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CAPILLARY LIQUID CHROMATOGRAPHY-ION TRAP-MASS SPECTROMETRY METHODOLOGY FOR THE SIMULTANEOUS QUANTIFICATION OF FOUR ANGIOTENSIN-CONVERTING ENZYME-INHIBITORY PEPTIDES IN *PRUNUS* SEED HYDROLYSATES

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ABSTRACT

Prunus genus fruit seeds are sources of highly angiotensin-I-converting enzyme (ACE)-inhibitory peptides. The presence of peptides IYSPH, IYTPH, IFSPR, and VAIP seems to be related to this activity but no previous work has demonstrated the direct relationship between the concentration of these peptides and the antihypertensive activity of hydrolysates. This work describes the development of a method for the quantification of these peptides in *Prunus* seeds hydrolysates based on capillary liquid chromatography-IT-MS/MS. The analytical characteristics of the method were evaluated through the study of the linearity, LOD, LOQ, presence of matrix interferences, precision, and recovery. The developed methodology was applied to the determination of the four peptides in seed hydrolysates from different *Prunus* genus fruits: peaches (7 varieties), plums (2 varieties), nectarines (3 varieties), apricots (2 varieties), cherry, and paraguayo. Peaches and plums seed hydrolysates yielded the highest concentrations of these peptides while paraguayo one showed the lowest concentrations. A high correlation between peptides concentrations was demonstrated suggesting that the four peptides could be released from the same seed proteins.

Keywords: ACE inhibitory peptides; quantification; capillary-HPLC; ion trap mass spectrometry; *Prunus* seeds.

1. INTRODUCTION

Bioactive peptides have usually between 2-20 amino acid residues exhibiting beneficial effects on body functions and being able to influence human health. Depending on their chemical structure, length, and amino acid composition, they could present different biological functions, such as antihypertensive, antioxidant, antimicrobial, etc [1]. Activity of antihypertensive peptides is mainly related to the renin-angiotensin system which is the main mechanism involved in the regulation of blood pressure. Briefly, the enzyme renin cleaves the angiotensinogen peptide, secreted by the liver, to form the decapeptide angiotensin I. Then, the angiotensin-I-converting enzyme (ACE) hydrolyzes angiotensin I into the octapeptide angiotensin II, which is a potent vasoconstrictor. At the same time, ACE is also involved in the degradation of the vasodilator peptide bradykinin [2]. Both effects can be hindered by the presence of antihypertensive peptides, which act in the active center of ACE, resulting in a decrease of blood pressure [3].

Bioactive peptides are usually released during food processing or gastrointestinal digestion [4]. Additionally, there is a growing interest in developing green strategies for the exploitation of food byproducts to obtain bioactive substances which substitute those extraction strategies that employ more expensive food sources. These bioactive compounds can be used to develop new functional foods and nutraceuticals. Food byproducts are low cost raw materials, easy to obtain and, in many cases, constitute an important problem for food producing companies since their incorrect disposal can result in a negative environmental impact [5]. This problem is even more worrying in the case of fruit residues due to their high fermentability.

Fruit industry byproducts represent a huge part of the total residues generated by the food industry [6]. Within these residues, stones and, especially, the seeds inside them,

64 are rich sources of proteins. Indeed, fruits from *Prunus* genus (plum, peach, nectarine,
65 cherry, apricot, etc.) are characterized by having seeds with up to 40% of proteins [7, 8],
66 which are discarded [9] or underused [10]. In recent years, different methodologies have
67 been developed for the extraction of these proteins and to obtain bioactive peptides [7,
68 8, 11-14]. As example, it has been demonstrated that peach seed hydrolysate [15] can
69 release four peptides (IYSPH, IYTPH, IFSPR, and VAIP) exerting high ACE inhibition
70 capacity. Additionally, these peptides have also been observed in other *Prunus* fruits
71 such as plum [11] and cherry [13]. Antihypertensive capacity of these seed hydrolysates
72 depends on the amount and distribution of these peptides within the seed and will
73 obviously vary among cultivars. Nevertheless, no previous work has determined the
74 distribution of these antihypertensive peptides in these fruit seeds and there is no
75 information on which variety is worthier to obtain these peptides. Moreover, in order to
76 evaluate the safety and the minimum consumption dose to produce a real effect, the
77 quantification of these peptides is mandatory.

78 Antihypertensive peptides are composed of 2-5 amino acid residues and are obtained by
79 hydrolysis with low specificity enzymes. This result in highly complex food matrices
80 and a high dynamic concentration range. Thus, the quantification of antihypertensive
81 peptides requires high resolving and high sensitivity separation techniques. The
82 preferred separation technique for the determination of bioactive peptides is high
83 performance liquid chromatography (HPLC) and, only in few cases, capillary liquid
84 chromatography has also been employed [16, 17]. Despite there are some works
85 employing UV or fluorescence detection [16, 18-20], MS detection is usually the best
86 choice. Although some works employ MS detection [21-23], the use of MS/MS [17, 24-
87 37] or MS^3 [38, 39] detection is preferred in order to improve the method sensitivity and
88 selectivity.

The aim of this work was to develop an analytical methodology for the simultaneous quantification of four ACE-inhibitory peptides (IYSPH, IYTPH, IFSPR, and VAIP) in different *Prunus* genus fruit seeds. For this purpose, a method based on capillary liquid chromatography hyphenated to an ion trap mass spectrometer will be developed. Both separation and MS parameters will be optimized and analytical characteristics of the method will be evaluated. Finally, the developed methodology will be applied to the determination of these antihypertensive peptides in seed hydrolysates from different *Prunus* fruits seeds and the relationship of these peptides concentrations with the ACE-inhibitory capacity of seed hydrolysates will be evaluated.

2. EXPERIMENTAL SECTION

2.1. Chemicals, standards, and samples.

All chemicals and reagents were of analytical grade. Water was daily obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Acetone, methanol (MeOH), and hexane were acquired from Scharlau Chemie (Barcelona, Spain). Tris(hydroxymethyl)aminomethane (Tris), sodium dihydrogen phosphate, hydrochloric acid (HCl), and sodium dodecyl sulphate (SDS) were purchased from Merck (Darmstadt, Germany). Dithiothreitol (DTT), ACE from rabbit lung, hippuryl-histidyl-leucine (HHL), 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid (HEPES), sodium chloride, and Thermolysin were from Sigma-Aldrich (Saint Louis, MO, USA). Acetonitrile and acetic acid, both of LC-MS grade, were acquired from Fisher Scientific (Waltham, MA, USA).

Peptides standards (IYSPH, IYTPH, IFSPR, and VAIP) (purity > 95 %) were synthesized by GenScript Corp. (Piscataway, NJ, USA).

Peach and nectarine stones were provided by the Instituto Murciano de Investigación y Desarrollo Agrario (IMIDA, Murcia, Spain). Commercial peach, plum, nectarine, paraguayo, cherry, and apricot were acquired in a market at Alcalá de Henares (Madrid, Spain).

2.2. Protein extraction.

Proteins from seeds of 16 *Prunus* genus fruits were extracted employing a method based on previous optimizations of our research group [7, 8]. Seeds were ground with a domestic miller and defatted with hexane. Defatted seed powder (30 mg) was mixed with 5 mL of extracting solution (100 mM Tris-HCl (pH 7.5) containing 0.5% (m/v)

SDS and 0.5% (m/v) DTT) and sonicated with a high intensity focused ultrasound probe (model VCX130, Sonics Vibra-Cell, Hartford, CT, USA) for 5 min at 30% amplitude to accelerate the extraction process. Samples were centrifuged (10 min, 4000 xg) and supernatants were mixed with cold acetone at a 1:2 ratio to precipitate proteins (1 h, -20 °C). Samples were centrifuged again (10 min, 4000 xg) and pellets were recovered and stored at -20 °C until use.

2.3. Protein digestion and ultrafiltration.

Seed proteins were digested with Thermolysin, following a method based on previous optimizations of our research group [7, 8]. Briefly, pellets were dissolved in 5 mM phosphate buffer (pH 8.0) at a final concentration of 5 mg/mL. Thermolysin was added to the sample at a ratio of 0.1 mg enzyme/mg protein and the mixture was incubated in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany) at 50 °C during 4 h. Afterwards, temperature was raised to 100 °C for 10 min to inactivate the enzyme. Hydrolysates were centrifuged and supernatants collected and ultracentrifuged through 3 kDa molecular weight cut-off (MWCO) filters (Amicon, Millipore) during 90 min at 7000 xg. Collected hydrolysates were reconstituted to the initial volume and kept at -20 ºC until use.

2.4. *In vitro* ACE inhibitory capacity.

In vitro antihypertensive capacity was evaluated through the measurement of the ability of peptides to inhibit ACE. The capacity was measured following the procedure described by González-García et al. [7] and results were expressed as the percentage of inhibition of ACE ± standard deviation corresponding to two independent hydrolysates measured by duplicate.

147 **2.5. HPLC analysis.**

148 Peptides were separated on a modular capillary chromatographic system (Agilent
149 Technologies, Pittsburg, PA) equipped with a micro vacuum degasser (model 1100), a
150 capillary LC pump (model 1100), a thermostated autosampler (model 1100), a
151 thermostated column compartment (model 1200), and a multiple wavelength UV
152 detector (model 1200). This system was connected to a mass spectrometry detector.
153 Peptides separation was carried out in a C18 Zorbax 300 SB (150 mm x 0.5 mm, 5 µm
154 particle size) column from Agilent Technologies. The following optimized
155 chromatographic conditions were used: mobile phase A, Milli-Q water containing 0.3%
156 (v/v) acetic acid; mobile phase B, ACN containing 0.3% (v/v) acetic acid; binary
157 gradient, 5–26% B in 20 min, 26–95% B in 2 min, 95% B for 3 min, 95–5% B in 2 min;
158 and 5% B for 15 min; flow rate, 20 µL/min; temperature, 25 °C; injection volume, 1 µL;
159 and UV detection at 210, 254, and 280 nm. Standards and samples were maintained at 4
160 °C in the thermostated autosampler until their injection.

161 **2.6. MS/MS analysis.**

162 An ion trap (IT) mass spectrometer model Amazon SL (Bruker Daltonics, Bremen,
163 Germany) with electrospray ionization (ESI) was used. HyStar software (version 3.2,
164 Bruker) was employed to control both HPLC and MS instruments. The mass
165 spectrometer was operated in the positive ion mode, in the UltraScan mode, and with a
166 mass scan range ranging from 70 to 750 m/z. Detection conditions were modified
167 during the run in order to detect every peptide (IYSPH, IYTPH, IFSPR, and VAIP)
168 under their optimal MS/MS conditions. Therefore, every run was divided into four
169 sections to monitor every peptide: 0–12.5 min (IYSPH), 12.5–15 min (IYTPH), 15–17.5
170 min (IFSPR), and 17.5–27 min (VAIP). Optimized IT conditions were: capillary

voltage, 5000 V (for IYSPH) and 5500 V (for rest of peptides); end plate voltage, -500 V; nebulizer pressure, 8 psi; dry gas flow, 3 L/min; dry gas temperature, 150 °C; ion charge control (ICC) target, 50,000; and collision energy, 0.7 amplitude. Extracted ion chromatogram (EIC) was obtained by the addition of the signals corresponding to the selected transitions of every peptide: IYSPH (308.5* → (503.0 + 340.0 + 321.9 + 299.4* + 276.9 + 248.8 + 155.8)), IYTPH (315.5* → (517.0 + 353.9 + 306.4* + 276.8 + 248.8 + 155.7)), IFSPR (310.0* → (506.0 + 359.0 + 341.0 + 300.9* + 260.8 + 232.8)), and VAIP (399.0 → (283.9 + 238.8 + 228.8 + 170.8)), where m/z marked with * present z = +2. The extraction window for all of them was ± 0.5.

2.7. Method validation.

Both standard additions and external standard calibration curves were established. The external calibration curve was obtained in the range from 0.1 to 10 µM. All calibration solutions were obtained by dilution of a stock solution containing the four peptides standards.

The limits of detection (LODs) and the limits of quantification (LOQs) for all peptides were obtained as the minimum concentration providing a signal to noise ratio (S/N) equal to 3 and 10, respectively. The method precision was evaluated through the calculation of the instrumental repeatability and inter-day and inter-sample reproducibility. For that purpose, the relative standard deviation (% RSD) was employed. Repeatability was evaluated by injecting, ten consecutive times, solutions containing standard peptides at two different concentration levels (0.1 and 5.0 µM) and by injecting, ten consecutive times, a peach hydrolysate (Calanda San Miguel (CSM)). Inter-day reproducibility was obtained by injecting, five consecutive times, the same CSM hydrolysate in two consecutive days. Inter-sample reproducibility was determined

by the injection of five independent CSM hydrolysates by triplicate. On the other hand, peptides recovery was evaluated by spiking the CSM hydrolysate with known amounts of peptide standards (0.4, 0.8, and 1.2 μM) just before the ultrafiltration process.

The evaluation of the presence of matrix interferences was made by comparing the slopes obtained by the external standard calibration method with the slopes obtained by the standard additions calibration method. The standard additions curve was obtained by the addition of known and increasing concentrations of peptide standards to a sample hydrolysate. Three independent peptide hydrolysates (Zaragozano Rojo peach, commercial nectarine, and commercial plum) were used in this study. Every calibration point was injected three times and its EIC was extracted, averaged, and plotted against the peptide concentration.

2.8. Statistical analysis.

All statistical analysis was carried out using Statgraphics Software Plus 5.1 (Statpoint Technologies, Inc., Warrenton, VA, USA).

3. RESULTS AND DISCUSSION

Previous works carried out by our research team reported the release of antihypertensive peptides from peach [15], plum [11], and cherry [13] seeds proteins when they were hydrolysed with Thermolysin enzyme. Among them, IYSPH, IYTPH, and IFSPR peptides were identified. In a more recent work, a further fractionation of antihypertensive peptides enabled to identify an additional antihypertensive peptide (VAIP) in the peach seed [15]. In that work, IC_{50} values were determined for every peptide (IYSPH (IC_{50} = 38 ± 5 μM), IYTPH (estimated IC_{50} = 794 ± 25 μM), IFSPR (IC_{50} = 50 ± 4 μM), and VAIP (IC_{50} = 358 ± 55 μM)). These IC_{50} values are in the range of those corresponding to peptides with demonstrated *in-vivo* antihypertensive activity [40].

3.1. Method development.

Separation and detection of peptides by capillary liquid chromatography-IT-MS was firstly carried out with a four-standard peptide mixture (containing every peptide standard at a concentration of 1 μM) using preliminary MS conditions based on a previous work of our research group that was aimed to the determination of a single peptide (VLIVP) in soybean [17]. These initial MS conditions were: capillary voltage, 5000 V; end plate voltage, -600 V; nebulizer pressure, 7 psi; dry gas flow, 2 L/min; dry gas temperature, 150 °C; ICC target, 100,000; and collision energy, 0.5 amplitude. These conditions enabled to obtain the MS and MS/MS spectra of the four peptides (**Figure 1**). IYSPH, IYTPH, and IFSPR presented the double charged ion ($[M+2H]^{2+}$) as main signal (308.5 m/z, 315.5 m/z, and 310.0 m/z, respectively), while the single charged ion ($[M+H]^{+}$) of these peptides showed lower intensity (616.1 m/z, 630.0 m/z, and 619.1 m/z, respectively). In the case of the peptide VAIP, just the single charged

234 ion with m/z of 399.0 was exhibited. Therefore, the double charged ions were
235 monitored for peptides IYSPH, IYTPH, and IFSPR and the single charged ion was
236 detected in the case of peptide VAIP. Regarding MS/MS spectra, they mostly showed *b*
237 or *y* ions and their neutral loss of a CO group. Additionally, ions corresponding to the
238 loss of a water molecule ($[M+2H-H_2O]^{2+}$) and an *a* ion (b ion with a neutral loss of CO)
239 with loss of NH_3 were also observed in some MS/MS spectra. Finally, the selected
240 transitions for the detection of peptides were: IYSPH (308.5 → (503.0 + 340.0 + 321.9
241 + 299.4 + 276.9 + 248.8 + 155.8), IYTPH (315.5 → (517.0 + 353.9 + 306.4 + 276.8 +
242 248.8 + 155.7), IFSPR (310.0 → (506.0 + 359.0 + 341.0 + 300.9 + 260.8 + 232.8), and
243 VAIP (399.0 → (283.9 + 238.8 + 228.8 + 170.8)).

244 Subsequently, the optimization of the elution gradient and the separation temperature
245 was carried out. Acetic acid was chosen as ion-pairing reagent since it usually enables a
246 compromise between separation resolution and sensitivity in comparison to other
247 ion-pairing reagents that result in strong signal suppression [41]. Therefore, Milli-Q
248 water containing 0.3% (v/v) acetic acid and ACN containing 0.3% (v/v) acetic acid
249 were chosen as mobile phases A and B, respectively. An initial gradient from 5 to 95%
250 B in 30 min did not enable the separation of the four peptides that eluted in the first half
251 of the gradient. The gradient optimization demonstrated that a shorter gradient, 5-26%
252 B in 20 min, was suitable for the separation of peptides. In order to reduce the
253 separation time, different temperatures were tried (25°C, 30°C, 35°C, 40°C, and 45°C).
254 Nevertheless, no significant improvement in the separation time was observed, so 25°C
255 was kept. Finally, the optimized chromatographic conditions were: mobile phase A,
256 Milli-Q water containing 0.3% (v/v) acetic acid; mobile phase B, ACN containing 0.3%
257 (v/v) acetic acid; binary gradient, 5–26% B in 20 min, 26–95% B in 2 min, 95% B for 3
258 min, 95–5% B in 2 min; and 5% B for 15 min; flow rate, 20 µL/min; temperature, 25 °C;

and injection volume, 1 µL. These conditions enabled the complete and rapid separation of peptides from the four-standard peptide mixture.

In order to prove that the developed method also enabled the detection of the four peptides in real samples, a randomly chosen Prunus seed was employed (Calanda San Miguel variety). The hydrolysate corresponding to the CSM seeds (5 mg/mL) was analysed using the chromatographic and detection conditions described above. Hydrolysate was ultrafiltered through 3 kDa MWCO filters to decrease the sample complexity. **Figure 2** shows the total ion chromatogram (TIC) using MS detection (A), the extracted ion chromatogram (EIC) using MS/MS signals at 308.5, 315.5, 310.0, and 399.0 m/z (characteristics for every peptide) (B), and the chromatogram obtained using MS/MS detection and the transitions selected in the previous section (C). Since the chromatogram obtained by MS/MS detection with the CSM hydrolysate revealed the occurrence of all the above-mentioned transitions, the presence of the four peptides in the sample was confirmed.

On the other hand, in order to find out whether the developed method suffered from signal suppression, the signals corresponding to a mixture of peptides standards were compared with those obtained when the CSM hydrolysate was spiked with the same concentration of peptides standards. Results showed a reduction in the peptides signals and, thus, the existence of matrix interferences. In order to avoid this signal suppression, CSM hydrolysate was diluted before its injection at different levels (2, 10, 20, 50, 100, and 200 times). Increasing the level of dilution resulted in an increase in the peak areas of peptides in the EIC up to 100 times dilution. Consequently, samples were diluted 100 times before their injection in the capillary liquid chromatography-IT-MS system in order to avoid matrix interferences.

283 The last step in the method development was the optimization of the detection
284 conditions for every peptide to increase the sensitivity of the method. For that purpose,
285 the run was divided into four sections (see **Figure 2**), observing the elution of every
286 peptide in a different section: 0–12.5 min (IYSPH), 12.5–15.0 min (IYTPH), 15.0–17.5
287 min (IFSPR), and 17.5–27 min (VAIP). Different parameters related to the ESI
288 (capillary voltage, end plate voltage, nebulizer pressure, dry gas flow, and dry gas
289 temperature) and to the IT performance (ion charge control (ICC) target and collision
290 energy) were optimized in every section. Results are shown in **Figure S1** in
291 **Supplementary Material** related to the initial conditions (marked with *) and
292 expressed as relative peak area for every peptide. Those values yielding the highest
293 sensitivity and, thus, chosen as optimal, were capillary voltage of 5000 V for the IYSPH
294 peptide and 5500 V for the IYTPH, IFSPR, and VAIP peptides, end plate voltage of -
295 500 V, nebulizer pressure of 8 psi, dry gas flow of 3 L/min, dry gas temperature of
296 150 °C, ICC target of 50,000, and collision energy of 0.7 amplitude. Optimization of
297 these parameters enabled significant increases in peak areas for all peptides,
298 highlighting the case of peptide IYSPH with an increase of 75%.

299 **3.2. Method validation.**

300 Different analytical characteristics were evaluated in the developed method: dynamic
301 concentration range, working concentration range, LOD and LOQ, precision, and
302 recovery. Moreover, although a first investigation with the CSM hydrolysate was
303 previously performed to test the presence of signal suppression due to the sample
304 matrix, a further study was performed now to evaluate the presence of matrix
305 interferences. Results obtained for each peptide are grouped in **Table 1**. All peptides
306 presented a wide dynamic concentration range (1 nM-100 µM). Working concentration
307 range was set from 0.1 to 10 µM observing a high linear correlation in all cases (R^2 >

0.999). LODs and LOQs ranged from 2 to 17 and from 7 to 54 ng/g of dried and defatted seeds, respectively, for all peptides. On the other hand, precision of the method was evaluated by the determination of the repeatability, inter-day reproducibility, and inter-sample reproducibility. Repeatability using peptides standards or the real sample was considered very acceptable (RSD < 3%). Moreover, inter-day reproducibility presented RSD < 3% and inter-sample reproducibility showed RSD < 4%. Furthermore, recoveries close to 100% were obtained when a plum seed hydrolysate was spiked with increasing and known concentrations of the standards peptides mixture. Finally, the presence of matrix interferences was evaluated for three different seed protein hydrolysates, corresponding to a peach seed, a nectarine seed, and a plum seed. Statistical analysis revealed no significant differences between slopes (p value > 0.05), demonstrating that the developed methodology did not suffer from matrix interferences.

3.3. Quantification of peptides in different fruit seeds hydrolysates from *Prunus* genus.

The optimized method was applied to the quantification of the four antihypertensive peptides (IYSPH, IYTPH, IFSPR, and VAIP) in seeds hydrolysates obtained from 16 different *Prunus* genus fruits and varieties (eight samples were provided by a germplasm bank and the other eight were from local markets). Peptides concentrations, expressed as ng/g of defatted seeds, are grouped in **Table 2**. Peach and plum seed hydrolysates presented the highest peptides concentrations, highlighting the CSM variety, while paraguayo showed the lowest contents. Moreover, in general, peptides IYSPH and IYTPH were more abundant than peptides IFSPR and VAIP. In the case of peptide IYTPH, this fact can be explained considering that it is released from two different proteins identified in a previous work [42]. Moreover, it was observed a high correlation among peptide concentrations. In fact, **Figure 3** shows that Pearson product-

moment correlation coefficients were close to 1, being the better correlation that established between the IYSPH concentration and the IYTPH concentration. This high correlation enables to think that all peptides could have been released from the same proteins. Moreover, this high correlation would also enable to determine the content of the four peptides just knowing the concentration of one of them.

3.4. ACE-inhibitory capacity.

The capacity to inhibit ACE was evaluated through the determination of the percentage of inhibition of this enzyme (see **Table 2**). As expected, those varieties with the highest concentrations of the four antihypertensive peptides presented also the highest inhibition capacities. In fact, the CSM peach, the commercial peach, and the commercial plums, that presented the highest peptide contents, yielded almost a total or a very high inhibition of ACE. In the other extreme, paraguayo, which had the lowest peptides concentrations, barely reached the 50% of ACE inhibition. Some exceptions were Zaragozano Rojo, Zaragozano Amarillo, and Lovell that, despite not having very high peptides concentrations, presented an inhibition as high as the CSM variety. An intermediate situation was observed for the nectarine, cherry, and apricot seed hydrolysates that presented moderate-high percentages of ACE inhibition, although their concentrations were not much high. This fact could be due to the possible presence of other antihypertensive peptides in addition to the four monitored. These results would suggest a certain correlation between the four peptides concentrations and the ACE inhibition capacity of seeds hydrolysates that could be very interesting for the prediction of ACE inhibition.

4. CONCLUSIONS

A new analytical methodology using capillary liquid chromatography-IT-MS/MS has enabled the simultaneous determination of four ACE-inhibitory peptides (IYSPH, IYTPH, IFSPR, and VAIP) in *Prunus* fruits seeds hydrolysates. Significant reduction of signal suppression effects was observed by dilution of hydrolysates up to 100 times. Signals intensity was significantly affected by the capillary voltage, the dry gas temperature, and the collision energy. The optimization of these parameters enabled to increase the sensitivity up to a 75% (in the case of peptide IYSPH). Optimal parameters permitted quantify as low as 53.4, 32.1, 12.7, and 6.97 ng peptide (IYSPH, IYTPH, IFSPR, and VAIP, respectively)/g of defatted seeds, which were far lower than the concentrations presented by the samples. The developed methodology demonstrated to be sensitive, selective, precise, accurate, and free of matrix interferences. The method was successfully employed to quantify the four ACE-inhibitory peptides in seeds hydrolysates from 16 varieties of *Prunus* genus fruits. Highest concentrations of peptides were observed in peach and plum seeds hydrolysates observing the existence of a clear correlation among the concentrations of the four peptides. Moreover, ACE-inhibition capacity of seeds hydrolysates was somehow correlated with the concentration of the four peptides observing, in general, a highest ACE inhibition capacity in those seeds hydrolysates yielded the highest peptides concentrations.

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522 **Table 1.** Analytical characteristics of the developed methodology.

Characterization parameter			IYSPH	IYTPH	IFSPR	VAIP
Extraction window			± 0.5 Da	± 0.5 Da	± 0.5 Da	± 0.5 Da
Dynamic concentration range			1 nM – 100 µM	1 nM – 100 µM	0.5 nM – 50 µM	0.1 nM – 50 µM
Working concentration range			0.1 – 10 µM	0.1 – 10 µM	0.1 – 10 µM	0.1 – 10 µM
External calibration	Slope		$3.77 \cdot 10^7$	$3.96 \cdot 10^7$	$7.24 \cdot 10^7$	$1.45 \cdot 10^8$
	Linearity (R^2)		0.9994	0.9996	0.9991	0.9998
Standard additions calibration	Slope	ZR peach	$3.76 \cdot 10^7$	$4.02 \cdot 10^7$	$7.32 \cdot 10^7$	$1.44 \cdot 10^8$
		Commercial nectarine	$3.85 \cdot 10^7$	$4.07 \cdot 10^7$	$7.29 \cdot 10^7$	$1.50 \cdot 10^8$
		Commercial plum A	$3.79 \cdot 10^7$	$4.03 \cdot 10^7$	$7.27 \cdot 10^7$	$1.47 \cdot 10^8$
	Linearity (R^2)	ZR peach	0.9960	0.9986	0.9984	0.9991
		Commercial nectarine	0.9987	0.9989	0.9986	0.9988
		Commercial plum A	0.9992	0.9988	0.9989	0.9990
LOD (ng/g defatted seeds)[a]			16.3	9.76	3.71	1.99
LOQ (ng/g defatted seeds)[b]			53.9	32.1	12.7	6.97
Repeatability (% RSD) (n=10)[c]	At 0.1 µM		2.5	2.9	2.8	2.7
	At 5.0 µM		0.4	0.2	0.8	0.8
	CSM peach		2.2	2.5	0.4	2.3
Inter-day reproducibility (% RSD) (n=10)[d]	CSM peach		2.6	2.1	1.6	2.9
Inter-sample reproducibility (% RSD) (n=15)[e]	CSM peach		3.8	3.6	3.1	3.4
Recovery	0.4 µM[f]		98.3	99.3	99.1	99.8
	0.8 µM		100.5	100.7	100.2	100.8
	1.2 µM		98.8	98.9	97.7	97.8

523 CSM: Calanda San Miguel; ZR: Zaragozano Rojo.
524 [a] Calculated as the minimum concentration yielding an S/N equal to 3. Determined relative to 1 g of defatted seeds.
525 [b] Calculated as the minimum concentration yielding an S/N equal to 10. Determined relative to 1 g of defatted seeds.
526 [c] Evaluated using peptides standards at two concentration levels and using a real sample by ten consecutive injections.
527 [d] Expressed as RSD (%) value obtained by injecting the peach seed hydrolysate five consecutive times in two consecutive days.
528 [e] Expressed as RSD (%) value obtained by injecting five independent peach seed hydrolysates in triplicate.
529 [f] Concentration of peptides standards added to the seed hydrolysate obtained from the commercial plum A.

530 **Table 2.** Concentration of peptides IYSPH, IYTPH, IFSPR, and VAIP in the different varieties of *Prunus* genus fruit seeds[a] and percentage of

531 inhibition of the angiotensin-I-converting enzyme.[b]

Fruit	Variety	µg IYSPH/g seed	µg IYTPH/g seed	µg IFSPR/g seed	µg VAIP/g seed	ACE inhibition (%)
Peach	Calanda San Miguel	4401 ± 94	5383 ± 85	1401 ± 11	510 ± 20	90.3 ± 0.2
	Lovell	963 ± 15	1222 ± 10	280 ± 5	76 ± 3	89 ± 1
	Zaragozano Rojo	1414 ± 35	1793 ± 36	483 ± 5	140 ± 4	89.8 ± 0.8
	Zaragozano Amarillo	1297 ± 14	1757 ± 49	441 ± 5	110 ± 1	90.3 ± 0.4
	Borracho de Jarque	2040 ± 81	2773.7 ± 0.4	743 ± 6	185 ± 5	87.9 ± 0.9
	Campiel	625 ± 11	831 ± 8	224 ± 1	62.6 ± 0.3	82.0 ± 0.1
	Commercial	3100 ± 100	3620 ± 37	1608 ± 13	244 ± 2	89.2 ± 0.1
Nectarine	Blanca	188 ± 3	366 ± 13	39.1 ± 0.2	25.3 ± 0.6	76.7 ± 0.2
	Amarilla	407 ± 4	748 ± 28	70 ± 4	50 ± 1	83.7 ± 0.5
	Commercial	298 ± 9	445 ± 3	93.9 ± 0.3	12 ± 1	85.9 ± 0.2
Paraguayo peach	Commercial	15.5 ± 0.3	24.9 ± 0.4	17.3 ± 0.2	6.8 ± 0.3	57 ± 1
Plum	Commercial A	2385 ± 24	2768 ± 28	967 ± 7	191 ± 4	85.7 ± 0.8
	Commercial B	2883 ± 64	3237 ± 83	1000 ± 8	215 ± 2	86.5 ± 0.7
Cherry	Commercial	253 ± 5	567 ± 9	132 ± 3	3.34 ± 0.04	86.1 ± 0.2
Apricot	Commercial A	607 ± 15	13.2 ± 0.4	357 ± 3	46 ± 1	86.7 ± 0.1
	Commercial B	319 ± 2	20.56 ± 0.01	112 ± 1	26.44 ± 0.05	81.2 ± 0.1

532 [a] Concentrations were calculated from two individually prepared protein hydrolysates injected by triplicate.
533 [b] Percentages were calculated from two individually prepared protein hydrolysates injected by duplicate.

FIGURE CAPTIONS

Figure 1. MS (left) and MS/MS (right) spectra of peptides IYSPH, IYTPH, IFSPR, and VAIP (standard solution, 1 μM) injected into the capillary-HPLC-IT system under the following conditions: chromatographic conditions: mobile phase A, Milli-Q water containing 0.3% (v/v) acetic acid; mobile phase B, ACN containing 0.3% (v/v) acetic acid; binary gradient, 5–26% B in 20 min, 26–95% B in 2 min, 95% B for 3 min, 95–5% B in 2 min; and 5% B for 15 min; flow rate, 20 μL/min; temperature, 25 ℃; and injection volume, 1 μL; MS conditions: capillary voltage, 5000 V (for IYSPH) and 5500 V (for the rest of peptides); end plate voltage, -500 V; nebulizer pressure, 8 psi; dry gas flow, 3 L/min; dry gas temperature, 150 ℃; ICC target, 50,000; and collision energy, 0.7 amplitude.

Figure 2. (A) Total ion chromatogram (TIC) of Calanda San Miguel (CSM) hydrolysate, **(B)** extracted ion chromatogram (EIC) of the CSM hydrolysate using MS/MS signals at 308.5, 315.5, 310.0, and 399.0 m/z, and **(C)** chromatogram of the CSM hydrolysate obtained using MS/MS detection at characteristic transitions for peptides IYSPH, IYTPH, IFSPR, and VAIP.

Figure 3. Table showing the Pearson product-moment correlation coefficients between each pair of variables. Variables are the concentrations of IYSPH, IYTPH, IFSPR, and VAIP expressed as μg/g seed.