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# Ion-exchange centrifugal partition chromatography: A methodological approach for peptide separation

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ABSTRACT

This article presents the scope and optimization strategies employed in ion-exchange centrifugal partition chromatography (IXCPC). Both the weak and the strong modes were used to separate the constituents of a model mixture of dipeptides. Thus, 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, and of two displacers: calcium chloride and hydrochloric acid, has proven to be efficient for the preparative separation of the model mixture of five dipeptides (GG, GY, AY, LV and LY, in the order they were collected). The separation was optimized by splitting the stationary phase into two sections that differed by their triethylamine concentration. Moreover, the chemical nature of the exchanger/analyte entities that were involved in the chromatographic process was determined by $^{31}$P and $^{1}$H DOSY NMR experiments.

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## 1. Introduction

Peptides constitute a large class of molecules with high potential as therapeutic agents (anti cancer, anti hypertensive for example), or as active substances in cosmetics or nutriceuticals [1]. Bioactive peptides can be produced by chemical synthesis, fermentation and extraction from natural sources. These techniques often yield mixtures that are chemically complex and frequently contain compounds with high structural similarity. Traditional processes for peptide purification generally involve a chromatographic step, such as reverse phase high performance liquid chromatography, or ion exchange solid phase chromatography. These methods are highly specific and lead to very good chromatographic selectivity, but their poor throughput constitutes the most common bottleneck in an industrial scale production process.

The technique of support free liquid–liquid chromatography [4], in its hydrostatic (centrifugal partition chromatography or CPC) and its hydrodynamic (Counter-Current Chromatography or CCC) declensions, has also been applied to peptide purification. Knight et al. developed specific solvent systems for the purification of short or hydrophobic peptides by elution High Speed Counter-Current Chromatography (HSCCC) [2,3]. In addition to the elution mode, the displacement mode was introduced in countercurrent chromatography (CCC) in 1994 and applied to the purification of protected dipeptides [5]. Basically, displacement chromatography in CCC and centrifugal partition chromatography (CPC) are performed by dissolving a displacer in the mobile phase and a retainer or an ion-exchanger in the stationary phase. By adding an acid or a base in the stationary phase as a retainer, Ito introduced the pH-zone refining mode [5]. For the first time in CCC and CPC, isotachic rectangular shaped blocks of analytes separated by steep boundaries corresponding to "mixing zones", the so-called shock layers [6], were observed. The original pH-zone refining mode (*i.e.* without any ion-pairing agent) is restricted to solutes that show a dramatic difference in polarity and, therefore, in solubility, between their neutral and ionized forms. This limits the application of pH-zone-refining to ionized or ionizable, strictly water-soluble, or amphoteric molecules. Ma and Ito purified protected peptides in the pH-zone refining mode using an HSCCC apparatus [7,8]. This method is highly productive and selective for the purification of organic molecules from many compound classes [9–11], but it cannot be applied to unprotected peptides due to their amphoteric nature.

A variant of pH-zone refining, called "affinity-ligand counter-current chromatography", was then developed by Ma and Ito for peptide purification [12,13]. In their approach, an ion-pairing reagent was added to the stationary phase to improve peptide extraction. This process probably inspired the first separations in

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CPC that were based on the weak ion-exchange displacement mode. These works concerned the purification of polysulfated polysaccharides (fucans and heparins) by means of the lipophilic secondary amine Amberlite LA2 (*N*-lauryl-*N*-trialkylmethylamine) [14–17]. More recently, a preparative purification method of hydrophilic anionic molecules based on strong ion-exchange centrifugal partition chromatography (SIXCPC) using a lipophilic quaternary ammonium salt such as Aliquat® 336 as a strong anion-exchanger was developed. This strategy was applied to the purification of the three isomers of hydroxycinnamic acid [18], of rosmarinic acid from *Lavandula vera* [19], of glucosinolates from various Brassicaceae [20,21], and of anthocyanins from *Vitis vinifera* [22]. Weisz et al. have also recently applied the affinity-ligand pH-zone refining strategy to the preparative separation of di- and trisulfonated components of Quinoline Yellow, using protonated dodecylamine as an ion exchanger and tetrabutylammonium hydroxide as an ion-pairing reagent [23].

This publication highlights the performance of the cation-exchange mode for the CPC purification of unprotected peptide mixtures. The methodology was applied to a synthetic mixture of five dipeptides of different polarity. The use of di(2-ethylhexyl)phosphoric acid (DEHPA) as exchanger [12,13], the sequential injection of two displacers ($Ca^{2+}$ followed by $H^+$) and the splitting of the stationary phase into two sections with different triethylamine concentrations lead to the resolution of the model mixture in just one run. This separation study also provided a good pretext for the thorough investigation of the different experimental parameters, such as the exchanger/analyte ratio, the nature and concentration of the displacer, the nature of the exchanger–analyte complex, and the ionization state of the exchanger.

## 2. Experimental

### 2.1. Reagents

Acetic acid (AcOH), acetonitrile ($CH_3CN$), *n*-butanol (*n*-BuOH), methyl-*tert*-butyl ether (MtBE), methanol (MeOH) were purchased as chromatographic grade solvents from Carlo Erba (Rodano, Italy). Trifluoroacetic acid (TFA), glacial acetic acid, calcium chloride ($CaCl_2$), potassium chloride (KCl), magnesium chloride ($MgCl_2$), manganese chloride ($MnCl_2$), di(2-ethylhexyl)phosphoric acid (DEHPA), triethylamine (TEA), and 35% hydrochloric acid were purchased from Acros Organics (Illkirch, France). Water was purified by de-ionization and reverse osmosis. Gly-Gly (GG, >99%) and Gly-L-Tyr (GY, >99%) were kindly provided by Lonza (Visp, Switzerland). L-Ala-L-Tyr (AY, >99%), L-Leu-L-Val (LV, >99%) and L-Leu-L-Tyr (LY, >99%) were purchased from TCI Europe (Zwijndrecht, Belgium).

### 2.2. 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 per 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 $120 \times g$ at 1000 rpm and $480 \times g$ 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).

### 2.3. Preparation of the biphasic solvent system for CPC separations

The biphasic system (2 L) was prepared by mixing MtBE, $CH_3CN$, *n*-BuOH and water in suitable proportions in a separatory funnel. The solvents 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 in appropriate amounts to activate DEHPA at the DEHPA/TEA molar ratio of 46.51 or 3.33. The mobile phase was prepared by adding the appropriate amount of displacers (solid $CaCl_2$ and/or HCl solution).

### 2.4. Preparation of sample solutions

A sample (20 mg) of each peptide (GG, GY, AY, LV and LY) 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.

### 2.5. CPC experimental conditions

The particular experimental conditions are contained in the figure captions. Before each experiment, the column was washed by 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) were then pumped in the descending mode at the same flow rate and rotation speed. The sample was injected through the sample loop at 2 mL/min at 1200 rpm. Displacer-free mobile phase (MP) (50 mL on average) was pumped at 2 mL/min in order to allow column equilibration. Finally, the aqueous mobile phase that contained the displacer was pumped at 2 mL/min, and the fractions were collected every minute. Effluent content was monitored at $\lambda = 215$ nm. Stationary phase retention was about 75% on average. The pressure was approximately 35 bars. The experiments were conducted at room temperature ($22 \pm 2$ °C).

### 2.6. Fraction analyses

All CPC fractions were checked by TLC on Merck 60 F254 silica gel plates. After elution with *n*-BuOH/AcOH/water (55:15:30, v/v), the analytes were revealed by spraying with a 1% ninhydrin solution in pyridine/glacial acetic acid (5:1, v/v). Under these conditions, the retention factors ($R_f$) for each dipeptide were: 0.10 for GG, 0.30 for GY, 0.35 for AY, 0.50 for LV and 0.55 for LY. The purity and recovery of the peptides were determined by HPLC according to the following procedure. Quantitative analysis were performed on a customized Dionex Summit HPLC system, equipped with a P580 pump, an ASI-100 automated injector, a STH column oven and a UVD340S diode array detector. The system was fitted with a Jupiter Proteo ($250 \times 4.6$ i.d., 4 μm particle size) column, itself protected by a C12 4 mm × 3.0 mm cartridge in a KJ0-4282 Security Guard Analytical Guard cartridge system (Phenomenex, Le Pecq, France). The mobile phases were 0.1% (v/v) TFA in water and 0.09% TFA in acetonitrile. The water/$CH_3CN$ gradient was set as follows: the initial $CH_3CN$ content was 0%; it was raised to 25% (v/v) in 15 min then to 50% (v/v) in 30 min and finally maintained for 5 min. The flow rate was 0.5 mL/min. The injection volume was 15 μL. UV detection was fixed at $\lambda = 215$ nm. The temperature of the column oven was set at 25 °C. All of the chromatographic data management was supervised by the Chromeleon software, version 6.0.1 (Dionex). Under these conditions, retention times for each dipeptide were: 6.47 min for GG, 19.44 min for GY, 19.35 min for AY, 23.52 min for LV and 23.03 min for LY. Calibration curves were established by

serial dilution of three independent stock solutions (0.1, 0.5, 1, 1.5, and 2 g/L) and by plotting the peak area recorded from HPLC chromatograms as a function of each peptide concentration. Recoveries were obtained on the basis of the calibration curves, by calculating the quantities of peptides obtained with purities greater than or equal to 95% as a percentage of the injected peptides.

### 2.7. Partition isotherm determination

Partition isotherms of each peptide in the MtBE/$CH_3CN$/*n*-BuOH/water (2:1:2:5, v/v) system were determined at room temperature (22 ± 2 °C) with DEHPA as the exchanger. Stock solutions of each peptide were prepared by dissolving an appropriate amount of the peptide in the aqueous phase of the biphasic solvent system, so that the concentration was 5 mM. In the same way, a stock solution of DEHPA was prepared by adding an appropriate amount of the exchanger in the organic phase to obtain a 200 mM concentration. In vials, 0.5 mL of the peptide stock solution was mixed with 0.5 mL of the organic phase of well defined DEHPA concentration (0, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 mM) that was obtained by dilution of the DEHPA stock solution. The phases were allowed to settle until they became limpid. Then 250 μL of each phase was mixed with 250 μL of fresh conjugate phase (without peptide or DEHPA) and 250 μL of MeOH to make the system monophasic. Peptide concentrations in each phase were determined by HPLC as described in Section 2.6. The partition curves were drawn by plotting $[\text{peptide}]_{org}/[\text{peptide}]^0_{aq}$ *vs* $[\text{DEHPA}]_{org}/[\text{peptide}]^0_{aq}$, in which $[\text{peptide}]^0_{aq}$ is the initial peptide concentration in the aqueous phase. Slopes were calculated by considering the first 4 points of each partition curve.

### 2.8. NMR

#### 2.8.1. Reagents

$D_2O$ was purchased from Eurisotop (Gif-sur-Yvette, France). Dimethylphenylphosphine, methyldiphenylphosphine, triphenylphosphine and tri-*n*-octylphosphine oxide were purchased from Sigma–Aldrich (Lyon, France). Trialkylphosphine internal references were dissolved at a concentration of 30 mM in the MtBE/$CH_3CN$/BuOH/$D_2O$ biphasic solvent system (2:1:2:5, v/v).

#### 2.8.2. $^1H$ and $^{31}P$ 2D-DOSY experiments and 31P D-FW analysis

All NMR experiments were performed at 298 K on a Bruker Avance AVIII-500 NMR spectrometer equipped with a 5 mm BBFO-PLUS probe. Static field gradient pulses were generated by a 10 A amplifier that delivered a nominal 0.535 T m$^{-1}$ gradient. The other necessary experimental details are reported in the Supplementary Material file.

## 3. Results and discussion

### 3.1. General strategy

Ion-exchange CPC purification of cationic molecules requires the presence of an exchanger in the organic stationary phase, namely a lipophilic anionic extraction agent. During sample injection, the analytes and the exchanger must preferentially form ion pairs in the organic stationary phase. Then, the displacer-containing aqueous mobile phase is pumped through the stationary organic phase, and performs the displacement process of the hydrophilic analytes in the mobile phase, thus allowing them to move along the CPC column [22]. 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 between the pH-zone refining mode, which involves multiple changes in the ionization state of the analytes through acid–base reactions. The chromatographic processes (schematized and adapted to the present separation in Fig. 3) and the corresponding chemical equilibriums of both the weak ion- [16] and the strong ion-exchange [18] modes have previously been described.

In each cell, the analyte that has the highest association constant for the cation-exchanger competitively excludes those with a lower one. Thus, it acts as a displacer by forcing the other analytes back into the aqueous mobile phase and to progress along the column. Narrow shock layers between analytes are always observed. This process creates steep analyte concentration rises and falls at the column outlet. The sharp front of the analyte train is formed by the competition generated during the association process with the exchanger between the less retained analyte and the exchanger counter-ion, whereas the end of the train is maintained as a shock layer by the displacer. Once the analytes are separated by mutual exclusion, they progress in the CPC column as neighboring segments.

A model mixture of five dipeptides: Gly-Gly (GG), Gly-Tyr (GY), Ala-Tyr (AY), Leu-Val (LV) and Leu-Tyr (LY) was chosen in order to evaluate the scope of ion exchange CPC for peptide purification. These peptides have very close isoelectric points that range from 6.08 to 6.1, so that they are electrically charged in the same way at the same pH. Nevertheless, they cover an hydrophobicity range, from the hydrophilic GG ($\log D = -4.35$ at pH = 6) to the hydrophobic LV ($\log D = -1.37$ at pH = 6) and LY ($\log D = -1.14$ at pH = 6). Both GY ($\log D = -2.90$ at pH = 6) and AY ($\log D = -2.55$ at pH = 6) have an intermediate hydrophobicity, and are structurally very close. Log *D* is defined by the logarithm of the ratio of the sum of concentrations of the solute's various forms in octanol, to the sum of the concentrations of its forms in water. It is routinely used by the pharmaceutical industry to evaluate the hydrophobic-lipophilic balance of ionized drugs. The log *D* values were calculated using the log *D* software (Advanced Chemistry Development, Inc., Ontario, Canada).

The model mixture we chose thus allowed us to investigate the chromatographic process selectivity in a non-trivial case.

#### 3.1.1. Starting conditions

The starting conditions were based on the literature [8]: 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 hydronium ions (hydrochloric acid) as displacer. DEHPA/peptides, DEHPA/TEA and DEHPA/HCl molar ratios, calculated on the basis of the experimental conditions described in Ref. [8], were 16.60, 46.51 and 46.50, respectively.

Under these conditions, GG was eluted close to the solvent front. As expected, GY and AY were well separated but the process was intentionally stopped because LV and LY were not eluted, in spite of an excessively long experimental time. Moreover, the chromatographic peaks did not show the typical profile of the displacement mode with a compressive front between each solute. Previous work on the separation of free dipeptides [8,13] suggested that adjustment of the ligand concentration (DEHPA) and/or the polarity of the solvent system was necessary to improve the separation. Nevertheless, it was clearly observed that the resolution of both hydrophilic (GY) and hydrophobic (LY) dipeptides in the same run would be a challenge. Thus, all of the different experimental parameters, such as the exchanger/peptide and exchanger/displacer ratios, the nature of the displacer and the deprotonation rate of the exchanger were examined and optimized in order to improve the separation performance.

#### 3.1.2. Exchanger and displacer concentration adjustment

The optimal DEHPA/peptide ratio was empirically found to be about five to three times less than in the initially tested conditions. A possible explanation could be that DEHPA concentration was too high to promote competition between the peptide analytes, which is a basic requirement of displacement chromatography. By decreasing the DEHPA concentration, the peptides can now compete for the exchanger, thus improving the separation.

In the same way, the best compromise for the DEHPA/HCl ratio was found to be about 5, which is tenfold less than in initial conditions.

The chromatogram that was obtained with these two ratios and experimental conditions is shown in Fig. 1A.

All peptides were displaced in less than 200 min. A compressive aspect of the shock layers was observed with GY and AY, thus proving that a displacement process took place.

Nevertheless, apolar peptides LV and LY presented a characteristic elution mode profile with a high overlapping. The peptide recoveries are shown in Fig. 2 (run 1).

This observation led us to draw partition isotherms (see Section 2.7) that represent the proportion of each peptide in the organic phase, $[\text{peptide}]_{org}/[\text{peptide}]^0_{aq}$, as a function of the $[\text{DEHPA}]_{org}/[\text{peptide}]^0_{aq}$ concentration ratio (Fig. 1B).

The slope of the tangent in the initial part of these curves represents the $[\text{peptide}]_{org}/[\text{DEHPA}]_{org}$ concentration ratio, and provides an estimate of the number of exchanger molecules that are required to cause the extraction of one peptide molecule. A slope value close to 1 characterizes a system in which one molecule of exchanger is necessary to extract one molecule of peptide. We clearly see in Fig. 1B that GG does not interact with DEHPA since the slope of the linear part is about 0. The other peptides are well extracted and the slopes, lower than 1, thus suggest that the “entities” that are responsible for the extraction phenomenon contain many DEHPA molecules. This observation is in good agreement with our previous results on the purification of hydroxycinnamic acids and glucosinolates [18,20]. More precisely, extraction is the most efficient when the DEHPA/peptide ratio is close to 5.5.

The asymptotic region of the curves in Fig. 1B reflects the affinity of the DEHPA–peptide species for the organic phase. The difference between the LV and LY isotherms should be sufficient to ensure a good selectivity during the extraction step.

Thus, the observed lack of selectivity between LV and LY could be due to the displacement step. Addressing this problem is possible in two ways, by either modifying the nature and/or the concentration of the displacer, or the deprotonation rate of the exchanger by adjusting the TEA concentration in the organic phase.

### 3.2. Optimization of the displacement step

#### 3.2.1. Choice of a cationic displacer

All of the previously described experiments were carried out in the weak ion exchange mode ($H^+$ as displacer). A significant improvement in the separation was observed when the sequential use of strong ion exchange and weak ion exchange modes was employed.

In the literature, DEHPA is known to be a good extractant of metallic cations such as calcium and magnesium but also nickel and rare earth elements [24–26]. Some of these cations were tested as displacers and calcium chloride was selected, due to its efficiency, low cost and low toxicity.

The calcium cation was only able to displace GY and AY into the aqueous mobile phase, and the overlapping zone was reduced compared to what was observed with protons as the displacer. The two other extracted dipeptides of the test mixture were left in the organic phase, thus providing a highly selective process.

In this case, the exchange process is said to be strong because the ionization state of DEHPA does not change. Conversely, the $H^+$ promoted exchange is considered as weak. Fig. 3 shows a schematic representation of the peptide separation using the weak ion-exchange ($H^+$ as displacer) or the strong ion-exchange ($Ca^{2+}$ as displacer) mode.

#### 3.2.2. Mixed ion exchange CPC (MIXCPC)

The separation of all 5 peptides was carried out by the sequential use of two displacers: first $Ca^{2+}$ and then $H_3O^+$. Both strong and weak ion-exchange processes occur during the same CPC run, resulting in an original mixed ion-exchange development mode (MIX-CPC).

Fig. 4A shows the resulting chromatogram that was obtained under the following experimental conditions: DEHPA/peptides = 5.4, DEHPA/$CaCl_2$ = 10.4 and DEHPA/HCl = 6.

Mildly polar peptides, GY and AY, were well separated, with the corresponding shock layer being reduced. At the same time, polar peptide separation was also improved, even though an important mixture zone remained (Fig. 4A). Moreover LV and LY no longer showed highly compressive profiles, due to an elution component in the chromatographic process. Even if the sequential action of the displacer did not solve the selectivity problem between the non-polar peptides, the MIX-CPC mode controlled the elution of the different peptide classes, the non-polar ones being collected only upon displacement by hydronium ions. Peptide recoveries are shown in Fig. 2 (run 2).

### 3.3. Deprotonation rate of the exchanger

#### 3.3.1. Impact of the DEHPA/TEA ratio

By testing different DEHPA/TEA molar ratios, it appeared that the extraction of the apolar peptides (LV and LY) was significantly improved when this ratio reached 3.33, but GY and AY were still not well separated.

#### 3.3.2. Use of a segmented stationary phase

In CPC, the liquid nature of the stationary phase enables a wide range of original solutions for the resolution of tough purification problems. Thus, even if this purification could certainly have been achieved by two successive CPC runs, we wished to find the right conditions to perform the separation in a single run. Indeed, this became possible by dividing the stationary phase into two zones, each one corresponding to a different DEHPA ionization state (Fig. 5).

It was first necessary to find the right conditions in order to exploit the separations observed with both a 46.51 and 3.33 DEHPA/TEA molar ratio. Using a 46.51 ratio, the GY and AY mildly polar peptides were well separated, and with the optimized 3.33 ratio, the isotachic train organization of the apolar peptides was observed. The head of the column (a quarter of the total inner volume, about 50 mL) was filled with the 3.33 DEHPA/TEA organic phase. In this part of the column, the isotachic train took place [16] and LV and LY were put in their optimal “train”. The other part of the column was filled with the 46.51 DEHPA/TEA organic phase: thus, in this part GY and AY were slowed down and so remained in the column until the injection of $Ca^{2+}$ ions. In these conditions and with DEHPA/peptides = 5.4; DEHPA/$CaCl_2$ = 10.4 and DEHPA/HCl = 6, the chromatogram in Fig. 4B was obtained.

Clearly, GY and AY were well separated in the new improved conditions. Moreover, the LV and LY apolar peptides were displaced in zones with highly compressive fronts and minimum overlap. As shown in Fig. 2 (run 3), this strategy led to a significant improvement in peptide recovery. This last optimization has lead to a new stationary phase design: a segmented stationary phase, which, to

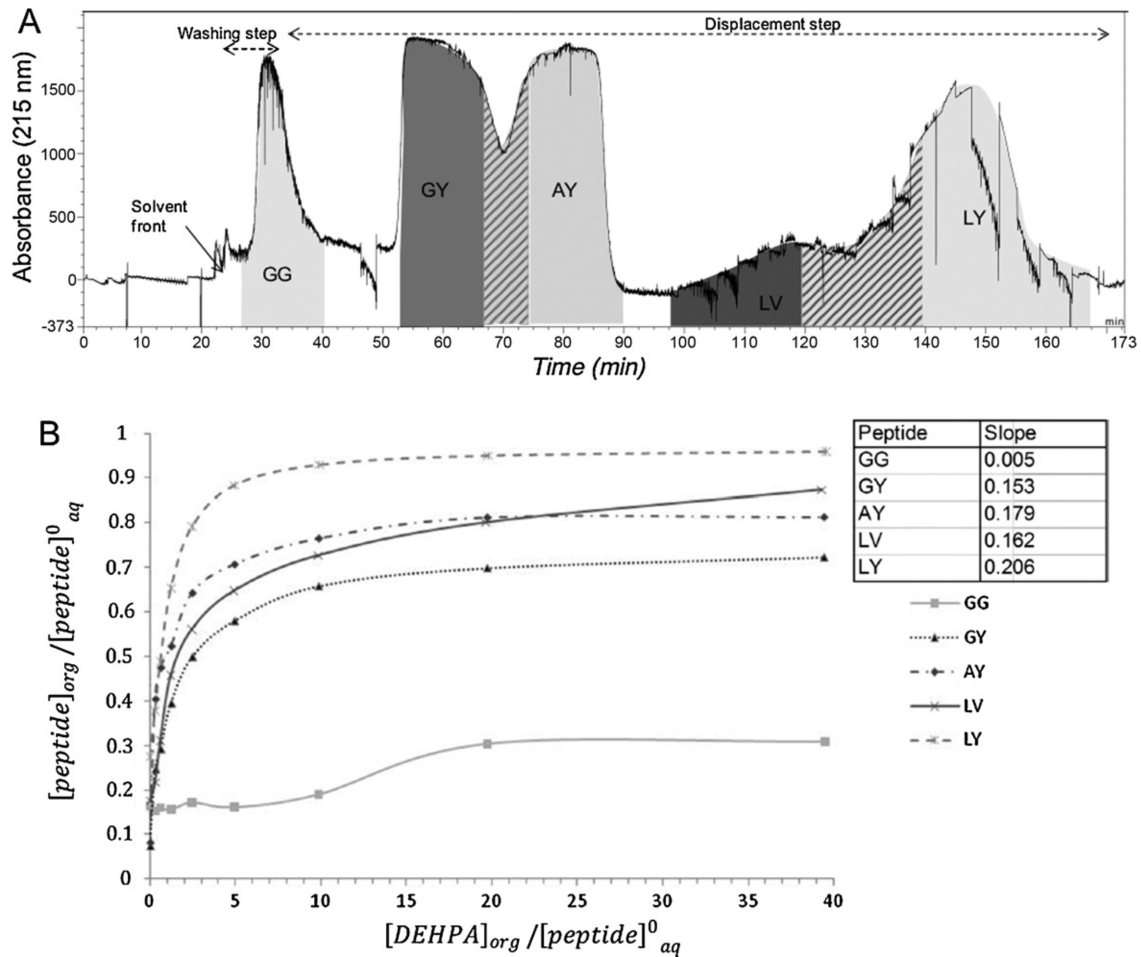


**Fig. 1.** (A) CPC chromatogram for the separation of 20 mg of each peptide (GG, GY, AY, LV and LY) with optimized DEHPA/peptide and DEHPA/HCl ratios, biphasic solvent system: MtBE/$CH_3CN$/n-BuOH/water (2:1:2:5, v/v), stationary phase: Upper organic phase + partially deprotonated DEHPA (DEHPA 15 mM, DEHPA/TEA = 46.51), mobile phase: lower aqueous phase (+ HCl 3.22 mM as displacer after 40 min). (B) Partition isotherms of GG, GY, AY, LV and LY (see Section 2.7).

the best of our knowledge, is an original concept in liquid/liquid chromatography.

The mass effect was then investigated by injecting a five-fold greater amount of peptides (100 mg of each,). The resulting chromatogram in Fig. 6 and the recoveries (Fig. 2, run 4) show that the quality of the separation was preserved and highlights the efficiency of the MIX-CPC mode as a preparative separation method.

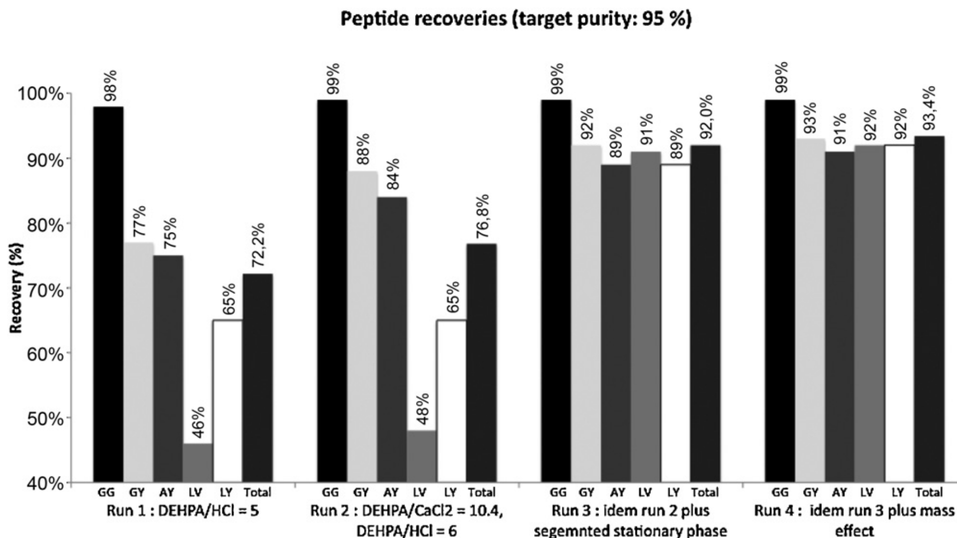


**Fig. 2.** Peptide recovery for each CPC run.

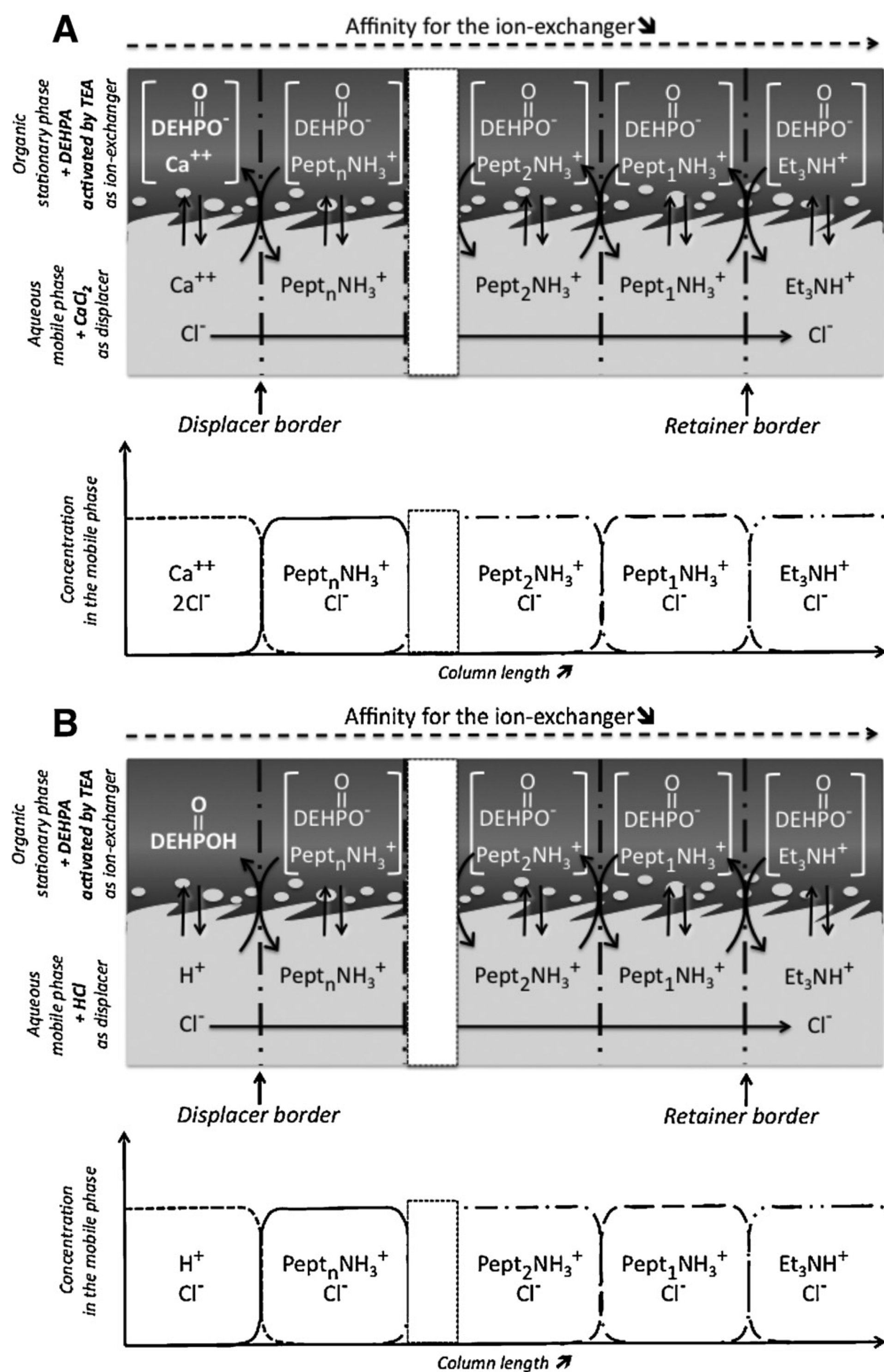


**Fig. 3.** Isotachic train (A) in the strong ion-exchange mode and (B) in the weak ion-exchange mode.

### *3.4. Exchanger/peptide interactions: aggregation of di(2-ethylhexyl)phosphoric acid (DEHPA) in non-polar solvents*

Ion-exchange CPC relies on interactions between the exchanger and the analytes. However, during process optimization, an excess of DEHPA was needed in order to properly extract the peptides into the organic phase. DEHPA with its hydrophobic chains and its hydrophilic phosphate core may form reversed micelles in certain solvents [27] that could be responsible for the extraction of the peptides during the CPC process. The aggregation of DEHPA molecules was suspected to influence separation efficiency when carried out in the MtBE/$CH_3CN$/BuOH/water biphasic solvent system, with or without triethylamine. For this purpose, the translational diffusion coefficients *D* of the exchanger at different concentrations and of all components of the organic phase were measured in 2D NMR DOSY spectra, since *D* values and molecular mass are correlated [28–31]. The DOSY NMR experiment yields a 2D spectrum with a chemical shift scale along the F2 axis and a *D*

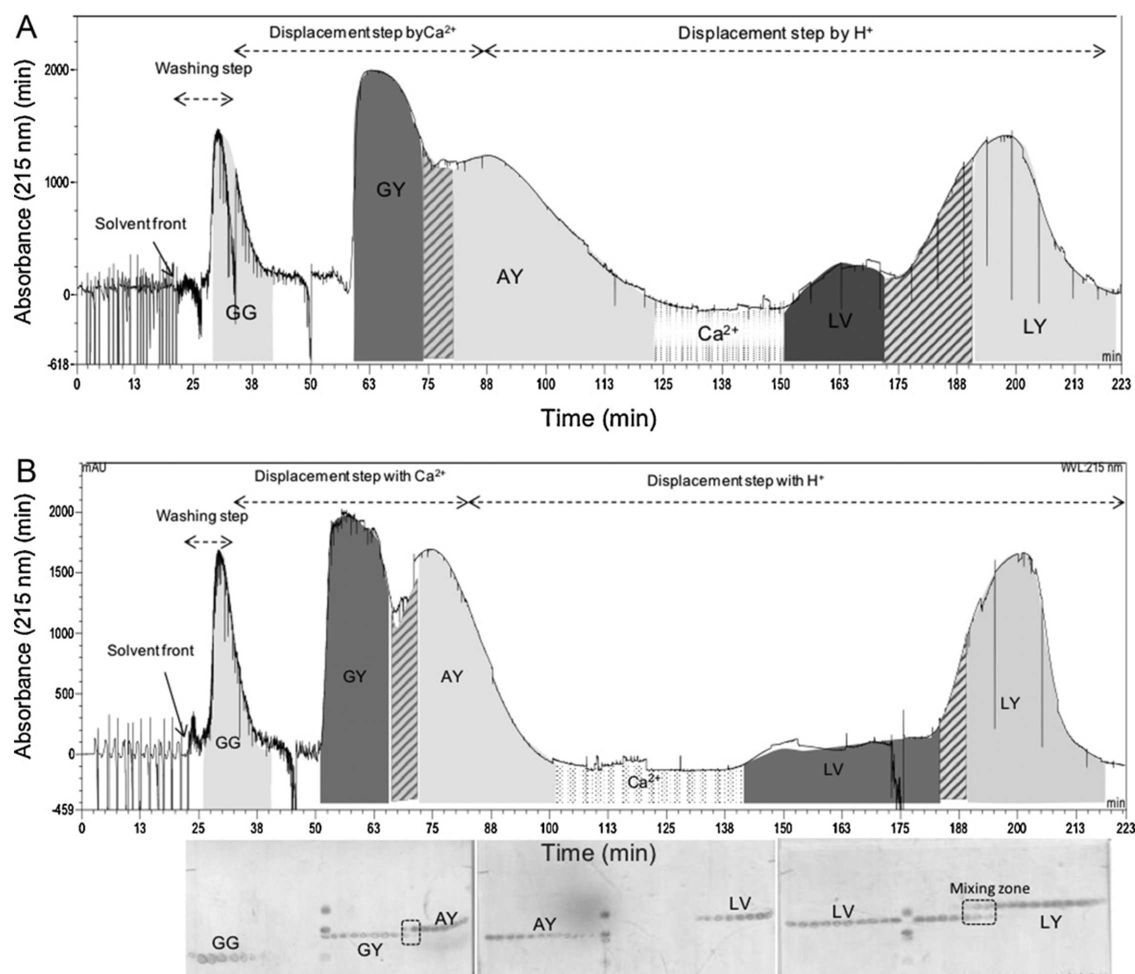


**Fig. 4.** MIXCPC chromatogram obtained for the separation of 20 mg of each peptide (GG, GY, AY, LV and LY). (A) Experimental conditions identical to those in Fig. 1A, except for the displacer: $CaCl_2$ 1.44 mM then HCl 2.5 mM. (B) MIXCPC chromatogram and TLC fractogram of the optimal peptide purification using a divided stationary phase, experimental conditions identical to those in (A), except for the 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).

scale along the F1 axis [28,30,32]. The size of the DEHPA-containing chemical species was studied by $^{31}$P diffusion coefficient-formula weight (D-FW) analysis [33–35]. The details of this study are reported in the Supplementary Material file. The main results are presented below. DEHPA, alone or in the presence of TEA, does not form reversed micelles in our biphasic solvent system. It is therefore unlikely that such entities could be involved in the extraction of peptides into the organic phase. Nevertheless, a diffusion study of

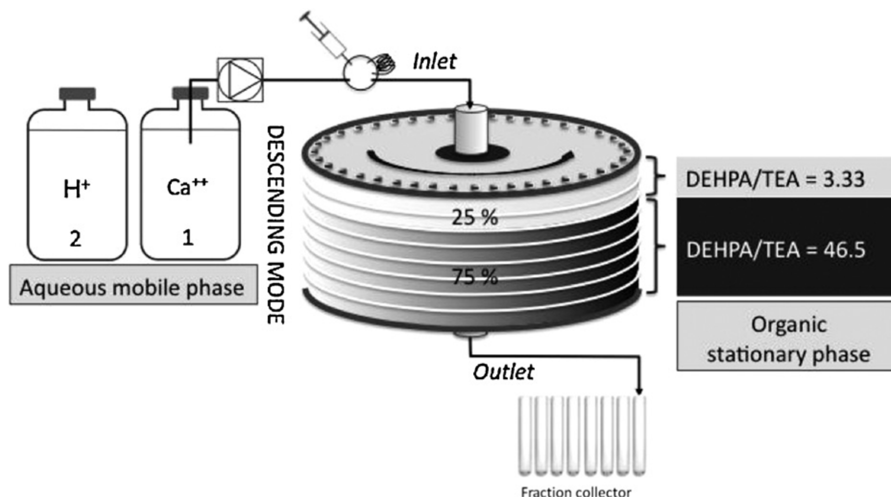


**Fig. 5.** Schematic representation of the MIXCPC mode (segmented stationary phase and two displacers).

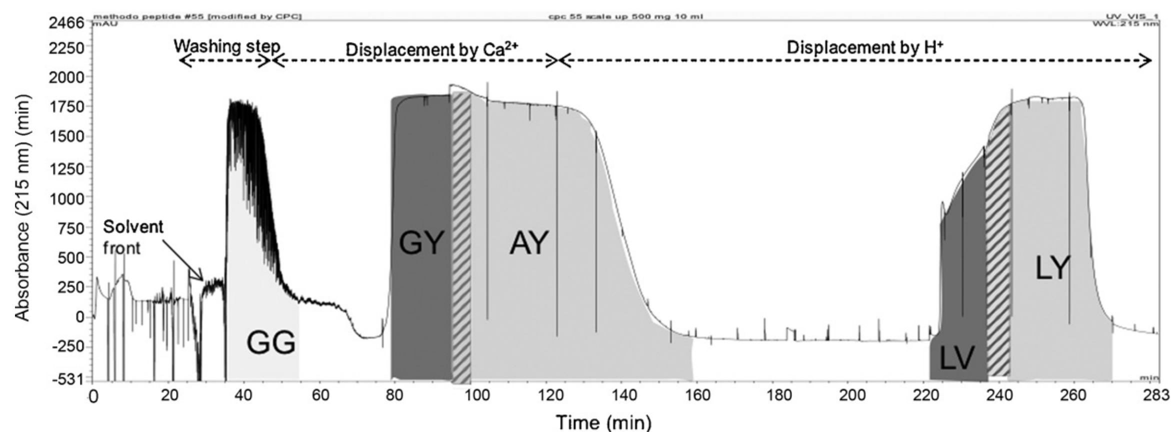


**Fig. 6.** Purification of 100 mg of each dipeptide by MIXCPC. Experimental conditions identical to those in Fig. 4B except for exchanger and displacer concentrations: DEHPA 75 mM, $CaCl_2$ 7.2 mM and HCl 12.25 mM.

DEHPA was conducted in the presence of dipeptides. In these conditions, the diffusion behavior of DEHPA varied very little. Thus, it can be concluded that DEHPA and the peptides do not interact through the formation of reversed micelles but by ion pair formation.

## 4. Conclusion

Ion-exchange CPC was successfully applied to the purification of dipeptides within a five component model mixture. The optimal separation conditions relied on a Mixed Ion eXchange (MIX) mode, which combined a strong displacer, calcium, and a weak displacer, proton. Moreover, the column was segmented in two parts, in which the exchanger was activated by triethylamine at two different concentrations. The application of this highly versatile and adaptable process to various purification problems, such as the fractionation of protein hydrolysates and the purification of a synthetic peptide, is currently under investigation. The DOSY NMR study allowed us to better understand the mechanisms that are involved in the peptide extraction during CPC experiments. We were able to confirm the hypothesis of ion pair formation at usual DEHPA concentrations. Analysis of the diffusion behavior of the involved chemical species appears to be a promising technique for the understanding of the ion-exchange CPC mechanisms.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.03.010.

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