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NEW HORIZONTAL FLOW-THROUGH COIL PLANET CENTRIFUGE FOR COUNTER-CURRENT CHROMATOGRAPHY

III. SEPARATION AND PURIFICATION OF DINITROPHENYL AMINO ACIDS AND PEPTIDES

YOICHIRO ITO

Laboratory of Technical Development, National Heart, Lung and Blood Institute, Bethesda, Md. 20205 (U.S.A.)

and

GERALD J. PUTTERMAN

Biological Markers Program, National Cancer Institute–Frederick Cancer Research Center, Frederick, Md. 21701 (U.S.A.)

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SUMMARY

The new type of horizontal flow-through coil planet centrifuge enables both preparative and analytical separations with partition efficiencies comparable to liquid chromatography. This counter-current chromatographic method was evaluated by the separation and purification of DNP-amino acids and various kinds of peptides using common two-phase solvent systems. The peptide samples separated include commercially available oligopeptides and two synthetic peptides of five and fifteen amino acids. A partition efficiency of up to several thousand theoretical plates was obtained with DNP-amino acids. Purification of the synthetic peptides, which produced problems with conventional liquid chromatography due to contamination and poor solubility, was successfully performed on a preparative scale with a reasonable degree of recovery.

INTRODUCTION

The new horizontal flow-through coil planet centrifuge can perform counter-current chromatography enabling the separation and purification of biological materials under a variety of conditions. The most recent version of this instrument contains two separation columns[1–4] rather than a single column with a counterbalance as in an earlier model[5]. As described in Part I[3], the new apparatus not only allows continuous elution through these coiled separation columns without the need for rotating seals, but it also gives each column a particular mode of synchronous planetary motion to produce a characteristic acceleration field for each column, thereby facilitating the partition process. The partition capability of each column

was examined in Part II[4] by determining the degree of stationary phase retention and partition efficiency under a variety of revolutional speeds and flow-rates. The results indicated that the gear-side column produces separations well suited for preparative-scale work, and the pulley-side column produces separations well suited for analytical-scale work. With both columns, partition efficiencies similar to those observed for column chromatography were obtained.

This report describes separations of dinitrophenyl (DNP) amino acids and peptides using both the preparative and analytical columns. Samples include commercially available oligopeptides and crude synthetic mixtures of peptides obtained by the Merrifield procedure[6]. Separations of DNP-amino acids and oligopeptides were evaluated by monitoring the absorbances of the eluates at 280 nm, while the purity and yield of the synthetic peptides were determined by thin-layer chromatography (TLC), fluorescence, and amino acid analysis.

EXPERIMENTAL

Apparatus

The design and function of the new horizontal flow-through coil planet centrifuge were described in detail in Part II[4]. In brief, the rotary frame of the centrifuge contains a pair of column holders; each is subjected to a specific mode of synchronous planetary motion provided by a set of gears and pulleys. The gear-side holder (coupled with gears) rotates about its own axis and simultaneously revolves around the central axis of the apparatus at the same angular velocity and in the *same* direction. The pulley-side holder (coupled with the pulleys and a toothed belt) undergoes a similar planetary motion except that it rotates about its own axis in the *opposite* direction. Each column holder is thus exposed to a characteristic pattern of acceleration. On the gear-side holder is mounted a large-bore column (2.6 mm I.D.) of PTFE tubing containing 1000 helical turns (1.25 cm helical diameter). This column has a total capacity of 270 ml and is used for preparative-scale separations. On the pulley-side holder is mounted a small-bore column (0.55 mm I.D.) of PTFE tubing containing 3500 helical turns (0.68 cm helical diameter). This column has a total capacity of 24 ml and is used for analytical-scale work. The apparatus is shown in Fig. 1. Procedures for preparing and mounting the columns are found in Part II[4].

Materials

DNP-Amino acids and oligopeptides were obtained from Sigma (St. Louis, Mo., U.S.A.). Two peptides having the sequence Ser–Ser–Ile–Ile–Arg (also referred to as peptide I) and Tyr–Ala–Ala–Nle–Ala–Ala–Met–Arg–Asp–Val–Val–Leu–Phe–Glu–Lys (also referred to as peptide II) were prepared by a modification of the Merrifield solid-phase method[6] using a Vega (Vega, Tuscon, Ariz., U.S.A.) Model 96 automated synthesizer. The benzyl group was used to protect Ser, Asp, and Glu; Arg, Tyr and Lys were protected by *p*-toluenesulfonyl, *o*-bromobenzyl and *o*-chlorobenzyloxycarbonyl groups, respectively. Peptides were custom-cleaved with HF by Peninsula Labs. (Belmont, Calif., U.S.A.). The level of desired peptide I in the crude was based on the amount of arginine obtained by subjecting a known weight of crude starting material to hydrolysis and amino acid analysis. The level of desired peptide

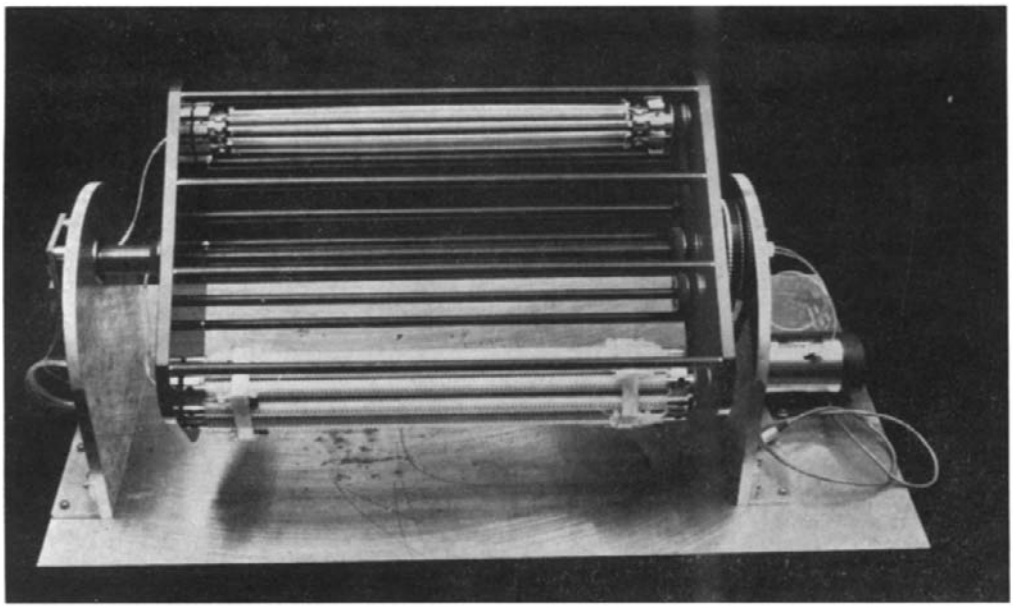

Fig. 1. Overall view of the apparatus.

II in the crude starting material was estimated from the amount of tyrosine obtained by amino acid analysis of starting material before and after hydrolysis since crude peptide II contained some free tyrosine. Crude samples were hydrolyzed in 12 *M* HCl–propionic acid (50:50, v/v) in evacuated sealed tubes at 140° for 3 h. These methods indicated that crude samples contained a maximum of 60% desired peptides.

The organic solvents, chloroform and *n*-butanol, were of a chromatographic grade (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) to facilitate absorbance monitoring at 280 nm. Other solvents such as glacial acetic acid and hydrochloric acid were of a reagent grade (J. T. Baker, Phillipsburg, N.J., U.S.A.).

Preparation of two-phase solvent systems and sample solutions

In Table I are listed the sets of samples and two-phase solvent systems used in this work. Each two-phase solvent system was thoroughly equilibrated in a separatory funnel at room temperature and separated before use.

For studies with DNP-amino acids and oligopeptides, mixtures were obtained by dissolving 4–10 mg (depending on solubility) of each component in 2 ml of stationary phase. For the analytical column, 50 μl of this solution were used in each run. For the preparative column, 1 ml of the sample solution was diluted with 9 ml of stationary phase, and the entire 10 ml were used for each run. Because the major goal of the work with the synthetic peptides was to obtain quantities of purified product, only the preparative column was employed. A sample of crude peptide I (100 mg) was dissolved in 5 ml of stationary phase (Table I) and added to the rotating column. The larger peptide (peptide II) had poor solubility in both phases of the solvent system given in Table I. However, 100 mg of crude peptide was dissolved in 13 ml of warmed upper phase, and this warm solution was slowly added to the rotating apparatus.

TABLE I

LIST OF SAMPLES AND TWO-PHASE SOLVENT SYSTEMS

Partition coefficient (PC) expressed as solute concentration in the mobile phase divided by that in the stationary phase

Samples	*Components (PC)*	*Two-phase solvent systems*		*Mobile phase*
DNP-Amino acids, I	N-DNP-δ-L-Ornithine (>100) N-DNP-L-Aspartic acid (3.8) N-DNP-L-Glutamic acid (1.9) N,N'-diDNP-L-Cystine (0.94) N-DNP-β-Alanine (0.71) N-DNP-L-Alanine (0.56) N-DNP-L-Proline (0.45) N-DNP-L-Valine (0.26)	Chloroform Acetic acid 0.1 *M* Hydrochloric acid	2 2 1	Upper aqueous phase
DNP-Amino acids, II	N-DNP-L-Leucine (5.6) N-DNP-L-Alanine (1.8) N,N'-diDNP-L-Cystine (1.1) N-DNP-L-Glutamic acid (0.53) N-DNP-L-Aspartic acid (0.26)	Chloroform Acetic acid 0.1 *M* Hydrochloric acid	2 2 1	Lower non-aqueous phase
Oligopeptides, I	L-Tyrosylglycylglycine (4.0) L-Prolyl-L-tyrosine (1.9) L-Leucyl-L-tyrosine (1.0) L-Tryptophyl-L-tyrosine (0.45) L-Tryptophyl-L-leucine (0.33)	*n*-Butanol Acetic acid Water	4 1 5	Lower aqueous phase
Oligopeptides, II	L-Tryptophyl-L-tryptophan (5.2) L-Tryptophyl-L-tyrosine (2.2) L-Leucyl-L-tyrosine (1.0) L-Valyl-L-tyrosine (0.53) L-Tyrosylglycylglycine (0.31)	*n*-Butanol Acetic acid Water	4 1 5	Upper non-aqueous phase
Synthetic peptide, I	Ser–Ser–Ile–Ile–Arg (4.0)	*n*-Butanol Acetic acid Water	4 1 5	Lower aqueous phase
Synthetic peptide, II	Tyr–Ala–Ala–Nle–Ala–Ala–Met–Arg–Asp–Val–Val–Leu–Phe–Glu–Lys (0.46)	*n*-Butanol Acetic acid Water	4 1 5	Upper non-aqueous phase

Separation procedure

For each separation a column was first filled with the stationary phase (either upper or lower phase). This was accomplished by pumping the stationary phase into the column at relatively high flow-rates (200–300 ml/h for the large-bore column and 40–60 ml/h for the small-bore column). The large-bore column occasionally trapped air bubbles which could be observed through the column wall. These bubbles were easily eliminated by waiting until the column was completely filled and running the apparatus at 200–300 rpm in a counter-clockwise direction (opposite direction to that used in a run) for several minutes while pumping the stationary phase at the same rate. The small-bore column was usually filled free of bubbles, and such a procedure was not needed. The sample solution was then injected through the sample port into the column which was either kept stationary or rotated at the

desired revolutional speed. Following sample addition, the mobile phase was pumped through the rotating column at the desired flow-rate using a Cheminert Metering Pump (Chromatronix). When appropriate, column eluates were continuously monitored with an LKB Uvicord III at 280 nm and fractionated with an LKB fraction collector. In monitoring a run, the trapping of the stationary phase in the flow cell was avoided by introducing the eluate from the bottom of the flow cell when the mobile phase was the lower phase and from the top of the flow cell when the mobile phase was the upper phase. After the desired solute peaks were eluted, the apparatus was slowly decelerated over a 1-min period and stopped to terminate the run.

When fractionation of the column contents from the large-bore column was required, the apparatus was rotated in the opposite direction at a speed of about 20 rpm while the column was eluted with the stationary phase at a flow-rate of 60–120 ml/h. The contents of the small-bore column could be collected by pumping the stationary phase at a flow-rate of 20–40 ml/h without column rotation. The fractions of column contents thus obtained consisted of both mobile and stationary phases with the volume ratio of the two phases determined by the percent retention of the stationary phase. An alternate approach of collecting the column contents in one fraction (not used here) consists of connecting the inlet of the column to a nitrogen tank and pushing out the column contents with a nitrogen pressure of 100 p.s.i. In this manner the column can be emptied in about 20 min. The sample still retained in the column is then recovered by injecting a small volume (30 ml for the large-bore column and 5 ml for the small-bore column) of a solvent miscible with both phases (such as methanol) through the sample port. The methanol is then pushed through the column by nitrogen pressure as described above. Emptying the large-bore column is facilitated by rotating it at about 20 rpm counter-clockwise (opposite direction from a normal run).

To prevent cross-contamination in consecutive runs, the preparative column was emptied by purging with nitrogen as described above. A small amount of methanol was injected and pushed through with nitrogen while the apparatus was rotated in the opposite direction at about 20 rpm. Within about 30 min the column was ready for the next separation. The analytical column was cleaned in a similar manner except that column rotation was not required.

TLC monitoring of column fractions

Tubes from planet centrifuge runs of synthetic peptides were vortexed whereupon aliquots were removed and spotted on MN cellulose 300 plates (Brinkmann, Westbury, N.Y., U.S.A.). TLC was performed with the solvent system butanol–acetic acid–water (4:1:5, v/v). Peptides were detected by means of a collidine-containing ninhydrin reagent[7].

Fluorescence monitoring of column fractions

A fluorescence profile of the planet centrifuge run was obtained with an SPF-500 spectrofluorometer (American Instrument, Silver Spring, Md., U.S.A.) in the single-beam mode. The wavelength for excitation was 280 nm, and fluorescence emission was determined at 308 nm using a 5-nm bandpass for both monochromators.

Amino acid analyses

Amino acid compositions and yields of the synthetic peptides were determined with a Durrum Instruments (Sunnyvale, Calif., U.S.A.) D-500 amino acid analyzer equipped with a fluorescence detector. Amino acids were made fluorescent by reaction with *o*-phthalaldehyde.

Bio-Gel P-2 chromatography of Ser–Ser–Ile–Ile–Arg

Crude peptide (100 mg) was dissolved in 2 ml of 0.1 *M* acetic acid and applied to a 60 × 1.5 cm column of Bio-Gel P-2 (Bio-Rad Labs., Richmond, Calif., U.S.A.). Elution was performed with 0.1 *M* acetic acid, and 2-ml fractions were collected.

RESULTS AND DISCUSSION

Separation of DNP-amino acids

With optimum conditions of flow-rate and revolutional speed as determined previously[4], separations of DNP-amino acids were obtained with the large-bore column mounted on the gear-side holder. The upper pair of chromatograms shown in Fig. 2A were obtained by using the upper aqueous phase as the mobile phase, and the lower pair of chromatograms in Fig. 2A were obtained by using the lower non-aqueous phase as the mobile phase. In all of these chromatograms, the DNP-amino acids were well resolved and eluted as symmetric peaks with partition efficiencies ranging from 550–2500 theoretical plates. It is quite apparent from these chromatograms that a flow-rate of 60 ml/h gave better peak resolution than a flow-rate of 120 ml/h, but with the faster flow-rate, separations were completed in a shorter period of time. These results allow a choice of flow-rates for the separation of biological materials depending on their stability and their relative partition coefficients.

The chromatograms in Fig. 2A can also serve as a guide to the choice of a mobile phase. For example, DNP-L-aspartic acid and DNP-L-glutamic acid have partition coefficients which favor partition to the upper aqueous phase (see Table I). When these compounds are eluted with the aqueous phase as shown in the upper chromatograms of Fig. 2A, they appear shortly after the solvent front and form very sharp peaks. When the same samples are eluted with the lower non-aqueous phase as shown in the lower chromatograms of Fig. 2A, they are retained in the column for much longer periods of time and produce broader but more resolved peaks. In separations of several components having similar partition coefficients which favor a particular phase, higher resolution should be obtained by using that phase as the *stationary* phase and eluting with the other phase. In this manner, the solutes are subjected to the partitioning process for longer periods of time and yield fractions of higher purity.

Fig. 2B shows typical chromatograms obtained with the small-bore column mounted on the pulley-side holder using the optimum conditions of flow-rate and revolutional speed previously determined[4]. The top chromatogram was obtained by pumping the upper aqueous phase and that at the bottom by pumping the lower non-aqueous phase, each at a rate of 6 ml/h, under a revolutional speed of 500 rpm. All components were separated with much greater resolution as compared to the separations obtained with the large-bore column. The partition efficiencies calculated from these chromatograms range from 1800–4000 theoretical plates. Comparison

between the two chromatograms shown in Fig. 2B clearly indicates that the top chromatogram produced by eluting with the aqueous phase gives higher peak resolution and shorter retention time for the solvent front. This indicates the effects of solvent-wall interaction in the narrow-bore column on the retention of the stationary phase and the partition efficiency as described in Part II[4]. When the stationary phase has an affinity for the tubing wall, a greater amount of the stationary phase is retained in the column resulting in higher peak resolution. Therefore, for the analytical column, it is recommended that the non-aqueous phase be used as the stationary phase.

Separation of oligopeptides

Fig. 3A shows typical chromatograms for the separation of oligopeptides with the large-bore column mounted on the gear-side holder. Separations were performed at a revolutional speed of 500 rpm using flow-rates of 24 ml/h and 60 ml/h. The chromatograms in the figure were obtained with both aqueous and non-aqueous mobile phases. In all the chromatograms, the five peptide components were well resolved, and partition efficiencies of 200–2000 theoretical plates were obtained. In contrast to the runs with the DNP-amino acids, the peaks were quite asymmetric. This skewing resulted from non-linear distribution isotherms of the samples in the particular solvent system employed and could be significantly decreased by lowering the concentration of peptides in the sample. Similar results were obtained for the small-bore column mounted on the pulley-side holder (Fig. 3B).

Purification of synthetic peptides

The synthetic peptide Ser–Ser–Ile–Ile–Arg (peptide I) (100 mg crude material) was purified using the large-bore column mounted on the gear-side holder. The lower aqueous phase of the solvent system listed in Table I was pumped at a flow-rate of 16 ml/h while the apparatus was spun at 500 rpm. Fractions of 2 ml were collected. Because the peptide contained no chromophore, the run was monitored by removing aliquots and subjecting them to TLC. The results are shown in Fig. 4. Amino acid analyses clearly revealed that tubes 11–16 contained the desired peptide; tubes 3–6 contained serine (from insufficient washing of the synthesizer resin following the last coupling step) plus traces of other contaminants; tubes 7–10 contained both the desired peptide and serine. The yield of pure peptide was 65%, but this should be improved by rerunning tubes which contain both the peptide and serine contaminant. In four runs, the pure peptide was found 24 ± 2 ml after the solvent front. This excellent reproducibility allows the peptide to be located precisely by spotting only one TLC plate. The predictions made above for the DNP-amino acids concerning relative peak position and polarity of the mobile phase are confirmed since serine was eluted sooner than the less polar peptide when the aqueous phase served as the mobile phase. While this run was performed at a mobile phase flow-rate of 16 ml/h (to try to maximize separation), identical results were obtained with a run using a 24 ml/h flow-rate. No attempt has been made to expedite the purification by using faster flow-rates.

A particular advantage of employing counter-current chromatography to purify synthetic peptides is that this technique appears to remove non-peptide

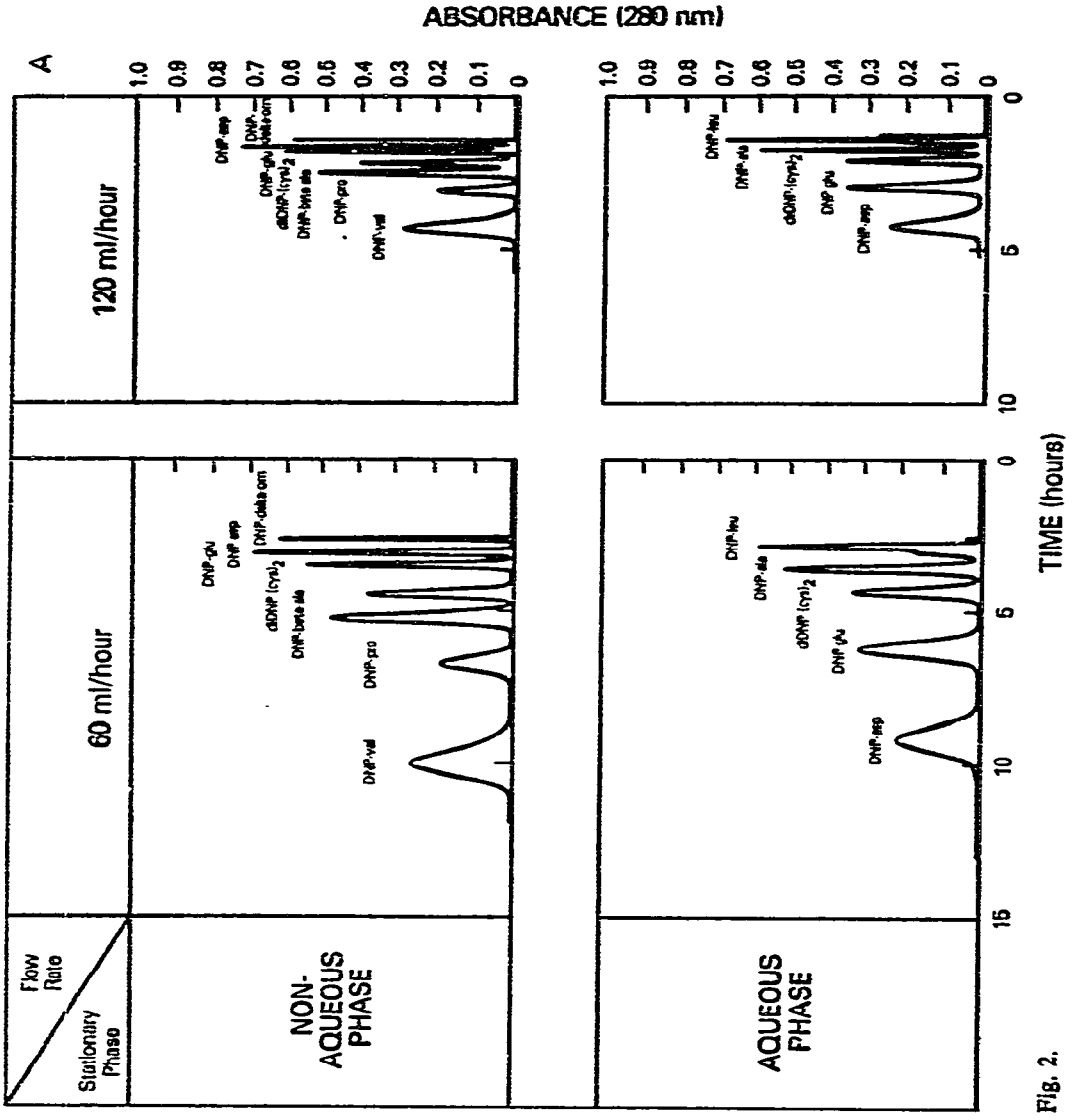
A
ABSORBANCE (280 nm)
Flow Rate
Stationary Phase
60 ml/hour
120 ml/hour
NON-AQUEOUS PHASE
AQUEOUS PHASE
TIME (hours)
DNP-asp
DNP-glu
DNP-ala
DNP-(cys)2
DNP-leu
DNP-pro
DNP-val

Fig. 2.

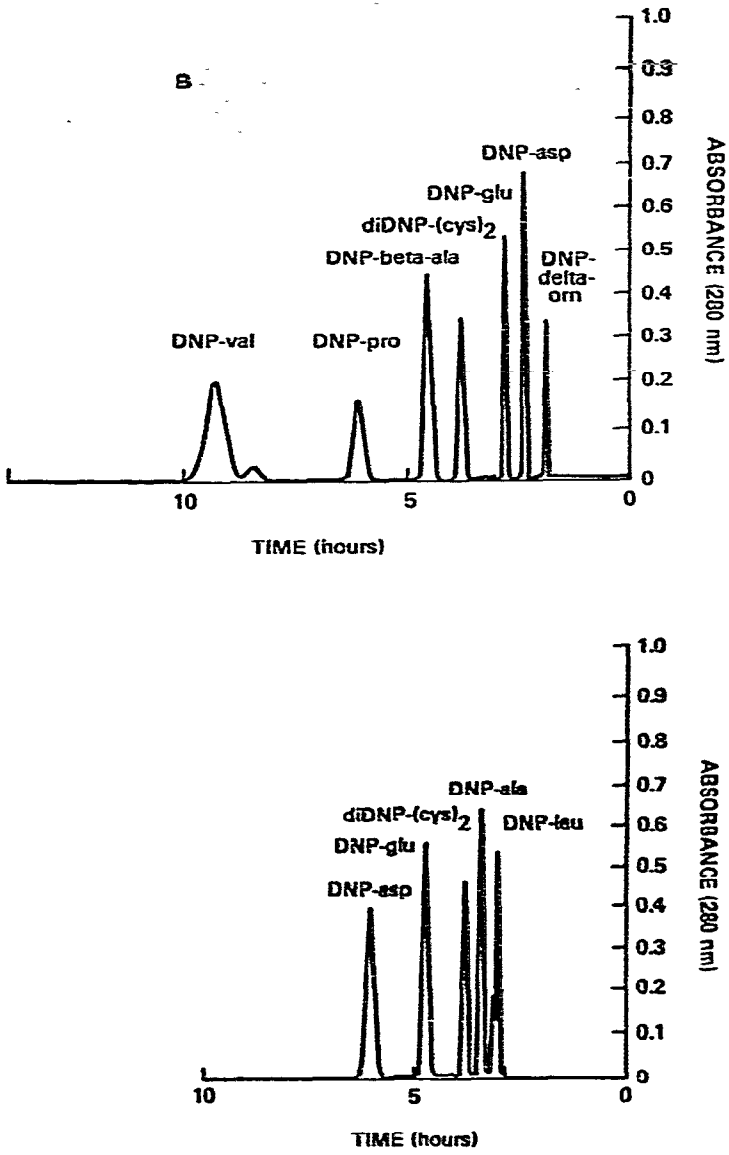

Fig. 2. Chromatograms of DNP-amino acids. A, Preparative-scale separations; solvent system: chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1); sample volume: 10 ml; revolution: 400 rpm. B, Analytical-scale separations. Column: 0.55 mm I.D.; 24 ml capacity, pulley-side; solvent system as in A; sample volume: 50 μl; revolution: 500 rpm; flow-rate: 6 ml/h; stationary phase: top, non-aqueous phase; bottom, aqueous phase.

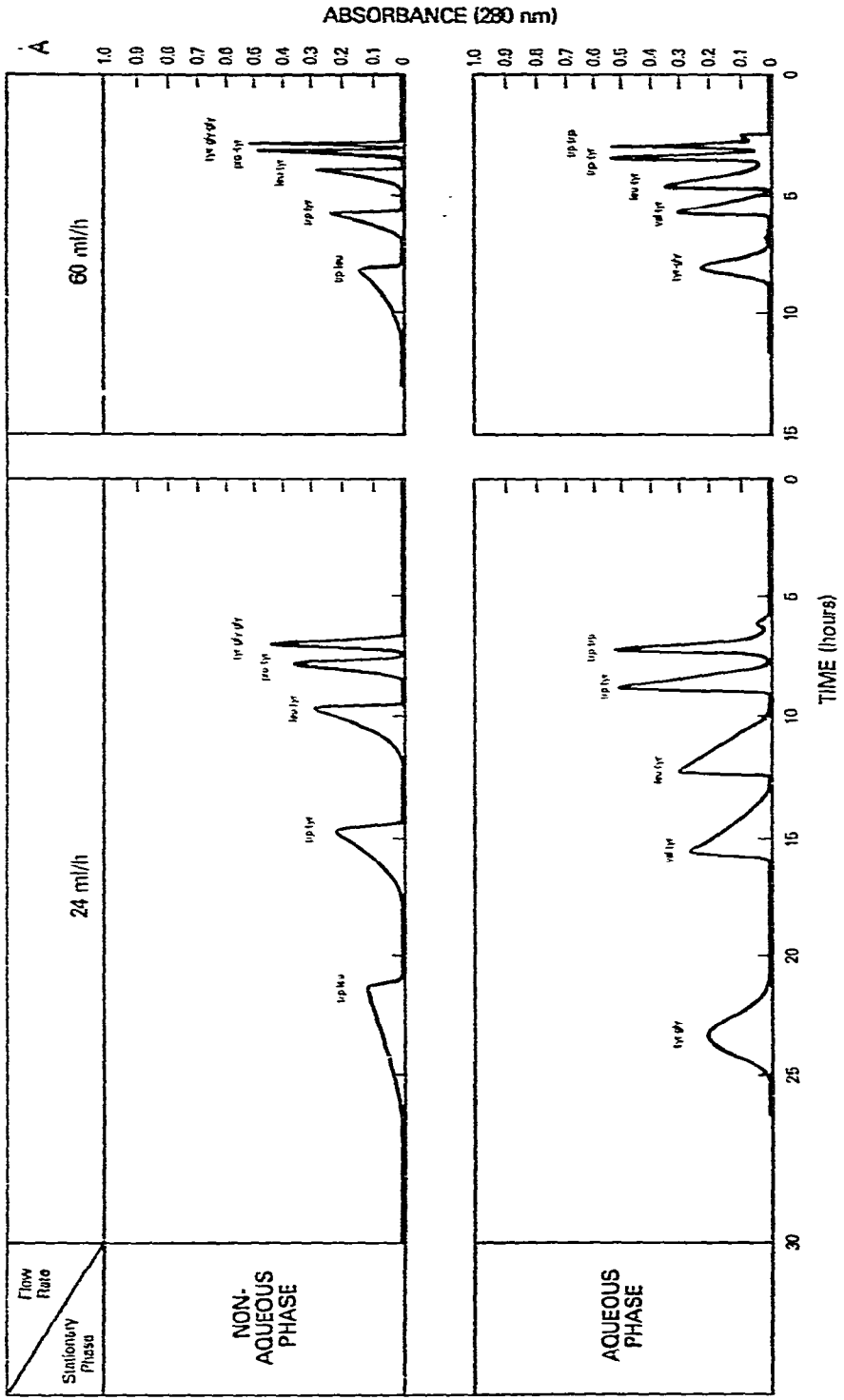
ABSORBANCE (280 nm)
A
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
60 ml/h
24 ml/h
Flow Rate
Stationary Phase
NON-AQUEOUS PHASE
AQUEOUS PHASE
0
5
10
15
20
25
30
TIME (hours)

Fig. 3.

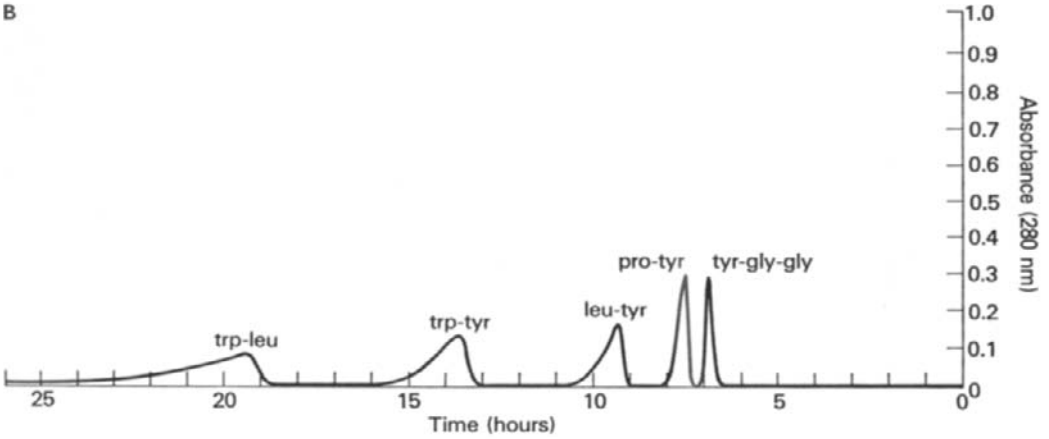

Fig. 3. Chromatograms of oligopeptides. A, Preparative-scale separations; solvent system: *n*-butanol–acetic acid–water (4:1:5); sample volume: 10 ml; revolution: 500 rpm. B, Analytical-scale separations. Column: 0.55 mm I.D.; 24 ml capacity, pulley-side; solvent system as in A; stationary phase: non-aqueous phase; sample volume: 50 μl; revolution: 450 rpm; flow-rate: 2.4 ml/h.

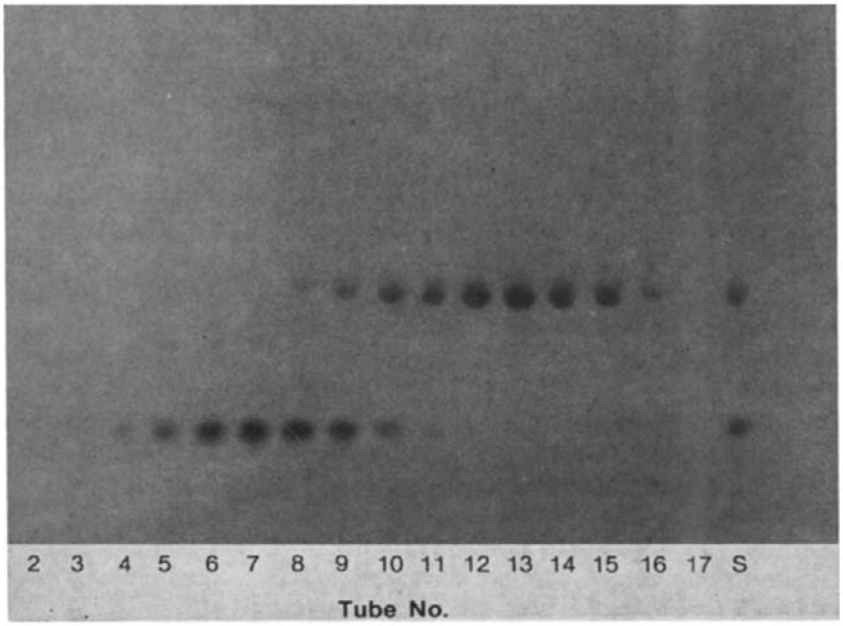

Fig. 4. TLC monitoring of the purification of peptide I. Note that tube 1 is that which immediately followed the tube containing the solvent front; S refers to 16 μg of crude starting material. Reprinted with permission of Pierce Chemical Company from E. Gross and J. Meienhofer (Editors), *Peptides: Structure and Biological Function*, 1979 (ref. 2).

contaminants (salts, resin by-products, products of de-blocking) which may not be removed by other procedures. For example, Fig. 5 shows TLC monitoring of the purification of 100 mg of this crude peptide by Bio-Gel P-2 chromatography (see Experimental). As expected, the peptide was eluted sooner than serine. However, tube

TABLE II

AMINO ACID ANALYSES OF FRACTIONS OBTAINED FROM PLANET CENTRIFUGE RUN OF PEPTIDE II

Samples were hydrolyzed in 12 *M* hydrochloric acid–propionic acid (50:50, v/v) in evacuated sealed tubes at 140° for 3 h. Note that amino acids are listed in the order in which they are added to the synthesizer resin rather than in the order in which they are eluted from the analyzer resin. Numbers in parentheses give the composition of peptide II.

Amino acid	*Molar ratio*																			
	Tube number																			
	6	*7*	*38*	*40*	*42*	*44*	*46*	*48*	*50*	*52*	*54*	*56*	*58*	*60*	*62*	*64*	*66*	*68*	*70*	*72*
Lys (1)	1.03	1.04	0.92	0.89	0.89	0.97	1.12	1.11	1.08	0.99	0.96	0.98	0.98	0.95	1.01	1.07	1.08	1.04	1.07	1.02
Glu (1)	0.95	1.08	1.09	1.01	1.06	0.96	0.94	0.99	1.01	0.99	1.02	1.04	1.02	1.02	1.02	1.06	1.00	1.08	1.13	0.98
Phe (1)	1.04	1.04	0.91	1.05	0.97	1.04	1.02	1.08	1.04	1.01	1.09	1.09	1.05	0.97	1.04	1.11	1.09	1.05	1.02	0.99
Leu (1)	0.98	0.90	1.16	1.06	0.97	0.99	1.06	1.03	0.99	1.09	0.99	0.99	0.97	1.12	1.02	0.87	0.91	1.00	0.98	1.15
Val (2)	1.40	1.89	1.76	1.95	1.38	1.86	1.50	1.91	1.68	1.97	2.03	2.00	2.03	1.75	1.61	1.99	1.90	1.62	1.86	1.68
Asp (1)	0.63	0.87	0.93	1.02	0.93	0.90	1.01	0.92	0.94	0.93	0.94	0.94	0.97	0.94	0.88	0.95	0.88	0.90	0.90	0.95
Arg (1)	0.28	0.44	1.07	0.97	1.19	0.93	0.86	0.92	0.93	0.99	1.00	0.97	1.00	0.95	1.04	1.09	1.05	0.93	0.90	0.90
Met (1)	0.56	0.38	0.65	0.57	0.49	0.68	0.55	0.87	0.86	1.97	0.73	0.58	0.48	0.63	0.57	0.58	0.77	0.67	1.03	0.53
Ala (4)	1.34	1.65	3.38	3.80	3.51	3.60	3.63	3.95	3.87	3.86	3.98	3.99	3.92	3.71	3.59	3.91	3.59	3.48	3.70	3.26
Nle (1)	0.22	0.42	0.97	1.09	0.84	0.97	1.05	1.10	1.00	1.09	1.07	1.08	1.04	1.04	1.07	1.06	0.96	0.94	0.97	0.96
Tyr (1)	0	0.17	0.94	0.84	0.74	0.86	0.88	0.95	0.92	0.96	1.06	1.10	1.13	1.06	1.04	1.11	0.98	0.90	0.93	0.79
Yield/tube (μmole)	0.28	0.45	0.21	0.28	0.32	0.35	0.45	0.52	0.57	0.60	0.57	0.55	0.47	0.38	0.35	0.29	0.27	0.27	0.25	0.25

30 of this run contained not only the desired peptide as evidenced by positive ninhydrin and Sakaguchi (Arg test) reactions[7], but also white material which remained at the origin and did not stain with these reagents. Either a much larger column or repetitive runs would have to be used to obtain good yields of uncontaminated peptide.

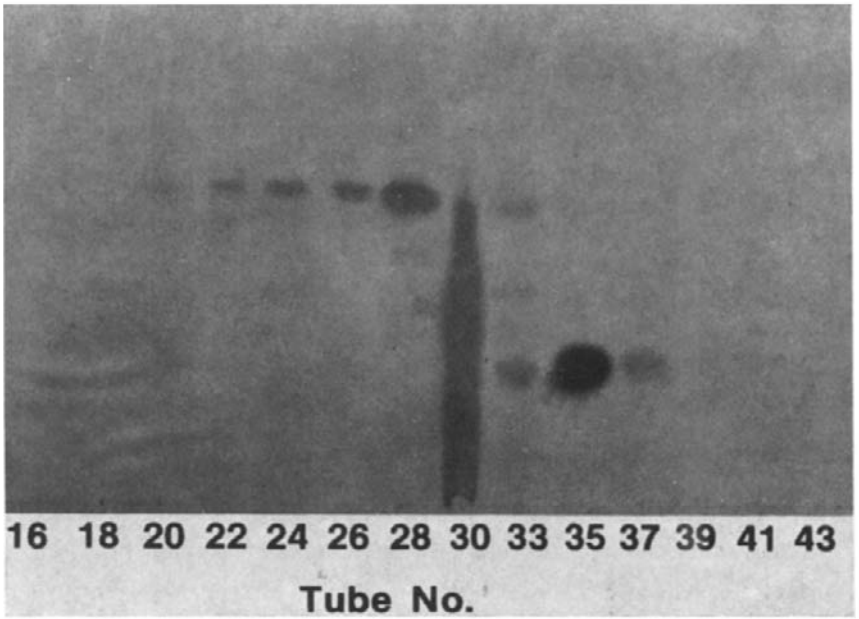

Fig. 5. TLC monitoring of the purification of peptide I by Bio-Gel P-2 chromatography.

The synthetic peptide II (see *Materials*) (100 mg crude material) was purified as described above for the pentapeptide except that the non-polar upper phase was used as the mobile phase. Although monitoring of ultraviolet absorbance was used to locate tubes of interest, improved sensitivity was obtained by monitoring with fluorescence (Fig. 6A), yield of peptide (Fig. 6B and Table II), amino acid composition (Table II), and behavior on TLC (Fig. 7). The results indicate that the run could be divided into three main areas of interest. The high levels of fluorescence for the contents of tubes 5–11 (Fig. 6A) and their lack of reaction with ninhydrin when run on TLC (results not shown) suggest that these tubes contained blocked peptides and aromatic by-products of deblocking. This hypothesis is further supported by the realization that, by using the non-polar phase as the mobile phase, the least polar products would appear in the early tubes. Amino acid analyses (Table II) also suggest that this fraction contained abbreviated sequences. Tubes 38–49 contained the pure peptide as indicated by amino acid analyses (Table II) and uniform TLC behavior (Fig. 7). Tubes 50–66 contained peptide contaminated with tyrosine (from insufficient washing of the resin following the last coupling), since an increase in fluorescence for these tubes (Fig. 6A) was accompanied by an increase in the number of moles of tyrosine/mole of peptide (Table II) on the descending side of the peptide concentration curve (Fig. 6B). Furthermore, the distinct, fairly intense spot in tubes

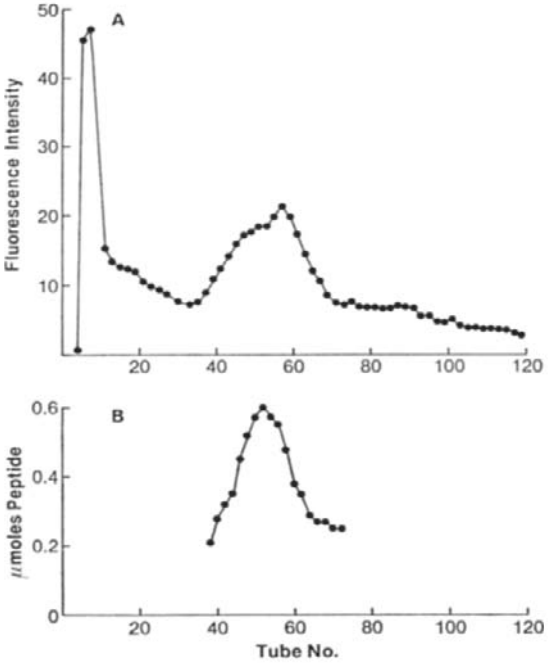

Fig. 6. Monitoring of the purification of peptide II by fluorescence intensity (A) and yield of peptide (B). Note that tubes are numbered as described for Fig. 4. Reprinted with permission of Pierce Chemical Company from E. Gross and J. Meienhofer (Editors), *Peptides: Structure and Biological Function*, 1979 (ref. 2).

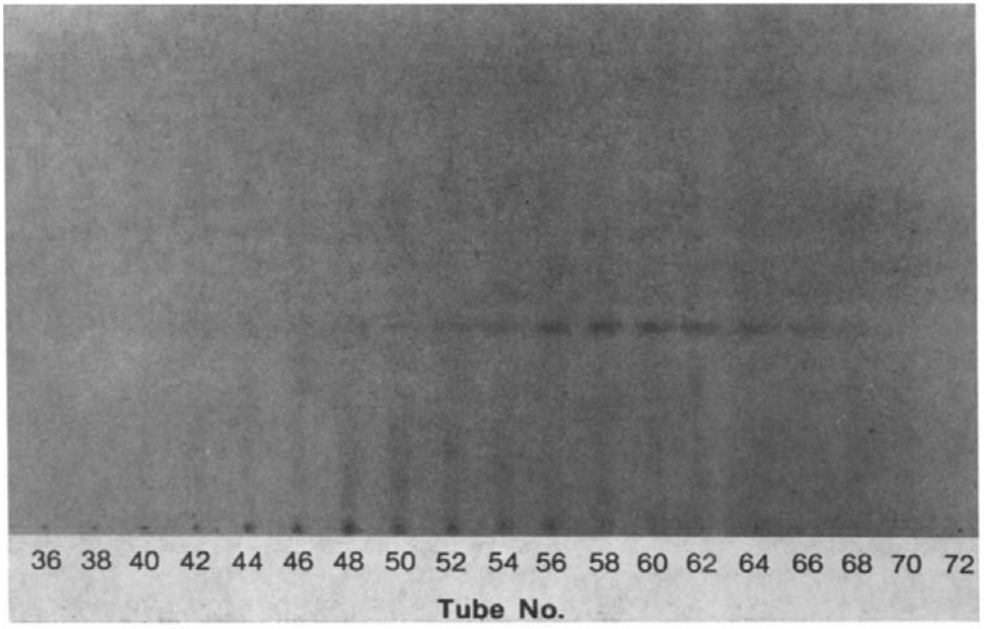

Fig. 7. TLC monitoring of the purification of peptide II. Note that tubes are numbered as described for Fig. 4. Reprinted with permission of Pierce Chemical Company from E. Gross and J. Meienhofer (Editors), *Peptides: Structure and Biological Function*, 1979 (ref. 2).

50–66 migrates on TLC and thin-layer electrophoresis in 5% formic acid identically to tyrosine.

The recovery of the desired peptide (including fractions containing free tyrosine) was only 38%. Solubility was a problem with this peptide (see Experimental) and was probably responsible for both a low yield and the streaking on TLC. To test whether some of the peptide was precipitating upon its addition to the stationary phase at the start of the run, the column contents were collected in fractions which were evaporated to dryness and analyzed by TLC. Aliquots from four tubes which appeared to contain the desired peptide without free tyrosine were hydrolyzed and analyzed. Excellent molar ratios were obtained, and the overall recovery of the peptide was raised to 53%. A yield of 60% (without measuring column contents) was also obtained by running a 10-mg sample. At this level the percent of soluble peptide was increased, and less material was lost to the column contents. To confirm that the low yield of peptide was due to its poor solubility in the butanol–acetic acid–water (4:1:5) solvent system, a separation was performed using the solvent system chloroform–acetic acid–water (2:2:1) with the aqueous phase as the mobile phase. The crude peptide was soluble in this solvent system, appeared in the first six tubes following the solvent front and was recovered in 95% yield. Traces of tyrosine also appeared in these fractions, but levels of this contaminant were vastly reduced.

CONCLUSIONS

The new horizontal flow-through coil planet centrifuge can perform both preparative- and analytical-scale separations of various biological samples. The results indicate that counter-current chromatography with the planet centrifuge has a number of advantages over conventional partition methods. Compared with the Craig counter-current distribution method (CCD), the present method provides higher partition efficiency in shorter periods of time without excessive dilution but with the retention of high reproducibility and predictability inherent with CCD. The present method also avoids the use of solid supports which are a necessity for most column chromatographic procedures. Thus, such problems as sample loss, tailing of solute peaks, and extraction of contaminants from a support medium are reduced or completely eliminated.

Among a variety of applications, separation and purification of natural and synthetic peptides may be the most useful. With synthetic peptides, a planet centrifuge run appears to free the desired peptides of salts, resin by-products and contaminating peptides. Even with a peptide possessing low solubility, yields can be improved by recovering the column contents without the complication of the peptide precipitating on a support medium. Furthermore, the peptide which was poorly soluble in the solvent system described in Table I was even less soluble in the aqueous solvents typically used for ion-exchange chromatography and gel filtration. However, counter-current chromatography allows a choice from a variety of solvent systems. Thus the solvent system chloroform–acetic acid–water (2:2:1) gave a high yield for this peptide, while the yield for the same peptide with the solvent system *n*-butanol–acetic acid–water (4:1:5) was merely adequate.

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