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Identification by hydrophilic interaction and reversed-phase liquid chromatography-tandem mass spectrometry of peptides with antioxidant capacity in food residues

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Abstract

HILIC- and RP-HPLC-ESI-Q-TOF identification of bioactive peptides with antioxidant capacity in peach by-products was carried out. Peach seeds contain more than 40% of proteins (as dried and defatted basis) and could constitute a cheap source of bioactive peptides. Extracted proteins were digested using four different commercial enzymes. Five assays based on different antioxidant mechanisms were employed for a reliable evaluation of the antioxidant capacity of the extracts. Thermolysin enzyme originated the extract with the most favorable antioxidant capacity. Probably due to a synergic effect among antioxidant peptides, it was not possible to find a peptide fraction with a higher antioxidant capacity than the whole extract. Eighteen peptides were identified in the whole hydrolysate when combining HILIC- and RP-HPLC-ESI-Q-TOF. A high percentage of hydrophobic amino acids were observed within their sequences which is a characteristic feature of the antioxidant nature of peptides.

Keywords: hydrophilic interaction, reversed-phase, liquid chromatography-tandem mass spectrometry, peptides, antioxidant, peach seed.

1. Introduction

Bioactive peptides have many advantages against synthetic drugs since they have low toxicity and different bioactivities, they exhibit less side effects, and they are more bioavailable [1]. Nevertheless, the complexity and the high cost of the production and purification of natural peptides are the main factors limiting the large scale production and commercialization of these peptides. An approach to reduce these costs is the use of food by-products for that purpose. Peptides with biological activities can be released from protein sequences. The enzymatic hydrolysis of protein by-products is the most efficient technology to recover bioactive peptides [2, 3]. Bioactive peptides normally show short amino acid chains (approximately 2-30 amino acids) [4, 5]. Moreover, antioxidant peptides possess a high level of hydrophobic amino acid residues within their sequence [6].

Peptides exerting antioxidant capacity can prevent oxidative processes occurring in both organisms and foodstuff. Accumulation of reactive oxygen species (ROS) in the organism plays a key role in the development of some pathologies and aging and they have been related with the development of degenerative diseases [7]. ROS are constituted by a large amount of reactive molecules derived from molecular oxygen and free radicals formed in organisms by oxygen consumption, water reduction, lipid oxidation, glycosylation, and environmental causes such as smoking and exposition to irradiation and air pollutants [7, 8]. On the other hand, food oxidation processes, especially lipid oxidation, leads to food degradation and reduction of food quality. Synthetic antioxidants preventing lipid autooxidation (e.g., tert-butylhydroquinone, tert-butyllatedhydroxyanisole, butylatedhydroxytoluene, and propyl galate) are allowed in food industry within certain regulation limits but there is a negative consumer perception against them [9]. Natural antioxidants constitute an alternative to prevent

oxidation in foodstuff. Most natural antioxidant compounds that have been extracted from residual sources are polyphenols [10]. Antioxidant peptides from animal, egg or seaweed by-products have also been found [4, 11].

Peach (*Prunus Persica (L.) Batsch*) processing industry leads to a large amount of waste (around 22-28 % of raw material) mainly constituted by the peel and the stone [12]. Taking into account that peach seeds have 43 g of protein per 100 g of dried and defatted milled seeds [13], this by-product could be a potential source of bioactive peptides. Our research group has obtained peptides extracts with antioxidant capacity from olive, plum and cherry seeds [14-16] but this is the first time that antioxidant peptides have been recovered from peach seeds.

Identification of peptides and proteins is mainly performed using reversed-phase (RP)-HPLC coupled to tandem mass spectrometry. Nevertheless, RP sometimes results usefulness for the separation of most polar molecules that elute very early or even in the dead volume. Bioactive peptides are characterized by having short chains and their comprehensive identification would require the use of a chromatographic mode complementary to RP-HPLC. Hydrophilic interaction liquid chromatography (HILIC) is suitable for the separation of small size peptides [17]. Unlike RP mode, gradient elution in HILIC is performed by increasing the hydrophilicity of mobile phase. In fact, elution in HILIC involves the use of a high concentration of a low polarity organic solvent (usually acetonitrile) at the beginning followed by an increasing water content. These mobile phases are highly compatible with electrospray mass spectrometry [18].

The objective of this work was the comprehensive identification of bioactive peptides with antioxidant capacity in peach by-products by tandem mass spectrometry

(Q-TOF-MS/MS) coupled to reversed-phase chromatography (RP-HPLC) and hydrophilic chromatography (HILIC).

2. Materials and Methods

2.1. Chemical and samples

All reagents were of analytical grade. Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid, sodium phosphate, and sodium dodecyl sulfate (SDS) were purchased in Merck (Darmstadt, Germany). Ammonium bicarbonate (ABC), sodium hydroxide, dithiothreitol (DTT), o-phthaldialdehyde (OPA), methanol (MeOH), sodium tetraborate, 2-mercaptoethanol (2-ME), glutathione (GSH), acetic acid (AA), Thermolysin, bovine serum albumin (BSA), pepsin, pancreatin, 2,2'-azino-bis(3-ethylbenzothiazonile-6-sulphonic) acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethanol (EtOH), potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 1,10-phenantroline, ferrous sulfate, hydrogen peroxide, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), linoleic acid, ammonium thiocyanide (NH_4SCN), and ferrous chloride ($FeCl_2$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Alcalase 2.4 L FG and Flavourzyme 1000 L were kindly donated by Novozymes Spain S.A. (Madrid, Spain). Protease P was kindly donated by Amano Enzyme Inc. (Nagoya, Japan). *Sencha* green tea and peaches were purchased in a local market (Alcalá de Henares, Madrid, Spain).

2.2. Protein extraction

Peaches were manually pitted and seeds were extracted from stones using a nutcracker. Ground seeds were defatted with hexane for 30 min (three times) before protein extraction. The protein extraction procedure was developed in a previous work

by Vásquez-Villanueva *et al.* [13]. Briefly, 30 mg of defatted peach seed was treated with 5 mL of extracting buffer (100 mM Tris-HCl (pH 7.5), 0.5 % (w/v) SDS, and 0.5 % (w/v) DTT) with a high intensity focused ultrasound (HIFU) probe (Sonic Vibra-Cell, Hartford, CT, USA) during 1 min at 30 % of amplitude. After centrifugation (4000g for 10 min), proteins in the supernatant were precipitated with cold acetone (10 mL) in the fridge for 15 min.

2.3. Protein hydrolysis

Four different enzymes (Alcalase, Thermolysin, Flavourzyme, and Protease P) were employed for the digestion of peach seed proteins. Buffer, substrate concentration, enzyme:protein ratio, temperature, and time employed in every case have been grouped in Table 1. Incubation was carried out in a Thermomixer Compact (Eppendorf, Hamburg, Germany) with shaking at 600 rpm. The digestion was stopped by boiling at 100 °C for 10 min. After centrifugation (4500g for 5 min), the supernatant was collected and the degree of hydrolysis (DH, %) and antioxidant capacity were estimated. Enzyme autolysis blanks in absence of sample were also measured. The DH was measured following the method described by Vásquez-Villanueva *et al.* [13].

2.4. Fractionation

Whole peptide extracts were fractionated by ultrafiltration using molecular weight cut-off (MWCO) filters Amicon® Ultra of 5 kDa and 3 kDa from Merck Millipore (Tullagreen, Ireland). Whole extracts sequentially passed through each filter in decreasing size order, for 1.30 h by centrifugation at 10000 rpm. Fractions with molecular weights above 5 kDa, from 3 to 5 kDa, and below 3 kDa were obtained. Every fraction was dissolved in the corresponding digestion buffer up to the initial volume.

Table 1. Optimal digestion conditions used for the hydrolysis of peach seed proteins using four different enzymes.

Enzyme	Alcalase	Thermolysin	Flavourzyme	Protease P
Substrate concentration (mg/mL)	5	5	5	7.5
Enzyme/substrate	0.1 AU/g protein	0.1 g enzyme/g protein	25 AU/g protein	0.5 mg enzyme/g protein
Buffer (pH)	PB (5mM) pH 8.0	PB (5mM) pH 8.0	ABC (5 mM) pH 6.5	PB (5mM) pH 7.5
Temperature (°C)	50	50	50	40
Hydrolysis time (h)	4	4	3	7

PB: Phosphate buffer; **ABC**: Ammonium bicarbonate

2.5. Evaluation of antioxidant capacity

Antioxidant capacity of peptides seed extracts was estimated by measuring the ability to scavenge free radicals as DPPH·, $ABTS^{\cdot+}$, and hydroxyl and by measuring the capacity to inhibit the lipid peroxidation of linoleic acid (all methods based on hydrogen atom transfer). Furthermore, the ability to reduce Fe (III) (method based on electron transfer) was also assayed. All measurements were carried out in three individual samples measured, at least, by triplicate.

2.5.1. DPPH radical assay

The capacity of extracted peptides to scavenge DPPH radicals was measured following the method described by You *et al.* [19] with some modifications. A 50 µL volume of a DPPH· solution (0.1 mM DPPH· in 95 % (v/v) MeOH) was mixed with 50 µL of sample and kept in the dark. After 30 min, the absorbance corresponding to the

DPPH radical was measured at 517 nm. GSH (0-5 mg/mL) was employed as positive control. DPPH· scavenging capacity was calculated using the equation:

$$DPPH\ radical\ scavenging\ capacity\ (\%) = \left(1 - \frac{Abs_{sample} - Abs_{control}}{Abs_{blank}}\right) \times 100 \quad (1)$$

where Abs_{sample} is the absorbance of the sample with DPPH· solution, $Abs_{control}$ is the absorbance of the sample with 95 % (v/v) MeOH and without DPPH·, and the Abs_{blank} is the digestion buffer with DPPH· solution.

2.5.2. ABTS radical assay

The capacity to scavenge $ABTS^{\cdot+}$ was measured according to Wiriyaphan *et al.* [20]. Briefly, an $ABTS^{\cdot+}$ stock solution was prepared by mixing 7.4 mM $ABTS^{\cdot+}$ and 2.6 mM potassium persulfate in 10 mM phosphate buffer (PB) (pH 7.4). This solution was kept in the dark for 16 h at room temperature. An $ABTS^{\cdot+}$ working solution was daily prepared, just before measuring, by dilution of the $ABTS^{\cdot+}$ stock solution (with 10 mM PB pH 7.4) and adjustment of the absorbance at 0.70 ± 0.01 UA. Next, 1 µL of sample was mixed with 100 µL of $ABTS^{\cdot+}$ working solution, incubated in the dark for 6 min, and the absorbance corresponding to ABTS radicals was measured at 734 nm. Trolox (synthetic analog of vitamin E) from 0 to 1.5 mM was employed as positive control. Scavenge capacity of samples was determined as:

$$ABTS\ radical\ scavenging\ capacity\ (\%) = \left(\frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}}\right) \times 100 \quad (2)$$

where Abs_{sample} is the absorbance of 1 µL of sample with 100 µL of $ABTS^{\cdot+}$ working solution and Abs_{blank} is the absorbance of 1 µL of the digestion buffer with 100 µL of $ABTS^{\cdot+}$ working solution (without peptides).

2.5.3. Hydroxyl radical assay

This assay estimates the capability to inhibit the hydroxyl radicals generated by Fenton's reaction in which Fe (II) is oxidized by peroxide hydrogen into Fe (III) and hydroxyl radicals are generated. The method was carried out according to Ajibola *et al.* [6] with slight modifications. The reagents were added in the following order: 50 µL of hydrolyzated sample, 50 µL of 3 mM 1,10-fenantroline in 0.1 M PB (pH 7.4), 50 µL of $FeSO_4$, and 50 µL of H_2O_2. The mixture was incubated at 37 °C for 1 h with shaking. The absorbance corresponding to the complex formed between the remaining Fe (II) and orthophenantroline was measured at 536 nm. Tripeptide GSH (0-5 mg/mL) was used as positive control. The percentage of scavenged hydroxyl radical was calculated by the following equation:

$$Hydroxyl\ Radical\ Scavenging\ Capacity\ (\%) = \left(\frac{Abs_{sample} - Abs_{blank}}{Abs_{control} - Abs_{blank}}\right) \times 100 \quad (3)$$

where Abs_{sample} is the absorbance of the hydrolysed sample, Abs_{blank} is the absorbance of the digestion buffer (without peptides), and $Abs_{control}$ is the absorbance of a control solution in absence of hydrogen peroxide.

2.5.4. Ferric Reducing Antioxidant Power (FRAP)

This method was reported by Ajibola *et al.* [6] and was employed with some modifications. This method measures the capacity of peptides to reduce Fe (III) from the ferricyanide complex into Fe (II). An aliquot of 12.5 µL of sample was mixed with 12.5 µL of 0.2 M PB (pH 6.6) and 25 µL of 1% (w/v) potassium ferricyanide. After incubation at 50 °C for 20 min, the reaction was stopped by addition of 25 µL of 10 % (w/v) TCA. For the assay, 25 µL of this solution was mixed with 20 µL of Milli-Q water and 10 µL of 2.5 (w/v) $FeCl_3$ and, after 3 min incubation, the absorbance

corresponding to the ferrous complex was measured at 700 nm. GSH (0-1 mg/mL) was used as positive control. The reducing power was calculated following this equation:

$$Reducing\ power = \frac{Abs_{sample}}{Peptide\ content} \quad (4)$$

where, Abs_{sample} is the absorbance of the hydrolysate and the peptide content was determined using the OPA assay [13]. The percentage of antioxidant power was calculated taking as maximum reducing power that corresponding to 1 mg/mL of the tripeptide GSH.

2.5.5. Inhibition of the peroxidation of linoleic acid

This assay was carried out according to the method described by Chen *et al.* [21] with some modifications. This method evaluates the capability of peptide extract to inhibit lipid peroxidation. A 20 µL volume of sample was mixed with 20 µL of 0.13 % (v/v) linoleic acid solution (dissolved in EtOH) and 10 µL of Milli-Q water and incubated for 144 h (6 days). For that purpose, 2.5 µL of every solution was mixed with 175 µL of 75 % (v/v) EtOH, 2.5 µL of 30 % (v/v) NH_4SCN, and 2.5 µL of 20 mM $FeCl_2$ in 3.5 % (v/v) HCl and the mixture was incubated at room temperature for 3 min. The degree of lipid peroxidation was evaluated by measuring the formed hydroperoxyde. Hydroperoxides oxidize Fe (II) to Fe (III) and the latter can form ferric thiocyanate that absorbs at 500 nm. GSH (1 mg/mL) was employed as positive control. The lipid peroxidation inhibition capacity of peptides was calculated according to the equation below:

$$Lipid\ peroxidation\ inhibition\ capacity\ (\%) = \left(1 - \frac{Abs_{sample\ 144h} - Abs_{sample\ 0h}}{Abs_{blank\ 144h} - Abs_{blank\ 0h}}\right) \times 100 \quad (5)$$

where, $Abs_{sample\ 144h}$ and $Abs_{blank\ 144h}$ are the absorbances of the sample and the buffer (without peptides), respectively, after incubation for 144 hand $Abs_{sample\ 0h}$ and $Abs_{blank\ 0h}$ are the absorbances of the sample and the buffer (without peptides), respectively, without incubation.

2.6. Peptide resistance to simulated gastrointestinal digestion

The evaluation of the resistance of most active peptide fractions to *in vitro* gastrointestinal digestion (GID) was carried out following the method described by Vásquez-Villanueva *et al.* [13].

2.7. Identification of antioxidant peptides by HILIC and RP-HPLC-ESI-Q-TOF

The identification of peptides in most active fractions was carried out using a high sensitivity mass spectrometry Quadrupole-Time-of-Flight (Q-TOF) (Series 6530) coupled to a liquid chromatograph (model 1100), both from Agilent Technologies (Pittsburgh, PA, USA). Peptides were separated using two different chromatographic modes: RP-HPLC and HILIC. For the RP-HPLC separation, an Ascentis Express Peptide ES-C18 column (100 mm x 2.1 mm I.D., 2.7 µm particle size) and guard column (5 mm x 2.1 mm, 2.7 µm particle size) from Supelco (Bellefonte, PA, USA) were employed. For the HILIC separation, an Ascentis Express column (100 mm x 2.1 mm I.D., 2.7 µm particle size) and guard column (5 mm x 2.1 mm, 2.7 µm particle size), also from Supelco, were chosen. An injection volume of 10 µL, a flow rate of 0.3 mL/min, and a column temperature of 25 °C were used in both separations. Furthermore, RP-HPLC separation was carried out using 0.3% (v/v) AA in water and 0.3 % (v/v) AA in ACN as mobile phases A and B, respectively, and a gradient from 3 to 35 % B in 40 min. The HILIC separation was performed using 65 mM ammonium

acetate in ACN and 65 mM ammonium acetate in water as mobile phases A and B, respectively, and a gradient from 60 to 95 % B in 30 min.

Detection by ESI-Q-TOF was performed in the positive ion mode using a mass range from 100 to 1500 *m/z*. ESI Jet Stream source conditions were: nebulizer pressure, 50 psig; fragmentator voltage, 200 V; capillary voltage, 500 V; sheath gas temperature, 400 °C; sheath gas flow, 12 L/min; drying gas flow, 12 L/min; drying gas temperature, 350 °C. MS/MS was carried out using *Auto* mode and collision induced dissociation was set at 4 V per each 100 Da molecular weight. PEAKS Studio software (Version 7) from Bioinformatics Solutions Inc. (Waterloo, Canada) was employed for the *de novo* peptide sequencing. Identified peptides showed an average local confidence (ALC, expected percentage of correct amino acids in the peptide sequence) ≥ 90% and a good precursor fragmentation pattern. Identifications were carried out using two individual samples injected by duplicate. Since it is not possible to differentiate I (Iso) from L (Leu) by MS due to their equal molecular masses, only isoforms with L were presented in our results, although peptide sequences containing I amino acid instead of L are also possible.

3. Results and discussion

Proteins extracted from peach seeds were hydrolysed using four different commercial enzymes (Alcalase, Thermolysin, Flavourzyme, and Protease P). The extraction and hydrolysis methodologies were optimized previously in order to obtain an efficient extraction of peach seed proteins and an efficient production of peptides [13].

3.1. Evaluation of the antioxidant capacity of peach seed hydrolysates

The antioxidant capacity of peptides obtained by the hydrolysis of peach seed proteins was evaluated. Antioxidant capacity of peptides depends on their amino acid composition and sequence. Moreover, it is also very important to consider the antioxidant assay itself. Indeed, the system composition, the oxidizing substrates or the interaction with other antioxidant components can influence the resulting antioxidant capacity of a peptide. Therefore, a reliable measurement of antioxidant capability involves to use different antioxidant assays. Figure 1 shows the antioxidant capacity evaluated using five different *in vitro* antioxidant assays for the extracts obtained using the four enzymes and for the control used in every assay. In general, all peptide extracts exhibited antioxidant capacity. The antioxidant capacity observed when using the FRAP assay (method based on electron transfer) was always lower than the antioxidant capacity showed when using those assays based on hydrogen atom transfer (ABTS, DPPH, and hydroxyl radical scavenging assays and the assay measuring the inhibition of the peroxidation of the linoleic acid). Unlike the FRAP assay, the assay measuring the inhibition of lipid peroxidation showed for every enzyme the highest antioxidant capacity with percentages ranging from 60 %, obtained with the Protease P extract, to 92 %, obtained with the Thermolysin extract. Regarding enzymes, Protease P seemed to be the enzyme yielding the lowest antioxidant capacity in most assays, while Thermolysin and Flavourzyme resulted in the highest antioxidant capabilities. These results were compared with the obtained with olive, plum, and cherry seeds [14-16]. Although in these cases Thermolysin yielded peptide extracts with high antioxidant capacity, the highest antioxidant power was observed when using Alcalase enzyme. The antioxidant capacity of peach peptide extracts was similar to the observed with the olive and the plum seeds and a bit higher than the corresponding to the cherry seeds.

The antioxidant capacity of peptide extracts obtained from peach seed proteins was compared to the obtained with a popular antioxidant beverage, the green tea (*Sencha* green tea) [22]. For that purpose, a solution of green tea at a concentration of 6 mg/mL (identical to the concentration of isolated peach seed proteins) was prepared and the ABTS, DPPH, and hydroxyl radical scavenging capacities of that solution were evaluated (see Figure 1). ABTS, DPPH, and hydroxyl radical scavenging capacities were within the values obtained for the peach seed hydrolysates.

In order to isolate fractions containing peptides with high antioxidant capacity, the peptide extract obtained with Thermolysin was next fractionated by ultrafiltration and the antioxidant capacity of peptide fractions was evaluated.

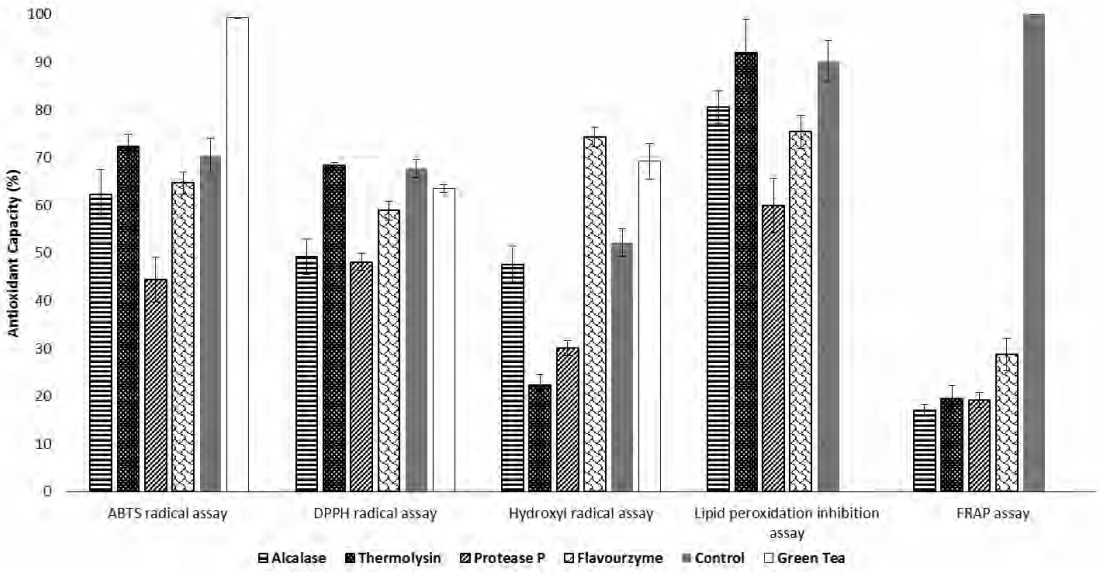

Figure 1. Comparison of the antioxidant capacity of the whole peptide extracts obtained with four different enzymes using five different assays 3.2. Evaluation of the antioxidant capacity of peptide fractions.

Fractions with theoretical molecular weights below 3 kDa, 3-5 kDa, and above 5 kDa were obtained and the peptide concentration in every fraction was evaluated. A total of 2.73 mg/mL of peptides were collected in the three fractions: 2.24 ± 0.32 mg/mL in the fraction < 3 kDa, 0.23 ± 0.06 mg/mL in the fraction from 3-5 kDa and

0.26 ± 0.09 mg/mL in the fraction > 5 kDa. Taking into account that the peptide concentration in the whole Thermolysin extract was 3.21 ± 0.20 mg/mL, it is possible to say that 15% of peptides were retained in the ultrafiltration filters and that most peptides showed molecular weights below 3 kDa. The antioxidant capacity was evaluated in every fraction by the ABTS and hydroxyl radical scavenging assays, by measuring the capacity to inhibit the peroxidation of the linoleic acid, and by the FRAP assay. Figure 2 summarizes all results in comparison with the antioxidant capacity shown by the whole Thermolysin extract. All fractions exhibited antioxidant capacity observing the lowest value in the fraction with peptides between 3-5 kDa. Antioxidant capacity obtained by the hydroxyl radical scavenging assay, FRAP assay, and the assay evaluating the inhibition of lipid peroxidation for the fraction below 3 kDa and for the fraction above 5 kDa was similar despite the fraction below 3 kDa contained 8.6 times more peptides than the fraction above 5 kDa. Nevertheless, the antioxidant capacity for all fractions was always lower than the observed for the whole Thermolysin extract. This result can be explained taking into account that antioxidant systems work in a synergistic way [23]. Indeed, an antioxidant avoids oxidation of a molecule by turning into a radical. Other components within the antioxidant system probably play a key role in the reduction of the reactivity of the formed antioxidant radicals and the recovery of the reduced antioxidant. Therefore, the deficiency of a component in an antioxidant system can affect the efficiency of other. Consequently, the whole Thermolysin extract was used in further studies. Similar situations were observed in the case of the olive and cherry seeds [14-16].

3.3. Evaluation of the resistance of peptides to *in vitro* gastrointestinal digestion

Bioactive peptides orally administrated must resist gastrointestinal digestion in order to reach target organs in an intact state.

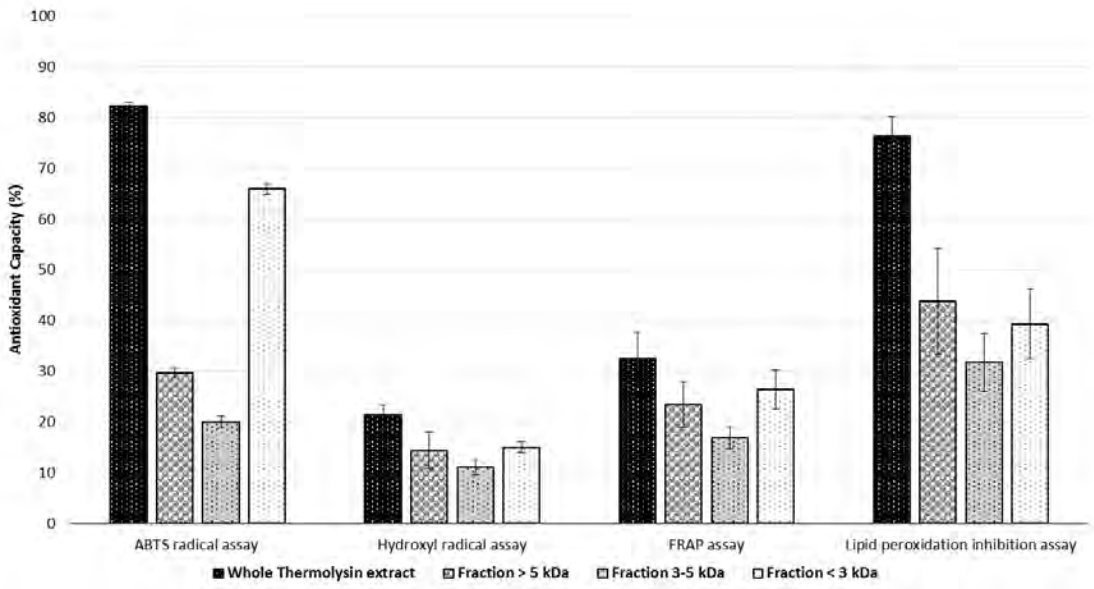

Figure 2. Comparison of the antioxidant capacity of the whole peptide extract obtained with Thermolysin and its corresponding fractions (fraction with peptides above 5 kDa, fraction wit peptides between 3- 5 kDa, and fraction with peptides below 3 kDa) employing four different antioxidant assays.

Whole Thermolysin extract was submitted to a simulated gastrointestinal digestion with pepsin and pancreatin and the antioxidant capacity (using ABTS radical assay) of the resulting extract was assayed in order to evaluate the resistance of peptides to this process. As expected, peptide concentration in the whole extract before GID (3.21 ± 0.20 mg/mL) was lower than the obtained after simulated GID (3.80 ± 0.20 mg/mL). Nevertheless, this variation did not result in a significant variation in antioxidant capacity after GID ($p > 0.05$).

3.4. Identification of peptides by HPLC-ESI-Q-TOF-MS/MS

Peptides present in the whole Thermolysin extract were identified by HPLC-ESI-Q-TOF-MS/MS. Chromatographic separation of peptides was carried out by RP-HPLC and HILIC modes in order to assure that both most hydrophobic and less hydrophobic peptides are separated and identified. Figure 3 shows the TIC (Total Ion

Chromatogram) corresponding to the whole peptide extract obtained by RP-HPLC and HILIC. Moreover, Figure 3 also shows as an example, the fragmentation spectrum obtained for a peptide identified in each case (Figure 3A and Figure 3B, respectively). TIC obtained by RP-HPLC was more crowded that obtained by HILIC. Furthermore, peptides separated by HILIC eluted within the first 10 min from the 30 min gradient. This could mean that the peptides obtained by the hydrolysis for peach seed proteins by Thermolysin were mainly hydrophobic and that a small amount of them were slightly hydrophilic. Also, Table 2 shows the peptides identified in both separations. All identified peptides showed molecular weights lower than 1 kDa and were monocharged. A total of 18 different peptides were identified: 12 peptides by RP-HPLC-MS/MS, 3 peptides by HILIC-MS/MS and 3 additional peptides that were identified by both modes (LYSPH, LYTPH, and ALPDEV). A high amount of hydrophobic and aromatic amino acids were present among peptides sequences. Peptides LYTPH, LYSPH, LLNDE, and LAGNPENE were also observed in cherry and plum seeds but not in the olive seed [14-16].

The presence of hydrophobic and aromatic amino acids is common among antioxidant peptides. In fact, peptides containing amino acids residues such as Trp (W), Tyr (Y), His (H), Leu (L), Iso (I), Pro (P) and Cys (C), have been reported to exert radical scavenging and metal chelating capacity [24]. Aromatic amino acids (eg. W, Y and H) allow the electron transfer to deficient radicals, therefore, converting radicals to a stable form. On the other hand, hydrophobic amino acid residues (L, P, I) allow hydrogen-transfer, lipid peroxyl radical trapping due to their high solubility in hydrophobic radical species. Peptides containing H amino acid residues also allow hydrogen-transfer, lipid peroxyl radical trapping and/or metal ion-chelating due to imidazole contained in their chains [25, 26].

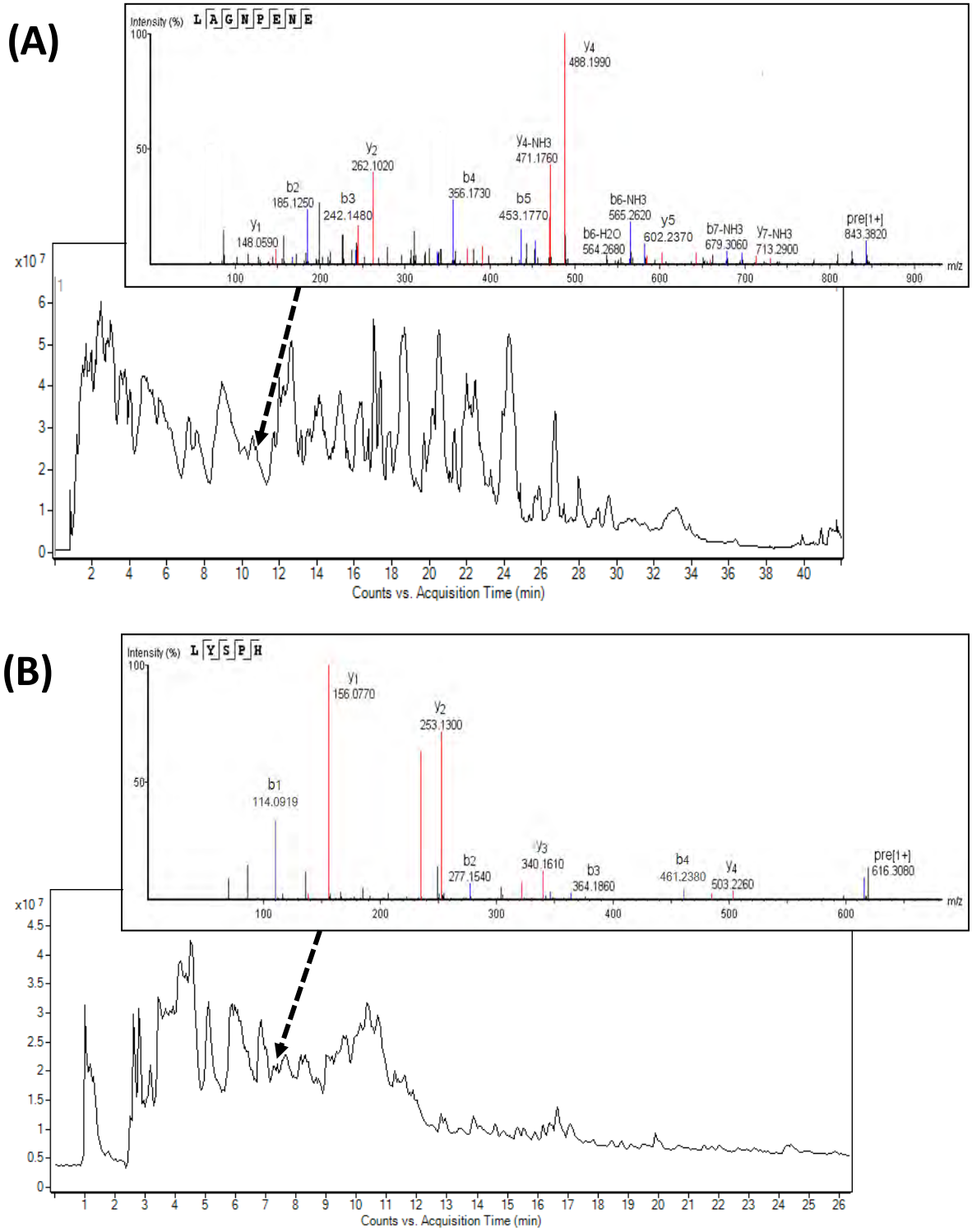

Figure 3. Total ion chromatograms (TIC) obtained by HPLC-ESI-Q-TOF using RP-HPLC (A) and HILIC (B) modes for the whole peptide extract obtained with Thermolysin. As an example, the spectra corresponding to LAGNPENE peptide using RP-HPLC mode (A) and LYSPH peptide using HILIC mode (B) are shown.

Table 2. Peptide sequence, ALC, and molecular mass of the peptides identified in the whole Thermolysin extract before simulated GID.

RP-HPLC			HILIC		
Peptide sequence	**ALC (%)**	**Molecular mass (Da)**	**Peptide sequence**	**ALC (%)**	**Molecular mass (Da)**
VLYL	97	615.3016	**LYSPH**	98	478.2903
LYTPHW	97	720.3918	**LYTPH**	96	849.5687
FVPTQQ	96	629.3173	**HLLP**	96	629.3173
LLDQE	92	508.2281	**ALPDEV**	95	557.3537
FEYL	95	571.2802	**LHLPS**	91	566.3262
VLYN	92	508.2744	**LTAH**	92	441.2462
VLTQ	91	460.2808			
YGPQQQE	91	849.3668			
ALPDEV	94	643.3342			
LAGNPENE	95	843.3763			
YLESQQSSQQ	94	1197.5054			
LLNDE	98	603.3031			
LYSPH	97	616.3128			
LYTPH	96	630.3289			
LLPGANH	95	721.4003			

Conclusions

HILIC- and RP-HPLC-ESI-Q-TOF have enabled the identification of bioactive peptides with antioxidant capacity in peach by-products. It is the first time that antioxidant peptides have been extracted from a by-product of the processing of peach, the peach seeds. The method involved the enzymatic hydrolysis of seed proteins and different enzymes were tested. The selection of five different antioxidant assays based on different oxidation mechanisms enabled the reliable evaluation of the antioxidant capacity of hydrolysates. Despite peptide extracts obtained with Alcalase, Protease P, Thermolysin, and Flavourzyme showed antioxidant capacity, most favorable enzyme was Thermolysin. Fractionation by ultrafiltration did not guarantee the complete recovery of peptides and did not yield fractions with higher antioxidant capacity than the whole hydrolysate. The antioxidant capacity of the hydrolysate obtained with Thermolysin was not affected when it was submitted to a simulated gastrointestinal digestion. A comprehensive identification of peptides in the hydrolysate obtained with Thermolysin has been possible by combining HILIC- and RP-HPLC-ESI-Q-TOF. Eighteen different peptides with a high percentage of hydrophobic amino acids residues such as L/I and P and aromatic amino acids as H were identified.

Acknowledgments

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