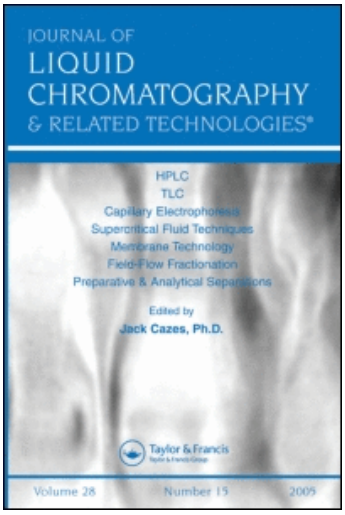

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Amino Acid Analysis and Enzymatic Sequence Determination of Peptides by an Improved o-Phthaldialdehyde Precolumn Labeling Procedure

Barry N. Jones[a]; Svante Pääbo[a]; Stanley Stein[a]
[a] Roche Institute of Molecular Biology, Nutley, New Jersey

To cite this Article Jones, Barry N. , Pääbo, Svante and Stein, Stanley(1981) 'Amino Acid Analysis and Enzymatic Sequence Determination of Peptides by an Improved o-Phthaldialdehyde Precolumn Labeling Procedure', Journal of Liquid Chromatography & Related Technologies, 4: 4, 565 — 586
To link to this Article: DOI: 10.1080/01483918108059956
URL: http://dx.doi.org/10.1080/01483918108059956

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(4), 565-586 (1981)

AMINO ACID ANALYSIS AND ENZYMATIC SEQUENCE DETERMINATION OF PEPTIDES BY AN IMPROVED o-PHTHALDIALDEHYDE PRECOLUMN LABELING PROCEDURE

Barry N. Jones, Svante Pääbo and Stanley Stein

Roche Institute of Molecular Biology
Nutley, New Jersey 07110

ABSTRACT

Primary amino acids react with o-phthaldialdehyde in the presence of mercaptans to form intensely fluorescent derivatives. By the use of reverse-phase high-performance liquid chromatography, a mixture containing 26 of these derivatives was efficiently resolved with an analysis time of less than 35 minutes. The quantitation of the individual amino acids was reproducible with an average relative deviation of ± 1.4% and had a detection limit of approximately 50 femtomoles. Improvements in the stability and fluorescence response of lysine and hydroxylysine were obtained by the incorporation of sodium dodecyl sulfate in the derivatizing medium. Applications of the chromatography system involving the amino acid analysis of peptides after either acid or enzymatic hydrolysis are presented. Methods for the sequence analysis of picomole quantities of peptides by time course hydrolysis with exopeptidases were also developed, which employed the above separation procedure for the identification and quantification of the released amino acids.

INTRODUCTION

The ability to separate and quantitate amino acids is essential for the characterization and structural elucidation of peptides and proteins. This is usually accomplished by separating amino acid mixtures using classical ion-exchange chromatography (1) followed by post-column derivatization with ninhydrin (2,3) or a fluorogenic reagent (4-7). This technique resolves most amino acids with good detection limits, especially if either fluorescamine or o-phthaldialdehyde is used as the post-column derivatizing

reagent. However, this procedure generally requires specialized equipment and an analysis time greater than an hour.

In order to improve the efficiency and decrease the analysis time for the resolution of amino acid mixtures, several precolumn techniques have been developed which utilize reverse-phase high-performance liquid chromatography. Two of the most widely employed methods are the formation of dansyl (8) or phenylthiohydantoin (9,10) derivatives of the amino acids prior to high-performance liquid chromatographic analysis. Recent studies have demonstrated that o-phthaldialdehyde can also be used successfully as a precolumn derivatizing reagent. o-Phthaldialdehyde in the presence of either 2-mercaptoethanol or ethanethiol reacts rapidly with primary amino acids to form highly fluorescent, thio-substituted isoindoles (11,12). These derivatives are then analyzed with good selectivity and sensitivity by reverse-phase high-performance liquid chromatography (13-17). In this report, the use of o-phthaldialdehyde as a precolumn derivatizing reagent for primary amino acids is evaluated further. In addition, its applicability for the amino acid analysis of peptides, as well as for the determination of their amino acid sequence, is investigated.

MATERIALS AND METHODS

Apparatus

Two separate chromatography systems were employed in the present studies. One consisted of a high-pressure Milton Roy minipump (Lab Data Control), an LKB Ultrograd unit for generation of elution gradients, and a model FS970 Liquid Chromatographic Fluorometer (Schoeffel Instrument Corp.). The following fluorometer settings and characteristics were used for detection: 5 μL flow cell, excitation monochrometer at 330 nm, the emission measured with a 418 nm cut-off filter, time constant of 0.5 sec, and a sensitivity dial setting at 7.00 units. The other chromatography system consisted of two Altex Model 110A high-performance liquid chromatography (HPLC) pumps, an Altex Model 420 microprocessor

for generation of elution gradients, and a model FS950 Liquid Chromatographic Filter Fluorometer (Schoeffel Instrument Corp.). The following fluorometer settings and characteristics were used: 20 µL flow cell, a FSA 403 excitation filter and a FSA 111 lamp for excitation in the 330-375 nm range, the emission was measured with a FSA 426 (418 nm cut-off) filter, time constant of 0.5 sec., and a sensitivity dial setting at 8.00 units. The two chromatography systems gave essentially identical results with respect to the resolution of amino acid mixtures. The only major difference between the two systems was in sensitivity. Using the above fluorometer settings, the FS970 detection unit was approximately ten times more sensitive.

All sample injections were performed with a Rheodyne injection valve (Model 7120) equipped with a 20 µL sample loop. An Altex Ultrasphere ODS column (250 x 4.6 mm; particle size, 5 µ) fitted with a guard column (80 x 4.6 mm) packed with CO:PELL ODS sorbent (particle size, 40 µ) (Reeve Angel) was used for chromatographic separations. The guard column was packed downwards at a pressure of 3000 psi with a Haskel pump (DST-150). Chromatographic peaks were integrated by a Spectra-Physics Minigrator and recorded on a two-channel Linear recorder operated on the 10 mV and 100 mV scales. Gradients were formed between two degassed solvent mixtures. Solvent A was tetrahydrofuran:methanol:0.05M sodium acetate (pH 5.9) 1:19:80 and solvent B was methanol:0.05M sodium acetate (pH 5.9) 8:2. Further details of the chromatographic procedure are given in the figure legends.

Reagents and Standards

All HPLC solvents were distilled-in-glass grade (Burdick and Jackson) and used without further treatment. High purity water was obtained with a system from Hydro Service and Supplies (Durham, N.C.). Solutions of amino acid standards (2.5 µmol/mL), 2-mercaptoethanol, ethanethiol, o-phthaldialdehyde (Fluoropa), 4N methanesulfonic acid (MSA), and sodium dodecyl sulfate (SDS)

were obtained from Pierce Chemical Co. Individual amino acid standards were purchased from Sigma Chemical Co. All other chemicals were reagent grade. Enzymatic digestion buffers contained 0.01% pentachlorophenol (Pierce) as a preservative.

Peptides and Enzymes

Met-enkephalin-Arg^6-Phe^7 was synthesized by Peninsula Laboratories. Met-enkephalin, porcine glucagon, carboxypeptidase B, and carboxypeptidase Y were products of Boehringer Mannheim Co. Aminopeptidase M was purchased from Sigma Chemical Co. Oxidized Met-enkephalin was prepared according to the procedure of Savige and Fontana (18) and purified by reverse phase HPLC (19).

Preparation of Amino Acid Standards

Amino acid standard solutions were prepared in water in the following concentrations: 1.25, 6.25, 25, and 50 pmol/μL and stored at -20^{0}C. The standard solutions were stable for approximately 3 weeks of continual use. After this time, appreciable amounts of methionine were lost with a concomitant increase in the methionine sulfoxide content of the standard. The glutamine peak in the standard runs also decreased with time, presumably due to the conversion of glutamine to pyroglutamic acid. For the ammonia standard, a 50 pmol/μL solution in water was prepared from ammonium acetate.

Preparation of the o-Phthaldialdehyde/2-Mercaptoethanol Derivatizing Solution

Fifty milligrams of o-phthaldialdehyde were dissolved in 1.25 mL of absolute methanol. 2-Mercaptoethanol (50 μL) and 0.4M sodium borate (pH 9.5) (11.2 mL) were then added and the solution mixed. The mixture was flushed with nitrogen and stored in the dark. The solution was allowed to stand for 24 hours before use. This was found to reduce the background amino acids which are contributed by the derivatizing reagent. 2-Mercaptoethanol

(10 µL) was added every 2 days to help maintain the reagent strength. Using these precautions, the derivatizing reagent was usable for approximately two weeks before serious contaminants appeared in the elution profiles. The o-phthaldialdehyde/ethanethiol derivatizing solution was prepared daily by the procedure of Hill et al. (13).

Derivatization Procedure

The general procedure for derivatization was as follows: 5 µL aliquots of standards or unknown samples were mixed with 5 µL of a 2% sodium dodecyl sulfate (SDS) solution in 0.4M sodium borate (pH 9.5) and then 5 µL of the derivatizing solution added. The resulting solution was mixed thoroughly. After 1 minute, 10 µL of 0.1M potassium phosphate (pH 4.0) was added, the solution mixed, and 5-20 µL injected onto the reverse phase column.

Acid Hydrolysis of Peptides

For HCl hydrolysis, peptides (50-300 pmol) were dried in a hydrolysis tube and the tube sealed under vacuum after the addition of 200 µL of constant boiling HCl containing 0.1% thioglycolic acid. Following hydrolysis, the HCl was removed by lyophilization and the resulting residue dissolved in 25-50 µL of 2% SDS in 0.4M sodium borate (pH 9.5). Analyses were then performed as above, usually on 5 µL aliquots. For hydrolysis with methanesulfonic acid (MSA), peptides (500 pmol - 1 nmol) were hydrolyzed with 50 µL of 4N methanesulfonic acid. After hydrolysis, the acid was neutralized with 50 µL of 4N NaOH. Aliquots (5 µL) were removed, mixed with 2% SDS in borate, derivatized, and analyzed by HPLC. All acid hydrolyses were performed at 110^{0}C for 22 hours.

Total Enzymatic Hydrolysis of Peptides

Peptides (200 pmol - 1 nmol) were dissolved in 24 µL of 0.2M sodium phosphate (pH 7.0) buffer containing 4-aminobutyric acid (usually 10 pmol/µL) as an internal standard. To this solution,

1 µg of aminopeptidase M (1 µL) was added and the resulting mixture incubated at 37°C for 24 hours. Aliquots (5 µL) were removed and analyzed. A digest blank containing 24 µL buffer and 1 µL aminopeptidase M was also prepared and analyzed.

Hydrolysis of Peptides with Carboxypeptidase B

Peptides (20-200 pmol) were dissolved in 24 µL of 0.2M sodium phosphate (pH 8.5) buffer containing 4-aminobutyric acid (5 pmol/µL) as an internal standard. To this solution, 25 ng of carboxypeptidase B (1 µL) was added and the resulting mixture incubated at 37°C for 1 hour. Aliquots (5 µL) were removed and analyzed. As an added precaution during enzymatic hydrolyses, the peptide solution was usually analyzed for free amino acids before the addition of the enzyme. Enzyme blanks were always prepared and analyzed.

Time Course Hydrolysis with Aminopeptidase M

Peptides (200 pmol - 1 nmol) were dissolved in 49 µL of 0.2M sodium phosphate (pH 7.0) buffer containing 4-aminobutyric acid as an internal standard. To this solution, 100 ng of aminopeptidase M (1 µL) was added and the resulting mixture incubated at room temperature. Timed aliquots (5 µL) were removed, mixed with 2% SDS in borate (5 µL), and then frozen in a dry ice/methanol bath. The aliquots were stored at -20°C until analyzed.

Time Course Hydrolysis with Carboxypeptidase Y

Peptides (200 - 1 nmol) were dissolved in 49 µL of 0.05M sodium acetate (pH 5.5) buffer containing 4-aminobutyric acid as an internal standard. To this solution, 1 µg of carboxypeptidase Y (1 µL) was added and the resulting mixture incubated at room temperature. Timed aliquots (5 µL) were removed and analyzed. Carboxypeptidase Y digestions were also performed in the presence of 5M urea.

RESULTS AND DISCUSSION

Preliminary separations of the o-phthaldialdehyde/2-mercaptoethanol derivatives of primary amino acids were conducted using mixtures of methanol and sodium or potassium phosphate buffer as the mobile phase according to the procedures of Lindroth and Mopper (14). Satisfactory resolution was obtained for all derivatives tested except for glycine and threonine which co-eluted even under isocratic conditions. In an attampt to resolve these two derivatives, the pH of the mobile phase was varied between 5.0 and 7.5 and the ionic strength was varied between 0.05M and 0.2M; however, glycine and threonine did not resolve under any of these conditions. Citrate and acetate buffers were then tested but did not help to resolve these two amino acids. Following the suggestion of Hodgin (16), tetrahydrofuran was added to the mobile phase an an organic modifier; this resulted in the immediate resolution of glycine and threonine. The final conditions chosen for HPLC analysis are given in Figure 1 which illustrates the resolution obtained for a mixture of 26 amino acids plus ammonia. Excellent resolution was obtained for the components in the mixture. Of a total of 30 amino acids tested, only two were not resolved by these conditions. Norleucine co-eluted with leucine and methionine sulfone eluted as a shoulder on the threonine peak.

The stability of the o-phthaldialdehyde/2-mercaptoethanol derivatives was determined by reacting a mixture of amino acids (40 pmol each) with the derivatizing reagent for varying time periods before injection. Only the derivatives of glycine, lysine, and hydroxylysine showed appreciable decay of fluorescence after an eight minute reaction time. In addition, the lysine and hydroxylysine derivatives gave a very low response when compared to the other amino acid derivatives. According to Benson and Hare (6), the addition of the surfactant, Brij 35, to the o-phthaldialdehyde reagent enhances the fluorescence of lysine during post-column derivatization procedures. Gardner and

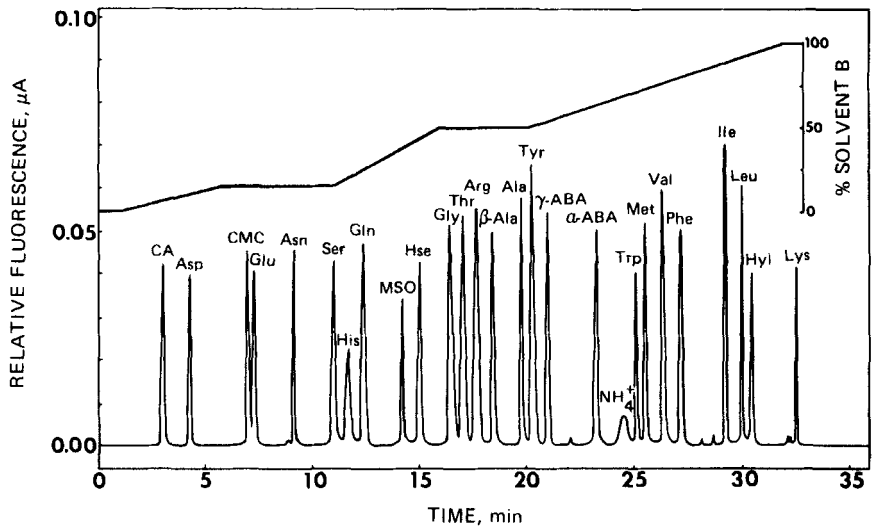

Figure 1. Elution profile of amino acid standards derivatized by the reaction with o-phthaldialdehyde/2-mercaptoethanol. Each peak represents 5.0 pmoles except for the ammonia peak which represents 20.0 pmoles. Conditions: Solvent A, tetrahydrofuran:methanol: 0.05M sodium acetate (pH 5.9), 1:19:80; Solvent B, methanol:0.05M sodium acetate (pH 5.9), 8:2; gradient program, 0%B for 1 min from the initiation of the program, linear step to 14% B in 5 min, isocratic step at 14% B of 5 min duration, linear step to 50% B in 5 min, isocratic step at 50% B of 4 min duration, linear step to 100% B in 12 min; flow rate of 1.7 mL/min; Schoeffel FS950 fluorescence HPLC detector. Non-standard abbreviations used: CA, cysteic acid; CMC, carboxymethyl cysteine; MSO, methionine sulfoxide; Hse, homoserine; γ-ABA, 4-aminobutyric acid; α-ABA, 2-aminobutyric acid.

Miller (17) found that the addition of Brij 35 also increased the fluorescence response of lysine in their precolumn derivatization procedure. In the present study, sodium dodecyl sulfate (SDS) was evaluated for its effect on the fluorescence of o-phthaldialdehyde-derivatized amino acids (Figure 2). An approximately 50% increase in the response of lysine was obtained and approximately a 40% increase for hydroxylysine. The stability of these two derivatives was also increased by the presence of SDS. These observations are in agreement with those reported by Chen

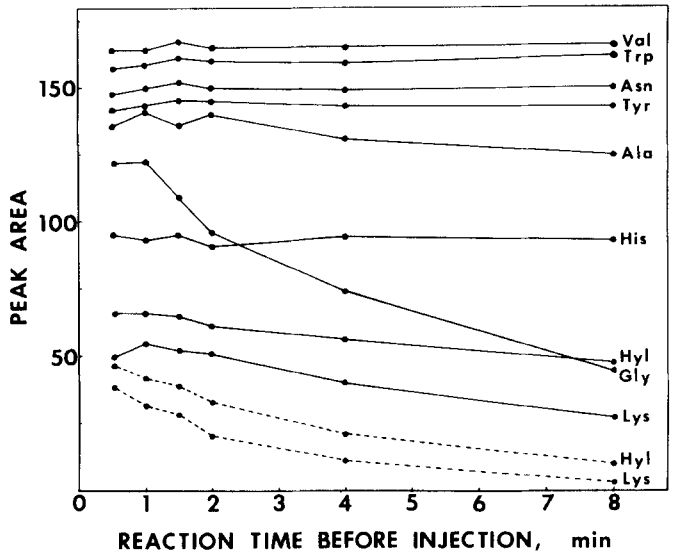

Figure 2. Influence of reaction time before injection on the fluorescent intensity of ten amino acids. Peak areas are given in arbitrary units. The effect of the presence of sodium dodecyl sulfate (SDS) on the fluorescent intensity and stability of the lysine and hydroxylysine derivatives is also shown. Derivatizations in the presence of SDS are given by the solid lines and in the absence of SDS by dashed lines.

et al. (20). No observable effect on the retention times and fluorescence response of the other amino acids tested (see Figure 1) was detected. The large decrease in the fluorescence response of glycine was minimized by injection of all derivatized samples after a 1 minute reaction time (see Figure 2),

In order for the present chromatographic system to be of value for the quantitative analysis of amino acids, the technique must yield reproducible results, possess high sensitivity, and give a linear fluorescence response in the general concentration range required for analyses. The precision of the procedure was evaluated by injection of a 22-component amino acid standard using the chromatographic conditions presented in Figure 1. Five consecutive injections were performed following a reaction time

of one minute and the resulting retention times, peak heights, and peak areas of the individual components measured. Analysis of the data indicated that the average deviation of the retention times was ± 1.1 second. The largest deviation observed was for methionine sulfoxide (± 2.6 sec.) and the smallest deviation obtained was for methionine (± 0.2 sec.). The total analysis time was 34 minutes. The average relative deviation in the measured peak heights was ± 6.4%. However, part of this deviation is due to pipetting errors that may occur during the derivatization procedure. When results from all five runs were normalized to an internal standard (4-aminobutyric acid) the relative deviation of the peak heights was calculated to be ± 1.4%. The average relative deviation of the normalized peak areas was ± 1.7%. The largest deviation in the normalized peak height values was observed for cysteic acid (± 2.8%) whereas the lowest was for arginine (± 0.4%). From the above results, the incorporation of an internal standard is recommended for a quantitative analysis of an amino acid mixture.

The detedtion limits for the chromatography system were determined by serial dilution of an amino acid standard solution followed by derivatization and analysis. A detection limit of approximately 50 fmol was obtained for the Schoeffel FS 970 detection unit whereas the Schoeffel FS950 unit displayed a detection limit of approximately 500 fmol for the individual amino acid components. The sensitivity of detection can be increased somewhat by using ethanethiol instead of 2-mercaptoethanol in the derivatizing reagent (12,13). However, in the present study, the added sensitivity was not required and the use of ethanethiol was abandoned because of its unpleasant odor. Additional sensitivity (approximately a six-fold increase) can be obtained by excitation at 229 nm instead of 330 nm (13), but interference from extraneous components may be a problem at this lower wavelength when biological fluids are examined.

The linearity of response was examined in the concentration range of 5-100 pmol for a number of amino acid derivatives

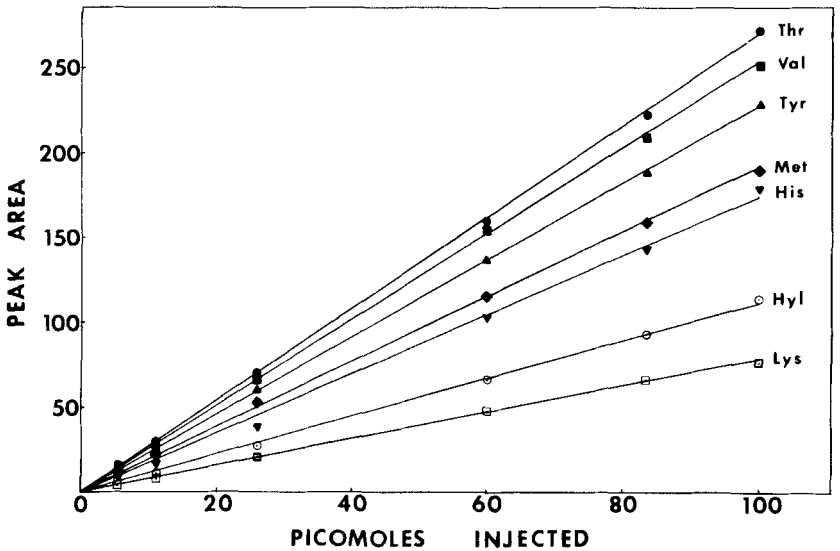

Figure 3. Linearity of response for seven amino acid derivatives. The chromatography conditions were identical to those in Figure 1.

(Figure 3). At concentration levels below 5 pmol, large deviations from linearity were obtained, especially for serine, glycine, and alanine. These deviations from linearity are most probably due to the background presence of these amino acids. This can be partially corrected by the subtraction of the background amino acids which are determined by "blank" runs. Even this procedure does not completely correct for the presence of glycine and serine whose presence vary considerably during a series of analyses. To minimize this effect when quantitative analysis was desired below the 5 pmol level, all reaction vessels (1 mL polypropylene centrifuge tubes) and pipet tips were acid washed before use. Furthermore, plastic surgical gloves had to be worn to prevent "fingerprint" contamination.

Three different types of HPLC columns were evaluated for their use in the separation of the o-phthaldialdehyde derivatives of amino acids. The three were Altex Ultrasphere columns, octadecylsilane (ODS), octadecylsilane/ion-pairing (ODS I/P.), and

cyanopropylsilane (CN). The amino acid derivatives shown in Figure 1 were resolved on the CN column, but variable peak heights and retention times were obtained and its use was discontinued. The ODS and ODS I.P. columns gave identical results. The Ultrasphere ODS column was used in all chromatographic separations used in this report. Over 600 analyses have been performed on this column without any detectable loss of resolving power. However, the column is protected with a guard column since many analyses (see below) require the injection of derivatized amino acid mixtures which contain enzymes and partially digested peptides. The guard column was replaced after every 200 injections as a precaution.

The applicability of the chromatography system for the determination of the amino acid composition of peptides was evaluated. Three different hydrolysis procedures, HCl hydrolysis, methanesulfonic acid (MSA) hydrolysis, and total enzymatic hydrolysis with aminopeptidase M, were used and found to be compatible with the o-phthaldialdehyde derivatization technique. The results obtained for the amino acid analysis of porcine glucagon using each of these hydrolysis procedures is summarized in Table I. The elution profile of the enzymatic hydrolysate is given in Figure 4. The resulting compositions are in good agreement with the reported amino acid sequence of porcine glucagon (21). It should be noted that the acid/amide content of glucagon was readily obtained by the enzymatic hydrolysis procedure. Detection and quantitation of asparagine and glutamine usually require special buffers (lithium citrate) and modified chromatography conditions (22) when analysis is performed by classical ion-exchange methods.

The quantitation of methionine sulfoxide is also a problem for many amino acid analyzer systems. However, methionine sulfoxide content of peptides is easily determined by the precolumn derivatization with o-phthaldialdehyde. This is illustrated in Figure 5 by the elution profile of a MSA hydrolysate of purified oxidized Met-enkephalin. The methionine sulfoxide elutes as a

TABLE I

AMINO ACID COMPOSITION OF PORCINE GLUCAGON

Amino Acid	HCl Hydrolysate[a]	MSA Hydrolysate[b]	Enzymatic Hydrolysate[c]	From Sequence[d]
Asp[e]	4.0	3.8	3.2	3
Glu[f]	3.0	3.1	0.1	0
Asn	---	---	1.1	1
Ser	3.8	3.6	3.8	4
His	0.9	0.7	1.1	1
Gln	---	---	3.2	3
Gly	1.2	1.1	1.0	1
Thr	2.9	2.5	3.0	3
Arg	2.0	2.1	2.0	2
Ala	1.0	1.2	1.0	1
Tyr	2.0	2.0	1.9	2
Trp	0.3	0.8	0.9	1
Met	0.9	0.9	1.1	1
Val	1.0	1.0	1.0	1
Phe	2.0	2.0	1.9	2
Ile	0.0	0.0	0.0	0
Leu	2.0	2.1	2.0	2
Lys	1.3	1.0	0.9	1

[a] 300 pmol of peptide were hydrolyzed by HCl. Results are the average of three analyses.

[b] 1 nmol of peptide was hydrolyzed with methanesulfonic acid (MSA). Results are the average of two analyses.

[c] 500 pmol of peptide were digested with aminopeptidase M. Results are the average of two analyses.

[d] The amino acid sequence used is from Bromer et al. (21).

[e] Asp + Asn for HCl and MSA hydrolysates.

[f] Glu + Gln for HCl and MSA hydrolysates.

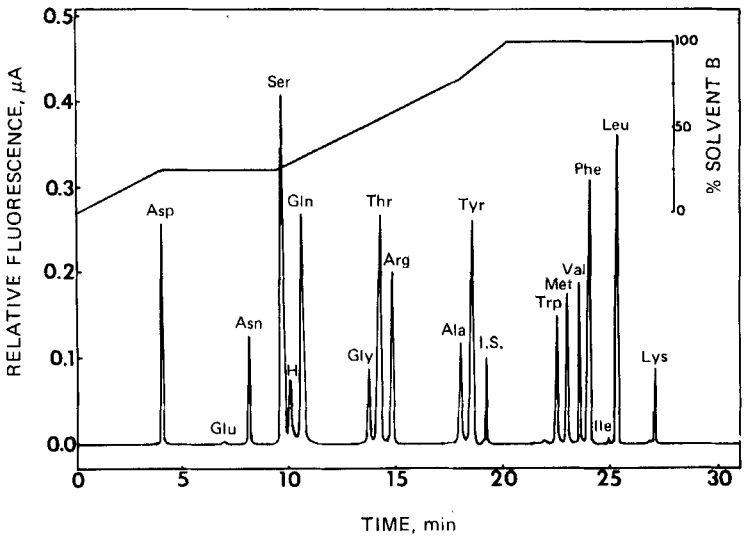

Figure 4. Elution profile of a total enzymatic hydrolysate of glucagon. Digestion was accomplished by incubating 500 pmoles of the peptide with 1 μg of aminopeptidase M. A 5 μL aliquot representing 10% of the hydrolysate was derivatized and 30% of the resulting derivatization mixture applied to the HPLC column. Conditions: Solvents A and B, same as in Figure 1; gradient program, linear step to 25% B in 4 min, isocratic step at 25% B of 5 min duration, linear step to 80% B in 9 min, linear step to 100% B in 2 min; flow rate of 2.0 mL/min; Schoeffel FS950 fluorescence HPLC detector. Internal standard (I.S.) was 4-aminobutyric acid. The single letter code, H, was used for histidine.

characteristic doublet. However, in the elution profile shown in Figure 1, it elutes as a singlet. The discrepancy appears to result from the two diastereomeric forms of methionine sulfoxide. The amino acid standards in Figure 1 were obtained from Pierce and presumably contained only one of the diastereomeric forms of methionine sulfoxide. This was further investigated by testing a methionine sulfoxide standard from Sigma tha- contained a mixture of the two diastereomers. Analysis of this standard yielded the characteristic doublet. Figure 5 also indicates that some of the methionine sulfoxide was reduced to methionine during the hydrolysis procedure.

In addition to the use of this chromatography system for amino acid analysis, its applicability in obtaining sequence data was also evaluated. The methods developed include digestion of peptides with carboxypeptidase B (CPB) plus aminopeptidase M (APM) and carboxypeptidase Y (CPY) time course hydrolysis. The use of CPB digestion is illustrated in Figure 6. In this example, two tryptic peptides were obtained from enkephalin precursor molecules that gave identical compositions, Gly_2,Met,Tyr,Phe,Arg (23). However, their elution positions were different as determined by their chromatography on reverse phase columns. In order

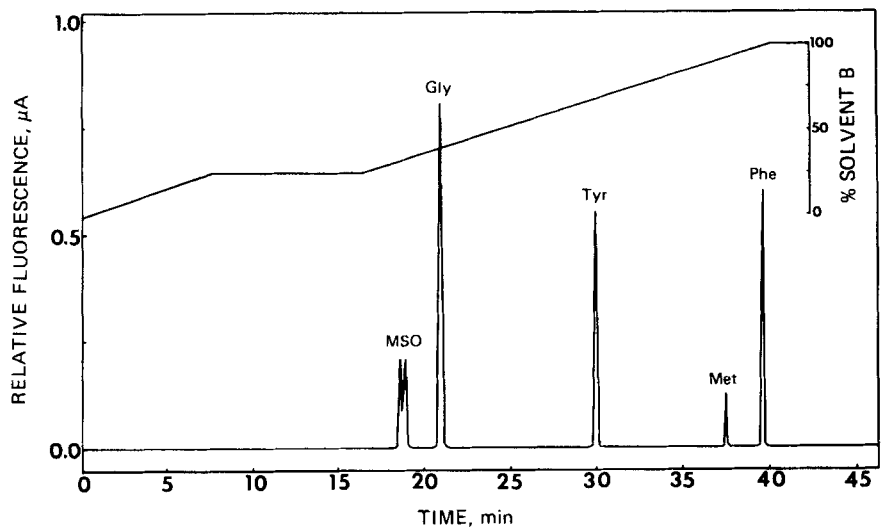

Figure 5. Elution profile of an acid hydrolysate of oxidized Met-enkephalin (Tyr-Gly-Gly-Phe-MSO). The peptide (1 nmole) was hydrolyzed in 50 μL of 4N methanesulfonic acid and then diluted to a volume of 100 μL with 4N NaOH. A 5 μL aliquot of the hydrolysate was derivatized and 20% of the resulting derivatization mixture applied to the HPLC column. Conditions: Solvents A and B, same as in Figure 1, gradient program, linear step to 25% B in 7.5 min, isocratic step at 25% B of 9 min duration, linear step to 100% B in 23 min; flow rate of 1.0 mL/min; Schoeffel FS970 fluorescence HPLC detector. Methionine sulfoxide (MSO) elutes as a doublet due to the resolution of its two diastereomeric forms. Composition was calculated to be MSO, 0.9 residues; Gly, 1.9 residues; Tyr, 1.1 residues; Met, 0.2 residues; Phe, 0.9 residues.

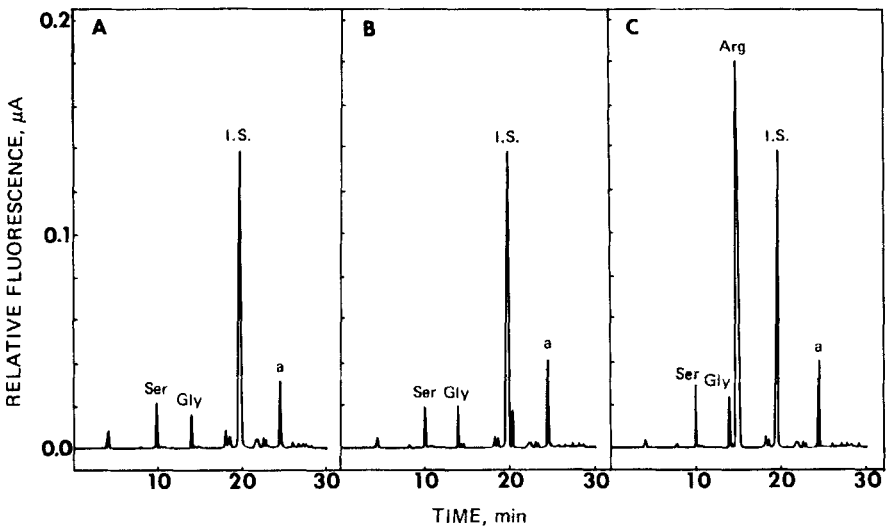

Figure 6. Elution profiles of carboxypeptidase B (CPB) digests of two Met-enkephalin-containing peptides. A, Digest blank: 25 ng CPB in 25 μL of digestion buffer, 5 μL aliquot derivatized and applied to HPLC column. B, Digest of Arg-Tyr-Gly-Gly-Phe-Met, 125 pmoles of peptide in 25 μL of digestion buffer containing 25 ng CPB, 5 μL aliquot derivatized and analyzed. C, Digest of Tyr-Gly-Gly-Phe-Met-Arg, 150 pmoles of peptide in 25 μL of digestion buffer containing 25 ng CPB, 5 μL aliquot derivatized and analyzed. The digestion buffer contained 4-aminobutyric acid (5 pmol/μL) as an internal standard (I.S.). Peak "a" is contributed by the derivatizing reagent. Conditions: identical to those in Figure 3.

to determine the structural difference in the two peptides, each was digested with CPB. For one of the peptides, no detectable amino acids were released (Figure 6B) after comparison to the analysis of the enzyme blank (Figure 6A), but CPB did release one residue of arginine from the other tryptic peptide indicating that this was the carboxyl-terminal residue. These results were later confirmed by sequence analysis which determined the amino acid sequence of each tryptic peptide (23) (see Figure 6).

Amino-terminal sequencing with APM is illustrated in Figure 7. In this example, Met-enkephalin was digested with APM and timed aliquots removed for analysis. From Figure 7, it can be seen what tyrosine is the first amino acid to be released

followed by glycine. Methionine and phenylalanine are released simultaneously. Thus, the release data are consistent with the known amino acid sequence of Met-enkephalin, Tyr-Gly-Gly-Phe-Met.

Carboxyl-terminal sequencing with CPY is illustrated in Figure 8. In this example, Met-enkephalin-Arg^{6}-Phe^{7} was digested with the enzyme and the released amino acids identified and quantitated. The data obtained from the HPLC analyses are presented in Figure 9 in the form of a time course release plot and established the carboxyl-terminal sequence of the peptide as -Gly-Phe-Met-Arg-Phe.

This chromatography system has other applications. For example, free amino acids released during tryptic digestion of peptides and proteins are readily identified and quantitated. In

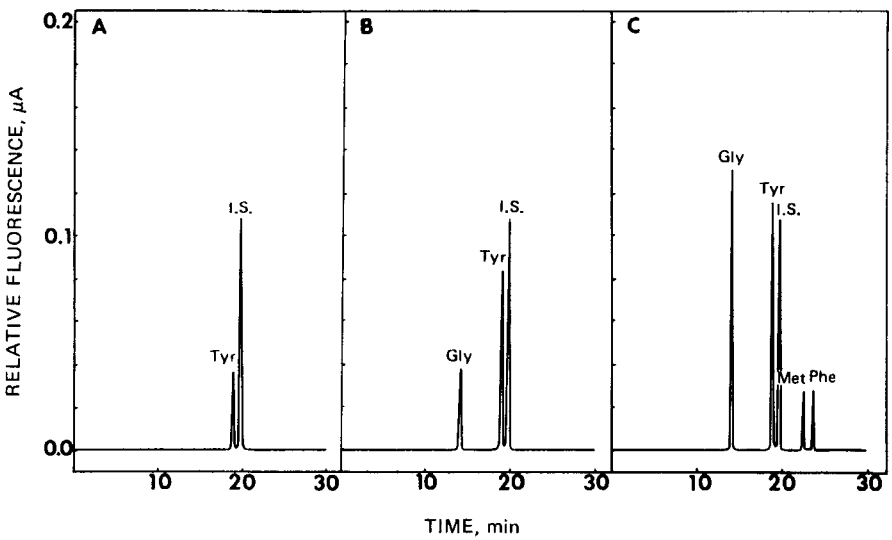

Figure 7. Elution profiles of aminopeptidase M (APM) time course hydrolysis of Met-enkephalin (Tyr-Gly-Gly-Phe-Met). Peptide (1 nmole) was dissolved in 50 μL of digestion buffer containing 50 ng APM and 20 pmol/μL 4-aminobutyric acid as internal standard (I.S.). Timed 5 μL aliquots were derivatized and 10% of the resulting derivatization mixtures applied to the HPLC column. A, 4-min aliquot; B, 30-min aliquot; C, 180-min aliquot. Conditions: identical to those in Figure 3.

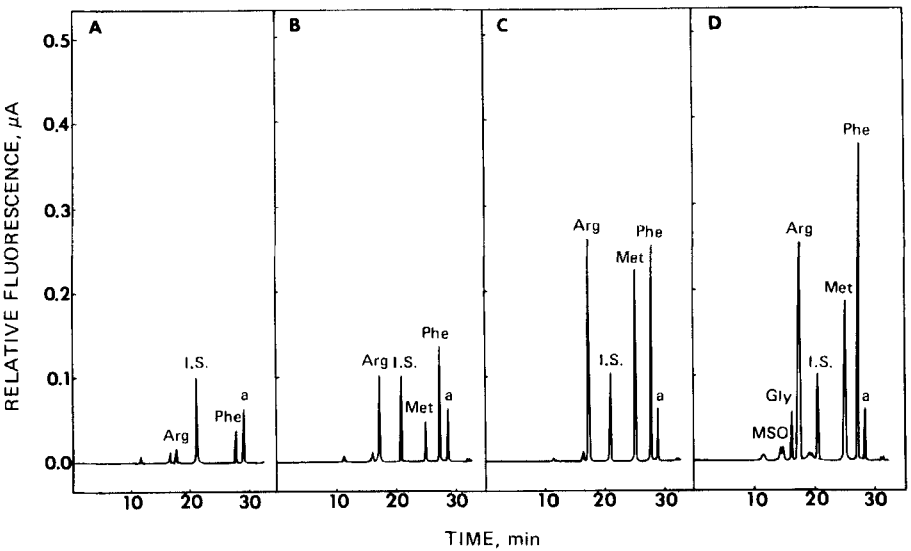

Figure 8. Elution profiles of carboxypeptidase Y (CBY) time course hydrolysis of Met-enkephalin-Arg^6-Phe^7 (Tyr-Gly-Gly-Phe-Met-Arg-Phe). Peptide (360 pmol) was dissolved in 60 μL of digestion buffer containing 500 ng CPY and 2 pmol/μL 4-aminobutyric acid as internal standard (I.S.). Timed 5 μL aliquots were derivatized and analyzed. A, 2-min aliquot; B, 8-min aliquot; C, 60-min aliquot; D, 600-min aliquot. Peak "a" is contributed by the derivatizing reagent. MSO represents methionine sulfoxide. Conditions: identical to those in Figure 1.

addition, the homoserine content of cyanogen bromide fragments is readily determined by amino acid analysis using this precolumn derivatization procedure. The method also has the potential for many other applications such as the determination of free amino acids in biological fluids (13,14). A variety of primary amines of biological importance could conceivably be identified and quantitated by this method. These include taurine, 4-aminobutyric acid, ornithine, citrulline, histamine, serotonin, and epinephrine as well as others. Several reports have already appeared describing procedures for the analysis of some of these components (13-15,24,25).

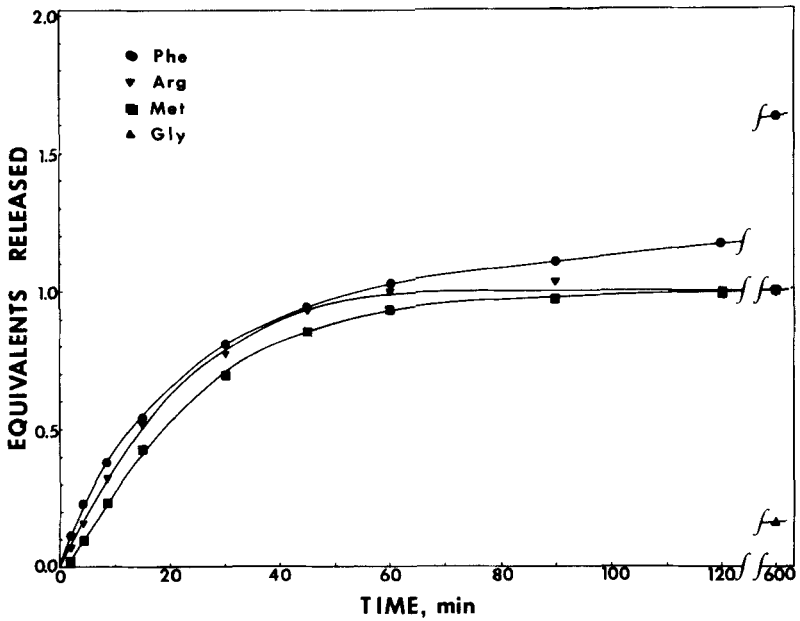

Figure 9. Time course release plot for the carboxypeptidase Y digestion of Met-enkephalin-Arg^6-Phe^7. Carboxyl-terminal sequence established by release data is -Gly-Phe-Met-Arg-Phe-COOH.

CONCLUSIONS

The use of precolumn derivatization of amino acids with o-phthaldialdehyde and their subsequent separation by reverse phase HPLC provides an excellent method for the amino acid analysis and sequence determination of peptides. The derivatization procedure is rapid, is performed in an aqueous medium, requires few transfer steps, and yields strongly fluorescent derivatives. The separation procedure is highly reproducible and rapid when compared to current amino acid analyzers. The cost per analysis is considerably less in comparison to post-column procedures in which the derivatizing reagent is added in a continuous manner.

Three major disadvantages to this procedure are the low response of cysteine, lysine, and hydroxylysine, the lack of re-

action with the secondary amino acids, proline and hydroxyproline, and the decay in fluorescence of the derivatized amino acids. The response of lysine and hydroxylysine was improved by the addition of sodium dodecyl sulfate to the derivatizing reagent. Cysteine can be readily detected as cysteic acid or carboxymethyl cysteine following performic acid oxidation or carboxymethylation (26). The problem of the detection of proline and hydroxyproline requires further investigation. The effect of fluorescence decay can be minimized by strict attention to the reaction time before injection.

ACKNOWLEDGEMENTS

We wish to thank Dr. Sidney Udenfriend for his support and advice as well as Drs. John Moschera, R.V. Lewis, A.S. Stern, and Mr. L. Brink. We also wish to thank Ms. D.V. Torres for the typing of this manuscript. Dr. S. Pääbo's present address is Svartagatan 36, S-12159 Johanneshov, Sweden.

REFERENCES

1. Spackman, D.H., Moore, S. and Stein, W.H., Automatic Recording Apparatus for Use in the Chromatography of Amino Acids, Anal. Chem., 30, 1190, 1958.

2. Samejima, K., Dairman, W. and Udenfriend, S., Condensation of Ninhydrin with Aldehydes and Primary Amines to Yield Highly Fluorescent Ternary Products II. Application to the Detection and Assay of Peptides, Amino Acids, Amines, and Amino Sugars, Anal. Biochem., 42, 237, 1971.

3. Hamilton, P.B. and Anderson, R.A., Ion Exchange Chromatography of Amino Acids. Semiautomatic Method for Operation with Cationic Exchange Resin Columns, Anal. Chem., 31, 1504, 1959.

4. Roth, M., Fluorescence Reaction for Amino Acids, Anal. Chem., 43, 880, 1971.

5. Stein, S., Böhlen, P., Stone, J., Dairman, W. and Udenfriend, S., Amino Acid Analysis with Fluorescamine at the Picomole Level, Arch. Biochem. Biophys., 155, 203, 1973.

6. Benson, J.R. and Hare, P.E., o-Phthaladehyde: Fluorogenic Detection of Primary Amino Acids in the Picomole Range. Comparison with Fluorescamine and Ninhydrin, Proc. Natl. Acad. Sci. USA, 72, 619, 1975.

7. Böhlen, P. and Mellet, M., Automated Fluorometric Amino Acid Analysis: The Determination of Proline and Hydroxyproline, Anal. Biochem., 94, 313, 1979.

8. Schmidt, G.J., Olson, D.C. and Slavin, W., Amino Acid Profiling of Protein Hydrolysates Using Liquid Chromatography and Fluorescence Detection, J. Liq. Chromatogr., 2, 1031, 1979.

9. Zimmerman, C.L., Appella, E. and Pisano, J.J., Rapid Analysis of Amino Acid Phenylthiohydantoins by High Performance Liquid Chromatography, Anal. Biochem., 77, 569, 1977.

10. Johnson, N.D., Hunkapiller, M.W. and Hood, L.E., Analysis of Phenylthiohydantoin Amino Acids by High Performance Liquid Chromatography on DuPont Zorbax Cyanopropylsilane Columns, Anal. Biochem., 100, 335, 1979.

11. Simmons, S.S. and Johnson, D.F., The Structure of the Fluorescent Adduct Formed in the Reaction of o-Phthalaldehyde and Thiols with Amines, J. Am. Chem. Soc., 98, 7098, 1976.

12. Simmons, S.S. and Johnson, D.F., Ethanethiol: A Thiol Conveying Improved Properties to the Fluorescent Product of o-Phthalaldehyde and Thiols with Amines, Anal. Biochem., 82, 250, 1977.

13. Hill, D.W., Walters, F.H., Wilson, T.D. and Stuart, J.D., High Performance Liquid Chromatographic Determination of Amino Acids in the Picomole Range, Anal. Chem., 51, 1338, 1979.

14. Lindroth, P. and Mopper, K., High Performance Liquid Chromatographic Determination of Subpicomole Amounts of Amino Acids by Precolumn Fluorescence Derivatization with o-Phthaldialdehyde, Anal. Chem., 51, 1667, 1979.

15. Stuart, J.D., Wilson, T.D., Hill, D.W., Walters, F.H. and Feng, S.Y., High Performance Liquid Chromatographic Separation and Fluorescent Measurement of Taurine, A Key Amino Acid, J. Liq. Chromatogr., 2, 809, 1979.

16. Hodgin, J.C., The Separation of Pre-Column o-Phthalaldehyde Derivatized Amino Acids by High Performance Liquid Chromatography, J. Liq. Chromatogr., 2, 1047, 1979.

17. Gardner, W.S. and Miller, W.H., Reverse-Phase Liquid Chromatographic Analysis of Amino Acids After Reaction with o-Phthalaldehyde, Anal. Biochem., 101, 61, 1980.

18. Savige, W.E. and Fontana, A., Interconversion of Methionine and Methionine Sulfoxide, in Methods in Enzymology (Hirs, C.H.W. and Timasheff, S.N., eds.) 47E, 453, 1977.

19. Lewis, R.V., Stein, S. and Udenfriend, S., Separation of Opioid Peptides Utilizing High Performance Liquid Chromatography, Int. J. Peptide Protein Res., 13, 493, 1979.

20. Chen, R.F., Scott, C. and Trepman, E., Fluorescence Properties of o-Phthaldialdehyde Derivatives of Amino Acids, Biochim. Biophys. Acta, 576, 440, 1979.

21. Bromer, W.W., Sinn, L.G. and Brehrens, O.K., The Amino Acid Sequence of Glucagon, J. Am. Chem. Soc., 79, 2807, 1957.

22. Rothgeb, T.M., Jones, B.N., Hayes, D.F. and Gurd, R.S., Methylation of Glucagon, Characterization of the Sulfonium Derivative, and Regeneration of the Native Covalent Structure, Biochemistry, 16, 5813, 1977.

23. Jones, B.N., Stern, A.S., Lewis, R.V., Kimura, S., Stein, S., Udenfriend, S. and Shively, J.E., Structure of Two Adrenal Polypeptides Containing Multiple Enkephalin Sequences, Arch. Biochem. Biophys., 204, 392, 1980.

24. Mell, L.D., Dasler, A.R. and Gustafson, A.B., Pre-Column Fluorescent Derivatization for High Pressure Liquid Chromatography with o-Phthalaldehyde: Separation of Urinary Catecholamines, J. Liq. Chromatogr., 1, 261, 1978.

25. Davis, T.P., Gehrke, C.W., Gehrke, C.W., Jr., Cunningham, T.D., Kuo, K.C., Gerhardt, K.O., Johnson, H.D. and Williams, C.H., High Performance Liquid Chromatographic Separation and Fluorescence Measurement of Biogenic Aimes in Plasma, Urine and Tissue, Clin. Chem. 24, 1317, 1978.

26. Lee, K.S. and Drescher, D.G., Derivatization of Cysteine and Cystine for Fluorescence Amino Acid Analysis with the o-Phthaldialdehyde/2-Mercaptoethanol Reagent, J. Biol. Chem., 254, 6248, 1979.