Journal of Chromatography B, 905 (2012) 23–30

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# Concentration and selective fractionation of an antihypertensive peptide from an alfalfa white proteins hydrolysate by mixed ion-exchange centrifugal partition chromatography

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ARTICLE INFO

*Article history:*
Received 20 April 2012
Accepted 24 July 2012
Available online 3 August 2012

*Keywords:*
Bioactive peptide
Centrifugal partition chromatography
Ion exchange
HPLC–MS
Protein hydrolysate fractionation
Antihypertensive peptide

ABSTRACT

This article reports a promising use of the mixed ion-exchange centrifugal partition chromatography (MIXCPC) technique in the field of downstream processes. A complex alfalfa white protein concentrate hydrolysate (AWPC hydrolysate) showing anti-hypertensive properties was successfully fractionated by MIXCPC to yield a L-valyl-L-tryptophan (VW) enriched fraction in one run. This dipeptide shows an interesting anti-angiotensin converting enzyme (anti-ACE) activity. An analytical method based on RP-LC/MS-MS was developed to quantify the target VW peptide in both the starting material and the enriched fractions. The best results for the MIXCPC fractionation were obtained by the combined use of the quaternary biphasic solvent system, methyl-*tert*-butylether/acetonitrile/*n*-butanol/water (2:1:2:5, v/v) in the descending mode, of the lipophilic di(2-ethylhexyl)phosphoric acid (DEHPA) cation-exchanger with an exchanger (DEHPA)/peptides ratio of 15, and of two displacers: calcium chloride and hydrochloric acid. The complexity of the starting material involved the selectivity optimization by splitting the stationary phase into two sections that differed by their triethylamine concentration. From 1 g of AWPC hydrolysate containing 0.26% of VW, 30.7 mg of a VW enriched fraction were recovered with a purity of 10.9%, corresponding to a purification factor of 41 and a recovery of 97%.



## 1. Introduction

Bioactive peptides obtained from food proteins by enzymatic hydrolysis (using trypsin, chymotrypsin, pancreatin, pepsin, termolysin, *etc.*) are of major interest for both academic and industrial sectors due to their beneficial health effects [1]. Indeed, these compounds combine (i) a wide variety of bioactivity (anti-hypertensive [2], morphinomimetic [2] or anti-microbial [3] for example), (ii) good development perspectives in middle to high value-added markets (such as nutrition, health, food safety, *etc.*) and (iii) the renewable aspect of their sources. Nevertheless, these hydrolysates are usually composed of about one hundred different peptides and therefore, an enrichment of raw mixtures in bioactive peptides must be envisaged in order to increase the target biological activity. In most cases, the enrichment is achieved by a tangential ultrafiltration step combined to at least two low-pressure liquid chromatographic steps (ion-exchange chromatography then size exclusion chromatography) [4–6]. The purification factor observed after such a process combination classically ranges from 10 to 15 and is costly and time consuming. This is currently one of the main bottlenecks of product development based on bioactive peptides.

Support free liquid–liquid chromatographic techniques, in its hydrostatic (centrifugal partition chromatography or CPC) and its hydrodynamic (counter-current chromatography or CCC) versions are both based on the use of biphasic solvent systems [7,8]. They have emerged as interesting alternative tools for the purification of biomolecules such as peptides. Different development modes defining different ways to implement such processes have been proposed. For instance Knight et al. developed specific solvent systems for the purification of short or hydrophobic peptides by elution high speed counter-current chromatography (HSCCC) [9,10] using for example, the quaternary biphasic system MtBE/acetonitrile/*n*-butanol/water with 1% TFA. In another work, protected peptides were purified in the pH-zone refining

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http://dx.doi.org/10.1016/j.jchromb.2012.07.034

mode using an HSCCC apparatus [11,12]. This method was highly productive and selective for the purification of organic molecules from many compound classes, but it cannot be applied to unprotected peptides due to their amphoteric nature. More recently, an improvement of the pH-zone refining process, called "affinity-ligand countercurrent chromatography", and closed to the ion-exchange process, was developed by Ma and Ito for the purification of unprotected peptide or di- and trisulfonated components of Quinoline Yellow using HSCCC devices [13–15]. In their approach, an ion-pairing reagent was added to the stationary phase in order to improve analyte extraction in the organic stationary phase. At the same time, the weak ion-exchange mode was developed on CPC apparatuses for the purification of sulfated polysaccharides [16]. More recently the strong ion-exchange centrifugal partition chromatography (SIXCPC) [17] was successfully applied to the purification of natural products such as rosmarinic acid from *Lavandula vera* [18] and glucosinolates from various Brassicaceae [19,20]. For this purpose, the lipophilic ion-exchanger (Aliquat® 336 in the case of the SIXCPC mode) was dissolved in the organic stationary phase in order to extract the hydrophilic analytes through ionic associations during the injection step. A displacer-containing aqueous mobile phase was pumped through the stationary phase, thus forcing the ionic analytes to be displaced in the aqueous mobile phase and to move along the column. As in any other displacement chromatography technique, the analytes proceed in the CPC column as an isotachic train, with the ionization state of the analytes being maintained throughout the whole chromatographic process. This last point is the main difference with the pH-zone refining mode, which involves multiple changes in the ionization state of the analytes through acid–base reactions.

Very recently, ion-exchange CPC was successfully carried out using the lipophilic di(2-ethylhexyl)phosphoric acid (DEHPA) cation-exchanger, for the purification of dipeptides within a five component model mixture [21,22]. The optimal separation conditions relied on a mixed ion exchange (MIXCPC) mode, which combined in a same experiment a strong ion-exchange process with $Ca^{2+}$ as displacer and a weak ion-exchange process with $H^+$ as displacer. Fig. 1 shows a schematic representation of the peptide separation using the weak and the strong ion-exchange mode. Selectivity is induced by the stability of ion pairs that is in turn dependent on amino-acid number, amino-acid structure and molecular geometry. As suggested by Chevolot et al. this procedure seems a good and simple method for fractionation of complex mixtures of ionic species, which are water soluble [16,17].

In order to improve the selectivity, the column was segmented in two zones, each one corresponding to a different DEHPA ionization state. The exchanger (DEHPA) was then activated by triethylamine at two different concentrations. Indeed, the liquid nature of the stationary phase in CPC (or in CCC) enables a wide range of original solutions for the resolution of tough purification problems (Fig. 2). By testing different deprotonation rates of DEHPA, corresponding to different DEHPA/TEA molar ratios, the separation of the apolar peptides was significantly improved when this ratio was low (about 3), whereas polar peptides required a higher ratio (about 45).

Then, the aim of this work was to assess the performance of the MIXCPC strategy in the field of downstream processes and more specifically for bioactive peptide separation. This purification strategy was applied to the capture of the L-Val-L-Trp (VW) antihypertensive dipeptide from an alfalfa (*Medicago sativa*, Fabaceae) white protein hydrolysate. This hydrolysate, composed of more than one hundred different peptides [23] was shown to have a strong *in vivo* antihypertensive activity [2], probably due to presence of the potent anti-angiotensin converting enzyme (anti-ACE) peptide VW [23].

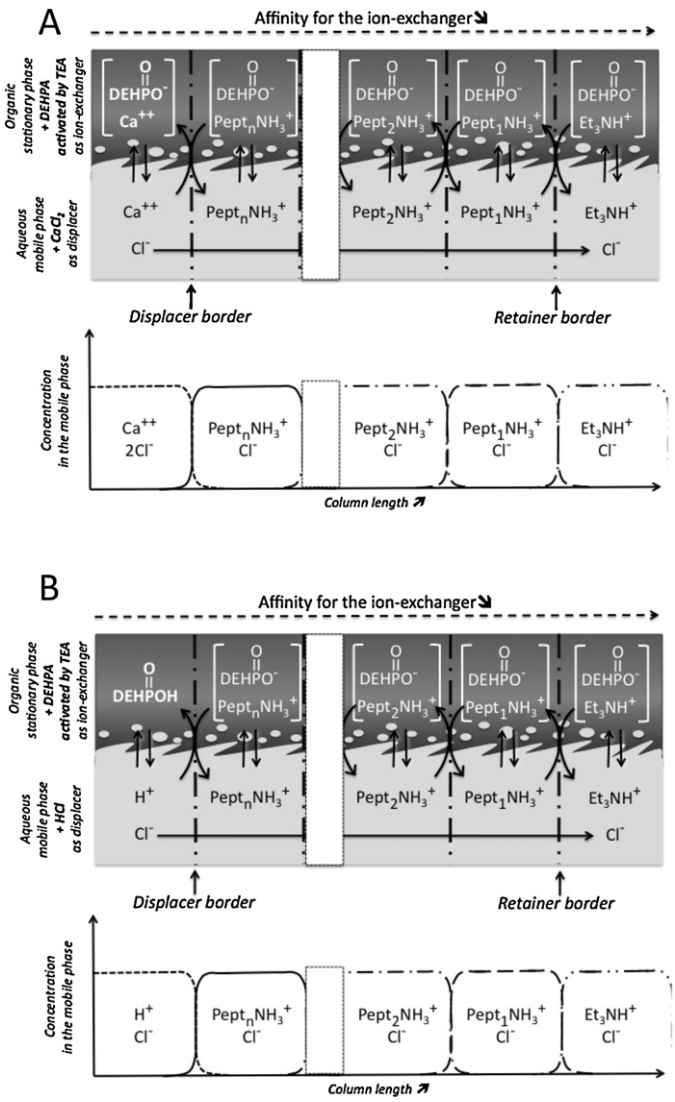


**Fig. 1.** Isotachic train A: in the strong ion-exchange mode, B: in the weak ion-exchange mode.

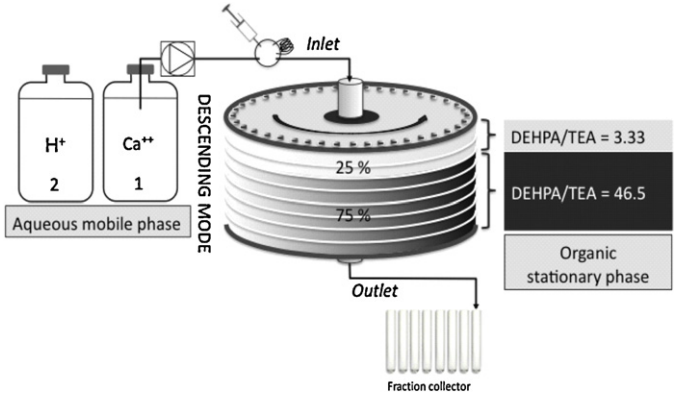


**Fig. 2.** Schematic representation of the MIXCPC mode (segmented stationary phase and two displacers).

## 2. Materials and methods

### 2.1. Reagents

Acetonitrile ($CH_3CN$), *n*-butanol (*n*-BuOH), methyl-tert-butyl ether (MtBE), were purchased as chromatographic grade solvents from Carlo Erba (Rodano, Italy). Trifluoroacetic acid (TFA), calcium chloride ($CaCl_2$), di(2-ethylhexyl)phosphoric acid (DEHPA), triethylamine (TEA), and 35% hydrochloric acid were purchased from Acros Organics (Illkirch, France). Water was purified by deionization and reverse osmosis. Pure Val-Trp (VW) and Gly-Val-Gly (GWG) from chemical synthesis used for the VW HPLC/MS assay calibration were from Bachem (Bubendorf, Switzerland).

### 2.2. Alfalfa white protein concentrate (AWPC) hydrolysate

The hydrolysate used in this study was obtained at pilot-plant scale according to Kapel et al. [2]. Spray-dried alfalfa white protein concentrate 3% (w/v) was hydrolyzed in a 32 L continuous reactor thermostated at 40 °C coupled with two tubular ultrafiltration modules containing $ZrO_2$ mineral membranes of 10 kg mol$^{-1}$ nominal molecular weight cut-off (6 mm inner diameter, 1.2 m long, 0.16 m$^2$ filtering area, Carbosep M5). Volume, pH and temperature were kept at 32 L, 9.5 and 40 °C respectively. The chosen space–time of 8 h was obtained holding the filtrate flow at 4 L h$^{-1}$. The ultrafiltrate obtained (100 L) is still charged in polyphenolic compounds. They were eliminated on an ion exchange step using a pilot plant scale column BPG 100/950 (Amersham biosciences, Uppsala, Sweden) filed with an anion exchanger resin (Amberlite IRA 900 Cl Rhöm & Haas, Germany). The column was equilibrated with an acetic acid/sodium acetate buffer (50 mM, pH 5.3) and eluted with the ultrafiltrate (100 L, adjusted at pH 5.3) at a 0.33 L min$^{-1}$ flow rate. At the column outlet, the ultrafiltrate appeared discolored, due to polyphenols adsorption on the resin.

The discolored filtrate was then desalted by electrodialysis (EUR2D-5P11, Eurodia industrie S.A., Wissous, France) at a constant 70 V voltage and spray dried (Niro atomizer minor production type, Rueil, France) at a 10 L h$^{-1}$ flow rate with a 170 °C inlet temperature and a 90 °C outlet temperature. The hydrolysate is a powder containing 7% water (w/w). Its dry matter consisted of about 95% of peptides (on the basis of Kjeldahl analysis).

### 2.3. CPC apparatus

The separations were performed on a FCPC® Preparative 200 Kromaton Technologies apparatus (Angers, France) using a rotor made of 20 circular partition disks (1320 partition cells: 0.130 mL/cell; total column capacity: 205 mL, dead volume: 32.3 mL). Rotation speed could be adjusted from 200 to 2000 rpm, thus producing a centrifugal force field in the partition cells of about 120 × *g* at 1000 rpm and 480 × *g* at 2000 rpm. The solvents were pumped by a semi-preparative Dionex P580HPG 4-way binary high-pressure gradient pump (Sunnyvale, CA, USA).

The samples were introduced into the column through a PEEK dual mode preparative scale sample injector 3725 (Rheodyne, Rohnert Park, CA, USA) equipped with a 10 mL sample loop. Effluent content was monitored by a Dionex UVD 170S detector equipped with a preparative flow cell (6 μL internal volume, path length of 2 mm). Fractions were collected by a Pharmacia Superfrac collector (Uppsala, Sweden).

### 2.4. Preparation of the biphasic solvent system for CPC runs

The biphasic system (2 L) was prepared by mixing MtBE, $CH_3CN$, *n*-BuOH and water in suitable proportions in a separatory funnel. They were vigorously shaken and then allowed to settle until the phases became limpid. After phase separation, DEHPA was added to the organic stationary phase. TEA was then added to activate DEHPA at the DEHPA/TEA molar ratio of 46.5 or 3.3. The mobile phase was prepared by adding the appropriate amount of displacers (solid $CaCl_2$ and/or HCl solution).

### 2.5. Sample preparation for CPC runs

The Alfalfa white protein hydrolysate (250 mg or 1 g) was dissolved in 7.5 mL of the fresh aqueous phase (without displacer). This aqueous solution was equilibrated with 2.5 mL of DEHPA-free organic phase to restore the saturation of the aqueous phase.

### 2.6. CPC experimental conditions

The particular experimental conditions are reported in Table 1. Before all experiments, the column was washed with MeOH/water (50:50, v/v) in the ascending mode at 20 mL/min with a 200 rpm rotation speed. Two column volumes (410 mL) of the organic stationary phase (SP) containing DEHPA and TEA with DEHPA/TEA = 46.5 were pumped in the descending mode at the same flow rate and rotation speed. Then, 50 mL of the second stationary phase with a higher concentration of TEA (DEHPA/TEA = 3.33) was pumped in the same conditions. The sample was then injected through the sample loop at 2 mL/min at 1200 rpm. Displacer-free mobile phase (MP) (50 mL on average) was pumped in at 2 mL/min for column equilibration. Finally, the aqueous mobile phase containing the displacer ($Ca^{2+}$, then $H^+$) was pumped at 2 mL/min, and the fractions were collected every minute. A schematic representation of the experimental procedure is shown in Fig. 2. The aqueous effluent was monitored by on-line UV absorbance measurement at λ = 215 nm. Stationary phase retention was 75% on average. The pressure was approximately 30 bars.

### 2.7. Peptide content

The total peptide amount in CPC fractions was evaluated on the basis of nitrogen assay by Kjeldahl analysis. The sample (1 mL) was mineralized with 4 mL $H_2SO_4$ 95% (v/v) and 1 mL $H_2O_2$ 30% at 400 °C for 150 min and cooled to room temperature. At this stage, all organic nitrogen is converted into ammonium salts, then converted into ammoniac by adding concentrated alkali. Ammonia is finally distilled and titrated by HCl 0.5 M. The amount of nitrogen in the sample was corrected by a 6.25 factor to get a proteic matter amount as recommended for alfalfa proteins.

### 2.8. VW quantification

A RP-HPLC/MS-MS method with an internal standard (peptide GWG) was used to quantify the VW peptide both in hydrolysate and CPC fractions. For the analysis, 10 μL samples (of hydrolysate or CPC fractions) were injected onto a C18 column (2.1 mm × 250 mm, 5 mm diameter beads, Prospher) connected to a Surveyor HPLC-MS pump (Thermoelectron corporation). Prior to injection, mixtures (AWPC hydrolysate or CPC fractions) were half-diluted with GWG solution at 50 mg L$^{-1}$ in solvant A ($H_2O$ at 0.1% TFA (v/v)). For RP-HPLC elution, solvant A and solvant B (acetonitrile 0.1% TFA in acetonitrile (v/v)) were used. The starting condition was 100% A. A first gradient slope was applied to reach 28% of solvent B in 50 min. A second gradient slope was then applied to have a final eluent composition of 60% B in 20 min.

After each run, the column was washed with 100% B for 10 min and re-equilibrated with 100% A for 20 min. Quantitation of peptide VW was performed using an ion trap as mass analyzer (LTQ, *i.e.* Linear Trap Quadripole, Thermoelectron corporation). Data were processed using Xcalibur software (version 2.1, Thermoelectron

**Table 1**
.

| | Run 1 | Run 2 | Run 3 | Run 4 |
|---|---|---|---|---|
| Sample | 254.6 mg | 252.9 mg | 254.9 mg | 1.07 g |
| Biphasic solvent system | MtBE/$CH_3CN$/*n*-BuOH/water (2:1:2:5, v/v) | | | |
| Stationary phase | Upper organic phase + DEHPA with DEHPA/TEA = 3.33 (25% of the column volume) then DEHPA/TEA = 46.51 (75% of the column volume) | | | |
| Mobile phase | Lower aqueous phase (+displacer after 35 min) | | | |
| [DEHPA] | 11.2 mM | 33.6 mM | 95 mM | 135 mM |
| DEHPA/peptides ratio | 5 | 15 | 42 | 15 |
| [$CaCl_2$] | 1.06 mM | 3.18 mM | 9 mM | 12.8 mM |
| [HCl] | 2 mM | 7.5 mM | 16 mM | 30.1 mM |
| VW content in the fractions of interest % m/m | 2.7 | 11.0 | 6.6 | 10.9 |
| Concentration factor | 10.2 | 41.3 | 24.8 | 41.0 |
| Recovery (%) | 97 | 97 | 96 | 97 |

corporation). The mass spectrometric conditions were as follows: ESI positive ionization mode (ElectroSpray Ionization) was used; source gases were set (in arbitrary units $min^{-1}$) for sheath gas, auxilliary gas and sweep gas at 20, 5 and 5, respectively; capillary temperature was set at 250 °C; capillary voltage was set at 26 V; tube lens, split lens and front lens voltages were set at 90 V, −42 V and −5.75 V, respectively.

The calibration curve was drawn using the L-Val-L-Trp (VW, peptide to be quantified) and Gly-L-Trp-Gly (GWG, internal standard) signal after MS fragmentation. Fig. 3 shows the RP-HPLC elution chromatogram of a VW/GWG mixture (both at 25 mg $L^{-1}$). Two peaks detected at 27.6 and 33.8 min revealed *m/z* signals at 319 and 304, respectively. Thus, in this elution condition, GWG (MW 318 Da) and VW (MW 303 Da) corresponded to retention times of 27.6 and 33.8 min respectively. VW and GWG MS/MS fragmentation showed that VW produces a fragment having a *m/z* of 205 whereas GWG yields a fragment of *m/z* 244 (Fig. 3).

The 205 fragment corresponded to a hydrated tryptophan released from the lysis of the VW peptide bond (fragment [$M_{Trp}$+1]+18). The fragment at *m/z* 244 fragment released from GWG, was a dehydrated GW fragment ([$M_{Gly\text{-}Trp}$+1]−18). These MS signals were then selected for VW quantification in the CPC fractions. VW/GWG mixtures were prepared at concentration ratio of 3.125/25, 6.25/25, 12.5/25, 25/25 and 50/25 mg $L^{-1}$ of VW/mg $L^{-1}$ of GWG. These ratios were chosen from expectations of the VW concentrations in both hydrolysate and CPC fractions. The total ion current (TIC) obtained from the elution of these mixtures at window searches of 204.5–205.5 (VW fragment) and 243.5–244.5 (GWG fragment) are presented in Fig. 4.

A calibration curve was established from MS signals in order to quantify VW in hydrolysate or CPC fractions. From these quantifications, three performance criteria of the separation process were calculated: (i) the purity of a fraction in VW (defined as the amount of VW upon mixture dry matter), (ii) the purification factor of a CPC step (defined as the ratio between the VW purity in the fraction over the VW purity in the hydrolysate) and (iii) the recovery.

## 3. Results and discussion

### 3.1. General strategy

The alfalfa white protein hydrolysate used in this study contained 7% (w/w) of water. The content of its dry matter in peptide, assessed from a Kjeldahl analysis, was about 95% (w/w). This complex hydrolysate, containing more than one hundred peptides [23], is known to have a significant *in vivo* anti-hypertensive activity after oral administration on an animal model (spontaneously hypertensive rats) [2]. It was hypothesized that this bioactivity was due to the presence of the potent ACE inhibitory peptide VW [23]. The analysis of the raw AWPC hydrolysate and the enriched fractions involved the development of a highly sensitive and specific analytic method in order to quantify fraction purity, purification factor and recovery.

Thus, the first part of the study was dedicated to the quantification of the VW target peptide in the raw AWPC hydrolysate using a specific LC/MS-MS method. Then, the mixed ion-exchange centrifugal partition chromatography (MIXCPC) mode [22] was applied to the capture of the peptide of interest from the crude AWPC hydrolysate. The exchanger/peptide ratio was adjusted to improve the purification factor of VW. The peptide content in the injected samples (in moles) was determined by considering an average molar mass of 600 g $mol^{-1}$ for the peptides, a peptide content of 95% and a water content of 7%. The DEHPA concentration was then calculated on the basis of 75% stationary phase retention. In each case, the VW content in the different CPC fractions, the purification factor and the recovery were calculated from LC/MS-MS analysis. Finally, the impact of the injected mass of the crude hydrolysate was also evaluated.

### 3.2. VW quantification by RP-HPLC–MS/MS

Considering the complexity of the hydrolysate, RP-HPLC coupled with on-line MS-MS analysis was required for VW quantification. In our case, the peptide GWG was selected as an internal standard since it contains a W residue, which guarantees a similar UV response coefficient as compared to the target peptide. The two glycines (slightly apolar) were chosen in order to obtain a slightly lower retention time than for VW. Retention time of GWG and VW standards were about 27 and 33 min, respectively (Fig. 3). The RP-HPLC chromatogram in Fig. 5A, corresponding to the crude hydrolysate doped with GWG standard, confirmed the complexity of the peptide mixture. A prominent peak at a retention time of about 33 min was obtained. Nevertheless, its on-line MS analysis yielded 6 significant *m/z* signals (including *m/z* 304.8), indicated its co-elution with other peptides. GWG was detected as a well-individualized peak composed of a single signal at *m/z* 319.

The signals obtained after MS-MS fragmentation were used for quantification in order to improve the selectivity of the detection step. The analysis of pure VW and GWG yielded specific fragments at *m/z* 205 and 244 respectively, thus allowing the establishment of a calibration curve (Fig. 4). Fig. 5B and C shows the total ion current of the hydrolysate mixed with GWG with *m/z* ranges from 243.5 to 244.5 (Fig. 5B) and 204.5–205.5 (Fig. 5C). In both cases, a single peak was observed at the retention time of VW and GWG, allowing the quantification of VW in complex mixtures. From the calibration curve, it was concluded that the hydrolysate was composed of 0.26% of VW (on dry matter basis). This assay was then used to quantify the VW content in CPC fractions.

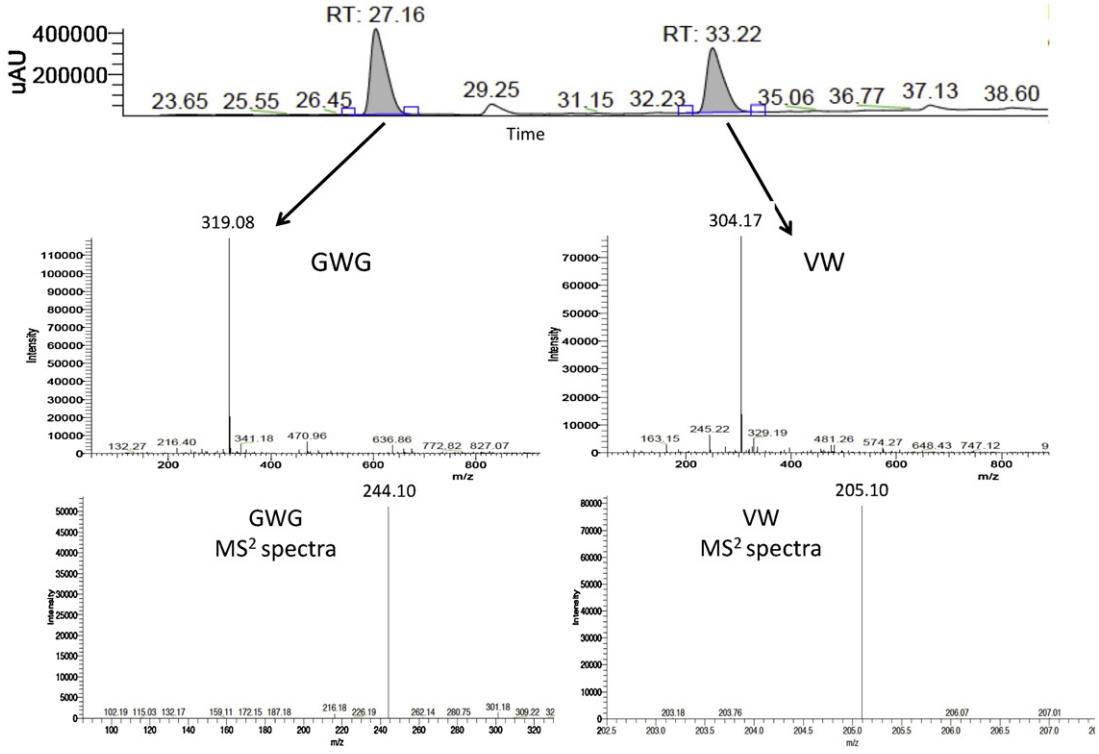


**Fig. 3.** RP-HPLC chromatogram of a VW/GWG mixture at a concentration ratio of 25 mg $L^{-1}$/25 mg $L^{-1}$. The MS spectra of the content of the two peaks obtained (at 27.65 min and 33.81 min) are displayed below the chromatogram.

### 3.3. AWPC hydrolysate fractionation by mixed ion-exchange CPC

#### 3.3.1. Starting conditions

The VW target peptide is a relatively apolar peptide ($\log D = -1$ at pH = 6, isoelectric point = 6.09) showing physico-chemical properties close to those of the peptide model mixture tested to develop the MIXCPC process [22]. Thus, the starting conditions were a polar MtBE/$CH_3CN$/*n*-BuOH/water (2:1:2:5, v/v) solvent system, di(2-ethylhexyl)phosphoric acid (DEHPA) as exchanger, TEA as DEHPA activator through acido-basic reaction, and two displacer carried out sequentially: $Ca^{2+}$ ($CaCl_2$) then $H^+$ (hydrochloric acid). DEHPA/TEA, DEHPA/$CaCl_2$ and DEHPA/HCl molar ratios were calculated on the basis of the optimal experimental conditions described in reference [22]. The head of the column (a quarter of the total inner volume, about 50 mL) was filled with the 3.3 DEHPA/TEA organic phase. The other part of the column was filled with 46.5 DEHPA/TEA organic phase (Fig. 2 and Table 1). This original solution involving a stationary phase step gradient had proven to be a promising solution to enhance the selectivity in the case of delicate peptide purifications [22]. In addition, DEHPA/$CaCl_2$ and DEHPA/HCl molar ratios were about 10 and about 5, respectively (Table 1). The last parameter to define was the DEHPA/peptide molar ratio.

#### 3.3.2. Exchanger/peptide concentration ratio adjustment

In ion-exchange CPC, the exchanger/analyte ratio is an important parameter to control, especially in the case of peptides which can possess several different ionized sites. In our previous work, we demonstrated that a ratio of about 5 for a dipeptide is generally

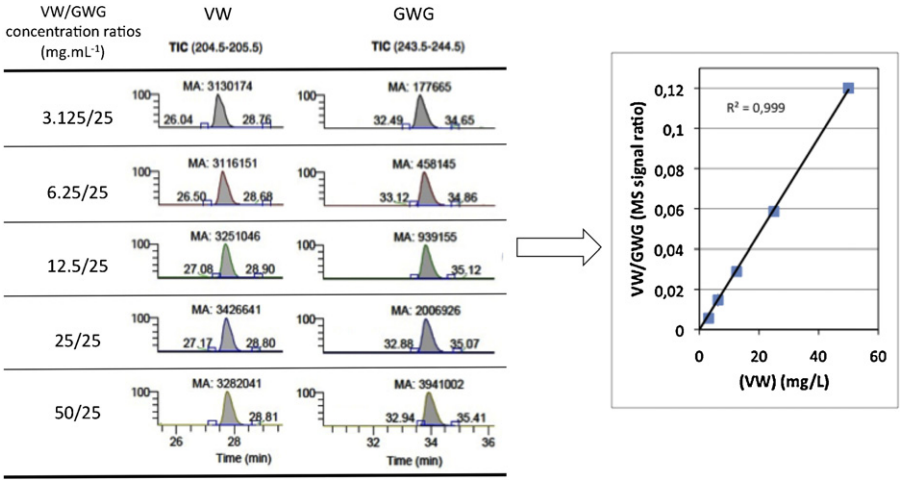


**Fig. 4.** Total ion current plot of VW/GWG mixtures at ratio ranging from 3.125/25 to 50/25 (in mg $L^{-1}$ of VW/mg $L^{-1}$ of GWG). To the right, the calibration curve (VW/GWG MS signal as a function of VW concentration) is displayed.

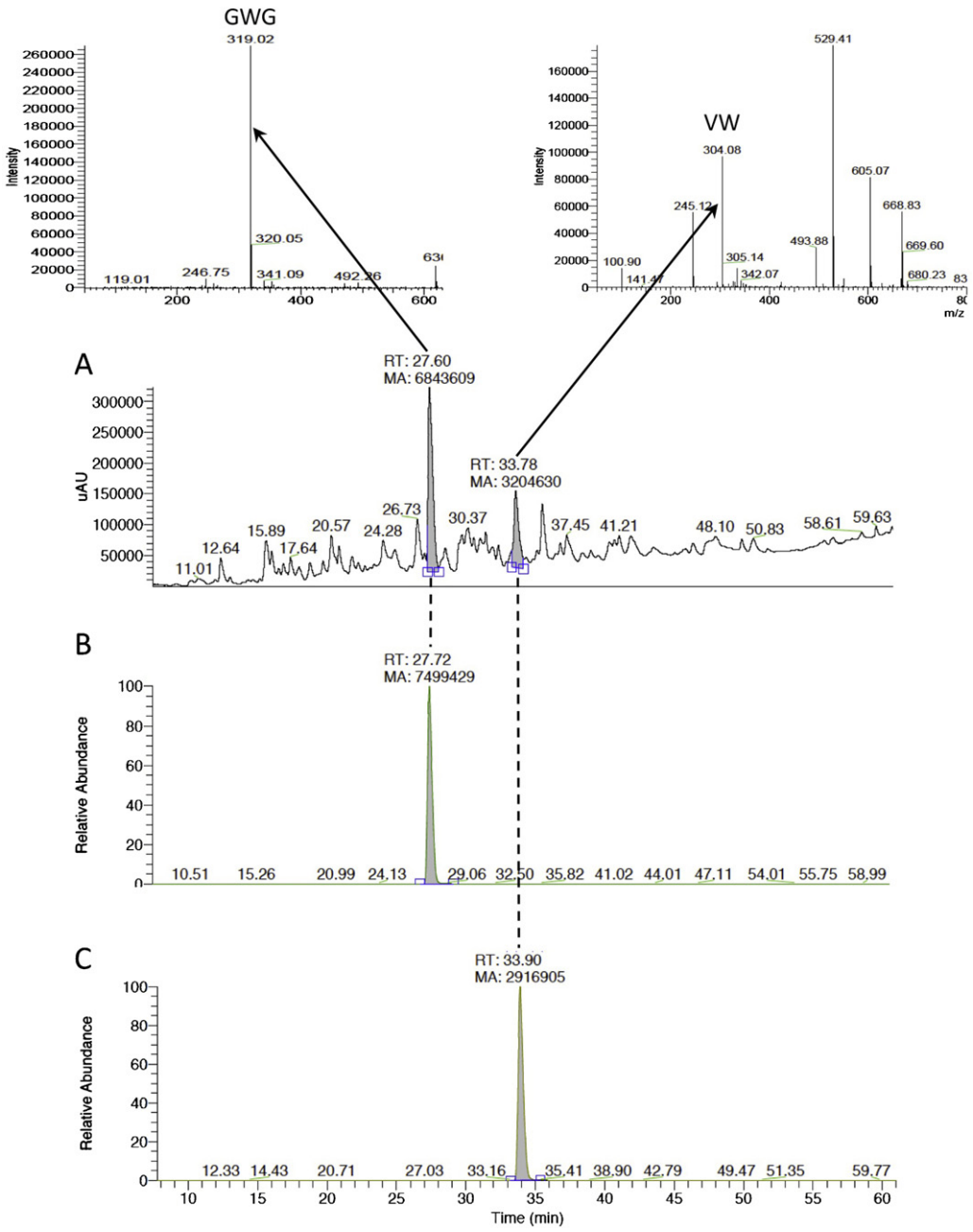


**Fig. 5.** (A) RP-HPLC chromatogram of the alfalfa white proteins hydrolysate dopped with GWG. The concentration ratio used was 10/25 (in g $L^{-1}$ of hydrolysate/mg $L^{-1}$ of GWG). MS spectra of the peak content at around 27 and 33 min are presented above the chromatogram and (B and C) total ion current in a 243.5–244.5 (b) and 204.5–205.5 (c) range.

optimal to provide a good separation [22]. Nevertheless the VW target peptide is contained in a very complex matrix, containing about 90% of peptides with an average molar mass of 600 g $mol^{-1}$ (corresponding to about 5–6 amino acid residues) [22]. Nevertheless, if a ratio DEHPA/peptides of about 5 is optimal for dipeptide mixtures (2.5 DEHPA per amino acid residue), it seems judicious to test also a ratio of about 15 ($2.5 \times 6$) for the AWPC, this hydrolysate containing peptides with an average amino acid number of about 6. Finally, a ratio of 42 was also tested to evaluate the effect of a large excess of exchanger on the quality of the fractionation. Ratios lower than 5, corresponding to an exchanger lack, were not used. Indeed, preliminary partition essays in vials showed an incomplete extraction of the peptides in the DEHPA containing organic phase. For all these preliminary CPC runs 250 mg of the hydrolysate were injected. All experimental conditions are presented in Table 1.

In each case, VW was displaced in the aqueous mobile phase only when the second displacer ($H^+$) was introduced, which is not surprising since VW is a relatively hydrophobic peptide ($\log D = -1$ at pH = 6). Indeed, in previous work we demonstrated that "apolar" peptides are displaced only during the weak displacement step involving hydroniums as displacer [22]. The VW behavior confirmed the potential of the capture strategy using the ion-exchange mode since part of the peptides were eliminated during both the washing step involving fresh aqueous mobile phase and the strong ion-exchange step involving $Ca^{2+}$ as displacer.

For run 1 (Table 1), a DEHPA/peptide ratio of 5, optimized for dipeptide separation, was used. Although a majority of peptides had

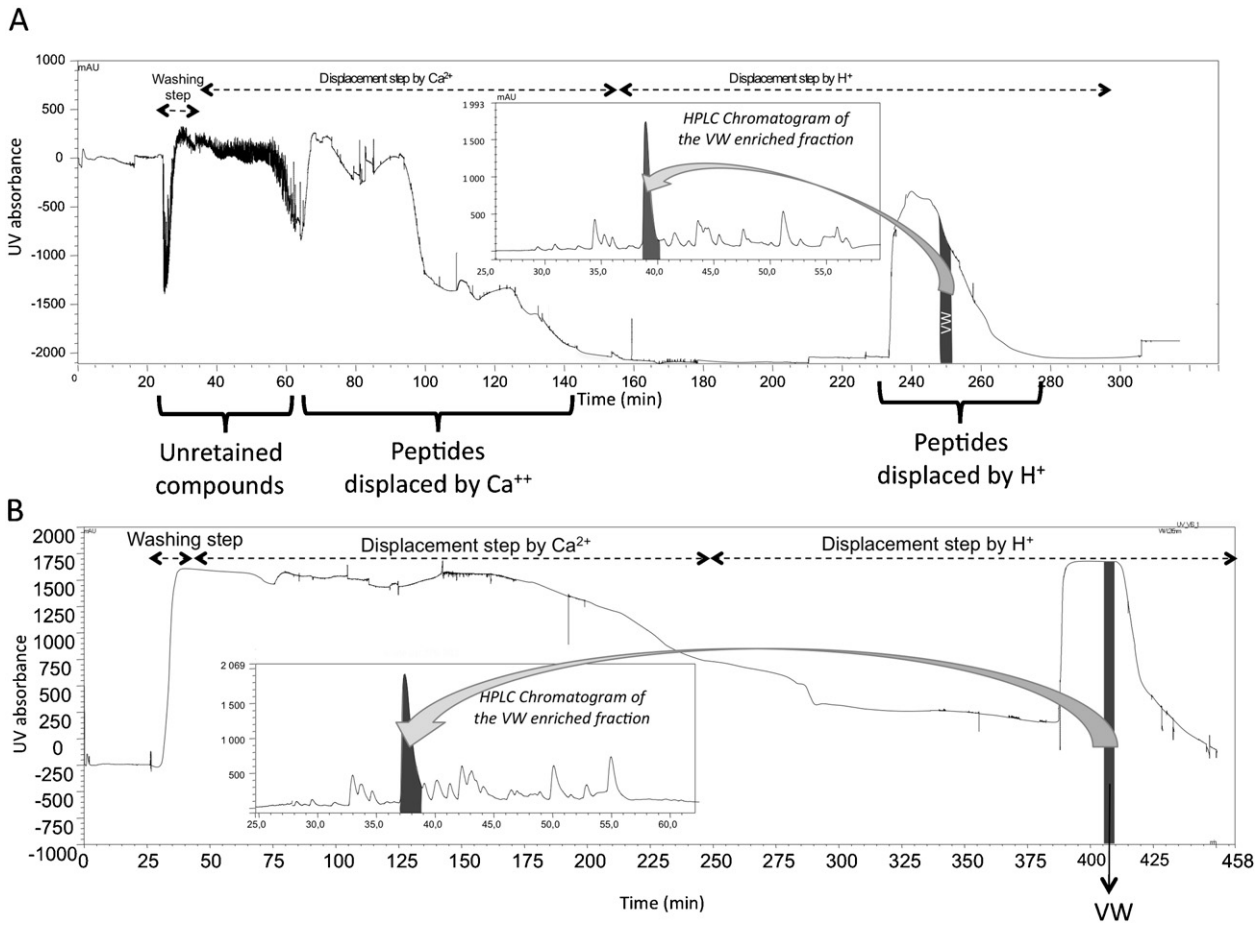


**Fig. 6.** (A) Purification by MIXCPC of the anti-ACE VW peptide from 250 mg of the AWPC hydrolysate, DEHPA/peptides molar ratio = 15 (see Table 1 for all experimental conditions) and (B) purification by MIXCPC of the target VW peptide from 1 g of AWPC hydrolysate DEHPA/peptides molar ratio = 15 (see Table 1 for all experimental conditions).

been eliminated during both the washing and the $Ca^{2+}$ displacement steps, VW being only displaced by $H^+$ action, the enrichment factor of 10.2 was lower than expected (see Table 1). This experiment yielded 24.3 mg of a VW containing fraction, with a purity of 2.7% and a VW recovery of 97%.

In the second experiment, the DEHPA/peptide ratio was increased to 15 to be theoretically adapted to the average amino acid number, which was about 6 (run 2, Table 1 and Fig. 6A) [22]. The first displacer $Ca^{2+}$ was introduced only 10 min after the solvent front, since VW was not displaced during the strong ion exchange process. The second displacer ($H^+$) was only introduced when the UV signal returned to baseline. This run yielded 6.5 mg of a VW enriched fraction (collected between 249 min and 252 min), corresponding to a purity of 11%, an enrichment factor of 41.3 (Table 1) and a VW recovery of 97%. Such a result is enough if neutraceutical applications are considered. The interest for the pharmaceutical industry, which markets highly pure products is more limited, this dipeptide being easily synthetized. This result was promising considering the complexity of the starting material and highlights the importance of the exchanger concentration on the separation quality, especially in terms of enrichment factor. In comparison, an ACE inhibitory peptide was recently purified with an enrichment factor of about 10, the separation process involving a sequential use of tangential filtration, ion exchange- and size exclusion-chromatography [4]. Thus, the purification factor (more than 40) obtained by MIXCPC is very high for a single separation run. Moreover, a classical chromatography sequence would probably have taken a full day and required different columns filled with high-cost stationary phases.

In a third experiment, a large excess of DEHPA was tested (DEHPA/peptides = 42, run 3 Table 1) yielding a VW purity of 6.6% in the enriched fraction, which is lower than for the run 2. This behavior had been previously observed [21]: in this case, the exchanger/analytes ratio is too high to promote competition between the peptide analytes, which is a basic requirement of displacement chromatography. By decreasing the DEHPA concentration (for example 15 for the run 2), the peptides can compete for the exchanger, thus improving the separation.

In the fourth MIXCPC run, 1 g of the starting material was injected, thus increasing the sample mass by a factor of 4. All the ratios (DEHPA/peptides, DEHPA/displacers) were kept constant (experiment 4, Table 1). In comparison, low-pressure liquid chromatographic supports, often used in downstream processes, are classically loaded with raw hydrolysates at concentrations ranging from 1 to 10 g $L^{-1}$. Thus, a 1 g injection of AWPC hydrolysate corresponds to an injection volume of several hundred milliliters in classical low pressure chromatography, involving then at least 1 L of solid stationary phase. The resulting MIXCPC chromatogram and the HPLC analysis of the VW enriched fraction are shown in Fig. 6B. An increase of the run duration was observed, due to the delay before $H^+$ introduction as second displacer. This delay was a consequence of the increasing amount of unretained compounds, these compounds not being involved in the displacement chromatographic process. According to HPLC analysis, fractions between

406 and 410 min containing the target peptide VW were pooled together, evaporated to dryness, and then analyzed by HPLC/MS to quantify the VW content. Thus, a 30.7 mg VW enriched sample (HPLC purity: 10.9%, enrichment factor = 41, Table 1) was obtained, these results being consistent with experiment 2.

## 4. Conclusion

The aim of the present study was to highlight the potential of mixed ion-exchange centrifugal partition chromatography when applied in the field of downstream processes. By using the MIXCPC method, fractions enriched in the ACE inhibitory peptide VW were obtained from an Alfalfa white protein concentrate hydrolysate showing interesting anti-hypertensive properties. The quantification method based on RP-HPLC–MS/MS showed the feasibility of the process, the latter yielding a purification factor of about 40 with a recovery of 97% for the target VW peptide. Further studies involving molecular modeling approaches will soon be undertaken to better understand selectivity criteria which govern the MIX-CPC process. Nevertheless, the weak point of this protocol is the run duration, which affects the process productivity. This problem should be overcome by transposing the MIXCPC process on a support-free liquid-liquid device with less partition cells, such as the recently developed centrifugal partition extractor FCPE® [24,25]. The purity of the fraction of interest could be increased, if necessary, by the subsequent use of a technique having an orthogonal selectivity criterion such as preparative reversed phase HPLC.

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