JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

LC-ESI-TOF MS Method for the Evaluation of the Immunostimulating Activity of Soybeans via the Determination of the Functional Peptide Soymetide

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Supporting Information

ABSTRACT: Bioactive peptides content in foodstuffs can seriously vary with many factors such as crop variety, food processing, animal breeding, etc. Because of this variability, quantitative methodologies are required to evaluate the content of bioactive peptides in foodstuffs. Progress in liquid chromatography and mass spectrometry technologies offer a great opportunity for the quantitation of bioactive peptides. This study undertook the development of a liquid chromatography−electrospray ionization−time-of-flight mass spectrometry method using a fused-core technology column for the sensitive and unambiguous determination of the immunostimulating peptide soymetide in soybean varieties. Soymetide precursor protein (α' subunit of β-conglycinin) was extracted with a Tris−HCl buffer (pH 8.0) containing urea and digested with trypsin. Soymetide separation conditions by reversed phase liquid chromatography (ion-pairing reagent, organic modifier, separation column, and elution gradient) and detection by MS were optimized, and a study of soymetide stability was also conducted. The method selectivity having been demonstrated, the linearity, accuracy, precision, and limits of detection and quantitation were evaluated. The developed method enabled the detection and quantitation of soymetide concentrations in the ppb range (7.5 ng/mL and 25 ng/mL, respectively), and about 30 times lower than those detected and determined in a previous work by capillary liquid chromatography with UV detection. These values could allow the quantitation of only 17 μg of soymetide per gram of soybean. The developed methodology was applied to the quantitation of soymetide in different soybean varieties from Europe, Japan, and USA observing great differences in soymetide content that ranged from 40 to 600 μg per gram of soybean depending on the soybean variety.

KEYWORDS: *soybeans, soymetide, immunostimulating peptide, liquid chromatography−mass spectrometry, time-of-flight, quantitation*

INTRODUCTION

Functional foods containing bioactive peptides or proteins are nowadays a very interesting area of research. Many efforts are now focused on the exploration of new functional proteins and peptides in foods and on the design of functional foods containing these bioactive ingredients.[1−4] Nevertheless, the number of works devoted to the quantitative determination of functional proteins or peptides is very low despite that their content can significantly vary with many factors such as crop variety, food processing, animal breeding, etc.

Quantitative determination of a peptide encrypted in a precursor protein requires seriously considering the treatment of the sample.[5−7] In addition to the initial extraction of the parent protein, peptides inside a precursor protein need to be previously released, usually by enzymatic digestion of the parent protein.[5−8] This digestion normally yields a highly complex extract containing up to hundreds of different peptides at different concentration levels.[9−11] The quantitation of a target peptide in this complex mixture requires its suitable separation and detection. Progress in liquid chromatography (LC) and mass spectrometry (MS) technologies offers a great opportunity for this purpose.[9−12]

LC column developments have been focused on accelerating chromatographic separations and increasing efficiency and sensitivity. Fused-core stationary phases consisting of superficially porous particles have attracted much attention in this regard.[13−16] Generally, these particles consist of a solid core (between 1.7 and 3.3 μm) surrounded by a small porous shell (between 0.5 and 0.6 μm). Compared to totally porous particles, fused-core particles exhibit high column efficiency due to shorter diffusion paths, which reduces mass transfer resistance and minimizes peak broadening. These features, in addition to a very tight particle size distribution and a high packing density, result in columns with comparable efficiency to sub-2 μm particle columns and nearly twice the efficiency obtained with 3 μm particle columns.[17−19] An additional advantage of fused-core columns is that, unlike sub-2 μm particle columns, they do not require special instrumentation to cope with high backpressures.

Soybean (*Glycine max.* (L.) Merr.) is a highly valuable legume and constitutes a cheap source of proteins (40−50%). Different bioactive proteins and peptides have been discovered in soybean such as the well-known lunasin. Another singular peptide in soybean is soymetide, with a sequence of 13 amino acids (MITLAIPVNKPGR). Soymetide is the only food peptide exhibiting immunostimulating properties. This peptide is encrypted between the residues 173−185 of the α' subunit of

Received: December 4, 2012
Revised: March 13, 2013
Accepted: March 17, 2013
Published: March 17, 2013

7S globulin (β-conglycinin).[20−22] Therefore, unlike lunasin and other bioactive peptides, soymetide only shows immunostimulating activity when it is released from its parent protein by trypsin digestion.[23,24] Released soymetide exhibits affinity for the *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) chemotactic receptor presents on the surface of neutrophils and macrophages. Since soymetide is an fMLP receptor agonist, a dietary ingestion of soybeans can send to the immune system signals similar to a bacterial infection, stimulating and strengthening it. This fact seems to contribute to a rapid response to bacterial infection, leading to bacterial death by phagocytosis and ROS-induced bactericidal effects.[25] Although different works have demonstrated soymetide capabilities, not much attention has been focused on the determination of this peptide in soybean and derived products. Indeed, only a previous work developed by our research team has determined this peptide in soybean dairy-like products (powdered milks and infant formulas). In that case, a capillary high performance liquid chromatography method using UV detection was employed.[21] Despite the interest of this first quantitative approach, lower detection and quantitation limits would enable a further knowledge on soymetide contents in other samples. Moreover, the study of the effect of the soybean genotype on the soymetide content would also be of great interest to select those varieties yielding higher soymetide content. These varieties would be preferred for the isolation of soymetide for the manufacture of functional foods and nutraceuticals.

The aim of this work was to develop a selective and sensitive analytical methodology enabling the determination of the bioactive peptide soymetide in different soybean varieties by liquid chromatography−electrospray ionization−time-of-flight (LC-ESI-TOF) mass spectrometry (MS) using a fused-core technology column.

MATERIALS AND METHODS

Chemicals and Samples. Acetonitrile, methanol, trifluoroacetic acid, and acetic acid of HPLC grade (Sigma, St. Louis, MO, USA) were used for the preparation of mobile phases. Calcium chloride was from Panreac (Barcelona, Spain). Hydrochloric and formic acids, hydroxymethylaminomethane (Tris), and urea were from Merck (Darmstadt, Germany). Dithiothreitol, iodoacetamide, bovine serum albumin, and trypsin type IX-S from bovine pancreas were from Sigma. Nine different soybean varieties [Fred (France), Flora (France), Zolta Przebedowska (Poland), Tokachi (Japan), Tokachi Napaha (Japan), Nagaha-jiro (Japan), Mrit (USA), Harosoy 63 (USA), and Evans (USA)] were from the germplasm collection of the CRF-INIA (Centro de Recursos Fitogenéticos del Instituto Nacional de Investigaciones Agrarias, Madrid, Spain). A soybean protein isolate (SPI) with a protein content of 89.1% (determined by Kjeldahl analysis) 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).

Protein Extraction and Digestion. Soybeans were ground using a domestic miller followed by partial moisture removal in a thermostatted oven for 3 h at 50 °C. Ground soybeans were sieved using different mesh sizes to obtain particle sizes ranging 0.05−0.6 mm.

Extraction of soybean proteins was carried out using a method previously described.[21] For that purpose, 180 mg of ground soybean were dissolved in 3 mL of 50 mM Tris−HCl buffer at pH 8.0 containing 8 M urea. After sonication for 3 min, samples were centrifuged for 7 min at 4000*g*. The supernatant fraction was collected for its enzymatic digestion.

Protein digestion was performed following a procedure described previously.[26] The procedure consisted of treating 1 mL of protein extract with 100 μL of 50 mM dithiothreitol for 20 min at 50 °C. After cooling to room temperature, alkylation of free thiol groups was performed by the addition of 110 μL of 100 mM iodoacetamide for 5 min. The resulting solution was diluted 10 times in 11 mM CaCl2 (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 for 12 h at 37 °C. Finally, the digestion reaction was stopped by adding 50 μL of trifluoroacetic acid, and final solutions were filtered through 0.45 μm pore size regenerated cellulose Titan 2 filter membranes (MicroSolv Technology Corp., Eatontown, NJ, USA) prior to injection into the LC system.

LC Separation. An 1100 series LC (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode-array detector, an automatic injector, a quaternary pump, and a thermostatic column compartment was employed. Preliminary experiments were performed with a 100 mm × 2.1 mm i.d., 10 μm, POROS R2/10 perfusion column (Perseptive Biosystems, Framingham, MA, USA). For that purpose, acetic acid and formic acid at three concentrations (0.1, 0.3, and 0.5%) and two different organic modifiers (acetonitrile or methanol) were employed with the following gradient: 5−30% B in 10 min, 30−95% B in 3 min, 95% B for 5 min to clean the column, 95−5% B in 1 min, and 5% B for 5 min for the re-equilibration of the column at the initial conditions; mobile phase, water-organic modifier containing an ion-pairing reagent; flow rate, 0.5 mL/min; injected volume, 3 μL; temperature, 50 °C. Final quantitation was carried out with a 100 mm × 2.1 mm i.d., 2.7 μm, Ascentis Express Fused-Core peptide ES-C18 column with a 5 mm × 2.1 mm i.d. peptide ESC18 Ascentis Express guard column of the same material (Sigma, St. Louis, MO, USA). The optimized conditions with this column were as follows: flow rate, 0.5 mL/min; mobile phases, 0.3% (v/v) acetic acid in water (phase A) and 0.3% (v/v) acetic acid in acetonitrile (phase B); binary gradient, 15−20% B in 4 min, 20−95% B in 3 min, 95% for 5 min to clean the column, 95−15% B in 1 min, and 15% B for 5 min for the re-equilibration of the column at the initial conditions; injected volume, 3 μL; temperature, 50 °C. The dead volume of the system was equivalent to 0.5 min, and the delay time in the gradient was 2.6 min.

In order to ensure the identity of soymetide, hydrolysates and spiked hydrolysates were injected and compared.

MS Detection. MS detection was performed in a quadrupole time-of-flight (Q-TOF) series 6530 (Agilent Technologies, Palo Alto, CA, USA) equipped with Agilent Mass Hunter software that was used for MS control, data acquisition, and data analysis. Optimized ESI parameters were as follows: capillary voltage, 3500 V; nozzle voltage, 1500 V; drying gas conditions, 10 L/min and 350 °C; nebulizer pressure, 2.7 bar; sheath gas conditions, 12 L/min and 400 °C. Other optimized MS parameters were as follows: fragmentator, 150 V; skimmer, 60 V; octapole voltage, 750 V. Spectra were acquired in the positive ion mode at 2 GHz over the range m/z 100−1700 (extended dynamic range) with an acquisition rate of 2 spectra/s. Purine with an $[M + H]^+$ ion at m/z 121.0509 and an Agilent compound (HP0921) yielding an ion at m/z 922.0098 were simultaneously introduced, and used as internal standards throughout the analysis.

Data Treatment. Soymetide identification and amino acid sequencing were carried out from the MS/MS spectra using Mascot database.

Detection and quantitation was performed from the extracted ion chromatogram (EIC) obtained by the extraction of signals from the most intense isotopic peak of the $(M + 2H)^{2+}$ and $(M + 3H)^{3+}$ ions of soymetide (m/z 470.6171 and m/z 705.4206) using as extraction window ±25 ppm.

Signal-to-noise (S/N) ratio was calculated by Mass Hunter MS software establishing the peak height as the signal and the noise as five times the standard deviation of the background. Detection (LOD) and quantitation (LOQ) limits were calculated as the concentration yielding a S/N ratio of 3 and 10, respectively.

RESULTS AND DISCUSSION

LC/MS Method Development. A previous research work of our group described the separation of soymetide from a

tryptic soybean digestion extract using micro-LC and UV detection.[21] Experimental conditions selected in that case consisted of a mobile phase containing trifluoroacetic acid as ion-pairing reagent. Since trifluoroacetic acid is a known strong signal suppressor in MS,[27] some preliminary experiments using other ion-pairing reagents were first conducted to select separation conditions that were compatible with MS detection. Different mobile phases used in reversed phase LC/MS were optimized based on the sensitivity of the soymetide standard peak, estimated from the S/N ratio in the EIC, and the percentage of mobile phase B at which soymetide eluted (see Supporting Information). For that purpose, mobile phases containing acetic acid or formic acid as ion-pairing reagents at three different concentrations (0.1, 0.3, and 0.5%) and with two different organic modifiers (acetonitrile or methanol) were employed. A 0.3% (v/v) acetic acid solution in acetonitrile yielded the highest sensitivity and recovery. These conditions were suitable to observe the mass spectrum of the soymetide standard shown in Figure 1. The two highest peaks

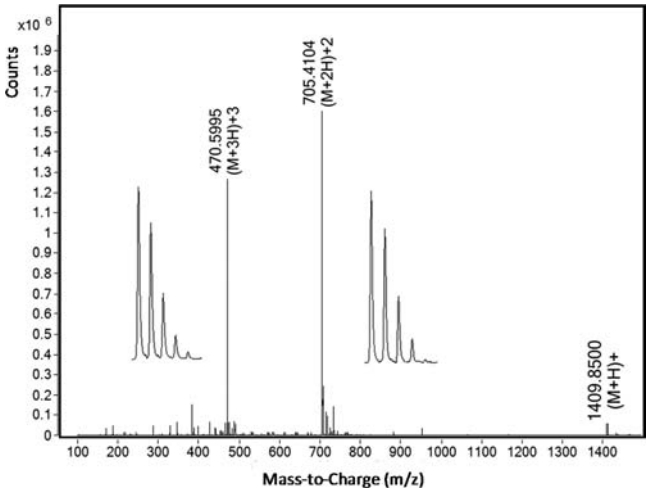

Figure 1. Mass spectrum corresponding to a soymetide standard with acetonitrile as organic modifier and 0.3% (v/v) acetic acid as ion-pairing reagent.

corresponded to ions $(M + 3H)^{3+}$ (m/z 470.6171) and $(M + 2H)^{2+}$ (m/z 705.4206). Moreover, a tiny signal attributed to ion MH^+ (m/z 1409.8284) was also observed. In addition, the isotopic clusters of the ions $(M + 3H)^{3+}$ and $(M + 2H)^{2+}$ showed that the highest peaks corresponded to the smaller m/z values.

Second, optimization of ESI parameters was carried out to obtain the best S/N values. For that purpose, a digested extract of SPI was employed. The studied range for parameters that exclusively depended on mobile phase flow rate and composition were as follows: nebulizer pressure (2.0−3.5 bar), drying gas flow rate (8−12 L/min), sheath gas flow rate (6.5−12 L/min), and capillary voltage (3000−4000 V). Other studied parameters also depending on mobile phase flow rate and composition but limited by analyte thermal stability were as follows: drying gas temperature (250−350 °C) and sheath gas temperature (300−400 °C). The optimized ESI parameters obtained with the previously selected mobile-phase composition and a flow rate of 0.5 mL/min were as follows: nebulizer pressure, 2.7 bar; drying gas flow rate, 10 L/min; sheath gas flow rate, 12 L/min; capillary voltage, 3500 V; drying gas temperature, 350 °C; and sheath gas temperature, 400 °C. Finally, ESI parameters only depending on analyte were also studied: nozzle voltage (0−2000 V) and fragmentator voltage (100−200 V). Optimal nozzle and fragmentator voltages were 1500 and 150 V, respectively. Skimmer and octapole voltages were automatically tuned by the instrument, and their values were 60 and 750 V, respectively.

Extracted compound chromatograms (ECC) obtained by Mass Hunter MS software show all coeluting compounds found using a deconvolution process by molecular features. Figure 2A shows the ECC and spectrum obtained for the retention time of soymetide with the optimized LC/MS conditions when a digested extract of SPI was employed to verify the chromatographic separation. As observed in the spectrum, there are two main ions (m/z 464.6309 and m/z 696.4416) in addition to the two ions of soymetide (m/z 470.6167 and m/z 705.4206). These two new signals corresponded to a compound with a molecular mass 18 units lower than soymetide (MITLAIPVNKPGR). MS/MS experiments attributed these signals to a peptide with an amino acidic sequence (LITLAIPVNKPGR) differing in just one amino acid (leucine) with a molecular mass 18 Da lower than methionine. In order to avoid these coelutions, another column with different selectivity and higher efficiency than the perfusion column was tested. Figure 2B shows how the use of a new fused-core column enabled the separation of soymetide from the peptide with 18 Da lower molecular mass and yielded narrower chromatographic peaks. However, the new spectrum obtained along the elution peak of soymetide showed additional smaller ions also coeluting with soymetide. In order to improve the selectivity in the separation of soymetide, the elution gradient was optimized. Figure 2C shows how the optimization of the gradient (15−20% B in 4 min) enabled the separation of soymetide with enough selectivity, there being only a small coelution with another compound whose ions (m/z 488.5936 and m/z 732.3827) were significantly different from those of soymetide.

In order to select an optimum extraction window for maximum selectivity while preserving sensitivity and chromatographic fidelity in the quantitation of soymetide, different extracting widths of ions used as quantifiers in the EICs were tested. According to Figure 1, the highest peak in the $(M + 3H)^{3+}$ isotopic cluster corresponded to m/z 470.6171 and in the $(M + 2H)^{2+}$ isotopic cluster corresponded to m/z 705.4206. These signals were used as quantifier ions, their baseline widths being approximately 120 ppm. Narrower extracting windows were more selective and yielded higher S/N ratios (see in Supporting Information), but resulted in lower calibration slopes and worse determination coefficients (R^2 < 0.9). Therefore, an extracting width of 50 ppm (±25 ppm symmetric extraction window) was established as a good compromise between selectivity and sensitivity for the quantitation of soymetide.

On the other hand, due to the narrower peaks using fused-core column, another important parameter to take into account in quantitative analysis is the data acquisition speed. Generally, 15 measurement cycles across a chromatographic peak are considered adequate for good peak precision (relative standard deviation [RSD] < 1%). Taking into account the selected scanned mass range (m/z 100−1700), a data acquisition speed of 2 spectra/s allowed 18 cycles per peak, which is enough for a suitable peak precision. Higher data acquisition speeds will lead a reduction in the accumulation time of transitions/spectra and, therefore, in a loss of sensitivity.

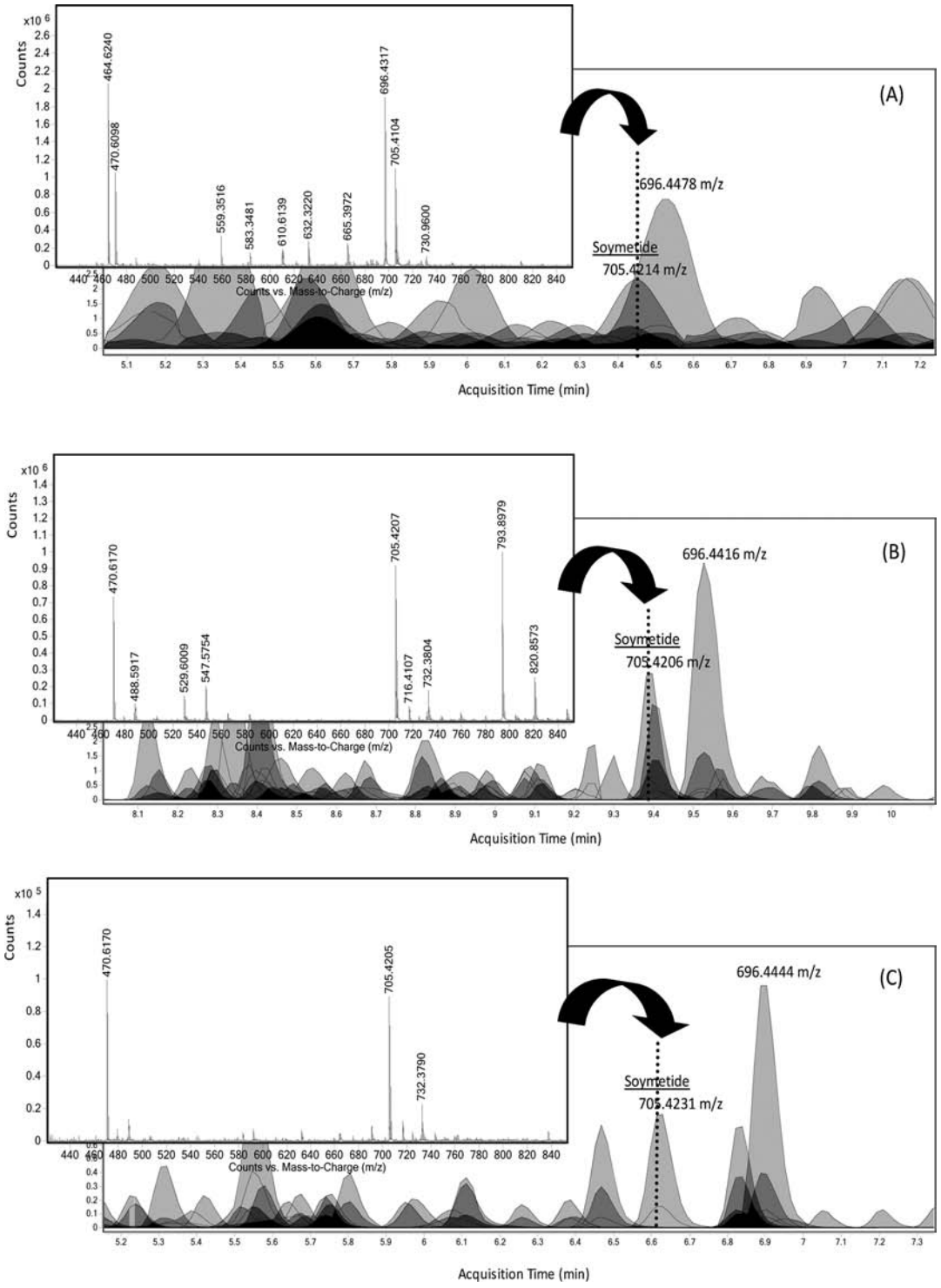

Figure 2. Extracted compound chromatograms generated by deconvolution and mass spectra over elution time of soymetide corresponding to digested extract of SPI analyzed with different columns and gradients. (A) POROS R2/10 perfusion column. (B) Ascentis Express Fused-Core peptide ES-C18 column with the same gradient as in panel A. (C) Ascentis Express Fused-Core peptide ES-C18 column with a new optimized gradient.

Finally, we investigated the possibility of improving the sensitivity in the quantitation of soymetide using MS/MS experiments and ions m/z 470.6 and m/z 705.4 as precursors. For that purpose, a collision cell energy providing few but intense fragment ions while preserving 10% of intact precursor ion was selected within the range 15−30 V. Since the best sensitivity obtained by MS/MS (energy at 17 V in collision cell) was less than that observed in the MS mode, the MS/MS experiments for the quantitation of soymetide were discarded due to the high chromatographic selectivity and best S/N in MS mode.

Development and Analytical Characterization of the Quantitative Method. In the study of soybean sample, the results showed a decrease in soymetide signal when mesh sizes

dx.doi.org/10.1021/jf305159m | J. Agric. Food Chem. 2013, 61, 3611−3618

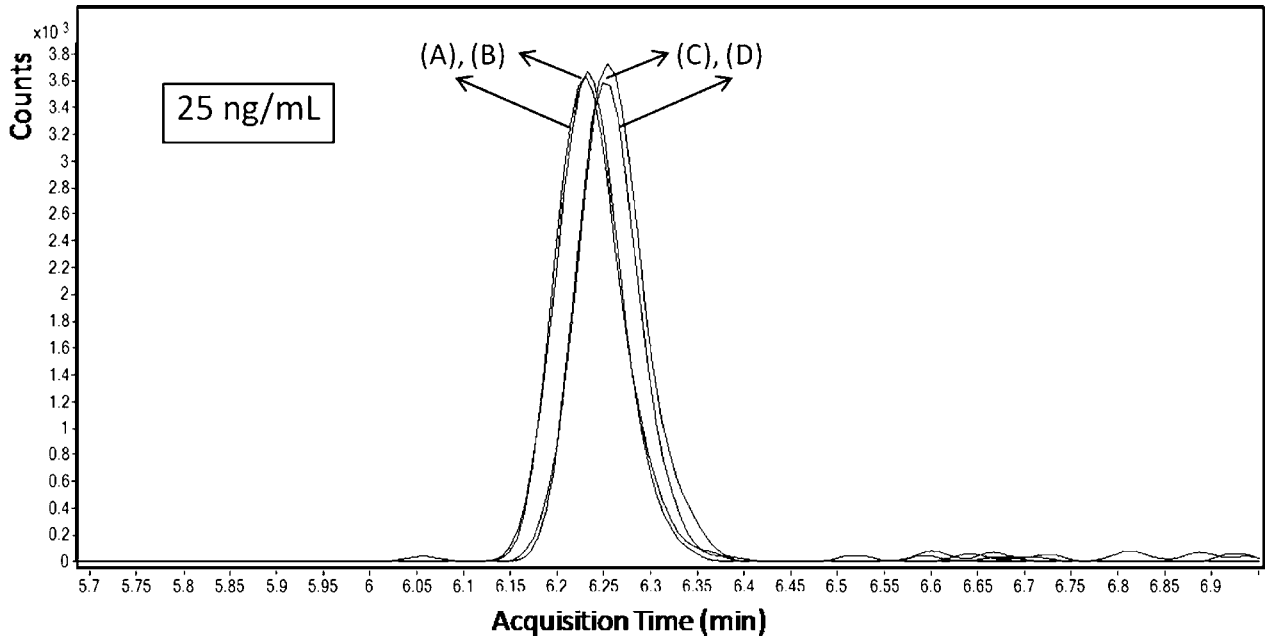

Figure 3. Extracted ion chromatograms corresponding to two replicates (A and B) of a soybean sample without content in soymetide (Harosoy 63) spiked with soymetide standard (25 ng/mL), and two replicates (C and D) of a soymetide standard solution (25 ng/mL). Experimental conditions as in Figure 2C.

higher than 0.2 mm were employed. Therefore, a mesh size ranging from 0.05 to 0.2 mm was selected.

Moreover, a stability study of the soymetide standard (100 ng/mL prepared in an aqueous solution) and the digested soybean sample was carried out. For that purpose, standards and digested samples were kept at room temperature over the range 0−72 h. No significant difference was observed in the case of the digested soybean sample up to 72 h storage time. However, a significant loss of signal was observed in the case of the soymetide standard which was attributed to its adsorption on the vial walls, regardless of the use of plastic or glass containers. In order to avoid this problem, different soluble media were tried: water, acetic acid (0.3%), Tris−HCl (pH 8.0), acetonitrile, and bovine serum albumin solutions (100 ng/mL and 1000 ng/mL). Best results were obtained using 0.3% (v/v) acetic acid in glass vials. Furthermore, three different storage temperatures were also tested (room temperature, 4 °C, and −22 °C), with observation of no signal reduction when the standards were kept at least 24 h at 4 °C or more than 72 h at −22 °C.

Once method selectivity was demonstrated, the following analytical characteristics were studied (see Supporting Information): linearity, accuracy, precision, and LOD and LOQ. Linearity was assessed using six standard solutions in the range 25−1000 ng/mL and observing a good linear correlation (R^2 = 0.9991). Moreover, intercept did not significantly differ from zero (P < 0.05). LOD and LOQ for soymetide were 7.5 ng/mL and 25 ng/mL, respectively. These values could allow the quantitation of 17 μg of soymetide per gram of soybean, which indicates a reduction of more than 27 times in the limits of detection and quantitation previously obtained by our research group.[21] Presence of matrix interferences was evaluated by comparing the slopes obtained by the external standard and the standard addition calibration methods, using three different soybean varieties (Fred, Nagaha-jiro, and Harosoy 63, one of each geographic region). Results showed no significant differences between the slopes (with p-values between 0.2925 and 0.7238), confirming the absence of matrix interference and allowing the use of the external standard calibration method for the quantitation of soymetide in soybean. The accuracy of the analytical method was assessed by evaluating the recovery of different amounts of soymetide added to a soybean sample in which soymetide was not detected, observing recoveries very close to 100% as shown in Figure 3 (between 100.0% and 101.7% with a RSD ≤ 3.7%). Finally, precision was evaluated in terms of instrumental repeatability and intermediate precision. Instrumental repeateability was obtained from six consecutive injections of soymetide standard solutions to LOD and LOQ levels and two soybean samples (Fred and Evans). RSD values lower than 3.0% for standard solutions and 4.2% for soybean samples were obtained. Intermediate precision was obtained by injecting three replicates during three consecutive days of soymetide standard solutions to LOD and LOQ levels and two soybean samples (Fred and Evans). RSD values lower than 4.1% for standard solutions and 6.6% for soybean samples were obtained.

In summary, a LC-ESI-TOF method has been developed enabling the selective and sensitive determination of the immunostimulating peptide soymetide in soybeans in about 15 min. The main advantage of this method over the previous one is that high resolution MS experiments, performed with the TOF analyzer, together with a fused-core technology column and a suitable method optimization, have made possible the unambiguous identification and determination of soymetide in tryptic digestions of soybean. Also the new method presented good accuracy and precision and was able to quantitate 17 μg of soymetide per gram of soybean, which significantly improves the detection and quantitation limits previously obtained for soymetide.

Soymetide Quantitation in Different Soybean Varieties. The developed method was next applied to the determination of soymetide in different soybean varieties: three from Europe, three from Japan, and three from USA. As

dx.doi.org/10.1021/jf305159m | J. Agric. Food Chem. 2013, 61, 3611−3618

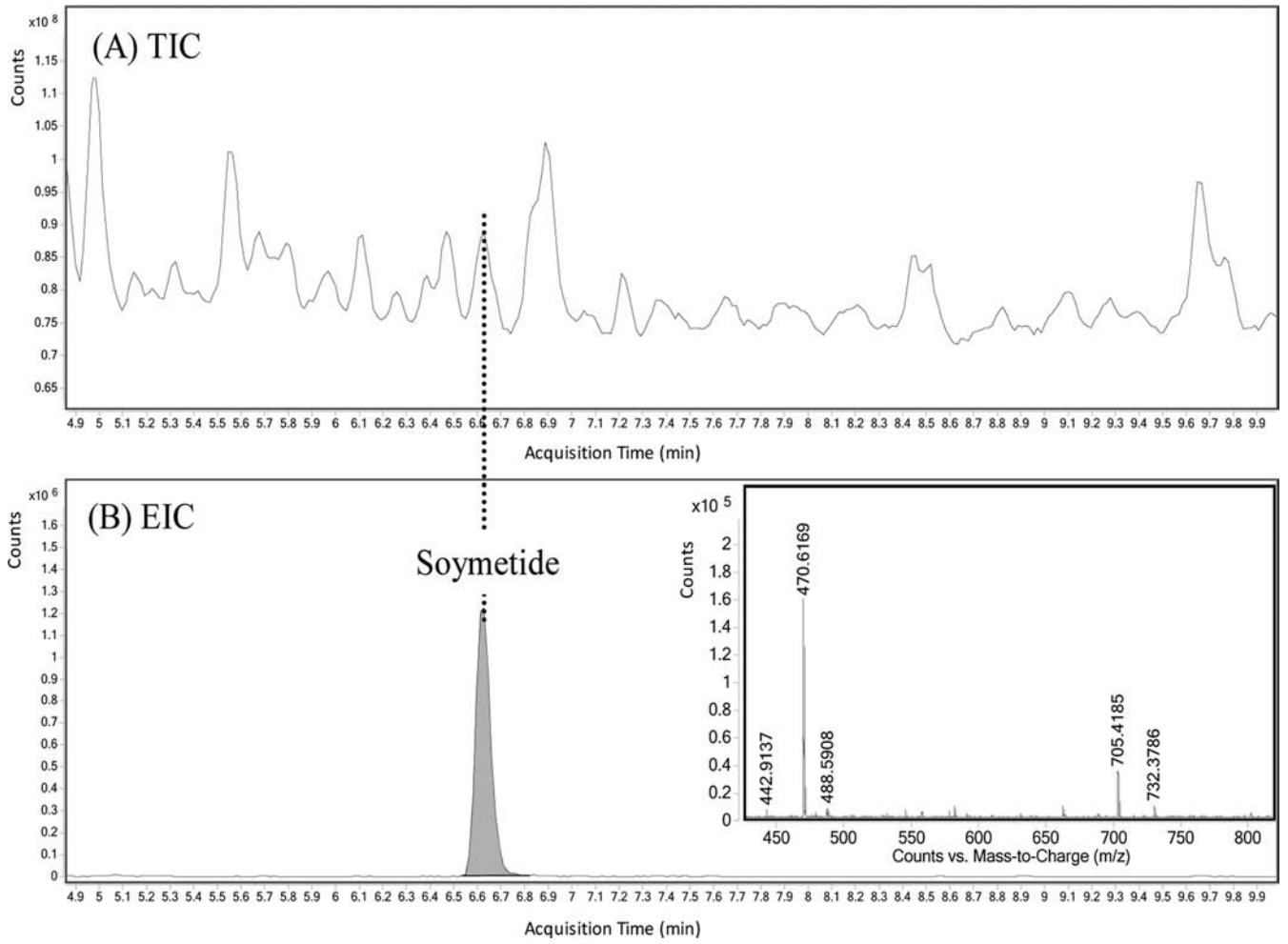

Figure 4. Chromatograms corresponding to a soybean sample (Fred) and the mass spectrum of soymetide peak under the optimized LC and MS conditions. (A) TIC and (B) EIC corresponding to ions m/z 470.6169 and m/z 705.4191 using an extracted width of 50 ppm. Experimental conditions as in Figure 2C.

an example, Figure 4 shows the signal obtained for a soybean sample. Despite sample complexity, the selectivity of the method was demonstrated in the EIC and in the spectrum corresponding to the soymetide peak. This method allowed the unequivocal determination of the immunostimulating peptide soymetide in soybeans and the re-equilibration of the column at the initial conditions in only 15 min.

Results obtained, grouped in Table 1, show that soymetide concentrations ranged from 41.3 to 597 μg/g soybean with the exception of the Harosoy 63 variety, in which case the signal was below the LOD. Great differences in soymetide contents were observed when the method was applied to the determination of soymetide in different soybean varieties. This demonstrated that not all soybean varieties showed the same immunostimulating activity or were equally suitable for subsequent use in the preparation of functional foods or nutraceuticals. No correlation between the soymetide content and the soybean geographical origin was observed. Nevertheless, it should be noted that growing conditions and soybean processing may affect these values. Since the concentration required for 50% of maximum phagocytotic activation (IC50) has been established in approximately 1 μM soymetide in plasma, the ingestion of about 4 mg of soymetide would be necessary for an adult to reach this IC50 value.[20] Taking into account this fact, the required consumption of soybean to obtain an immunostimulating effect is about 100 g for the soybean variety with the minimum soymetide content. However, currently there are no pharmacokinetic studies that

Table 1. Determination of Soymetide in Different Soybean Varieties by LC-ESI-TOF MS

variety	country of origin	soymetide/product[a] (μg/g; av ± SD)	RSD %[b]	soybean consumption to achieve soymetide IC50[c] (g)
Fred	France	70.9 ± 3.9	5.6	63
Flora	France	428 ± 47	11	10
Zolta Przebedowska	Poland	92.1 ± 4.4	4.8	48
Tokachi	Japan	597 ± 59	10	7
Tokachi Napaha	Japan	358 ± 20	5.6	12
Nagaha-jiro	Japan	41.3 ± 2.4	6	108
Merit	USA	48.4 ± 4.7	10.2	92
Harosoy 63	USA	<LOD		
Evans	USA	207 ± 13	6.6	21

[a]Average values and standard deviations for three replicates of each bean extracted and digested each injected in triplicate. [b]RSD calculated with three replicates of each bean extracted and digested, injected in triplicate. [c]IC50: half maximal inhibitory concentration, 1 μM in plasma.[20]

can confirm these calculations. Moreover, soybean varieties Tokachi (Japan) and Flora (France) can be considered very suitable for the isolation of this bioactive peptide for the preparation of functional foods and nutraceuticals with immunostimulating activity. The method constitutes a very powerful tool to evaluate what soybean varieties and what growing conditions and soybean processing would be most suitable for isolation of the bioactive peptide for the preparation of functional foods and nutraceuticals with immunostimulatory activity and could also be applied for pharmacokinetic studies to confirm the immunostimulatory activity of soymetide in soybeans.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1 shows the effect of different mobile phases used in reversed phase LC/MS on the sensitivity of soymetide standard peak, estimated from the S/N ratio in the EIC, and the percentage of mobile phase B at which soymetide eluted. Table S1 shows the relative slopes, square regression coefficient, and signal-to-noise ratio obtained with different extracting widths. Table S2 shows the analytical characteristics of the optimized LC-ESI-TOF MS method for the determination of soymetide. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

Financial support from the Spanish Ministry of Science and Innovation (Project CTQ2009-09022), the Comunidad Autónoma of Madrid (Spain) and European funding from FEDER program (Project S2009/AGR-1464, ANALISYC-II), and the University of Alcalá and the Comunidad Autónoma of Madrid (Project CCG10-UAH/AGR-5950) is gratefully acknowledged. M.G.-D. thanks the Ministry of Science and Innovation for his predoctoral grant.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Thanks to the CRF (Centro de Recursos Fitogenéticos del Instituto Nacional de Investigaciones Agrarias, Madrid, Spain) for donation of soybean varieties.

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