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First approach based on direct ultrasonic assisted enzymatic digestion and capillary-high performance liquid chromatography for the peptide mapping of soybean proteins

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ABSTRACT

This work proposes, for the first time, the use of a high intensity ultrasonic probe to accelerate the tryptic digestion of soybean proteins. Different digestion parameters were optimized: protein extracting solution, reduction, and alkylation conditions (time, concentration, and temperature), trypsin:protein ratio, and ultrasonic conditions (sonication amplitude and time). Separation of peptide profiles was carried out by capillary-HPLC. The effect of the variation of chromatographic conditions (elution gradient, column temperature, and injection volume) on peptide separation was also studied using two capillary-HPLC columns with different column diameters and particle sizes. Moreover, samples were focused at the top of the column in order to obtain an increasing sensitivity without loss of efficiency. This method was successfully applied to the profiling of soybean peptides from transgenic and non-transgenic soybeans and from different pigmented beans commercialized as soybeans.

1. Introduction

Soybean (*Glycine max* L.) is a well known source of proteins (48–50%) with high nutritional value, functional properties, and low cost [1]. These facts have promoted the production and consumption of soybean which has resulted in an increasing interest for the determination and characterization of soybean proteins [2–5]. Most of these works were focused on the characterization and determination of soybean proteins using HPLC and CE being very scarce the works aimed to the analysis of soybean peptides. Digestion of total proteome and analysis of resulting peptide profiles constitutes an alternative to protein analysis and, in addition, could provide new information about less abundant proteins that can be overlooked by the analysis of proteins, or about protein modifications as a consequence of food processing [6,7].

However, digestion of total proteome provides thousands of peptides and, thus, their analysis requires highly efficient and sensitive separations which are mostly difficult to obtain using conventional columns [6,8]. Miniaturization of chromatographic procedures using capillary columns offers substantial advantages over conventional HPLC such as the use of less mobile phase and a reduced waste [9,10]. Moreover, an advantage of using microflow through capillary columns is the higher sensitivity that can be achieved due to the lower dilution of the analyte that could be obtained when injecting large volumes based on on-column focusing [10,11].

Despite these advantages, analysis of digested proteins involves the use of tedious and time consuming methodologies (overnight for in-solution digestion and 24–72 h for in-gel digestion). Therefore, different strategies have been focused to accelerate that process. Recently, Capelo et al. have reviewed the new approaches to speed up protein digestions [12]. Although there are several methods available to accelerate protein digestions (heating, use of microspin columns, ultrasonic energy, high pressure, infrared energy, microwave energy, microreactors, and alternating electric fields) most of them are recent tools and more research work is needed. In this way, heating, microwave energy, and ultrasonication are the most promising approaches.

Regarding ultrasonication, there are three different commercial devices: ultrasonic bath, sonoreactor, and ultrasonic probe [13–15]. The ultrasound bath is the most available in laboratories, but the powerful of this device is not high, obtaining digestion times higher than 30 min [16]. Sonoreactors and ultrasonic probes (also known as high intensity focused ultrasound) offer high ultrasonic intensities and have provided significant reductions in digestion times (from 12 h up to 300 and 120 s, respectively) [15].

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Therefore, the aims of this work were the optimization, for the first time, of a digestion procedure for soybean proteins based on the use of an ultrasonic probe and the profiling of resulting soybean peptides using capillary-HPLC.

2. Materials and methods

2.1. Chemicals and samples

All reagents employed for the preparation of mobile phases were HPLC grade. Acetonitrile (AcN), methanol (MeOH), 2-propanol, and tetrahydrofuran (THF) were supplied by Scharlau (Barcelona, Spain) and trifluoroacetic acid (TFA) was from Sigma (St. Louis, MO, USA). Tris(hydroxymethylaminomethane) was from Merck (Darmstadt, Germany), urea was from Scharlau, and calcium chloride and hydrochloric acid were from Panreac (Barcelona, Spain). Guanidine hydrochloride (GnCl), dithiothreitol (DTT), iodoacetamide (IAA), and trypsin (type IX-S from bovine pancreas), employed for the digestion, were from Sigma. Bradford reagent and albumin from bovine serum (BSA), used in the Bradford protein assay, were also from Sigma. All solutions were prepared with ultrapure water from a Milli-Q system from Millipore (Bedford, MA, USA).

Six commercial beans commercialized as soybeans (tree yellow soybeans (SB1, SB2 and SB3), two green soybeans (SB4 and SB5), and one white soybean (SB6)) were purchased in local markets, in Madrid (Spain). Transgenic soybeans (SB7 and SB8) were kindly provided by INIA (Madrid, Spain) (http://www.inia.es/). The transgenic genotype of all soybeans was previously checked by DNA analysis using PCR.

2.2. Instrumentation

An ultrasonic probe consisting of a 3 mm stepped microtip (model CV-18) coupled to a 130 W ultrasonic processor (model VCX-130) from Sonic Vibra-Cell (Connecticut, USA) was employed.

The separations were performed in a modular capillary chromatographic system which consisted of a microvacuum degasser, a capillary LC pump, a microwell-plate autosampler (8 μL injection loop), a thermostatted autosampler model 1100, a thermostatted column compartment, and a multiple wavelength detector model 1200 from Agilent Technologies (Pittsburgh, PA, USA). An HP Chemstation software was used for instrument control and data acquisition. Two different reversed-phase HPLC (RP-HPLC) columns were employed: a C18 Zorbax SB (150 mm × 0.5 mm ID, 5 μm particle size, and 80 Å pore size) and a C18 Zorbax 300SB (150 mm × 0.3 mm ID, 3.5 μm particle size, and 300 Å pore size) from Agilent Technologies. After optimizing the experimental conditions, a suitable chromatographic separation of soybean peptides was achieved with the C18 Zorbax 300SB column using the following conditions: flow-rate, 14 μL/min; binary gradient, 5–75% B in 60 min; mobile phases, 0.1% (v/v) TFA in water (phase A) and 0.1% (v/v) TFA in MeOH (phase B). The injection volume was 1.5 μL, the separation temperature was 60 °C, and UV detection was performed at 210, 214, 254, and 280 nm.

2.3. Protein extraction from soybean seeds

Soybeans were ground using a domestic miller. Extraction of soybean proteins was performed by dissolving 600 mg of powdered soybean in 10 mL of 50 mM Tris–HCl (pH 8.0) and 8 M urea. The extraction was carried out in 3 min by sonication using an ultrasonic bath (J.P. Selecta, Barcelona, Spain). After centrifugation at 4000 × *g* for 10 min, the supernatant fraction was collected for its enzymatic digestion. The total protein content of this fraction was determined using the Bradford protein assay.

2.4. Protein digestion

Two different protocols were employed for the digestion of soybean proteins: a standardized procedure and a direct ultrasonic assisted procedure.

The standardized digestion was carried out treating 1 mL of the previous soybean protein extract with 100 μL of 50 mM DTT for 20 min at 50 °C for the reduction of disulfide bonds. After cooling to room temperature, alkylation of free thiol groups was performed with 110 μL of 100 mM IAA for 5 min. This solution was diluted ten times in 11 mM $CaCl_2$ (in 50 mM Tris–HCl at pH 8.0) in order to reduce the final urea concentration. Finally, 20 μL of 1 mg/mL trypsin solution were added (1:50, enzyme:substrate) and the solution was incubated at 37 °C overnight.

The ultrasonic assisted enzymatic digestion (USAED) was carried out following the same reduction, alkylation, and dilution steps detailed in the standardized protocol. The diluted sample was inserted into a glass cooling cell (4 cm × 1 cm ID) connected to a heating circulator set at 37 °C and 20 μL of 1 mg/mL trypsin solution were added. Finally, the tip of the probe was inserted and maintained at 3 mm from the bottom of the solution and digestion was performed in 1 min using the ultrasonic probe at 20% amplitude and without pulses.

In both protocols, the digestion reaction was stopped by adding 50 μL of TFA [17] and final solutions were filtered through 0.45 μm pore size regenerated cellulose filter membranes (Titan 2, Eatontown, NJ, USA) prior to injection into the capillary-HPLC system.

3. Results and discussion

3.1. Optimization of the digestion protocol

Obtaining an efficient digestion in real mixtures of proteins is a difficult task due to the high number of proteins present in a proteome and their different physico-chemical properties [6]. Therefore, a study of the best conditions enabling the tryptic digestion of soybean proteins was performed. For that purpose, obtained extracts were injected into the capillary-HPLC system using the following initial conditions: flow-rate, 20 μL/min; binary gradient, 5–95% B in 30 min; mobile phases, 0.1% (v/v) TFA in water (phase A) and 0.1% (v/v) TFA in ACN (phase B); injection volume, 0.5 μL; separation temperature, 30 °C. All these experiments were performed with the SB1 soybean. The digestion was firstly performed following the standardized procedure which involved the digestion of sample overnight and the following parameters were then optimized: protein extracting solution, reduction and alkylation conditions, and digestion conditions.

Different extraction buffers have been used in our research group to extract proteins from soybeans, based on organic/aqueous media [18,19] or aqueous buffered media [3,20]. However, the enzymatic digestion of proteins is more easily accomplished if proteins are denaturalized since denaturalized proteins are more soluble. Therefore, chaotropic agents were added (urea or guanidine hydrochloride) to the extracting solution observing the best results, from a quantitative point of view, when using a 8 M urea solution in Tris–HCl (pH 8.0).

Moreover, we demonstrated, in a previous work, the need of using reductive alkylation for soybean digestion [18] not only for the enhancement of the digestion itself but also to inactivate trypsin inhibitors present in soybeans. In this work, DTT has been the reducing reagent employed for the reduction of disulfide bonds and IAA has been the alkylating reagent used for the alkylation of free thiol groups. At this respect, the time needed to carry out the reduction (ranging from 10 to 60 min) and alkylation (ranging from 5 to 45 min), the concentration of DTT and IAA (50 and 100 mM

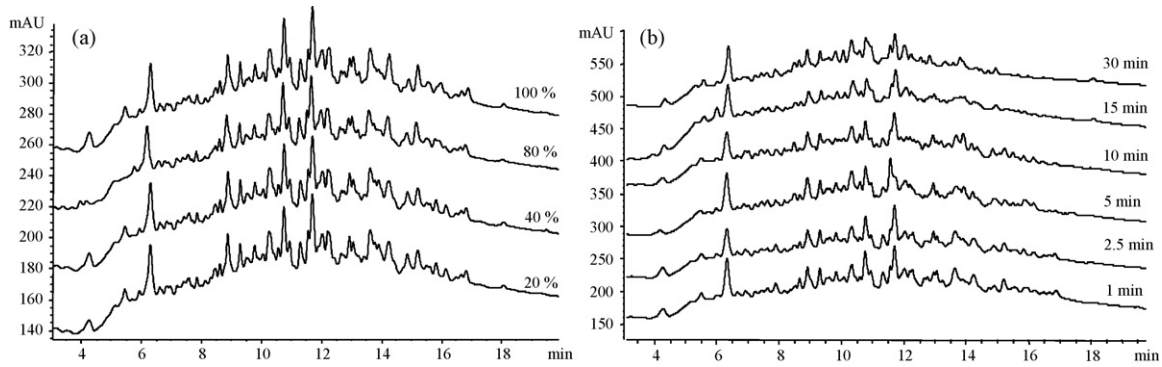

Fig. 1. Peptide separation of soybean proteins digested with an ultrasonic probe (a) at 5 min and at different ultrasonic amplitudes and (b) at 20% ultrasonic amplitude and at different sonication times. Chromatographic conditions: column, C18 Zorbax SB (150 mm × 0.5 mm and 5 μm particle size); mobile phase, 0.1% (v/v) TFA in water (solvent A) and in AcN (solvent B); gradient, 5–95% B in 30 min; flow-rate, 20 μL/min; injected volume, 0.5 μL; temperature, 30 °C; UV detection, 210 nm.

for DTT and 100 and 550 mM for IAA), and the reduction temperature (50 and 75 °C) were studied. The best conditions for reduction were 50 mM DTT at 50 °C for 20 min. After allowing the sample to cool at room temperature, alkylation of free thiol groups was best performed with 100 mM IAA for 5 min.

The optimized digestion conditions were next studied. The ratio trypsin:protein was optimized to avoid potential interferences from trypsin autodigestion. Two different trypsin:protein ratios (1:25 and 1:50) were tested observing best results when using a 1:50 (trypsin:protein) ratio. Furthermore, overnight digestion was accelerated by the use of a high intensity ultrasonic probe. Optimization of the ultrasonic probe conditions for soybean protein digestion involved to study the effect of temperature, amplitude of sonication, and time. Direct ultrasonic is based on cavitation phenomena that result in an increase in temperature [13,21]. This increase in temperature could cause different undesirables effects. By other hand, despite trypsin shows an increasing of activity at temperatures up to 60 °C, most of tryptic digestions are performed at 37 °C since other side reactions could occur at higher temperatures [22]. Moreover, the use of high temperatures could also affect the efficiency of the cavitation phenomena [23]. Consequently, the temperature of sonication was set at 37 °C by using a glass cooling cell connected to a heating circulator.

The effect of the sonication amplitude, which is directly related with the sonication energy, seems to depend on protein and enzyme types. Indeed, Rial-Otero et al. reported that the use of the ultrasonic probe for BSA digestion yielded the same protein sequence coverage regardless of the amplitude employed while when using the same protocol for α-lactalbumin digestion, the number of peptides, and thus the coverage, decreased as the amplitude increased up to 70% [15]. Moreover, Ishimori et al. reported a decrease in the enzymatic activity of α-chymotrypsin when increasing the amplitude of sonication [24]. For this reason, in this work, different amplitudes were tested for the digestion of soybean proteins (20, 40, 80 and 100%) observing the same peptide profile in all cases (see Fig. 1a). As a consequence, the lowest amplitude (20%) was selected. Moreover, sonication times ranging from 1 to 30 min were tested observing the profiles grouped in Fig. 1b. Despite separations were very similar up to 12 min, the last part of the chromatograms (from 12 to 18 min) showed differences. In fact, the longer the sonication time, the less crowded was this part of the chromatogram. For this reason, 1 min sonication time was selected to perform the soybean digestions.

By other hand, in order to confirm that peptides obtained came from the enzymatic digestion of soybean proteins and not from a possible protein degradation caused by the ultrasonic probe, further experiments were designed. Fig. 2 groups the chromatograms corresponding to the digested protein extract following the overnight protocol (Fig. 2a), the optimized protocol (Fig. 2b), to the extract submitted to the digestion protocol without reduction and alkylation (Fig. 2c), to the extract submitted to the digestion protocol without enzyme (Fig. 2d), to the undigested protein extract (Fig. 2e), and to the blank digestion (Fig. 2f). The comparison of Fig. 2d with b and f enabled to conclude that proteins were not degraded by the ultrasonic energy and that the obtained peptides (Fig. 2b) came from the protein digestion.

Finally, Lopez-Ferrer et al. [25] affirmed that the use of an ultrasonic probe enabled to remove reduction and alkylation steps for in-solution digestions. However, our results (comparison of chromatograms showed in Fig. 2b and c) demonstrated the need of using reduction and alkylation for a suitable tryptic digestion of soybean proteins.

3.2. Optimization of chromatographic conditions

Due to the complexity of the sample and the high number of co-migrating peptides obtained with the initial chromatographic conditions, the following chromatographic parameters were next optimized: injection volume, mobile phase composition, elution gradient, and separation temperature. All these experiments were performed using a Zorbax SB column (150 mm × 0.5 mm ID, 5 μm particle size) and injecting the digested protein extract obtained from SB1 using the previously optimized digestion protocol.

The use of high injection volumes to increase the sensitivity in capillary columns without a perceptible loss in efficiency is possible by the focusing of sample band on the top of the column [11]. In general, the on-column focusing is carried out by injecting the

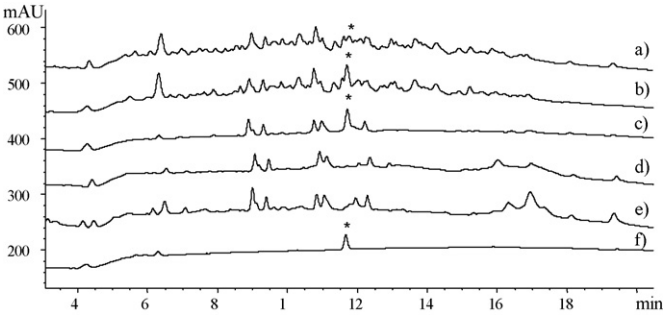

Fig. 2. Chromatograms corresponding to the digested protein extract following the overnight protocol (a), the optimized protocol (b), the optimized protocol without reduction and alkylation (c), and the optimized protocol without enzyme (d). Chromatograms corresponding to the undigested protein extract (e) and the blank digestion (f) are also included. Trypsin enzyme was signaled with and arrow. Chromatographic conditions were kept as in Fig. 1.

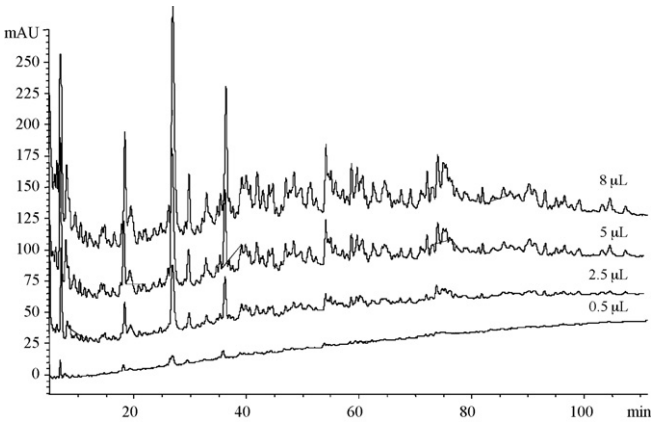

Fig. 3. Peptide profiles obtained for the digested extract corresponding to the SB1 soybean using different injection volumes. Ultrasonic conditions: amplitude, 20%; digestion time, 1 min. Chromatographic conditions: gradient, 5–43% B in 120 min. Other chromatographic conditions were kept as in Fig. 1.

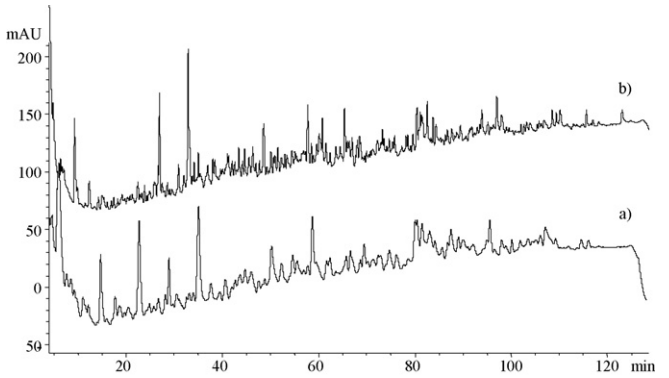

Fig. 4. Comparison of peptide separations obtained using different diameters reverse-phase capillary columns. Ultrasonic conditions as in Fig. 3. Chromatographic conditions: (a) capillary column, Zorbax SB (150 mm × 0.5 mm and 5 µm particle size); mobile phase, 0.1% (v/v) TFA in water (solvent A) and in MeOH (solvent B); gradient 5–75% B in 120 min; flow-rate, 20 µL/min; temperature, 60 °C; injection volume, 5 µL; (b) capillary column, Zorbax 300 SB (150 mm × 0.3 mm and 3.5 µm particle size); flow-rate, 7 µL/min; injection volume, 1.5 µL; other conditions such as in (a).

sample diluted in a solvent with a lower eluting strength than the elution solvent. In our case, the sample was diluted in a Tris–HCl buffer and the injected volume was varied from 0.5 to 8 µL. Fig. 3 shows a significant increase in sensitivity without loss in efficiency when increasing the injected volume. Thus, an injection volume of 5 µL was selected in order to avoid possible problems with column pressure and column blocking if using higher injection volumes.

On the other hand, despite AcN is the preferred organic modifier for peptide separation, due to the world shortage in AcN and its subsequent high cost, other organic solvents (THF, MeOH, and 2-propanol) were employed as alternatives in the mobile phase. The use of THF or 2-isopropanol did not enable a suitable peptide separation while MeOH resulted in a similar separation that the obtained with AcN. In addition, more sensitivity was obtained when MeOH was used in comparison with the other organic modifiers. Thus, mobile phases consisting of 0.1% (v/v) TFA in water (solvent A) and in MeOH (solvent B) were chosen. The gradient was next optimized for a better separation of peptides being a gradient from 5 to 75% B in 120 min the selected gradient. Moreover, the influence of column temperature on the separation was studied in the range 20–60 °C. Peptide retention decreased and efficiency enhanced at higher temperatures, selecting 60 °C for further experiments. Finally, the peptide profile obtained with the optimized conditions and the Zorbax SB column (150 mm × 0.5 mm ID, 5 µm particle size) is shown in Fig. 4. Despite the optimization of these chromatographic conditions enabled an improvement in comparison with initial chromatographic separation (Fig. 1), the complexity of the sample and the high number of peptides present required higher separation efficiency. Therefore, a column with a lower particle size (3.5 µm) and a smaller column diameter (d_c = 0.3 mm) was next tested. For an adequate comparison of these two columns the down-scaling approach $(d_{c_1}/d_{c_2})^2$ was used to calculate the parameters with the new 0.3 mm column, resulting a flow-rate of 7 µL/min and an injection volume of 1.5 µL. Fig. 4 compares the chromatograms obtained with both columns observing an important improvement in the efficiency of the separation with the smaller diameter column.

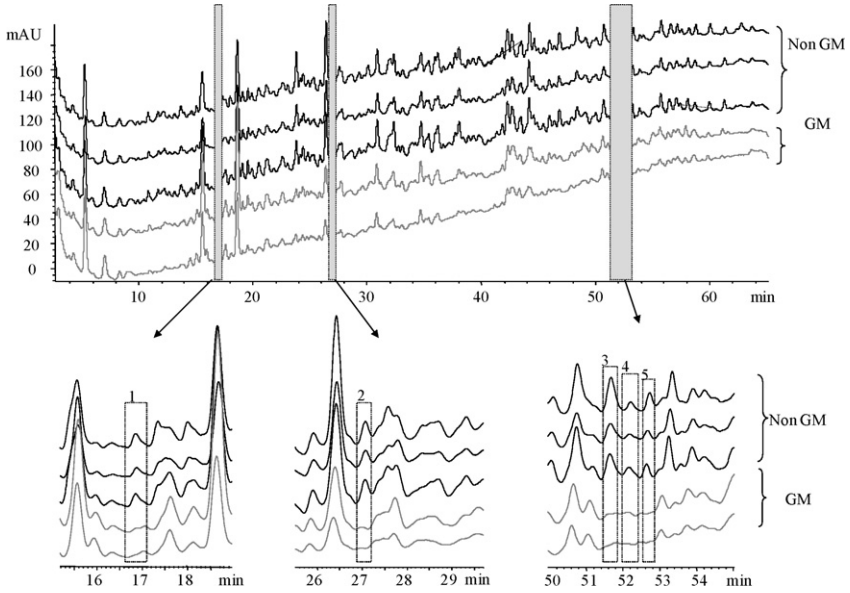

Fig. 5. Peptide profiles obtained from three conventional and two transgenic soybeans detected at a wavelength of 210 nm. Ultrasonic conditions as in Fig. 3. Chromatographic conditions: capillary column, Zorbax 300 SB (150 mm × 0.3 mm and 3.5 µm particle size); mobile phase, 0.1% (v/v) TFA in water (solvent A) and in MeOH (solvent B); gradient 5–75% B in 60 min; flow-rate, 14 µL/min; temperature, 60 °C; injection volume, 1.5 µL.

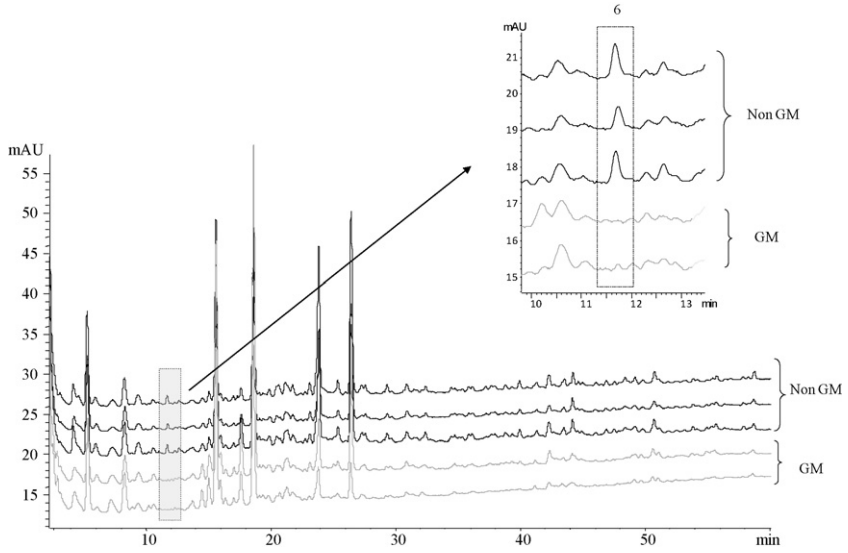

Fig. 6. Peptide profiles obtained with three conventional and two transgenic soybeans detected at a wavelength of 280 nm. Ultrasonic and chromatographic conditions were kept as in Fig. 5.

Furthermore, in order to decrease the separation time (from 120 to 60 min), the flow-rate was increased from 7 to 14 μL/min (with the subsequent decrease in the gradient time to keep the gradient slope).

3.3. Application of the optimized methodology

The developed methodology was next applied to the profiling of soybean peptides in different transgenic and non-transgenic soybean cultivars and in different pigmented beans commercialized as soybeans. In a previous work, we showed the substantial equivalence of genetically modified (GM) and conventional soybeans based on peptide profiles obtained employing a standardized digestion protocol and separating peptide extracts in a CE-MS system [18]. However, the high number of co-migrating peptides produced suppression in the ionization of less abundant peptides and, thus, the substantial equivalence was only demonstrated with the most abundant peptides. The optimized method was applied to the profiling of GM and conventional soybeans peptides observing in Figs. 5 and 6 the peptide maps obtained at 210 and 280 nm, respectively. At first glance, similar peptide profiles could be observed for GM and conventional samples. However, when a deeper comparison of peptide profiles was performed, different potential marker peptides were observed. In fact, there were five peaks that could be observed in conventional soybeans but not in transgenic soybeans at 210 nm (see Fig. 5), and one peak at 11.5 min that appeared only in conventional soybeans and not in GM soybeans at 280 nm (see Fig. 6). Thus, the optimized methodology could be a very interesting tool for the differentiation between transgenic and non-transgenic soybeans.

Moreover, the optimized method was also applied to the profiling of different pigmented seeds commercialized as soybeans: three yellow, one white and two green. Fig. 7 shows, as an example, the chromatograms corresponding to one yellow, one white, and one green bean. Yellow and white soybeans yielded similar profiles while beans commercialized as green soybeans yielded a different map. These results confirmed, again, the suspicion previously observed [26] that these green beans could not be soybean despite they were commercialized as it.

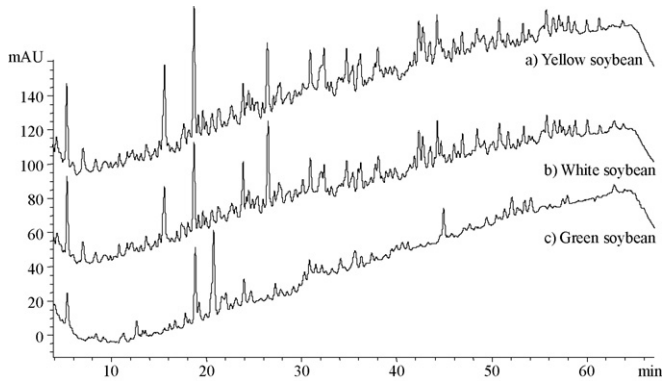

Fig. 7. Comparison of peptide profiles corresponding to different pigmented beans commercialized as soybeans. Ultrasonic and chromatographic conditions were kept as in Fig. 5.

4. Concluding remarks

A new methodology based on high intensity ultrasonic enzymatic digestion and capillary-HPLC has been developed for the profiling of soybean peptides. This method enabled to digest proteins in a record time of 1 min which constituted a significant reduction since tryptic digestions used to take overnight. The use of capillary-HPLC using a 300 μm diameter column packed with 3.5 μm particle size allowed obtaining a suitable efficiency in the separation of peptide extracts using the following chromatographic conditions: mobile phase, 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA in MeOH (solvent B); elution gradient, 5–75% B in 60 min; column temperature, 60 °C; flow-rate, 14 μL/min; injection volume, 1.5 μL. The on-column focusing enabled an increase in the sensitivity without a significant loss in efficiency. The potential of the method was demonstrated by its application to the differentiation between conventional and GM soybeans and to the study of different pigmented beans commercialized as soybeans.

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