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Fast determination of the functional peptide soymetide in different soybean derived foods by capillary-high performance liquid chromatography

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ABSTRACT

The determination of bioactive peptides derived from food sources is gaining special attention in last years, due to their ability to promote health and their potential to reduce the risk of chronic diseases. In this work, a new analytical methodology has been developed enabling for the first time the determination of soymetide, a new immunostimulating peptide derived from soybean, in different soybean derived foodstuffs. Capillary-HPLC was employed for peptide separation after accelerated tryptic digestion of soybean proteins. Two different capillary-HPLC columns were tested and chromatographic separation was optimized in each case. The use of a 300 μm fused-core technology C18 column enabled a suitable separation of soymetide from the other peptides in less than 18 min. Different analytical characteristics of the method were evaluated: selectivity, linearity, accuracy, precision, limit of detection and quantitation, and stability. The developed method was applied to the determination of soymetide content in different soybean dairy-like products for human consumption (powdered milks and infant formulas).

1. Introduction

Traditionally, the dietetic value of a protein was evaluated by its nutritional quality which was based on the bioavailability of essential amino acids and its digestibility. Nowadays, an additional value for the determination of the nutritional quality of proteins is based on their health promoting properties. In this sense, there are many proteins derived from food sources with biological activity, as for example lactalbumin from milk which presents immunomodulation and anticarcinogenic activities [1,2] or lectin and Bowman Birk trypsin inhibitor from soybean which have shown anticarcinogenic activity [3,4]. In addition to proteins, food peptides can also yield health promoting activities. In some cases, peptides are as such in the food (e.g. lunasin in soybean). In other occasions, peptides are encrypted in the amino acid sequence of a protein and are released during the industrial processing or by the *in vivo* digestion [5]. Several articles have been published describing bioactivities of different food-derived peptides [6–9]. This fact has made food to be in the spotlight in the search of this kind of therapeutic peptides.

Soybean is a leguminous with high protein content which constitutes one of the most consumed foods in the world [10]. In addition to be a very cheap source of proteins, soybean consumption has always been associated with health benefits. Soymetide-13 (MITLAIPVNKPGR) is a peptide present in soybean exhibiting immunostimulating properties. Soymetide is released by trypsin digestion of soybean β-conglycinin, which is one of the major components of soybean proteins [11]. This tridecapeptide exhibits affinity for the receptor of the chemotactic peptide fMLP (formyl-Met-Leu-Phe). This fact seems to contribute to a rapid response to bacterial infection, leading to bacterial death by phagocytosis and ROS-induced bactericidal effects [12]. Therefore, dietary ingestion of soybeans is expected to improve immune system functions. For this reason, once demonstrated the capability of soymetide to improve physiological functions, there is a clear need for its determination in soybean products for human consumption.

Determination of peptides in food products is challenging due to the huge quantity of peptides that can be obtained from a food matrix [13]. In comparison with the existing methodologies for purification and characterization of bioactive peptides, the works focused to the determination of these peptides are very scarce. In most cases, HPLC has been the technique most widely employed. More recently, powerful analytical techniques such capillary LC and mass spectrometry (LC–MS and MS/MS) have also been applied [14]. Capillary HPLC offers significant advantages as a reduced consumption of solvents, reduced waste, [15,16] and a greater sensitivity [16,17]. Another significant progress in relation with HPLC has been the advent of new stationary phases enabling

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higher resolutions and reduced analysis times. In fact, columns filled with sub-2 μm particles have resulted in improved efficiency, linear velocity, and mass transfer [18]. However, the pressure increase obtained with these sub-2 μm particles columns usually requires the use of special instrumentation (Ultra-performance liquid chromatography systems) able to support the delivered high backpressure. Fused-core particle technology has been introduced as an alternative to sub-2 μm particles to achieve high separation efficiencies with low backpressure [19,20]. On the other hand, the use of ultrasonic energy is a useful tool to accelerate enzymatic digestions [21]. This new approach is being implemented in proteomics labs due to the strong decrease on digestion times which generally can be reduced from overnight to some minutes [22,23]. However, the applications of these approaches to real samples, as food samples are still limited.

The aim of this work was to develop an efficient and sensitive analytical methodology enabling, for the first time, the determination of the bioactive peptide soymetide in different soybean derived foods for human consumption. Capillary-HPLC using a new solvent in combination with a fused-core technology column was employed for peptide separation after ultrasonic assisted enzymatic digestion of soybean proteins from soybean derived products.

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) and Extrachrom® were supplied by Scharlau (Barcelona, Spain). Trifluoroacetic acid (TFA) and β-mercaptoethanol were 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). Dithiothreitol (DTT), iodoacetamide (IAA), and trypsin (type IX-S from bovine pancreas), employed for the digestion, were from Sigma. Soybean, soybean flour, powdered soybean milk-like products and soybean-based infant formulas were purchased in local markets, in Madrid (Spain). The soybean protein isolate (SPI) with 89.1% of soybean protein (determined by Kjeldahl method) was from ICN (Aurora, OH, USA). Soymetide-13 standard was synthesized by SBS Genetech (Beijing, China). All solutions were prepared with ultrapure water from a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

2.2.1. Capillary high-performance liquid chromatography

The separations were performed in a modular capillary chromatographic system from Agilent Technologies (Pittsburg, PA, USA) consisting of a micro vacuum degasser (model 1100), a capillary LC pump (model 1100), a microwell-plate autosampler with 8 μL injection loop (model 1100), a thermostatted autosampler (model 1100), a thermostatted column compartment (model 1200), and a multiple wavelength detector (model 1200). An HP Chemstation software was used for instrument control and data acquisition. Two different reversed-phase HPLC (RP-HPLC) columns were employed: C18 Zorbax 300SB (150 mm × 0.3 mm ID, 3.5 μm particle size, and 300 Å pore size) from Agilent Technologies and C18 Ascentis Express Fused-Core (150 mm × 0.3 mm ID, 2.7 μm particle size, and 90 Å pore size) from Supelco (Bellefonte, PA, USA). The optimized conditions with Ascentis Express column were: flow-rate, 5 μL/min; binary gradient, 35–46% B in 30 min; mobile phases, 0.1% (v/v) TFA in water (phase A) and 0.1% (v/v) TFA in Extrachrom® (phase B). The injection volume was 1.5 μL, the separation temperature was 60 °C, and UV detection was performed at 205, 210, 214, and 280 nm.

2.2.2. Mass spectrometry

Mass spectrometry detection was performed with a Quadrupole Time-of-Flight (Q-TOF) LC/MS series 6530 from Agilent Technologies coupled with a liquid chromatograph (model 1100) from Agilent Technologies. A column Ascentis Express (50 mm × 2.1 mm and 2.7 μm particle size) was employed for peptide separation. The mass spectrometer operated with an ESI Jet Stream source in the positive ion mode and with the analyzer in mode MS (only TOF) scanning in the range 100–3000 *m/z*. The dry gas conditions were 10 L/min and 300 °C, the nebulizer pressure was 30 psi, and the sheath gas flow and temperature were 12 L/min and 400 °C, respectively. The MS conditions were: capillary voltage, 3500 V; fragmentator, 200 V; skim 1 V, 60 V; octapole voltage, 750 V. MS control, data acquisition, and data analysis were carried out using the MassHunter Software from Agilent technologies.

2.3. Protein extraction

2.3.1. Protein extraction from soybean samples

Extraction of soybean proteins was performed dissolving 600 mg of grounded soybean or 1200 mg of soybean dairy-like product in 10 mL of 50 mM Tris–HCl buffer (pH 8.0) and 8 M urea. After sonication for 3 min (J.P Selecta, Barcelona, Spain) samples were centrifuged for 10 min at 4000 × *g*. The supernatant fraction was collected for its enzymatic digestion.

2.3.2. Protein fractionation

Fractionation of soybean proteins was performed using Thanh and Shibasaki method [24]. 6 g of grounded soybean were dissolved in 60 mL of 63 mM Tris–HCl buffer containing 10 mM β-mercaptoethanol (pH 7.8) and sonicated for 20 min. After centrifugation for 20 min (10,000 × *g*, 20 °C) the resulting supernatant was adjusted to pH 6.4. Afterwards, the solution was centrifuged (10,000 × *g* for 20 min, 4 °C) and the supernatant was separated from the pellet (11S fraction). This supernatant was adjusted to pH 4.8 and kept at room temperature for 15 min. Subsequently, the mixture was centrifuged (10,000 × *g* for 20 min, 4 °C) and the resulting precipitate (7S fraction) was collected and dissolved in Tris–HCl buffer (pH 8.0) and 8 M urea for its enzymatic digestion.

2.3.3. Protein digestion

Protein digestion was performed following a procedure described previously [25]. The procedure consisted of treating 1 mL of the protein extract with 100 mL of 50 mM DTT for 20 min at 50 °C. After cooling to room temperature, alkylation of free thiol groups was performed with 110 μL of 100 mM IAA for 5 min. The resulting solution was diluted 10 times in 11 mM $CaCl_2$ (in 50 mM Tris–HCl at pH 8.0) in order to reduce the final urea concentration. Digestion was performed by adding 20 μL of 1 mg/mL trypsin solution to the diluted sample in a glass cooling cell (4 cm × 1 cm ID) connected to a heating circulator set at 37 °C. Finally, the tip of the probe was inserted and maintained at 3 mm from the bottom of the solution and was sonicated during 1 min using the ultrasonic probe at 20% amplitude without pulses. Finally, the digestion reaction was stopped by adding 50 μL of TFA 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.

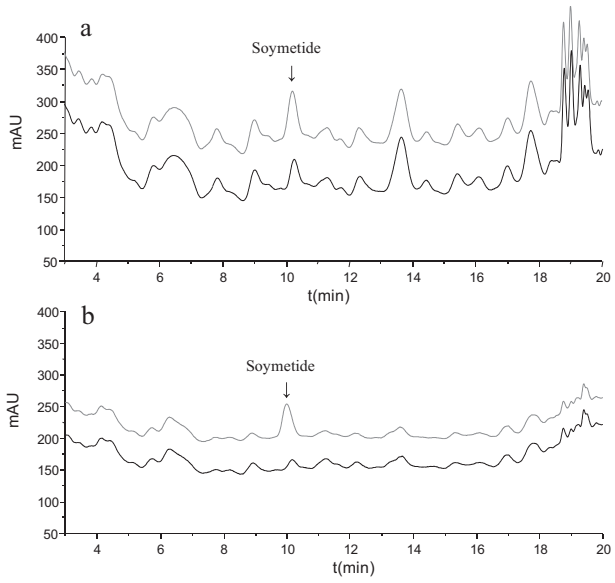

Fig. 1. Chromatograms corresponding to the digested total soybean protein extract (a) and its β-conglycinin fraction (b), spiked (grey line) and non-spiked (black line) with soymetide standard. Chromatographic conditions: gradient, 35–52% B in 15 min; mobile phases, 0.1% (v/v) TFA in water (mobile phase A) and 0.1% (v/v) TFA in MeOH (mobile phase B); flow-rate, 14 μL/min; temperature, 60 °C; injection volume, 1.5 μL; UV detection, 210 nm; column, C18 capillary column, Zorbax 300 SB (150 mm × 0.3 mm and 3.5 μm particle size).

3. Results and discussion

3.1. Evaluation of extraction and digestion conditions

Following the digestion procedure described in Section 2, soybean sample was digested and injected into the capillary-HPLC. The initial chromatographic conditions were: flow-rate, 14 μL/min; binary gradient, 35–52% B in 15 min; mobile phases, 0.1% (v/v) TFA in water (phase A) and 0.1% (v/v) TFA in MeOH (phase B); injection volume, 1.5 μL; separation temperature, 60 °C; UV detection, 210 nm. Fig. 1a shows the chromatograms obtained under these conditions for a digested soybean, spiked and non-spiked with soymetide peptide. The chromatograms obtained were very crowded and the assessment of selectivity for soymetide under these conditions was not possible. In order to decrease the complexity of the sample and to confirm the presence of soymetide in the peptide extract, two different strategies were evaluated: isolation of the protein fraction containing soymetide (β-conglycinin) and ultrafiltration of the peptide extract.

β-Conglycinin was isolated using the Thanh and Shibasaki procedure based on isoelectric precipitation of main soybean storage proteins [24]. Next, β-conglycinin fraction was digested and the chromatograms corresponding to the spiked and non-spiked extracts are shown in Fig. 1b. Despite observing a less crowded chromatogram, the assessment of selectivity was still not possible. Another approach to obtain a less crowded chromatogram was to filter the peptide extract through an ultrafiltration filter with a 3 kDa cutoff. Thus, soymetide with a 1.41 kDa molecular weight would pass through the filter while other molecules with molecular weights higher than 3 kDa will remain on the filter. However, no reduction in the number of peaks was observed in this zone of the chromatogram and this approach was rejected. Finally, a standard solution of soymetide was submitted to the same digestion procedure confirming that soymetide did not degrade during this process (data no shown).

In order to confirm the presence of soymetide in the digested extract, some additional experiments using mass spectrometry were conducted. Fig. 2 shows the total ion chromatogram (TIC), the extracted ion chromatogram (EIC) and the mass spectra obtained for a digested soybean protein isolate (SPI). Mass spectra of the peak at 6.99 min showed three major signals that were assigned to soymetide charged with +1, +2, and +3. These results confirmed the presence of this peptide in the digested samples.

3.2. Method optimization

In order to improve the separation and to assess soymetide selectivity, the chromatographic conditions initially established were optimized. The effect of the variation of the detection wavelength (205, 210, 214, 254 and 280 nm) was first evaluated observing the maximum sensitivity at 205 nm. Despite soymetide did not absorb at 280 nm, this wavelength was also registered in order to use it as an indicator of co-eluted peaks.

Because of the huge quantity of peptides obtained in the digestion, a new strategy of separation was evaluated. The use of columns with fused-core particles is a promising alternative to conventional columns. These types of columns are made with superficially porous particles, generally of 2.7 μm, with a 1.7 μm solid core and a 0.5 μm porous shell. Compared to totally porous particles, the fused-core particles have much shorter diffusion paths reducing axial dispersion of solutes and minimizing peak broadening. Other features, such as a very tight particle size distribution and high packing density, result in columns with comparable efficiency to sub-2 μm particle columns without the inconvenient of a high backpressure and nearly twice the efficiency possible with 3 μm particles [18]. For this reason, a new capillary column with fused-core particles was tested.

Fig. 3 compares the soymetide separation using a conventional column (C18 Zorbax SB 300 with 3.5 μm totally porous particles) and a fused-core technology column (C18 Ascentis Express with 2.7 μm superficially porous particles). In order to obtain an adequate comparison between these two columns, the same separation conditions were applied and the flow-rate of the new column was selected in order to maintain constant the system pressure (7 μL/min, 300 bar). Better efficiency was achieved with the new column and, as a consequence of the improved separation, soymetide peak splitted into two peaks with the fused-core column. As a result, this new column was selected, and the chromatographic conditions were optimized in order to obtain a suitable separation of soymetide from the other peptides.

Regarding the organic modifier, AcN and Extrachrom® were employed as alternative to MeOH in mobile phase. Worse separations of soymetide from the other peptides were observed with AcN. Extrachrom® is a new organic solvent consisting of MeOH and a mixture of modifiers. The use of this solvent did not improve the efficiency on peptide separation. However, this new solvent allowed the separation of soymetide from other peptides with an apparent suitable selectivity when comparing the signals obtained with both solvents at 280 nm (data no shown). For this reason, Extrachrom® was selected as organic modifier. Next, the elution gradient and the flow-rate were optimized. The use of a binary gradient from 35 to 44% in 30 min at a flow-rate of 5 μL/min was selected. Fig. 4 shows the chromatographic separation of soymetide in a digested soybean (SB) and a powdered soybean milk-like product (SM) under optimal conditions.

3.3. Analytical characteristics of the developed method

The parameters evaluated were selectivity, linearity, precision, accuracy, limits of detection and quantitation, and stability.

Selectivity was demonstrated by comparing the plots resulting from the injection of increasing concentrations of the soymetide standard with the plot obtained when injecting increasing con-

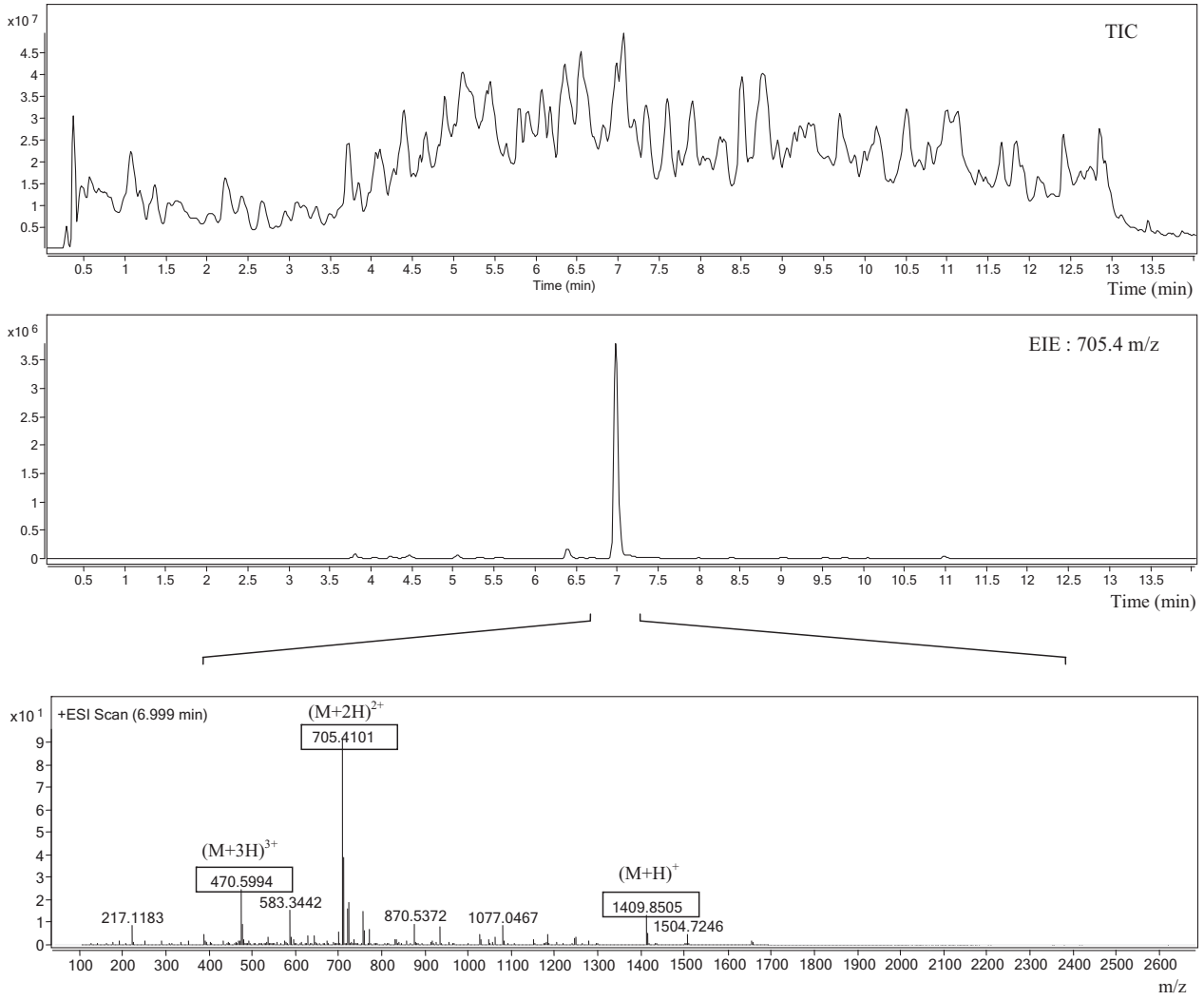

Fig. 2. Total ion chromatogram (TIC), extracted ion chromatogram (EIC) and mass spectra of soymetide obtained from a digested SPI. Chromatographic conditions: gradient, 5–30% B in 10 min; mobile phases, 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in AcN (mobile phase B); flow-rate, 0.5 mL/min; temperature, 60 °C; injected volume, 5 μL; column Ascentis Express (50 mm × 2.1 mm and 2.7 μm particle size), MS conditions as in experimental section.

centrations of a soybean product. Two different comparisons were conducted: one with a soybean sample (SB) and other with a powdered soybean milk-like product (SM). Table 1 groups the slopes and intercepts of the plots obtained using the soymetide standard and the soybean products. Comparison of slopes and intercepts by *t*-test (p value < 0.05) demonstrated that there was not any significant difference between the soymetide plot and the other plots. These results confirmed that the selected peak was soymetide and that no coelution could happen. Any impurity of this peak in the digested sample would have made that de slope of the plot when using the soybean products was different to the slope observed when using the soymetide standard. In addition, the slopes of the calibration plots obtained by the external standard method and by the standard additions method corresponding to two samples (SB1 and SM1) were compared concluding that the method did not show matrix interferences (see Table 1). Therefore, the external standard calibration method was used for the quantification of soymetide in different soybean dairy-like products for human consumption.

The *linearity* of the method was assessed in the range 0.7–100 μg/mL. Correlation coefficients higher than 0.999 were

Table 1
Calibration curves obtained by the external standard calibration method and by the standard additions calibration method for two representative samples: a soybean (SB) and a soybean milk-like product (SM). In the case of external calibration method the plots were obtained from the injection of increasing concentrations of soymetide standard (SS) and two soybean products, a SB and a SM.

	$b \pm t\, s_b$	$a \pm t\, s_a$	r
External calibration method			
With SS (n = 5)	237,061 ± 5580	−106 ± 229	0.9995
With SB (n = 5)	226,652 ± 16231	−31 ± 77	0.9991
With SM (n = 5)	228,727 ± 14153	−62 ± 65	0.9992
Standard additions calibration method			
With SB (n = 4)	235,251 ± 19688	294 ± 106	0.997
With SM (n = 4)	231,247 ± 10540	328 ± 57	0.9992

s_a, standard error of the intercept; s_b, standard error of the slope; r, correlation coefficient.

observed and the intercept did not significantly differ from cero (see Table 1).

Accuracy was estimated by means of recovery assays. The recovery of soymetide was evaluated by adding soymetide in every food sample at a concentration level of 2 μg/mL. The recovery values ranged from 88 to 103% with RSDs ≤ 6%.

Precision was evaluated as instrumental repeatability and intermediate precision (see Table 2). Repeatability was determined from six repeated injections of a standard solution of soymetide at two different concentration levels obtaining values of RSD for peak area of 2.9% for 0.7 μg/mL of soymetide and 0.4% for 100 μg/mL of soymetide (see Table 2). Intermediate precision was evaluated with soymetide standard solutions and with three different soybean samples: a soybean (SB1), a soybean milk-like product (SM1), and a soybean based infant formula (IF1). Three replicates of these solutions were injected by triplicate in three different days. RSD values were ≤ 8% in all cases (see Table 2).

The *limits of detection* (LODs) and *quantitation* (LOQs) were calculated as the minimum concentration yielding an S/N ratio equal to 3 and 10 times, respectively. LOD and LOQ for soymetide were 0.20 μg/mL and 0.66 μg/mL, respectively, for the solution injected These values allow quantifying 0.14 mg of soymetide per gram of product for soybean sources, and 0.07 mg of soymetide per gram of product in the case of commercial products.

Finally, *stability* of the sample was evaluated by injecting the same digested soybean sample after 0, 12, 24, and 48 h remaining at room temperature. No significant difference was observed in the sample in this period of time.

3.4. Analysis of soybean dairy-like products

Once demonstrated the suitability of the method for the determination of soymetide in food samples, different soybean dairy-like products for human consumption were analyzed. By one hand, three powdered soybean milk-like products and four soybean infant formulas and by other, different soybean sources employed for the manufacture of soybean foodstuffs (soybeans, soybean flour, and soybean protein isolate). Especial interest has the determination of the immunostimulating activity in infant formulas since they are used for babys who have not a mature immune system. Table 3 summarizes the quantitative levels of soymetide in the analyzed samples. The first column shows the mg of soymetide obtained per gram of product. Due to the differences in protein contents in each soybean product (ranging from 11 to 90% of product) the quantity of soymetide was also expressed as mg of soymetide per gram of protein in the product. As it can be observed, soymetide contents ranged from 1.31 to 1.85 mg soymetide per gram of protein in the soybean sources and from 0.28 to 1.29 mg soymetide per gram of protein in the soybean dairy-like products, in all cases values above or equal the LOQ of the method. In this point, it is important to highlight, that the concentration required for 50% of maximum phagocytotic activation (IC_{50}) has been established in approximately 1 μM in plasma [11] which can be corresponded to an ingestion of approximately 4 mg of soymetide for an adult. Therefore, in the case of powdered milks would need to consume

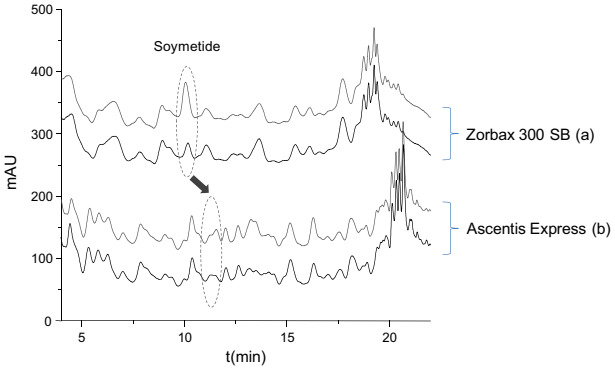

Fig. 3. Chromatograms corresponding to the digested soybean, spiked (grey line) and non-spiked (black line), obtained using different capillary-columns. Chromatographic conditions: (a) C18 capillary column, Zorbax 300 SB (150 mm × 0.3 mm and 3.5 μm particle size); mobile phases, 0.1% (v/v) TFA in water (solvent A) and in MeOH (solvent B); gradient 35–52% B in 15 min; flow-rate, 14 μL/min; temperature, 60 °C; injection volume, 1.5 μL; UV detection, 205 nm; (b) C18 Fused-Core capillary column, Ascentis Express (150 mm × 0.3 mm and 2.7 μm particle size); flow-rate, 7 μL/min; injection volume 1.5 μL; other conditions such as in (a).

Table 2
Precision of the developed method for the determination of soymetide in soybean food products.

	Concentration	Repeatability (n = 6)		Intermediate precision (n = 9)	
		Migration time (RSD %)	Area (RSD %)	Migration time (RSD %)	Area (RSD %)
Soymetide	0.7 μg/mL	0.54	2.9	3.9	4.9
	100 μg/mL	0.4	0.4	5.9	1.2
Soybean				2.5	3.9
Soybean milk-like product				0.6	4.8
Soybean infant formula				7.9	3.9

Table 3
Amounts of soymetide determined in different commercial soybean products for human consumption and soybean sources.

Soybean product	Soybean source	$mg_{soymetide}/g_{product}$ (average[a] ± s)	$mg_{soymetide}/g_{protein}$[b] (average[a] ± s)
Soybean (SB)		0.52 ± 0.01	1.33 ± 0.05
Soybean flour (SF)		0.54 ± 0.02	1.31 ± 0.03
Soybean protein isolate (SPI)		1.66 ± 0.02	1.85 ± 0.03
Soybean milk-like product			
SM 1	SB	0.18 ± 0.01	0.60 ± 0.05
SM 2	SPI	0.140 ± 0.007	0.96 ± 0.05
SM 3	–	0.070 ± 0.01	0.28 ± 0.03
Soybean infant formula			
IF 1	SPI	0.15 ± 0.01	1.29 ± 0.09
IF 2	SPI	0.13 ± 0.01	1.0 ± 0.1
IF 3	SPI	0.106 ± 0.004	0.90 ± 0.04
IF 4	SPI	0.131 ± 0.008	0.92 ± 0.06

[a] Average and standard deviation obtained by the injection of two individual samples by duplicate (n = 4).
[b] Grams of protein indicated in the label every product.

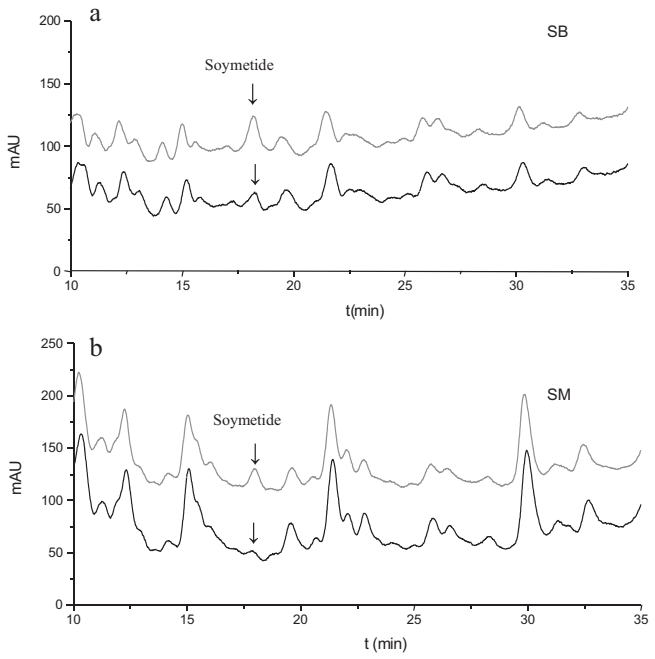

Fig. 4. Chromatograms obtained for the digested soybean (SB) (a) and powdered soybean milk-like product (SM) (b), spiked (grey line) and non-spiked (black line), under optimum conditions. Chromatographic conditions: capillary column, C18 Ascentis Express (150 mm × 0.3 mm and 2.7 μm particle size); mobile phases, 0.1% (v/v) TFA in water (solvent A) and in Extrachrom® (solvent B); gradient 35–46% B in 30 min; flow-rate, 5 μL/min; temperature, 60 °C; injection volume, 1.5 μL; detection, 205 nm.

between 22 and 57 g of product and in the case of infant formulas would only need to consume only between 27 and 38 g of product.

On the other hand, note that less quantity of soymetide was observed in the processed products. Moreover, less quantity of soymetide was obtained for those samples made from soybeans in comparison with those samples made from soybean protein isolate. Fig. 4 shows the chromatogram for a soybean milk-like product (SM) made from soybeans (a) and for a soybean variety (SB) (b), in which it can be observed the difference on soymetide quantity obtained in each case.

4. Conclusions

This work proposes, for the first time, an analytical methodology for the determination of soymetide, a new immunostimulating peptide derived from soybean, in different soybean foodstuffs. The development of a capillary-HPLC method using a fused-core technology column has enabled the separation of soymetide from other soybean tryptic peptides. Selectivity of the method was demonstrated by comparing the intercepts and slopes obtained when increasing concentrations of soymetide standard and soybean product were injected. Moreover, mass spectrometry analysis of the peptide in the standard sample and in soybean samples confirmed these results. The new method presented good accuracy and precision and was able to quantify only 0.14 mg of soymetide per gram of product for soybean sources, and 0.07 mg of soymetide per gram of product in the case of commercial products. The method was applied to the quantitation of soymetide in different soybean sources employed in the manufacture of soybean foodstuffs (soybean, soybean flour, and soybean protein isolate) and in different soybean dairy-like products (powdered milks and infant formulas). Soymetide contents ranged from 0.28 to 1.85 mg soymetide per gram of protein in soybean products is sufficient to present immunomodulating activity, observing less quantity of soymetide in the processed foods as well as in the products made from the soybean seed instead by soybean protein isolate. The developed method constitutes a very powerful tool to evaluate the immunostimulating activity of different soybean derived products for human consumption and the effects of the processing and the type of raw material employed for their manufacture on this bioactivity.

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