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Integrated extraction and purification of soy isoflavones by using aqueous micellar systems

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ABSTRACT

In this work, an integration of solid-liquid and liquid-liquid extractions by using aqueous micellar two-phase systems was evaluated as potential tool to purify soy isoflavones. Additionally, the proposed methodology aimed to preserve the protein content of the processed soy flour. The extractive assays were performed in AMTPS formed by Triton X-114 and sodium tartrate. In order to optimize the purification process, temperature and time were evaluated as independent variables. Under optimal working conditions, i.e. 100 min and 33 °C of incubation, IF were purified with a recovery percentage of 93 and a purification factor of almost 10. More importantly, the obtained sample presented an aglycone proportion superior to the reported by other methodologies. These results open perspectives to the use of aqueous micellar two-phase systems as an integrative methodology to extract, concentrate and purify isoflavones.

1. Introduction

Between the components of soy, isoflavones (IF) have been widely investigated due to their beneficial effects on human health (Baú & Ida, 2015). At present, the consumption of soy isoflavones is associated with reduced incidences of hyperglycemia, Alzheimer, menopause symptoms and diabetes mellitus (Andrade, Mandarino, Kurozawa, & Ida, 2016; Bedell, Nachtigall, & Naftolin, 2014). As a consequence of that, a plethora of pharmacologic formulations and dietary supplements based on soy isoflavones are being produced worldwide (Martínez-Domínguez, Romero-González, Arrebola, & Garrido Frenich, 2016; Zhao et al., 2011).

Isoflavones are polyphenolic molecules whose basic structure comprises a flavone nucleus, composed of two benzene rings linked to a hetero-cyclic ring (Valls, Millán, Martí, Borràs, & Arola, 2009). Soybeans and its derivatives, e.g. soy flour, represent the main sources of IFs (Ludueña, Mastandrea, Chichizola, & Franconi, 2007). Soy flour isoflavones occur primarily as β-glycosides with a small percentage of aglycones (2–3%, approximately). Even though aglycones are known to possess the highest estrogenic activity, it has been reported that conjugated isoflavones also present a considerable bioactivity (Islam et al., 2015).

Up to date, the methodologies commonly used to extract IF are not only expensive but also not friendly to environment because they require large quantities of toxic and flammable organic solvents (Azmir et al., 2013; Cho, Lee, & Park, 2009). Other methods, such as supercritical fluid extraction, microwave-assisted extraction or membranes, are not satisfactory for large scale processes because they require expensive equipment or high energy demand (Chen, Luo, Qi, & Wan, 2014; Li-Hsun, Ya-Chuan, & Chieh-Ming, 2004; Sun, Li, & Wang, 2011; Xu, Kumar, Lamb, & Layton, 2006). More importantly, the high temperatures required for some of these techniques may decrease the nutritional value of the used biomaterial. For example, Maillard reactions are associated with a reduced amino acid availability (Andrade et al., 2016; Li et al., 2015). Notice that if soy flour is used as IF source, there are important concerns about the preservation of proteins content (Li et al., 2015). Therefore, the systematic investigation of new methodologies to purify isoflavones is essential for a basis of new environmentally friendlier and non-destructive means of obtain natural isoflavones.

Over the last years, the application of liquid-liquid extraction by using surfactants has gained importance as an eco-friendly alternative to purify biomolecules (Schrader, Paasche, & Enders, 2014). This technique is based 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). Up to date, there are several

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works concerning to the application of this methodology to purify isoflavones (He et al., 2005; Mirzaei, Naeini, & Behzadi, 2012). For example, He and co-workers successfully purified daidzein from *Puerariae radix*. The proposed protocol involved several steps: solubilization and purification of isoflavones from solid materials into the aqueous surfactant solution, centrifugation of the obtained sample, separation of supernatant and preconcentration of isoflavones on the basis of phase separation by the cloud point methodology (He et al., 2005). Even though this methodology clearly resulted to be appropriate for analytical purposes, it may result expensive and time-consuming for larger scales.

On the other hand, the suitability of reverse micelles for extraction of soy isoflavones was also investigated (Zhao, Wei, Du, & Zhu, 2010). To accomplish that, several surfactant solutions (anionic and non ionic) were evaluated. According to the presented results, reverse micelles were able to extract a total amount of IF superior to the obtained by using organic solvents. Nevertheless, the selected solvent (isooctane) presents environmental concerns if used in high volume.

Taking into consideration the above mentioned, this work aimed to design a new methodology, based on AMTPS, to purify isoflavones from soy flour. The novelty of the proposed method consisted in an integration of solid-liquid and liquid-liquid extractions by using micellar two-phase systems in aqueous media. The main objective of this procedure is the reduction of both, extractive/separative steps and the use of organic solvent. By considering that proteins from soy flour could dissolve on water, the evaluated micellar systems were prepared at pH 5.00 to ensure low protein solubility (Rickert, Meyer, & Murphy, 2004) and preserve the protein content of the soy flour after IF extraction. Additionally, the obtained results were compared to an extraction with 80% ethanol in order to discuss the performance of the studied systems.

2. Materials and methods

2.1. Materials

Defatted non-deactivated soybean flour was obtained from the food processing company Molinos Río de la Plata SA (San Lorenzo, Argentina). Polyethylene glycol tert-octylphenyl ether (Triton X-114) and tartaric acid (Tart) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used as received. Isoflavone standards (Sigma Aldrich, St. Louis, MO, USA) were dissolved in pure methanol until obtaining the following concentrations: daizdin 1.5–80 μg/mL; daizdein 0.3–17 μg/mL; genistin 0.7–30 μg/mL and genistein 0.2–13 μg/mL. All the other reagents were of analytical grade and used without further purification.

2.2. Extraction of isoflavones from soybean flour

2.2.1. Experimental design

The extraction of soy isoflavones was performed with the aid of a central composite design 2^n (n = independent variables). In order to evaluate the effect of temperature (X_1, °C) and time (X_2, min) on the extractive process, the selected design consisted of 4 axial points and three replicates at the central point, for a total of 11 randomized experiments (Table 1). The analyzed responses were recovery (R%) and purification factor (PF) of total isoflavones.

2.2.2. Extraction of isoflavones with aqueous micellar two-phase systems

Isoflavones extraction with aqueous micellar two-phase systems was performed by using Triton X-114 (TX) and sodium tartrate (NaTart) 100 mM, pH 5.00 (Cordisco, Haidar, Goñi, Nerli, & Malpiedi, 2015). Surfactant concentration was kept at 5% w/w in order to achieve a low micellar phase volume, appropriate condition to concentrate the IF at this phase. Additionally, this concentration was selected in order to maintain similar conditions with other research groups (He et al., 2005; Luthria, Biswas, & Natarajan, 2007; Mirzaei et al., 2012). Notice that in these types of binodial diagrams, the tie line length is estimated as the difference between surfactant concentration at the micelle-rich phase and surfactant concentration at the micelle-poor phase (Liu, Nikas, & Blankschtein, 1996). Therefore, under the assayed conditions of this work, the tie line length was in the range of 12–17% w/w of TX, depending on the used temperature (33.0–47.0 ± 0.1 °C) (Cordisco et al., 2015).

Table 1
Variable values and results of IF purification at the micelle-rich bottom phase of AMTPS. Abbreviations: temperature (X_1), time (X_2), μg of IF (m), μg of soluble proteins (m_{SP}), bottom phase volume (V), IF recovery percentage (R%) and IF purification factor (PF).

Runs	Coded and independent variables values				m*	m_{SP}*	V (mL)*	R%*	PF*
	X_1	X_2	X_1 (°C)	X_2 (min)					
1	−1	−1	35	80	1849	980	1.60	43	9.68
2	+1	−1	45	80	1161	2410	0.90	27	2.47
3	−1	+1	35	120	4212	3590	1.70	98	6.01
4	+1	+1	45	120	1204	1860	0.90	28	3.32
5	**−1.41**	**0**	**33**	**100**	**3999**	**2110**	**1.80**	**93**	**9.72**
6	+1.41	0	47	100	989	2540	0.80	23	2.00
7	0	−1.41	40	72	1190	3110	1.35	28	1.96
8	0	+1.41	40	128	2666	3370	1.30	62	4.06
9	0	0	40	100	2064	3070	1.25	48	3.45
10	0	0	40	100	2107	3490	1.20	49	3.10
11	0	0	40	100	2193	3080	1.35	51	3.65

* The presented data are the average of triplicates with an error ⩽5%. The selected extractive condition (run 5) was indicated with bold letters.

The preparation of aqueous micellar two-phase systems of TX 5% w/w was performed as follows. Defatted soy flour (0.200 g) and TX (0.500 g of pure surfactant) were mixed in graduated glass tubes containing 9.300 g of sodium tartrate buffer 100 mM, pH 5.00 (final mass of 10.000 g). Notice that each component was added by direct weighing (Ohaus Pioneer™ Plus, 0.001 g). The prepared systems were then homogenized at 30 rpm for 1 h at room temperature using a tube rotator apparatus (Bioelec®, Argentina). Until this step, the same procedure was used for all the assayed systems. Then, in order to evaluate the effect of the different variables described in Table 1, the homogenized systems were incubated at different temperatures and time, according to the experimental design of Section 2.2.1. This procedure was accomplished by incubating the glass tubes on thermostated water bath (Dalvo Instrumentos, Argentina, ±0.1 °C). After incubating, both phases were conveniently separated by centrifuging at 4000 rpm for 10 min at constant temperature according to Table 1 (Sigma Laborzentrifugen, 3-18KS). Finally, samples from top and bottom phases were taken for the estimation of isoflavone (IF) and soluble protein (SP) content.

2.2.3. Extraction of isoflavones by using a methanol-water solution

The conventional extraction method with methanol 80% (v/v) was used as reference (Murphy, Barua, & Hauck, 2002). To accomplish that, 0.200 g of defatted soy flour was weighed (Ohaus Pioneer™ Plus, 0.001 g) and added into a 15-mL glass tubes. Then, 10 mL of extracting solution (pure methanol/water in 4:1 ratio) was added to the test tube. The solution was mixed using a tube rotator apparatus (Bioelec®, Argentina) at 30 rpm during 3 h at 35 ± 0.1 °C (Heater Sanjor, Argentina). After centrifuging (5000 rpm for 15 min at room temperature, Sigma Laborzentrifugen, 3-18KS), supernatant was used for isoflavone and soluble protein quantification.

2.3. Analytical methods

2.3.1. Estimation of total isoflavones

Total isoflavone content was estimated by a modification of the aluminum chloride-based colorimetric method (Chang, Yang, Wen, & Chern, 2002). In this assay, 0.5 mL of conveniently diluted phases (1:1 with distilled water) was mixed with 0.6 mL of pure methanol, 0.1 mL of 2% w/v of aluminum chloride and 1.9 mL of distilled water. After incubating at room temperature for 30 min, the absorbance of the reaction mixture was measured at 379 nm with a SPEKOL® 1200 diode-array spectrophotometer. A commercial supplement containing a natural mixture of soy isoflavones (Sojar, Argentina) was used to make a calibration curve. To accomplish that, one pill of supplement was dissolved in 4.0 mL of pure methanol. The mixture was then homogenized by using a tube rotator apparatus (Bioelec®, Argentina) at 30 rpm during 3 h at 35 ± 0.1 °C (Heater Sanjor, Argentina). After sample centrifugation (4000 rpm for 10 min at room temperature, Sigma Laborzentrifugen, 3-18KS), supernatant was diluted in pure methanol in order to obtain different concentrations of IF standards (0–400 μg/mL). The effect of AMTPS components was discounted by appropriate blanks (0.5 mL of diluted clean phases, 0.6 mL of pure methanol, 0.1 mL of 2% aluminum chloride and 1.9 mL of distilled water). The linear fitting equation, obtained by the software SigmaPlot version 10, was $A = 1.438C + 3.150e^{-3}$, $r^2 = 0.9986$, where A and C are the absorption value and the concentration of the isoflavones, respectively. All the experiments were run in triplicate and the obtained data were expressed in terms of μg of IF.

2.3.2. Estimation of soluble proteins

Soluble protein (SP) determination was carried out by using the Bicinchoninic Acid method (BCA, Sigma-Aldrich). To accomplish that, 50 μL of conveniently diluted phases (1:4 with distilled water) were mixed with 1.0 mL of BCA reagent (BCA and $CuSO_4$ 50:1). After incubation at 37.0 ± 0.1 °C for 30 min (Dalvo Instrumentos, Argentina), the absorbance of the reaction mixture was measured at 562 nm with a SPEKOL® 1200 diode-array spectrophotometer. Bovine Serum Albumin (BSA, Sigma-Aldrich) was used as standard (0–1000 μg/mL) and the medium effect was discounted by appropriate blank (50 μL of diluted clean phases). The linear fitting equation ($A = 0.477C + 8.691e^{-3}$, $r^2 = 0.9982$, where A and C are the absorption value and the concentration of the protein, respectively) was obtained by the software SigmaPlot version 10. All experiments were run in triplicate and the obtained data were expressed in terms of μg of SP.

2.4. Extraction performance parameters

Recovery percentage (R%) for total isoflavones was calculated in the micelle-rich (bottom) phase according to the following equation:

$$R\ \% = \left(\frac{m}{m^0}\right) \times 100\% \quad (1)$$

where m represents μg of IF in micellar phase and m^0 represents μg of IF obtained from the reference method (Section 2.2.3).

IF purification factor (PF) at micellar (bottom) phase was calculated as follows:

$$PF = \frac{m/m_{SP}}{m^0/m^0_{SP}} \quad (2)$$

where m_{SP} represents μg of SP in micellar phase and m^0 represents μg of SP obtained from the reference method (Section 2.2.3).

2.5. Identification of the extracted isoflavones

The quantitative identification of the extracted isoflavones was carried out by high-performance liquid chromatography (HPLC). To accomplish that, the micelle-rich (bottom) phase of the AMTPS corresponding to the run 5 (see Table 1) was conveniently diluted (1:3 with pure methanol) and submitted to chromatographic separation. This procedure was performed in a reverse phase column coupled with a guard column containing the same stationary phase (COSMOSIL 5C18-AR-II Packed Column – 4.6 mm I.D. × 250 mm). Separation of isoflavones was carried out with a mobile phase A – water and acetic acid (99.9:0.1, v/v) – and a mobile phase B – acetonitrile and acetic acid (99.9:0.1, v/v). The operating conditions were: gradient elution starts at 80% mobile phase A and ends at 0% after 25 min at a continuous flow of 0.80 mL/min, between 25 and 32 min phase A returns to 80% and remains at this percentage for 3 min (until 35 min). The separation analysis was performed using a Waters e2695 separation module system interfaced with a photodiode array UV/Vis detector (PDA 190–600 nm). Data acquisition and analysis were accomplished using Software Empower 3. The identification of each isoflavone was performed by comparing the absorption spectra and retention times of the respective individual patterns (see Supplementary Fig. 1). The quantification of each isoflavone was carried out by integrating the peak areas with external standardizations using the individual reference patterns. As the different forms of isoflavones have different molar mass values, the means and standard deviations were expressed in μg of isoflavones/g of dry soy flour.

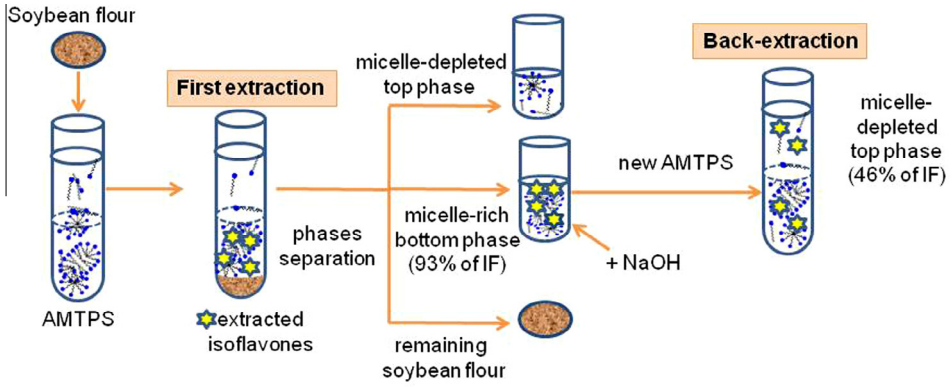

Fig. 1. Schematic representation of both extractive steps. In the first extraction, IF were mainly recovered at the micelle-rich, bottom phase. In the back-extraction procedure, IF were equally recovered in both phases.

2.6. Back extraction procedure

Back extraction assay was performed by using the micelle-rich (bottom) phase of the AMTPS with the best extractive performance (run 5 of Table 1). To accomplish that, 1.500 g of bottom phase was weighed (Ohaus Pioneer™ Plus, 0.001 g) into a 5-mL graduated glass tubes. Then, 0.750 g of basic solution (NaOH 0.01 M, pH 8.20) was added to the test tube. The resulting mixture was homogenized using a tube rotator apparatus (Bioelec®, Argentina) at 30 rpm during 15 min at room temperature. After that, the mixture was incubated at 60 ± 0.1 °C in a thermostated water bath (Dalvo Instrumentos, Argentina, ±0.1 °C) for the formation of a new AMTPS (see Fig. 1). Finally, samples from top and bottom phases were taken for the estimation of isoflavone (IF) and soluble proteins (SP). Recovery percentage and purification factor for total isoflavones were determined in each phase according to Section 2.4. All experiments were run in triplicate.

2.7. Statistical analysis

The statistical analysis was performed with the aid of Statistic 10.0 Software (StatSoft). Differences within means were determined using Least Significant Difference (LSD) multiple comparison analysis. Differences at a p-value <0.05 were considered significant. Graphs for the predictions and surface responses were also analyzed.

3. Results and discussion

3.1. Extraction of isoflavones by using aqueous micellar two-phase systems

The extractive performance of AMTPS was evaluated by analyzing both, IF recovery percentage (R%) and purification factor (PF). The obtained results concerning to IF recovery yield showed that these molecules could be successfully obtained at the micelle-rich (bottom) phase (recovery of almost 100%). A similar behavior has already been seen by other authors. For example, He and coworkers have demonstrated that the extraction efficiency of 5% Genapol X-080 was higher than that of several organic solvents (He et al., 2005). The same conclusions were reported by Zhao and colleagues, who evaluated the feasibility of using reverse micelles to extract isoflavones from soy flour (Zhao et al., 2010).

The high extractive capacity of micelles is generally attributable to hydrophobic and hydrogen-bonding interactions between isoflavones and surfactants. The work presented by Cao et al., clearly demonstrated this behavior. According to their reported results, at acidic working condition, isoflavones (mainly the aglycone forms) exist as neutral molecules capable of being solubilized in hydrophobic media (Cao et al., 2012). Thus, taking into account that the AMTPS evaluated in this work were prepared at pH 5.00, an efficient extraction of IFs with Triton X-114 and an uneven distribution of these IFs to the micellar phase occurred, as expected. In addition, a concentration of IFs took place simultaneously with its extraction, as a consequence of the noticeably low volume of micellar phase respect to that of the total one (see Table 1).

As mentioned above, the purification factor of isoflavones was also evaluated. Protein–polyphenol association is a well-known phenomenon (Liu, Wu, Wang, Li, & Huang, 2015). Particularly, soy isoflavones are believed to interact with proteins by hydrogen bonding. The reported information (Liu et al., 2015) suggests that working at high values of pH allows the separation between proteins and isoflavones. Nevertheless, it is also well known that this working condition generally result in a high protein solubilization, thus leading to a reduction of the nutritional value of the treated soy flour. In order to avoid this phenomenon, IF extraction was performed at acidic pH and the selection of working conditions was carried out by monitoring IF purification factor regarding soluble proteins. As it can be appreciated from Table 1, all PF values resulted to be superior to the unity, thus meaning that the obtained IF at the micellar phase have a higher purity degree than the obtained from methanolic extraction. This behavior can be related with both, a low solubility of soy proteins toward micellar phase or excluded-volume effect between micelles and hydrophilic proteins (Liu et al., 1996).

3.2. Statistical analysis

As can be appreciated from Table 1, both responses, i.e. recovery percentage and purification factor, resulted to be highly sensitive to the evaluated factors (temperature and time). Recovery percentage values varied from 23 to 98 while purification factor presented values from 1.96 to 9.72.

The performed ANOVA (see Supplementary Fig. 2) indicated that the selected model (linear/quadratic main effects +2-ways) for the responses recovery percentage and purification factor were statistically valid. The coefficients of determination (R^2) resulted to be 0.938 and 0.999, for purification factor and recovery percentage, respectively. Additionally, the lack of fit was not significant ($p > 0.05$) for both responses. These results indicated the suitability of the models for an appropriate representation of the real relationship among the studied factors. The statistical significance of

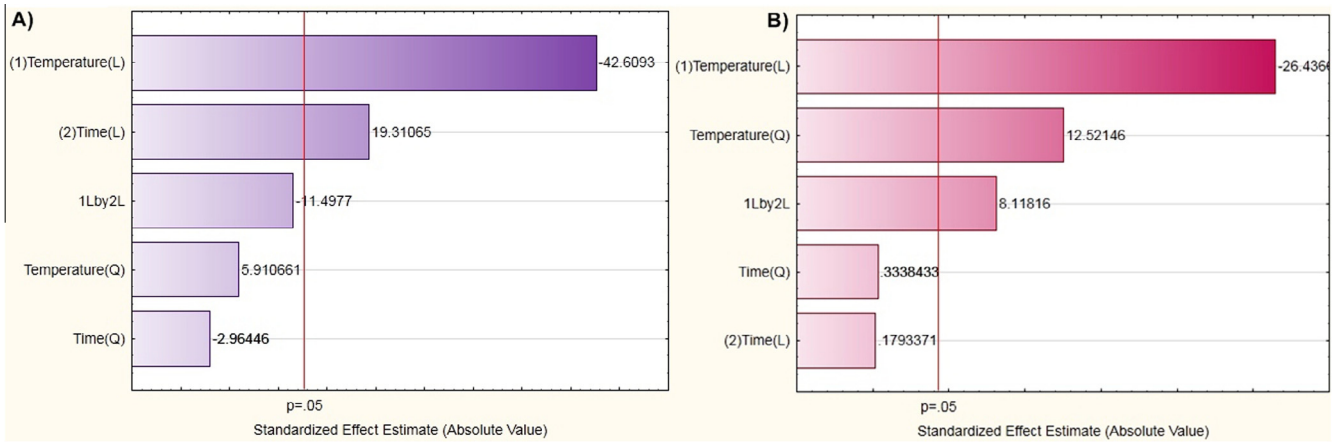

Fig. 2. Pareto charts for the effect of temperature (1) and time (2) on (A) IF recovery percentage (R%) and (B) IF purification factor (PF). Significance at a p *value* ⩽0.05. Both, lineal (L) and quadratic (Q) coefficients were considered.

each factor/interaction was visualized by using Pareto charts (Fig. 2). In Pareto charts, the bar lengths are proportional to the standardized value of the estimated effect. A vertical line corresponding to a p value of 0.05 is included in each chart, and any effect which exceeds this reference is considered significant with regard to the response.

The ANOVA test for recovery percentage (Fig. 2A) showed that temperature and time, both of them in lineal relationship, exerted the most significant effects on the studied response. The effect of temperature on the extraction of IF from solid materials has already been studied. According to the reported results, an increase in working temperature improved the extractive performance (Andrade et al., 2016; Rickert et al., 2004). This behavior was attributable to a decrease in both surface tension and viscosity, consequence of temperature raise, which leaded to an increased diffusion and mass transfer of the biomolecule (Cheigh, Yoo, Ko, Chang, & Chung, 2015). Nevertheless, as shown in Fig. 2A the temperature effect in this work has a negative sign, thus meaning that a temperature increase will result in a lesser IF recovery. To explain this effect, it should be taken into account that in this work, the solid-liquid extraction takes places simultaneously with the liquid-liquid equilibrium between micellar and aqueous phases. The notorious reduction of the micellar phase volume at increasing temperatures (see Table 1), would be responsible of the lower recoveries. This behavior which represents one of principal properties of AMTPS, has already been reported by other authors (Haga, Santos-Ebinuma, de Siqueira Cardoso Silva, Pessoa, & Rangel-Yagui, 2013; Rangel-Yagui, Pessoa-jr, & Blankschtein, 2004).

Analyzing the effect of time on IF recovery, it can be appreciated that this variable exerts a positive effect on the response, which indicate that an increase on the time extraction could result in a higher amount of recovered IF. A similar behavior has already been seen in previous reports, however, after reaching a maximum value of time, this variable starts to show an smaller effect or even can affect negatively the extraction performance (Cho et al., 2009; Rostagno, Palma, & Barroso, 2003). This behavior could be explained by considering a first extractive stage, in which IF are removed from the solid matrix, and a subsequent stage of saturation and/or degradation of the extracted IF (Rostagno et al., 2003). As depicted in Fig. 3A, the effect of time observed in this work depended on the temperature level. At the highest temperature values, a slightly decrease in IF recoveries was observed at higher time levels, thus suggesting a destabilization/degradation of the extracted IF. However, at lower temperature values the opposite effect was observed. This behavior could be indicating that working with low temperatures is more appropriate to preserve the integrity of the extracted IF.

On the other hand, the ANOVA analysis for PF is shown in Fig. 2B. In this case, it can be appreciated that temperature (in lineal relationship) exerts the most significant effect on this response. Its negative sign suggests that an increase in the working temperature, leads to a decrease in the purification factor. This behavior could be directly related with a higher protein solubility in the micellar phase, thus reducing IF purity (Cheigh et al., 2015; Rickert et al., 2004). As additional information, Fig. 3B showed the surface response of PF. As it can be appreciated, the obtained surface presents a minimum value at the central points of temperature and a deep increase toward the lowest values of this factor. Time extraction showed little effect on the response, agreeing with the observation of Fig. 2B.

In order to obtain an improved methodology to extract and purify IF, both response surfaces were combined to obtain the desirability function. The obtained analysis is showed in Fig. 4. As can be appreciated from the presented data, the desirability function did not present any maximum value. However, the observed increase of the combined surface toward the lowest levels of temperature, suggests that the desirability function could be maximized by lowering the working temperature. Besides, by analyzing the effect of time extraction, it could be notice that the maximization of recovery percentage and purification factor could be reached by both, lowering the working temperature and by extending the time extraction. Nevertheless, these conditions are either not experimentally accessible or not totally recommended in terms of large-scale processes. Firstly, lowering the temperature presents the risk of working close to the cloud point temperature (i.e., the minimal temperature at which the micellar systems separate into two phases) (Rangel-yagui et al., 2004), and therefore, small variations at the experimental conditions could lead to the AMTPS disruption (Malpiedi, Nerli, Abdala, Pessôa-Filho, & Pessoa, 2014). On the other hand, the extension of extraction time could affect both, isoflavone integrity (Domínguez-Perles, Teixeira, Rosa, & Barros, 2014) and soluble protein content of the treated soy flour. As it was mentioned above, the remaining amount of soluble protein in the treated soy flour should be preserved due to the fact

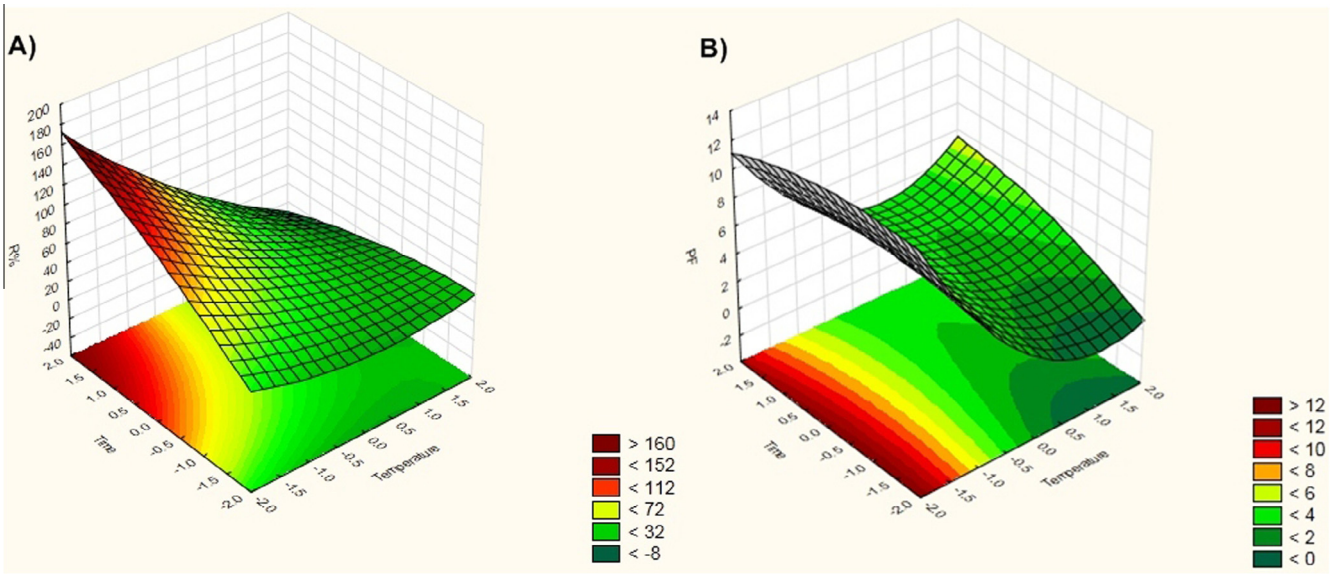

Fig. 3. Fitted surface responses for (A) IF recovery percentage (R%) and (B) IF purification factor (PF) as a function of temperature and time.

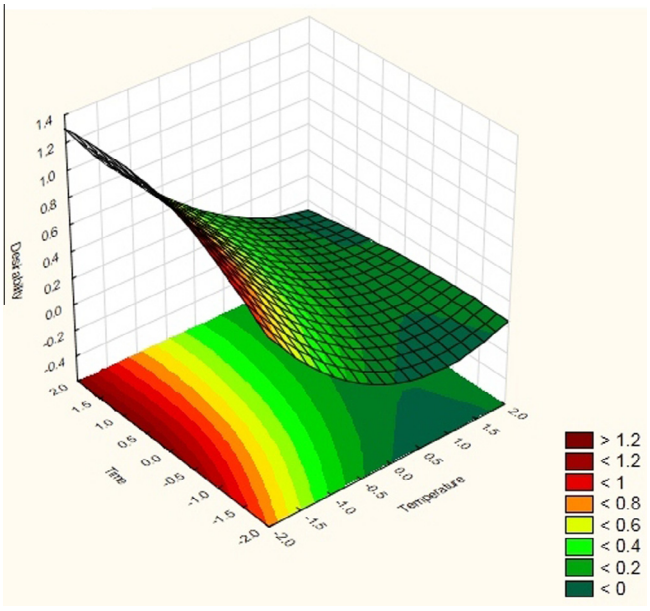

Fig. 4. Surface plot for the global desirability function.

that is raw material for the elaboration of different nutritional products (Capriotti et al., 2015).

On the basis of these considerations, it was concluded that in the range of the assayed variables the experimental conditions belonging to the run 5 of Table 1, i.e. extraction time of 100 min and a working temperature of 33 °C, are the most recommended for IF extraction. These conditions allow the extraction, preconcentration and purification of IF, simultaneously. Besides, the selected system presents the advantages of working with low temperature and a pH value of 5.00, thus preserving the nutritional value of the used soy flour.

3.3. Identification of the extracted isoflavones

Isoflavones extracted at bottom phase of the selected AMTPS were quantitatively identified by high-performance liquid chromatography. Additionally, the obtained results were compared with those of other methodologies. As can be seen from Table 2, the different samples presented different isoflavone composition, however, in all cases the highest content corresponded to the glycosides, daidzin and genistin. These results are consequence of the high content of β-glycosides with regard to that of aglycones in soybeans and derivatives. For example, it is well-known that aglycones from soy flour are just about 2–3% of total isoflavones, while non-conjugated β-glycosides represent the 34% of total IF (Andrade et al., 2016).

When comparing the obtained data in this work with those from other methodologies, it can be appreciated that the amount of aglycones recovered in this work was similar or even superior to most of them. The extraction with ethanol 70% was the methodology that extracted the highest amount of genistein and daidzein (Jankowiak, Kantzas, Boom, & Van Der Goot, 2014). At comparing the extractive performance of ethanol and pure water, it can be noticed that water extraction is not able to extract the most hydrophobic IF (Cao et al., 2012). On the other hand, it is also important to point out that the amount of IF (aglycones and β-glucosides) obtained in this work was superior to that of the reverse micelles extraction (Zhao et al., 2010), suggesting that the use of direct micelles improves the extractive performance.

3.4. Back extraction procedure

When target bioproducts are recovered in the micellar-rich phase of aqueous micellar two-phase systems, the high surfactant concentration may compromise the applicability of this product. In this case, a second extractive step tending to revert initial partitioning behavior represents a simple alternative to overcome this potential difficulty (Malpiedi, Picó, & Nerli, 2011). To accomplish that, a basic aqueous solution was added to the micellar phase of the selected AMTPS. This procedure had the aiming of converting IF into anionic forms, with higher solubility in the micelle-poor phase (Cao et al., 2012). According to the obtained results, just 50% of IF was recovered at the top micelle-depleted phase, thus giving a total recovery (considering both extractive steps) of 46% (see Fig. 1). Since these results are not totally satisfactory, a deeper investigation about the means to improve the back-extraction performance should be carried out. Nevertheless, the use of basic solution seemed to be an appropriate starting point to achieve the separation of IF from micelles.

4. Conclusion

In this work, aqueous micellar two-phase systems of Triton X-114 and sodium tartrate pH 5.00 were used to extract, concentrate and purify isoflavones from soy flour. The preservation of the protein content in the treated soy flour, ignored issue in other reported methodologies, was took into consideration when optimizing our extraction procedure. The selected experimental conditions, 100 min of incubation at 33 °C, allowed the recovery

Table 2
Soy isoflavones extracted with different methodologies.

Extractive method	Source	Isoflavone content				
		Daidzein	Genistein	Daidzin	Genistin	Reference
Aqueous micellar two-phase systems of Triton X-114	Soy flour	142.60 ± 2.60	69.60 ± 0.20	350 ± 8	736.50 ± 3.30	(**)
Water/acetone/ethanol solution	Soy flour	Nd	38.00 ± 0.01[a]	382 ± 12	467.00 ± 8.00	Andrade et al. (2016)
Acetonitrile 80%	Soybeans	36.60 ± 2.00[a]	36.30 ± 0.40[a]	670 ± 18	644.70 ± 22.40	Lee, Chung, Hunjung, & Jung (2015)
Methanol 80%	Soybeans	22.10 ± 2.50[b]	26.40 ± 1.30	631 ± 15	587.60 ± 2.20	Lee et al. (2015)
Water	Okara	22.00 ± 10.00[b]	3.00 ± 4.00[b]	108 ± 22	71.00 ± 16.00[a]	Jankowiak et al. (2014)
Ethanol 70%	Okara	173.00 ± 8.00	194.00 ± 14.00*	160 ± 7	171.00 ± 7.00	Jankowiak et al. (2014)
Reverse micellar systems of Triton X-100	Soy flour	Nd	79.03 ± 4.31	168 ± 5	118.24 ± 4.98	Zhao et al. (2010)
Water (pH 8.5)	Soy flakes	86.11*	152.55*	617*	996.66*	Rickert et al. (2004)
Methanol 50% with ultrasound assistance	Soybeans	–	–	217 ± 8	433.00 ± 14.32	Rostagno et al. (2003)
Supercritical carbon dioxide	Soy flour	30.93 ± 0.04[a,b]	1.71 ± 0.01[b]	–	53.64 ± 0.06[a]	Rostagno, Araújo, & Sandi (2002)

Values are expressed as mean ± SD (μg IF/g dry soy flour). Notice that some unities were adapted from the information reported by other authors. [a,b]Values within the column do not differ significantly with LSD test ($p > 0.05$). Nd: no detected.

* Values not included in the analysis due to lack of data (n, SD) or being outside the homocedasticity (Cochran's C test).

** Values obtained from AMTPS corresponding to run 5 of Table 1.

of 93% of total IF with a purification factor of about 10. The identification of extracted IF showed that the assayed methodology was able to extract 142.60 ± 2.60 μg/g of daidzein, 69.60 ± 0.20 μg/g of genistein, 350 ± 8 μg/g of daidzin and 736.50 ± 3.30 μg/g of genistin. The use of basic solution resulted to be a potential tool to separate the recovered IF from micelles.

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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.07.001.

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