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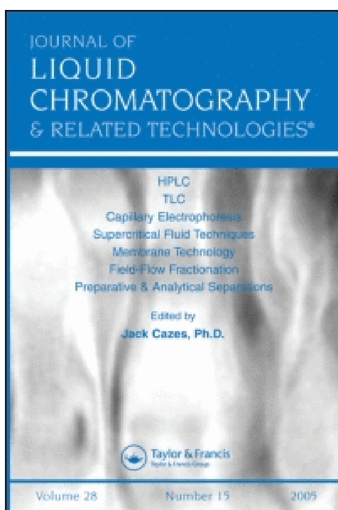

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Efficiency of Five Chromatographic Systems for the Separation of Casein Digest

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EFFICIENCY OF FIVE CHROMATOGRAPHIC SYSTEMS FOR THE SEPARATION OF CASEIN DIGEST

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ABSTRACT

Different chromatographic methods and packing supports have been proposed for the separation of protein hydrolysates. The selection of a method is often based on available equipment and quantity of sample and disposal time. Five liquid chromatographic systems were compared on the basis of their capability to resolve casein digest versus length of time and capacity.

INTRODUCTION

Different types of chromatographic systems have been developed for the separation of proteins and their hydrolysates. Among these, gel permeation[1] and ion-exchange[2] systems have been widely used. Efficiency of separation by ligand exchange or metal-chelate affinity gel chromatography has also been established for a tryptic hydrolysate into its peptide fragments[3]. However, a major limitation of the above-mentioned classical chromatography gel-type media is their lack of mechanical strength.

Recent techniques used to produce uniform microparticulate packings have allowed the development of high-performance liquid chromatography (HPLC)[4]. Hence HPLC has been extensively used for the separation of protein hydrolysates[5-6]. Among the various HPLC chromatographic systems including size-exclusion[7-8], reversed-phase[9-12] and ion-exchange[13-15], reversed-phase HPLC (RP-HPLC) has been extensively used. Also, further purification of hydrolysate fractions eluted from classical chromatographic systems has been achieved by RP-HPLC,

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since many nonionic, ionic and ionizable compounds can be separated by this technique using a single column and mobile phase with or without added salts[16].

For the separation of protein hydrolysates the selection of suitable stationary phase should be based on the characteristics and complexity of the samples. However, resolving capability of chromatographic systems has been shown to depend on the retention strength of the components on the column, on their selectivity, and also on the efficiency of the column (sharpness of the peaks)[17].

Milk protein hydrolysates have been recently proposed to contain fractions with potential physiological and biological activities such as bioavailability of oligo-elements, opioids, immunomodulation, antithrombosis and antihypertension[18]. However, no comparison study between separation systems for casein enzymatic digest is available to help for the selection of an appropriate and efficient system.

Therefore, casein hydrolysate phosphorylated (CHPS) and casein hydrolysate dephosphorylated (CHDS) have been separated using five different chromatographic systems: size-exclusion, reversed-phase and ion-exchange HPLC; combination of gel filtration and immobilized metal affinity. The objective of this study was to compare each chromatographic system on the basis of loading capacity, length of time and resolution. Moreover, HPLC chromatography has been studied on the basis of its advantages over classical chromatographic techniques.

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MATERIALS

Commercial casein hydrolysates, phosphorylated and dephosphorylated, (CHPS and CHDS) were provided by Laboratoire Sopharga, France. In the abbreviations used we have included the letter "S" to denote that it is a gift from Sopharga. A rationally designed and controlled hydrolysis of casein was carried out by proteases, chymotrypsin and trypsin, under precise temperature conditions in a continuous flow membrane enzymatic reactor[19]. The peptidic hydrolysate so obtained is called CHPS. Using the chelating properties of peptides, phosphoserine residues were isolated from peptidic hydrolysates in the presence of added calcium and phosphate ions. These peptides formed aggregates in the solution and were retained by the membrane; the non-phosphorylated peptides passed through the ultrafiltration membrane to give the CHDS.

N-ethylmorpholine was obtained from Sigma Chemicals Company (St. Louis, MO, U.S.A.). Acetic acid, ammonia, HPLC grade water, methanol, monobasic and dibasic sodium phosphates were purchased from Fisher Scientific Co. (Que, Canada). Trifluoroacetic acid was supplied by Pierce (U.S.A.). Acetonitrile

"Baker Analyzed" Reagent for chromatography was purchased from J.T. Baker Chemicals (B.V.-Deventer-Holland). Double-distilled water was purified by passing it through Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Tris-(hydroxymethyl)-amino-methane, ultrapure grade, gold label, was obtained from Janssen-Chemica (Belgium) and sodium acetate trihydrate R.P. Normapur was from Prolabo (France).

METHODS

Methods a, b and c used for peptide separation were previously described[8,20,21].

a) Size-Exclusion (SEC) and Metal-Chelate Affinity Chromatographies

Mixture of peptides was first separated on a semi-preparative size-exclusion chromatography (SEC) Pharmacia column (100 x 10 cm I.D.) packed with Sephadex G-25 superfine (Pharmacia) with the mobile phase (0.1 M N-ethylmorpholine acetate buffer, pH 8.0) run at a flow rate of 15 ml/h. After evaporation and freeze-drying, collected fractions were then injected on a Sephadex G-25-Cu coupled Biorad column (45 x 25 cm I.D.) and eluted with a 0.1 M ammonium acetate buffer (pH 5.0) at the rate of 27 ml/h. Polypeptides were monitored at 220 nm with a LKB 2210-011 recorder[20].

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High-Performance Liquid Chromatography (HPLC):

b) Size-Exclusion HPLC (SE-HPLC)

Size-exclusion HPLC was carried out on a TSK-G 2000 SW column (600 x 7.5 mm I.D.) with a guard column (60 x 7.5 mm I.D.) (Toyo Soda, Tokyo, Japan) using a LKB-HPLC system equipped with a 2150 pump, a 2152 controller, a 2151 variable wavelength monitor, a Rheodyne M 7010 sample injection valve with a 20 μl loop, a 3390 A integrator (Hewlett Packard). The mobile phase was 0.1% trifluoroacetic acid (TFA), 0.05 M phosphate buffer (pH 5.0) and 35% methanol. The system was run isocratically at a flow-rate of 0.75 ml/min under constant temperature[8]. Polypeptides were monitored at 214 nm with an absorbance scale of 0.05. The mobile phase was filtered through a 0.45 μm filter (Millipore) and sonicated before use.

c) Reversed-Phase HPLC (RP-HPLC)

Reversed-phase HPLC was performed on a μBondapak C_{18} column (10 μm, 300 x 3.9 mm I.D.) using a Waters Associates HPLC system equipped with two pumps F-6000A, a M 720 solvent programmer, a WISP automated sample injector, a M 441 fixed-wavelength detector (214 nm) and a M-730 two-channel chart recorder. A water-bath with a Thermonix 1420 (Braun, F.R.G.) was used to keep the column at 40°C. Polypeptides were eluted at a flow-rate of 2 ml/min using an

acetonitrile gradient in dilute aqueous trifluoroacetic acid and monitored at 214 nm with an absorbance scale of 2[21].

d) Anion-Exchange HPLC (AE-HPLC)

Anion-exchange HPLC was carried out on a Jobin and Yvon-Modulprep (France) column (20 mm I.D. x 13.7 cm), packed with a methyl iodine quaternized and epichlorhydrin reticulated polyvinylimidazole adsorbed on silica, using a HPLC system equipped with two 110A Beckman pumps, a Touzart and Matignon (France) damping device and Apple-II programmator, a home-made mixing chamber, a Rheodyne M 7010 sample injection valve with a 2 ml loop, a Varichrom detector set at 280 nm and a Pye-Unicam Philips recorder with an absorbance scale of 0.5. The system was run at a flow-rate of 5 ml/min using a solvent A + 0.5 M sodium acetate (pH 7.8) gradient in 0.015 M Tris (pH 8.0) [solvent A].

RESULTS

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Size-Exclusion (SEC) and Metal-Chelate Affinity Chromatographies

A. On the Sephadex G-25 superfine column, 150 ml of a 16% dephosphorylated casein hydrolysate solution (24 g of digest) were loaded and the elution was complete after collecting 9 fractions within 10 h (Fig. 1A). The chromatogram looked like an upside down funnel; peaks were not well-defined. Each fraction was resubmitted to a second chromatography on an immobilized copper ligand coupled to Sephadex G-25 support (Fig. 1 chromatograms of fractions 1-9). The time required for complete elution of each fraction in the second step chromatography was about 4 h; hence the time needed was 46 h for the separation of fractions without taking into account the time necessary for column equilibration and washings. With the G-25-Cu system, peaks were large and not well-defined.

B. In the SE-HPLC system, when TSK column was used, only 40 μg of digest was injected at a time. Each run required 40 min. Chromatographic patterns of dephosphorylated and phosphorylated digests of casein are presented in Fig. 2. Both chromatograms were not different from each other and the peaks were not sharp and well resolved.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

Chromatographies of 1.7 mg and 2.3 mg of CHPS and CHDS, respectively, were performed on a μBondapak C_{18} RP-column. Each run lasted 35 min. Chromatograms shown in Figs. 3 and 4 had large number of peaks with different areas, many of which appeared to separate well, but with indication of some overlaping.

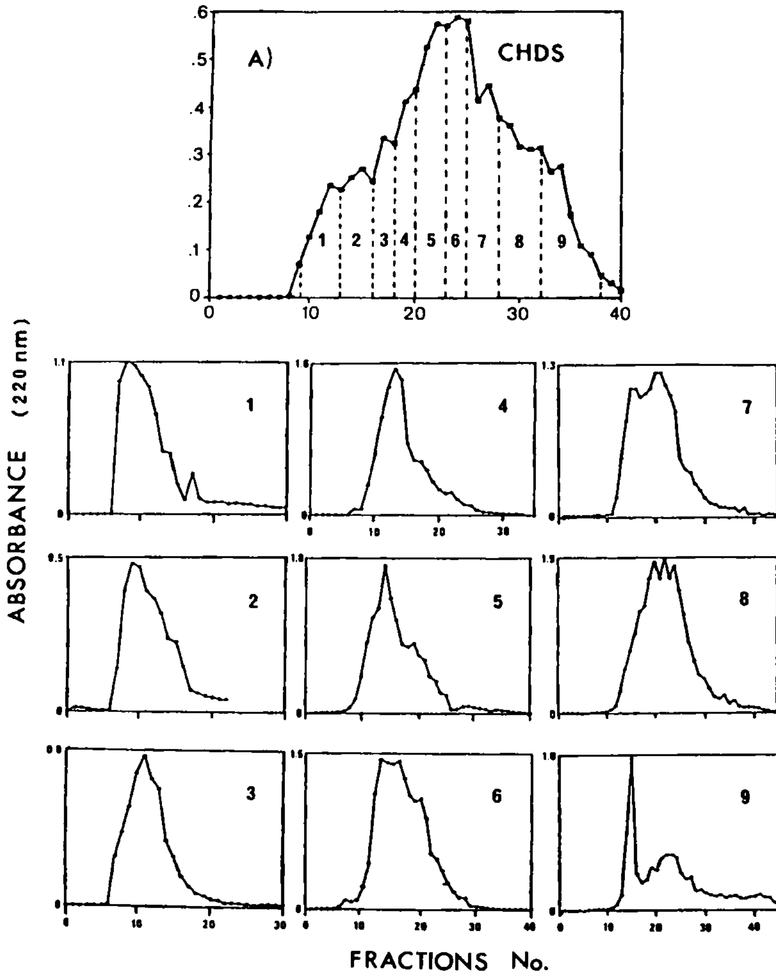

FIGURE 1. Elution profiles obtained from a two steps chromatography of casein hydrolysate dephosphorylated (CHDS). A: Sephadex G-25 column; 1-9: fractions from the first step chromatography collected and injected on an immobilized copper ligand coupled to the Sephadex G-25 support.

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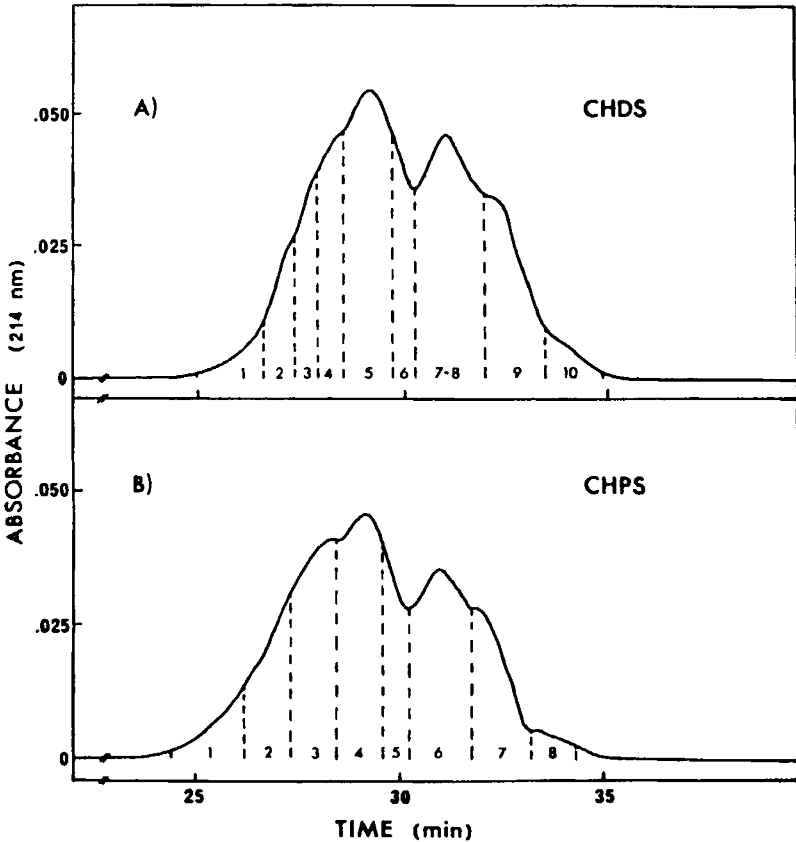

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FIGURE 2. Elution profiles of tryptic and chymotryptic digests of casein on a TSK G-2000 SW column. Each hydrolysate (2 mg) was dissolved in 1 ml of mobile phase (0.1% TFA, 0.05M phosphate buffer (pH 5.0) and 35% methanol). Injected volume, 20 µl; (A) CHDS, (B) CHPS; 1-10: fractions collected manually.

Anion-Exchange High-Performance Liquid Chromatography (AE-HPLC)

With AE-HPLC, 0.16 g of hydrolysate was separated into fractions within 55 min and the elution profiles are shown in Fig. 5. The separation by AE-HPLC was found to be efficient (Fig. 5) compared to SEC (Fig. 1A). Although Touraine et al.[22] used AE-HPLC system for the separation of peptides from milk proteins, the elution profile was not well resolved. This could be explained by the differences in the support and the mobile phase.

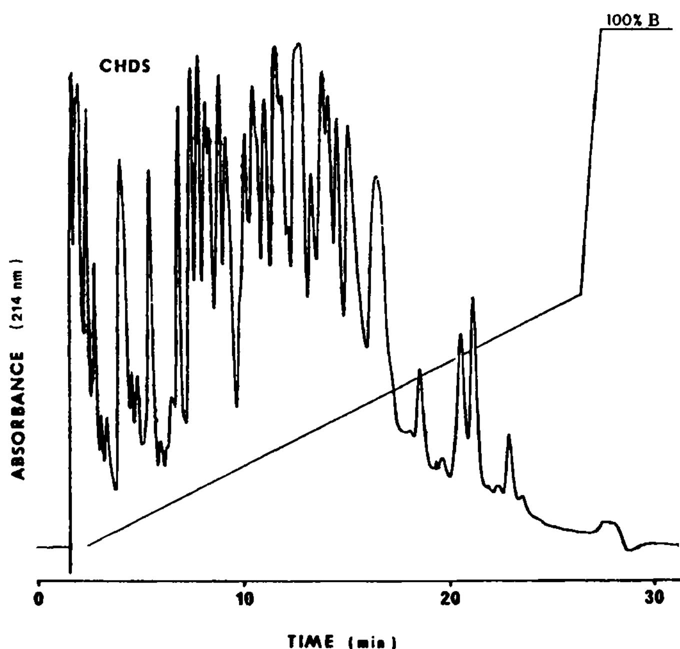

FIGURE 3. Reversed-phase (RP-HPLC) separation of an aliquot of 2.3 mg of a casein hydrolysate dephosphorylated (CHDS) on a µBondapak C_{18} column. After equilibration of the column with 0.115% TFA (solvent A) at a flow-rate of 2 ml/min, peptides were eluted by linearly increasing the concentration of solvent B (60% (v/v) acetonitrile in 0.1% TFA) as follows: 0-24 min (0-48% B), 24-25 min (48-100% B) and 25-25.5 min (100-0% B).

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The results obtained with the different chromatographic systems are summarized in Table 1. Based on the capacity, G-25, G-25-Cu and AE-HPLC appear to be the best for preparative separation. However, G-25 and G-25-Cu SEC systems are very time consuming compared to the other chromatographic systems, and the resolution obtained is very poor based on peak separation and sharpness.

Considering the ratio of column capacity and elution time per run ($\mu g.ml^{-1}.h^{-1}$) for each system, AE-HPLC appears to be the best, followed by RP-HPLC, G-25-Cu, G-25 and SE-HPLC.

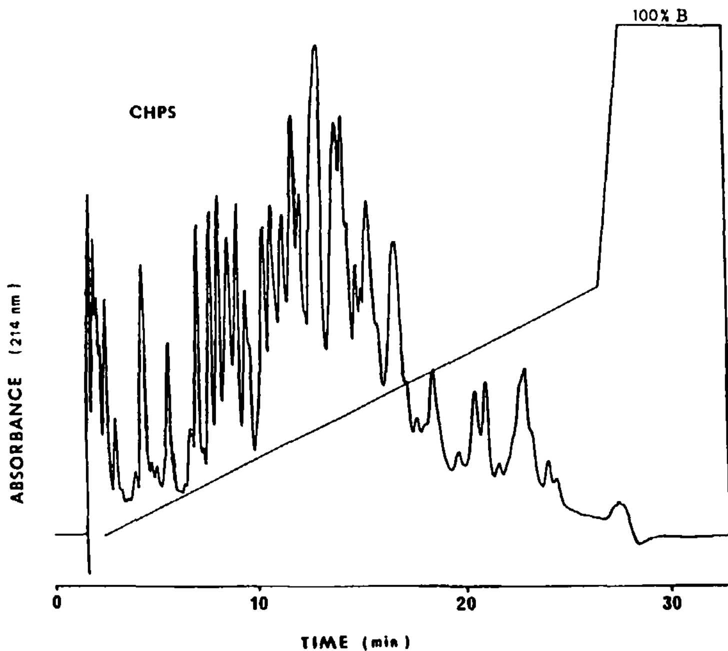

FIGURE 4. Reversed-phase (RP-HPLC) separation of an aliquot of 1.7 mg of a casein hydrolysate phosphorylated (CHPS) on a μBondapak C_{18} column following the elution conditions described in Fig. 3.

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The separation and resolution were most efficient with RP-HPLC compared to the rest of the systems. Further improvement on the separation could be achieved by using a second solvent system. Among the systems tested AE-HPLC was the second best on the basis of resolution.

DISCUSSION

HPLC systems consumed less time to perform chromatography for coarse separation of the hydrolysates compared to conventional SEC. Fischman et al.[23] reported that HPLC offers several advantages over the other methods of separation; amongst them are high resolution, nearly quantitative recovery and compatibility with the use of volatile buffer systems that allow direct assay of the effluent after lyophilization.

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TABLE 1

Comparison of Data Obtained from the Elution of Casein Hydrolysates from Five Chromatographic Systems.

Column type	Column volume (ml)	Quantity injected (mg)	Capacity (μg/ml)	Elution time per run (h)	Partition look appreciation*	Capacity/elution time per run (μg/ml-h)
Sephadex G-25	7854.00	24000.00	3100.0	10.00	4	310.00
Sephadex G-25-Cu	418.00	900.00	2000.0	4.00	3	500.00
TSK-G 2000 SW	26.50	0.04	1.5	0.67	3	2.24
μBondapakC_{18}	3.58	1.70 & 2.30	474.9 & 642.5	0.55	1	863.45 & 1168.18
Anion-exchange	43.04	160.00	3700.0	0.92	2	4021.74

* Peak resolution ranks from 1 to 4, 1 being the best based on peak separation and sharpness.

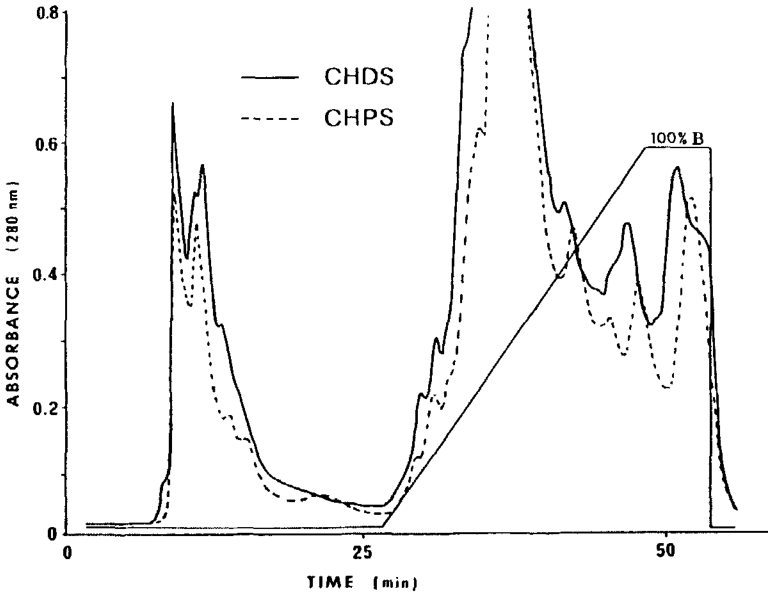

FIGURE 5. Chromatography of casein hydrolysates (phosphorylated, CHPS and dephosphorylated, CHDS) by anion-exchange HPLC. Details of AE-HPLC are provided in Experimental. After equilibration of the column with A, peptides were eluted with solvent B using a gradient as shown.

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The best system for molecular weight distribution study would be SE-HPLC (TSK-G 2000 SW) compared to SEC since elution time is low and the quality of separation is better. However the column capacity is too low to obtain sufficient amount of products for further study. This could be solved by using semi-preparative columns available in the market.

According to the results obtained (Table 1), AE-HPLC seems to be the most favourable system on the basis of elution time, capacity, and quantity injected. Similarly, Humphrey and Newsome[24] found that AE-HPLC was suitable for the separation of protein hydrolysates to obtain larger quantity of product for further analysis. Mychack and Benson[25] suggested ion-exchange chromatography for the separation of mixtures containing highly ionized peptides such as protein hydrolysates. It has been demonstrated[26-27] that tryptic digests and

closely related acidic and neutral peptides could be successfully resolved by HPLC on a weak anion-exchange bonded phase (AE-HPLC). The advantages of AE-HPLC method are in terms of sensitivity, peak symmetry, reproducibility, high capacity and recovery.

Combination of SE-HPLC and RP-HPLC has proven to be successful for the separation of casein hydrolysate phosphorylated and dephosphorylated into more than 200 identified peptides[21]. However, Dizdaroglu and Krutzsch[28] suggested that combination of AE-HPLC and RP-HPLC could provide a good resolving power and thus yield to a high probability for the complete separation of a given complex peptides mixture into its components.

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