ANFs constituted the analyzed responses. Selectivity (S) at top or bottom phases and partition coefficients (K_r) were also evaluated (equations described at section 2.8).

(Table 1- double columns fitting)

2.3. Liquid-liquid extraction assays

ANFs extraction with aqueous micellar two-phase systems was performed by using 50 g/L Genapol X-080 (GX) in sodium citrate (NaCit) 0.2 mol/L, pH 5.00. Notice that in this type of AMTPS the top phase is enriched in surfactant micelles while the

141 bottom phase presents scarce amount of these aggregates (Cordisco, Haidar, Goñi,
142 Nerli, & Malpiedi, 2015).
143 The preparation of the studied systems was carried out by weighing (analytical
144 balance Pioneer^{TM} Plus, Ohaus, Parsippany, USA) into graduated glass tubes each
145 system component: ND soy flour (0.100, 0.150 or 0.200 g, according to the run
146 number of Table 1), GX (0.250 g of pure surfactant) and sodium citrate buffer 0.2
147 mol/L, pH 5.00 (until reaching a final mass of 5.000 g). The prepared systems were
148 then mixed at 30 rpm for 1h at room temperature using a tube rotator apparatus
149 (Bioelec®, Santa Fe, Argentina). After that, the systems were incubated in a water
150 bath (Tecnodalvo, Santa Fe, Argentina) at the different conditions presented in
151 Table 1. At the end of the incubation step, both phases were conveniently separated
152 by centrifuging at 1,970 x g for 10 min (refrigerated benchtop centrifuge, Sigma
153 Laborzentrifugen 3-18 KS, Osterode, Germany) at the same temperature of
154 incubation. Finally, samples from top and bottom phases were taken for the
155 determination of partition coefficients and recoveries of ANFs (IT, IF and RFOs). The
156 treated soybean flour, which was totally recovered at the bottom of the test tube,
157 was dried and stored for further analysis.
158
159 *2.4. ANFs extraction with reference methods*
160 *2.4.1. Trypsin inhibitors*
161 The extraction of TI was performed by following the AOCS official method (AOCS,
162 2009; Coscueta et al., 2017). The obtained supernatant was used for determination
163 of TI activity.

164

165 *2.4.2. Isoflavones*

166 IF extraction was performed by suspending 1.000 g of ND soy flour into 50.0 mL of
167 extracting solution (pure methanol/water in 4:1 mL:mL). The suspension was
168 homogenized at 30 rpm (Age magnetic stirrer, Velp Científica, Usmate, Italy) for 3 h
169 at 35 ± 0.1 °C (thermostated incubator, San Jor, San Andrés, Argentina). After
170 centrifuging at 2,460 x g for 15 min at room temperature (refrigerated benchtop
171 centrifuge, Sigma Laborzentrifugen 3-18 KS, Osterode, Germany), supernatant was
172 used for isoflavone quantification.

173

174 *2.4.3. Raffinose family oligosaccharides*

175 The procedure consisted in adding 1.000 g of ND soy flour into 50.0 mL of
176 ethanol/water mixture (Dixit, Kumar, Rani, Manjaya, & Bhatnagar, 2011). The
177 resulting suspension was homogenized at 30 rpm (tube rotator, Bioelec®, Santa Fe,
178 Argentina) for 4 h at 80 ± 0.1 °C (thermostated incubator, San Jor, San Andrés,
179 Argentina). A final centrifugation step for 10 min at 2,460 x g (refrigerated benchtop
180 centrifuge, Sigma Laborzentrifugen 3-18 KS, Osterode, Germany) allowed
181 separating the supernatant for the determination of RFOs.

182

183 *2.5. Quantification of trypsin inhibitor activity*

184 The presence of trypsin inhibitory activity was analyzed by using a recently
185 developed continuous method (Coscueta et al., 2017). Aliquots (250 µl) of diluted
186 phases were mixed with 10 µL of bovine trypsin (100 mg/L,) and 2,240 µL of 0.85

187 mmol/L of BAPNA prepared in Tris buffer 0.050 mol/L pH 8.20. Each TI
188 determination required of two conditions to be measured: control (trypsin activity in
189 presence of clean top or bottom phases) and sample (trypsin activity in the presence
190 of top or bottom phases after soy flour partitioning). Immediately after mixing, the
191 Absorbance at 410 nm was monitored for 3 min recording measurements at time
192 intervals of 10 s. The reaction rate (Abs units/min) was obtained from the slope (m)
193 of Absorbance vs. time plot at both conditions ($m_{control}$, m_{sample}). Absorbance
194 measurements were carried out at room temperature in a JASCO V-550 (UV-VIS
195 spectrophotometer, Helmholtz Zentrum, Berlin, Germany) by using a thermostated
196 cell of 1 cm pathlength. The results were expressed in trypsin inhibitor units (TIU) in
197 order to compare them with those provided by the bibliography (AOCS, 2009). The
198 calculation was made by the following expression (eq. 1):

199 $$TIU/mL = \frac{100 \text{ x } (m_{control} - m_{sample}) \text{ x } 2.50 \text{ x } D}{0.25} \quad (1)$$

200

201 where 100 is the factor to convert 0.01 u. Abs in TIU units; $m_{control}$- m_{sample}, the
202 difference between the slopes of progress curves in absence and presence of TI
203 respectively; *D*, the dilution factor of each phase, calculated as the ratio between the
204 final volume and the aliquot taken to dilute the extract; 0.25, the aliquot (mL) used in
205 the current assay and 2.50, the final reaction volume (mL) in the cuvette.

206

207 2.6. *Estimation of total isoflavones*

Total isoflavones were estimated by using the aluminum chloride-based colorimetric method (Cordisco et al., 2016). A commercial supplement containing a natural mixture of soy isoflavones (Sojar S.A., Rosario, Argentina) was conveniently dissolved in methanol to make a calibration curve (0 to 400 mg/L). Absorbance measurements were carried out in a spectrophotometer (UV-VIS spectrophotometer, Helmholtz Zentrum, Berlin, Germany) by using a thermostated cell of 1 cm pathlength. The obtained data were expressed in mg of IF per gram of dried soy flour (see section 2.8).

2.7. *Estimation of raffinose family oligosaccharides*

Raffinose determination was performed by using an UV-test kit (Cat. No.: E 0428 167, R-Biopharm AG, Darmstadt, Germany). This method is based on the enzymatic hydrolysis of specific oligosaccharides, such as raffinose and stachyose, in presence of NAD^+ at pH 4.50 (eq. 2):

$$RFOs + H_2O \xrightarrow{\alpha\text{–galactosidase}} D\text{-}galactose + sucrose$$
$$D\text{-}galactose + NAD^+ \xrightarrow{\text{Gal–DH}} D\text{-}galacturonic\ acid + NADH + H^+ \quad (2)$$

At the end of the reaction, NADH concentration was determined by reading Absorbance at 340 nm (UV-VIS spectrophotometer, Helmholtz Zentrum, Berlin, Germany) by using a thermostated cell of 1 cm pathlength. Then, RFOs concentration at top or bottom phase was calculated as follows (eq. 3):

$$[ROFs]\ (g/L) = \frac{17.5}{\varepsilon}\, x\, \left[(A_2 - A_1)_{sample} - (A_2 - A_1)_{blanck} \right] \quad (3)$$

where ε represents NADH extinction coefficient and (A_2-A_1) represents the difference between absorbance values before (A_1) and after (A_2) enzymatic reactions. This difference was calculated for clean phases (blank) and for top and bottom phases after soy flour partitioning (sample). The amount of RFOs was also expressed as mg/g of dried soy flour (section 2.8).

2.8. *Determination of extractive performances*

ANFs partition coefficients were calculated according to the following equation:

$$K_r = \frac{[ANF]_T}{[ANF]_B} \quad (4)$$

where $[ANFs]_T$ and $[ANFs]_B$ represent antinutritional factor (TI, IF or RFOs) concentration at top (T) or bottom phase (B), respectively. TI concentration was expressed as trypsin inhibitor units (TIU)/mL, as described in section 2.5.

IF and RFOs content per gram of dried soy flour was calculated as follow (eq. 5):

$$ANFs_{T/B} = \frac{[ANFs]_{T/B} \times V_{T/B}}{m_{soy\ flour}} \quad (5)$$

where ANFs represents IF or RFOs, $V_{T/B}$ represents the volume of top or bottom phase, respectively, and $m_{soy\ flour}$ indicate the mass of ND soy flour that was used in each experiment (see section 2.3). The amount of TI was expresses as TIU/mg of dried soy flour.

The selectivity of ANFS extraction (S_T) was determined at top phases according to equation 6:

$$S_T = \frac{TI_T + RFOs_T}{IF_T} \quad (6)$$

where TI_T RFO_T and IF_T, represent the amount of each ANF at top phase.

2.9. In vitro gastrointestinal digestion

This assay was carried out for three different soy flour samples: non-deactivated (ND), deactivated with dry heat (D), and ND flour recovered after a liquid-liquid extraction, using the best separative conditions of Table 1 (AMTPS).

AMTPS sample was obtained from a two-phase system of 1 L of final volume (maintaining constant all the experimental conditions). Each sample (ND, D and AMTPS) was prepared as follows: 280 mg of dried soy flour was suspended into 7 mL of HCl solution (final pH of 2.00). The suspension was homogenized (Age magnetic stirrer, Velp Científica, Usmate, Italy) at 130 rpm for 20 min at 37 °C (thermostated incubator, San Jor, San Andrés, Argentina).

In vitro gastrointestinal digestion (IGD) was performed in two sequential steps:

A) Gastric digestion

Gastric digestion was initiated by adding 300 μL of gastric juice (25 g/L of pepsin, prepared in HCl 0.1 mol/L, pH 2.00) into the suspensions mentioned at section 2.9. Each sample was agitated at 130 rpm (tube rotator, Bioelec®, Santa Fe, Argentina) for 60 min at 37 °C (thermostated incubator, San Jor, San Andrés, Argentina). This

process was stopped by adding $NaHCO_3$ 0.1 mol/L until reaching a pH value of 6.50.

B) Intestinal digestion

To simulate intestinal digestion, pancreatin (2 g/L) and bile salts (12 g/L) were prepared in $NaHCO_3$ 0.1 mol/L (Laurent, Besançon, & Caporiccio, 2007). The experiment was initiated by adding de 1.5 mL of this pancreatic solution into the samples obtained from gastric digestion (section 2.9.1). This solution was then homogenized at 37 °C at 45 rpm (tube rotator, Bioelec®, Santa Fe, Argentina). After 90 min of incubation, the reaction was stopped by freezing at -30 °C. Finally, all samples were melted at room temperature, filtered through a 3 kDa membrane (Amicon® Ultra-4, Merck, Darmstadt, Germany) and stored at -20 °C for further analysis (IGD samples).

2.10. Analysis by gel filtration chromatography

Samples from IGD assays were also studied by gel filtration chromatography (FPLC system of AKTA pure 25 L, GE Healthcare Life Sciences, Uppsala, Sweden). The equipment configuration consisted of two high-performance pumps, a mixing chamber, a V9-IA motorized valve, a gel filtration column prepacked with Superdex® 200 10/300 GL connected with a Superdex Peptide 10/300 GL column (GE Healthcare Life Sciences, Uppsala, Sweden), and an UV U9-L detector. The column was operated at a flow rate of 0.5 mL/min with 0.025 mol/L phosphate buffer (pH 7.00) containing 0.15 mol/L NaCl and 0.2 g/L of NaN_3. Absorbance of the eluent was monitored at 280 nm. Standard proteins (Sigma-Aldrich, St. Louis, USA) with known

295 molecular weights (aldolase, 158 kDa; conalbumin, 75 kDa; ovoalbumin, 43 kDa;
296 carbonic anhydrase, 29 kDa; ribonuclease A, 13,7 kDa; aprotinin, 6.5 kDa) were
297 used to calibrate the system. The quantification of each peptide was carried out by
298 integration of the peak areas. Total peptide content was calculated by the sum of
299 individual peak areas and expressed as relative units per mg of dried soy flour
300 (RA^2/mg).
301
302 *2.11. Analysis of data*
303 The statistical analysis was performed with the aid of Statistic 10.0 Software
304 (StatSoft Inc., Tulsa, USA). Differences within means were determined using Least
305 Significant Difference (LSD) multiple comparison analysis. Differences at a *p*-value
306 <0.05 were considered significant.
307 The *in vitro* gastrointestinal digestion was assayed in duplicate. The mean values
308 were analyzed statistically by analysis of variance followed by the Tukey's post-hoc
309 test (Tukey, 1949). Separation of means was conducted by using the least
310 significant difference at the 5% level of probability.
311
312 **3. Results and discussion**
313 *3.1. ANFs partitioning by using aqueous micellar two-phase systems*
314 Partition coefficients of TI and raffinose family oligosaccharides (RFOs) are shown in
315 Table 2. Both ANFs presented a preferential affinity toward the micelle-poor bottom
316 phase (K_r<1). This behaviour could be attributable to their hydrophilic character,

leading to a higher solubility in the aqueous phase (Bordier, 1981; Duque Jaramillo et al., 2013).

Concerning IF, its concentration at the bottom phase was below the quantification limit at most of the evaluated conditions, thus its partition coefficient could not be calculated. This uneven partition towards the micelle-rich top phase agrees with other reported works (Cao et al., 2012; Cordisco et al., 2016; Zhao, Wei, Du, & Zhu, 2010), and it is attributable to a high affinity between IF and the hidrophobic tail of surfactants.

Taking into account the differential partitioning between IF and the rest of ANFs (TI and RFOs), the selection of extractive conditions was performed by prioritizing those AMTPSs that allow recovering IF at the top micelle-rich phase and TI and RFOs at the opposite phase.

(Table 2 - single column fitting)

3.2. Statistical analysis

Table 3 shows the recovered amount of each ANFs. The maximal amount of extracted TI was observed in run 3 (30.3 TIU/mg). Besides, 70% of total inhibitory activity was recovered at bottom phase, thus demonstrating a selective partitioning behaviour at this working condition. It is remarkable that total TIU obtained in this run represented a high percentage (84%) of the TIU value obtained thorough the reference method, described at section 2.4 (36.0 TIU/mg). These results

demonstrate that trypsin inhibitor activity can be drastically reduced by using AMTPS (more than 80%). This performance is comparable to those obtained from other conventional methodologies such as heat-induced inactivation and radiation (Chen, Xu, Zhang, Kong, & Hua, 2014; Coscueta et al., 2017; Vagadia et al., 2017).

(Table 3 - double columns fitting)

The significance and the magnitude of each variable on TI extraction are represented in the Pareto charts of Figure 1. The work temperature exerted the strongest effect on TI extraction. Its negative sign suggests that TI extraction with AMTPS can be maximized by working at lower temperatures. Solid to liquid ratio and time, also presented a significant effect on TI extraction, but showing a positive sign.

(Figure 1- double column fitting)

The maximum amount of extracted RFOs was 26 mg/g (run 11 of Table 3). This performance was comparable to that from reference method, described in section 2.4.3. (30 mg/g), and also similar to the that obtained from gamma irradiation (Dixit et al., 2011). The statistical analysis of total RFOs extraction is shown in Figure 2A.The strongest effect, with positive sign, was exerted by time. This behaviour suggests that the removal of these ANFs can be improved by using longer extractive times. With regard to RFOs recovery at bottom phase, solid to liquid ratio showed

363 the most significant effect, with negative sign. This phenomenon indicates that RFOs
364 may have reached their solubility limit at the micelle-poor phase.
365
366 **(Figure 2- double column fitting)**
367
368 Concerning to IF recovery at top phase, the highest yield (4.19 mg/g) was obtained
369 at the run 3 (Table 3). This value represented 97% of total IF recovered with the
370 reference method, described at section 2.4.2 (4.30 mg/g). This result is in
371 accordance with our previous work (Cordisco et al., 2016) that demonstrate the
372 feasibility of purifying IF by using micellar systems.
373
374 **(Figure 3- single column fitting)**
375
376 As shown in Figure 3, temperature exerted the strongest effect (with negative sign)
377 on IF recovery. Such behaviour is similar to that observed in previous work and can
378 be associated with a reduction in the micelle-rich phase volume (Cordisco et al.,
379 2016). Solid to liquid ratio and time has also affected significantly the response but
380 in a lower extent.
381 Apart from individual recoveries, the selectivity of the extractive process was also
382 analyzed (Table 2). The highest selectivity values between isoflavones and the rest
383 of ANFs are obtained at top phases of AMTPS belonging to runs 1 and 3. This
384 finding demonstrates the feasibility of recovering IF at the micelle–rich top phase
385 with little amount of other antinutritional factors (see Figure 4). More importantly, run

386 3 also presents the highest recovery of IF at top phase and the highest recovery of
387 TI at bottom phase (see Table 3). On the basis of these results, this AMTPS can be
388 considered as a suitable tool to extract different soy antinutritional factors in a
389 selective and non destructive manner.
390
391 **(Figure 4- Double columns fitting)**
392
393 *3.3. In vitro gastrointestinal digestion*
394 The nutritional value of soy flour mainly relies on its high protein content. Thus, this
395 assay was performed with the aim of evaluating soy proteins digestibility after
396 partitioning procedure. Liquid-liquid extraction was performed by following the
397 experimental conditions of run 3 (See Tables 1 and 3).
398 Figure 5 shows the molecular weight distribution of peptides obtained after *in vitro*
399 gastrointestinal digestion (IGD). Different chromatographic profiles were observed
400 for the distinct soy flour samples. This behaviour can be attributed to the lack of
401 specificity in protein hydrolysis, thus resulting in different peptides size distribution
402 (Capriotti et al., 2015).
403 **(Table 4 - single column fitting)**
404
405 Table 4 shows the statistical analysis of the integrated peak areas (see section
406 2.10). The highest peptide content ($p < 0.05$) was observed for the soy flour treated
407 with liquid-liquid extraction. This fact could be a consequence of different processes
408 such as the reduction of protease inhibitors, which result in a higher enzymatic

activity (Capriotti et al., 2015) or the increase in the exposure of inner amino acids, derived from the preferential interaction between hydrophobic patches of soy proteins and the surfactant (Malpiedi, Nerli, Abdalla, & Pessoa, 2014).

(Figure 5- single column fitting)

Conclusion

This work represents a pioneer study about the use of aqueous micellar two-phase systems as an alternative methodology to extract soy flour antinutritional factors. The best extractive condition allowed the extraction of 97% of total isoflavones in the top phase, while the rest of ANFs were recovered in the opposite phase. Notice that this selective extraction could facilitate later ANFs applications. Additionally, *in vitro* gastrointestinal digestion assays indicated that the treated soy flour improved its protein digestibility. This behaviour suggests that the proposed methodology preserves the biological source, maintaining, and even increasing, its economic value. These results allow us to conclude that AMTPS deserves being considered as a potential tool to selectively remove ANFs from soy flour. In our opinion, it is worth optimizing the extractive process and evaluating the use of AMTPS to remove ANFs from other nutritional soy sources.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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* These works were selected as reference of other methodologies to remove/reduce soy antinutritional factors. The performances obtained in our work were compared with that of this bibliography.

552 **Figure captions:**
553
554 **Figure 1:** Pareto charts for the effect of temperature (30 to 60 °C), time (10 to 40
555 min) and solid to liquid ratio (0.025 to 0.050 g/L) on the extraction of soy trypsin
556 inhibitor (TI) by using aqueous micellar two phase systems, prepared with 5 g/L of
557 Genapol X-080 and sodium citrate 0.2 mol/L, pH 5.00. Each variable and their
558 interactions are plotted in decreasing order and compared to the minimum
559 magnitude of a statistically significant factor with 95% of confidence (*p=0.05*),
560 represented by the vertical red line. A) Total TI (sum of top and bottoms yields). B)
561 TI obtained at bottom phase.
562
563 **Figure 2:** Pareto charts for the effect of temperature (30 to 60 °C), time (10 to 40
564 min) and solid to liquid ratio (0.025 to 0.050 g/L) on the extraction of soy raffinose
565 family oligosaccharides (RFOs) by using aqueous micellar two phase systems,
566 prepared with 5 g/L of Genapol X-080 and sodium citrate 0.2 mol/L, pH 5.00. Each
567 variable and their interactions are plotted in decreasing order and compared to the
568 minimum magnitude of a statistically significant factor with 95% of confidence
569 (*p=0.05*), represented by the vertical red line. A) Total RFOs (sum of top and
570 bottoms yields). B) RFOs obtained at top phase
571
572 **Figure 3:** Pareto charts for the effect of temperature (30 to 60 °C), time (10 to 40
573 min) and solid to liquid ratio (0.025 to 0.050 g/L) on the extraction of soy isoflavones
574 at the top phase of different aqueous micellar two phase systems, prepared with 5
575 g/L of Genapol X-080 and sodium citrate 0.2 mol/L, pH 5.00. Each variable and their
576 interactions are plotted in decreasing order and compared to the minimum
577 magnitude of a statistically significant factor with 95% of confidence (*p=0.05*),
578 represented by the vertical red line.
579
580 **Figure 4:** Schematic representation of the selective removal of soy antinutritional
581 factors by using aqueous micellar two-phase systems. Abbreviations: Genapol X-
582 080 (GX), isoflavones (IF), raffinose family oligosaccharides (RFOs), sodium citrate
583 (NaCit), trypsin inhibitor (TI).
584
585 **Figure 5:** Size exclusion chromatograms for different soy flour samples submitted to
586 *in vitro* gastrointestinal digestion assays. Solid curve, non deactivated soy flour;
587 dashed curve, soy flour deactivated with oven dry heat (80 °C for 1 h); dotted curve,
588 soy flour treated with liquid-liquid extraction with aqueous micellar two-phase system
589 (5 g/L of Genapol X-080, sodium citrate 0.2 mol/L, pH 5.00, 40 min of incubation at
590 30 °C and 0.050 g/L of solid to liquid ratio). Elution volumes of standard proteins:
591 aldolase: 10.11 mL; conalbumin: 0.91 mL; ovoalbumin: 11.37 mL; carbonic
592 anhydrase: 12.36 mL; ribonuclease A 13.71 mL; aprotinin: 15.48 mL.
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Table 1: Full factorial design 2^n (n: numbers of independent variables) for the study of soy flour antinutritional factors extraction by using aqueous micellar two-phase systems, prepared with 5 g/L of Genapol X-080 and 0.2 mol/L of sodium citrate pH 5.00. Independent variables: X_1= temperature (°C); X2= time (min); X_3= solid to liquid ratio (g/L).

Run	Coded and real independent variables					
	X_1	X_2	X_3	$X_{1\ (°C)}$	$X_{2\ (min)}$	$X_{3\ (g/L)}$
1	-1	-1	-1	30	10	0.050
2	+1	-1	-1	60	10	0.050
3	-1	+1	-1	30	40	0.050
4	+1	+1	-1	60	40	0.050
5	-1	-1	1	30	10	0.025
6	+1	-1	1	60	10	0.025
7	-1	+1	1	30	40	0.025
8	+1	+1	1	60	40	0.025
9*	0	0	0	45	25	0.033
10*	0	0	0	45	25	0.033
11*	0	0	0	45	25	0.033

* Central points.

Table 2: Effect of different liquid-liquid extraction conditions (temperature, 30 to 60 °C; time 10 to 40 min and solid to liquid ratio, 0.025 to 0.050 g/L) on antinutritional factor partition coefficients and on the selectivity of isoflavones extraction at top phase. Systems composition: 5 g/L of Genapol X-080 and 0.2 mol/L of sodium citrate pH 5.00. Abbreviations: partition coefficients (Kr), selectivity at top phase (ST), trypsin inhibitor (TI), raffinose family oligosaccharides (RFOs), least significant difference (LSD).

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Run	TI	RFOs	S_T*
	K_r *	K_r *	
1	0.06	0.67	0.24
2	1.03	0.80	0.12
3	0.25	0.78	0.19
4	1.09	0.73	0.10
5	0.68	0.51	0.13
6	1.59	0.98	0.07
7	0.38	0.78	0.11
8	0.91	0.87	0.03
9	0.37	0.67	0.13
10	0.36	0.80	0.11
11	0.33	0.80	0.11
LSD	0.03	0.21	0.02

* Data expressed as media of triplicate

Table 3: Dependent variables (responses) of the full factorial design accomplished with the aim to study soy antinutritional factors extraction by using aqueous micellar two-phase systems, prepared with 5 g/L of Genapol X-080 and 0.2 mol/L of sodium citrate pH 5.00. Independent variables: temperature, 30 to 60 °C; time 10 to 40 min and solid to liquid ratio, 0.025 to 0.050 g/L. Abbreviations: total trypsin inhibitor units (TIU_{Tot},), trypsin inhibitor units obtained at bottom phase (TIU_B), total raffinose family oligosaccharides($RFOs_{Tot}$), raffinose family oligosaccharides obtained at bottom phase ($RFOs_B$), isoflavones obtained at top phase (IF_T), least significant difference (LSD).

	Dependent variables (responses)				
Run	TIU_B (TIU/mg)*	TIU_{Tot} (TIU/mg)*	$RFOs_B$ (mg/g)*	$RFOs_{Tot}$ (mg/g)*	IF_T (mg/g)*
1	15.5	16.2	10.9	19	2.30
2	3.6	6.5	12.4	18	0.99
3	**21.2**	**30.3**	**8.9**	**22**	**4.19**
4	9.0	14.0	19.0	25	1.13
5	13.7	25.2	6.8	17	2.84
6	7.2	18.7	7.1	11	1.07
7	20.2	26.7	11.4	24	2.10
8	14.4	20.2	8.4	25	0.70
9	13.3	15.5	17.4	25	1.32
10	13.7	16.2	17.3	25	1.16
11	11.9	14.0	17.1	26	1.16
LSD	0.7	0.7	0.4	1	0.06

* Data expressed as media of triplicate, per gram of dry soy flour.

Table 4: Integrated peak areas (expressed as relative areas) obtained from the gel filtration chromatography applied over different soy flour samples previously submitted to in vitro gastrointestinal digestion. Abbreviations: Relative areas (RA^2), confidence interval for 95% of significance (CI).

Soy flour sample	$(RA^2/mg)^*$	CI
Non deactivated	1.3	0.2
Oven dry heat	1.7	0.2
Aqueous micellar two-phase systems	2.46**	0.09

* Data expressed as media of duplicate, per mg of dry soy flour.
**Values within the row differ significantly with Tukey's test ($p < 0.05$).

Figure 1

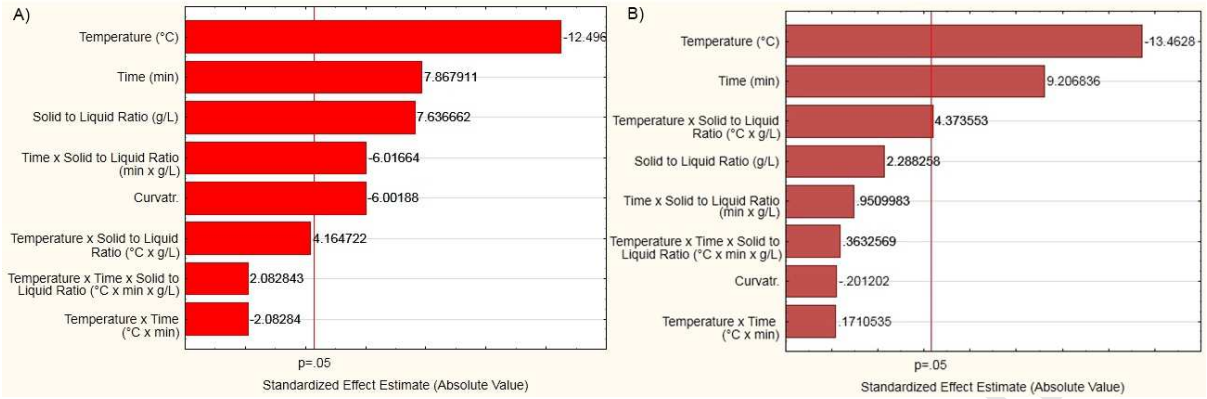


Figure 2

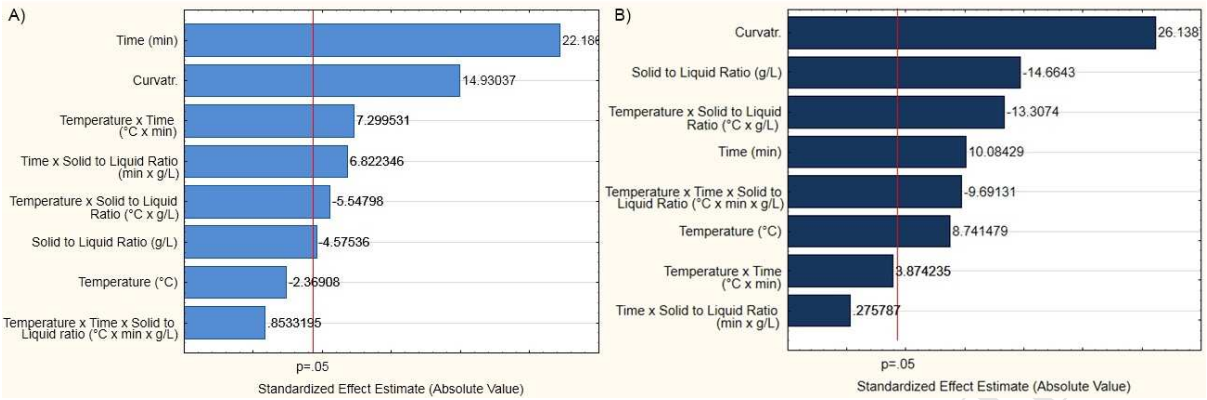


Figure 3

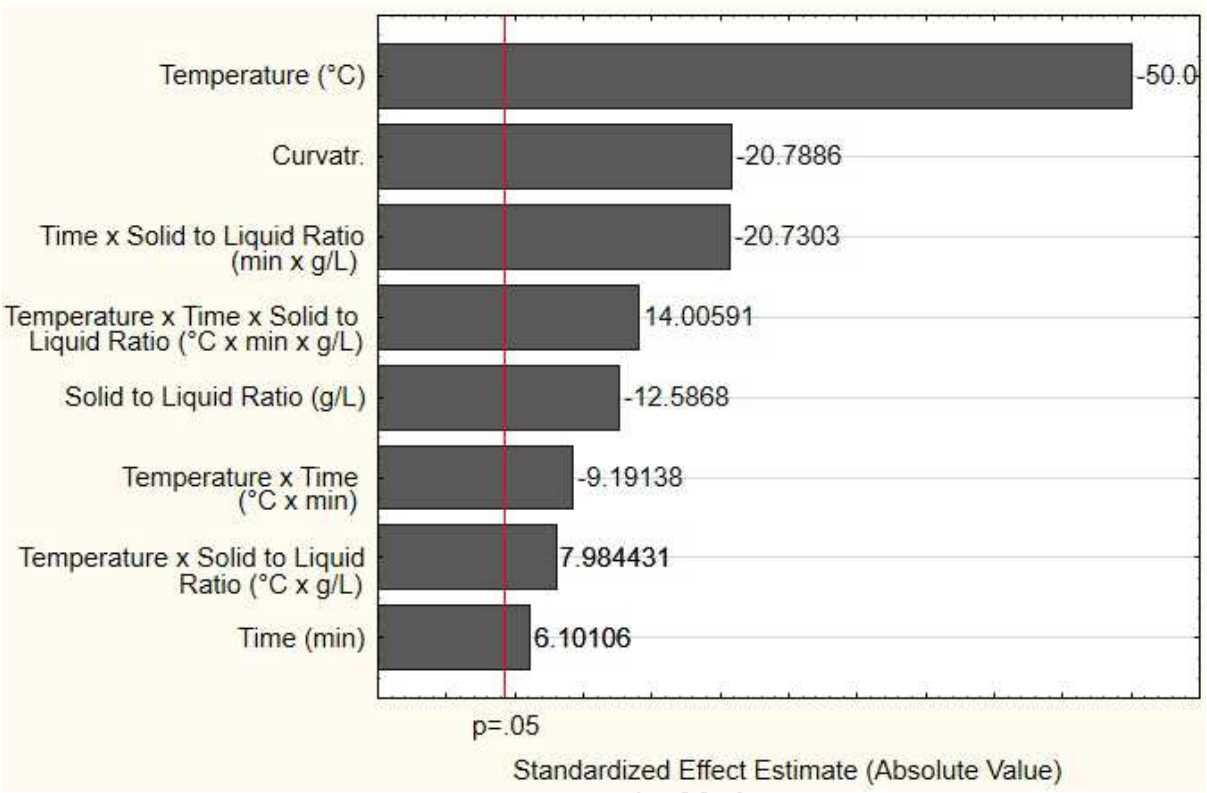


Figure 4

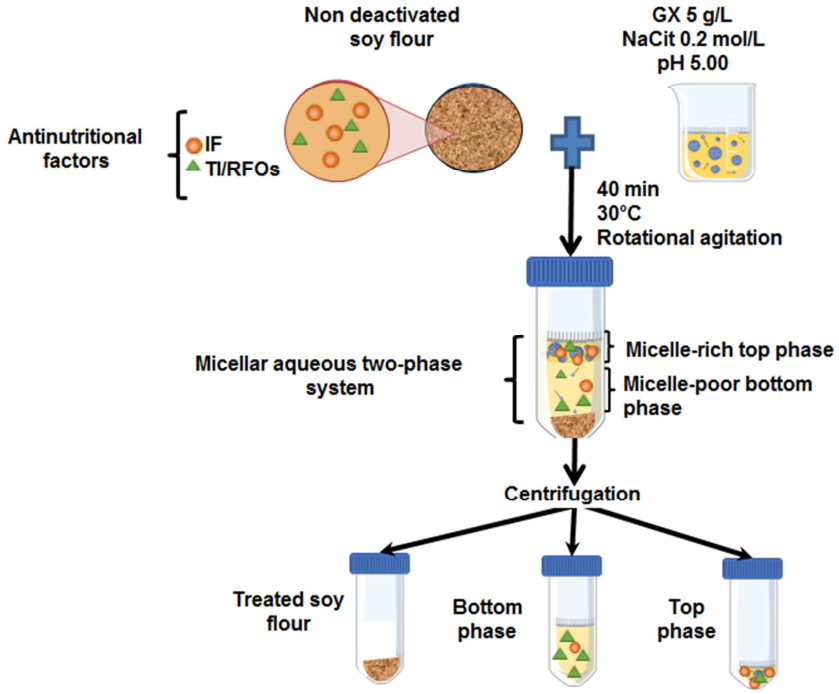
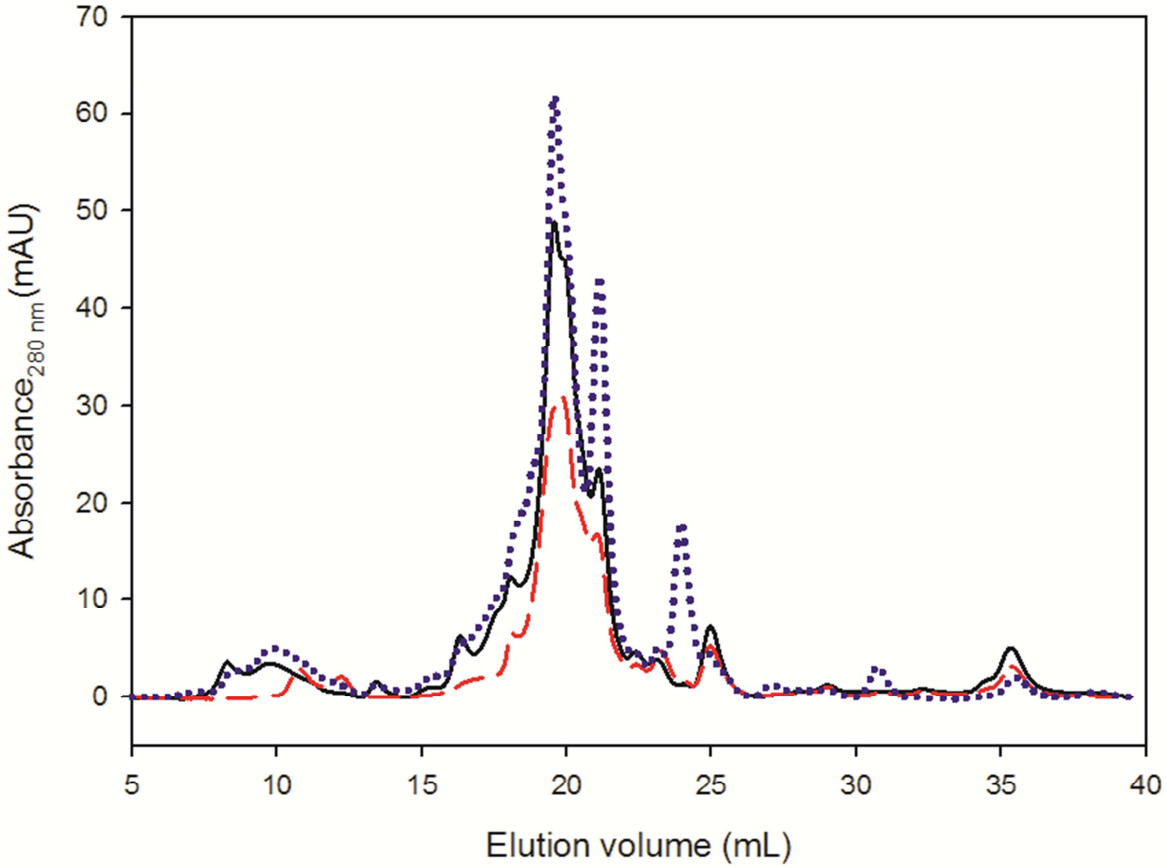


Figure 5



- Soy flour antinutritional factors were recovered in a selective manner.

- Trypsin inhibitor activity was reduced up to 84%.

- Raffinose family oligosaccharides were mostly obtained at bottom phase.

- Isoflavones were recovered at top phase with a yield of 97%.

- After liquid-liquid extraction the treated soy maintained its protein digestibility.