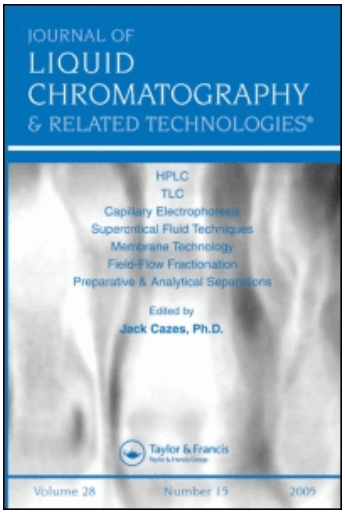

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High-Speed RP-HPLC/FL Analysis of Amino Acids After Automated Two-Step Derivatization with o-Phthaldialdehyde/3-mercaptopropionic Acid and 9-Fluorenylmethyl Chloroformate

T. Bartóak[a]; G. Szalai[b]; ZS. Lőrincz[a]; G. Bőurcsök[a]; F. Sági[a]

[a] Analytical Laboratory Cereal Research Institute, Szeged, Hungary [b] Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary

To cite this Article Bartóak, T. , Szalai, G. , Lőrincz, ZS. , Bőurcsök, G. and Sági, F.(1994) 'High-Speed RP-HPLC/FL Analysis of Amino Acids After Automated Two-Step Derivatization with o-Phthaldialdehyde/3-mercaptopropionic Acid and 9-Fluorenylmethyl Chloroformate', Journal of Liquid Chromatography & Related Technologies, 17: 20, 4391 — 4403

To link to this Article: DOI: 10.1080/10826079408013625

URL: http://dx.doi.org/10.1080/10826079408013625

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(20), 4391-4403 (1994)

HIGH-SPEED RP-HPLC/FL ANALYSIS OF AMINO ACIDS AFTER AUTOMATED TWO-STEP DERIVATIZATION WITH o-PHTHALDIALDEHYDE/3-MERCAPTOPROPIONIC ACID AND 9-FLUORENYLMETHYL CHLOROFORMATE

T. BARTÓK[1], G. SZALAI[2], ZS. LŐRINCZ[1], G. BŐRCSÖK[1], AND F. SÁGI[1]

[1]*Analytical Laboratory*
Cereal Research Institute
P.O. Box 391
H-6701 Szeged, Hungary
[2]*Agricultural Research Institute of the*
Hungarian Academy of Sciences
P.O. Box 19
H-2462 Martonvásár, Hungary

ABSTRACT

This paper describes an automated high-speed RP-HPLC/FL method, including two-step precolumn derivatization with o-phthaldialdehyde/3-mercaptopropionic acid (OPA/3-MPA) for primary amino acids and with 9-fluorenylmethyl chloroformate (FMOC-Cl) for secondary amino acids. The OPA/3-MPA derivatives eluted within 6.5 min, while the retention time of the last eluted amino acid derivative (FMOC-Pro) was 8.0 min. The total analysis time, including precolumn derivatization, separation, column washing and reequilibration cycles, was only 18 min. Therefore, 75-80 samples per day can be analysed during unattended operation. The efficiency of the method was demonstrated by the separation of amino acids extracted from potato tubers.

INTRODUCTION

The qualitative and quantitative determination of amino acids has become increasingly important in the chemical, pharmaceutical and food industries, and also in clinical chemistry and biotechnology. Because of their key role in these areas, increasingly more rapid and sensitive techniques have been developed for their analysis: thin-layer chromatography, gas chromatography, ion-exchange chromatography and high-performance liquid chromatography. Until the early eighties, the most widespread procedure was the cation-exchange chromatography combined with postcolumn derivatization with ninhydrin, as developed by Moore and Stein [1]. Although reliable, this technique is time-consuming and the derivatized compounds can be detected only in the nmol range. The most recent, frequently used methods are based on precolumn derivatization with a number of reagents, such as phenyl isothiocyanate (PITC) [2,3], 5-dimethylamino-naphthalene-1-sulphonyl chloride [4,5], 4-dimethylaminoazobenzene-4'-sulphonyl chloride [6,7], o-phthaldialdehyde (OPA) [8-10], or 9-fluorenylmethyl chloroformate (FMOC-Cl) [11,12]. The reactions of amino acids with PITC, OPA and FMOC-Cl can be automated by using computer-programmable autoinjectors and autosamplers, but phenylthiocarbamyl (PTC) derivative formation requires longer reaction time and these derivatives do not fluoresce. The PTC derivatives can generally be detected with a less sensitive UV detector, but the sensitivity can be improved by electrochemical detection [2]. OPA forms highly fluorescent isoindole derivatives from primary amino acids in the presence of mercaptans (e.g. 2-mercaptoethanol, ethanethiol, 3-mercaptopropionic acid (3-MPA) or 3-mercapto-1-propanol). Isoindole derivative formation needs a short reaction time (1 min) under basic conditions and takes place at room temperature. Unfortunately, the OPA reagent does not react with secondary amino acids such as Pro and HO-Pro and the derivatives have limited stability [13]. To overcome these disadvantages, an automatic two-step derivatization has been developed. In the first step, all primary amino acids react with the OPA reagent containing mercaptan. During the second step, the reaction mixture of isoindole derivatives is treated with FMOC-Cl to derivatize the unreacted secondary amino acids [8,9]. Both reactions are rapid and, since the derivatives are strongly fluorescent, the detection limits are in the low pmol range.

In this article we describe an efficient, high-speed RP-HPLC method for the analysis of amino acids. The method involves automated two-step precolumn derivatization, high-speed separation on a short cartridge-type column and fluorescence detection of the amino acid derivatives.

MATERIALS AND METHODS

Chemicals

For calibration, standard mixtures (20, 50, 100, 200 pmol/μl) of amino acids (Serva, Heidelberg, Germany) were prepared with 0.1 N hydrochloric acid. Each standard mixture contained 100 pmol/μl norvaline (Nval) as internal standard (ISTD). Acetonitrile (MeCN), methanol (MeOH) and tetrahydrofuran (THF) were HPLC grade from Farmitalia Carlo Erba (Milano, Italy). Triethylamine (TEA) was purchased from Sigma (St. Louis, MO, U.S.A.). Borate buffer (0.4 N, pH 10.4) was prepared from boric acid and potassium hydroxide (Reanal, Budapest, Hungary). Sodium acetate (NaOAc) was obtained from Reanal (Budapest, Hungary). For the preparation of buffers (borate and acetate), HPLC grade water was produced by using Nanopure II (Barnstead/Thermolyne Corporation, Dubuque, IA, U.S.A.) cartridge-type water purification equipment with a 0.2 μm final filter.

Preparation of Derivatization Reagents

The primary amino acids were derivatized with OPA/3-MPA reagent. 10 mg of OPA in an amber HPLC autosampler screw-cap vial (Sigma, St. Louis, MO, U.S.A.) was dissolved in 100 μl of HPLC grade MeOH and made-up to 1 ml with borate buffer (0.4 N, pH 10.4). Finally, 20 μl of 3-MPA was added to the vial. Secondary amino acids such as HO-Pro and Pro were derivatized with FMOC-Cl (Sigma, St. Louis, MO, U.S.A.) reagent. The FMOC-Cl (2.5 mg) was dissolved in 1 ml of anhydrous MeCN. Both reagents were stable for at least one week when stored in amber vials in a refrigerator at 4 °C under nitrogen.

Equipment

The analyses were performed on a Hewlett-Packard HP 1090 Series II/M liquid chromatograph supplied with a DR5 binary solvent-delivery system, a variable-volume autoinjector, an autosampler, a temperature-controlled column compartment and a stand-alone HP 1046A programmable fluorescence detector. To minimize noise, second-order or higher reflected light was removed by installing a 289 nm cut-off filter in front of the emission grating. The chromatographic separations were performed through a Hewlett-Packard 100x4 mm ID cartridge-type column filled with 3 μm Hypersil (Shandon, Cheshire, England) ODS particles. The guard column was attached to the analytical column without any capillaries.

Extraction of Free Amino Acids from Potato Tubers

Potato tubers (Solanum tuberosum L. cv. Kondor) were peeled and cut into pieces, and a representative 100 g sample was extracted with 100 ml of boiling water for 2 hours. Thereafter, the extract was centrifuged at 5000 g for 10 min and the supernatant was filtered through a 0.45 μm Millex FH membrane (Millipore, Bedford, MA, U.S.A.). Finally, 100 μl of extract was pipetted into a HPLC autosampler vial and diluted ten-fold with 900 μl of HPLC grade water containing 100 ng of Nval as internal standard.

Automated Precolumn Derivatization

A reliable computer-controlled autoinjector and an autosampler were employed to perform the derivatization of primary and secondary amino acids, with OPA/3-MPA and by FMOC-Cl. The borate buffer and the needle rinsing fluids, together with the reagents, were placed in the first magazine of the autosampler. The derivatization reactions were completed in the injection capillary of the autoinjector, in which the reaction mixture was mixed by moving the plunger of a 25 μl Hamilton syringe (Hamilton-Bonaduz AG, Bonaduz,

Switzerland) back and forth with the aid of a stepping motor. The different steps of the injector program are shown in Table 1.

Chromatographic Conditions

The gradient solvent-delivery system consisted of two solvents. Solvent A was 0.018 M NaOAc supplemented with 0.02% (v/v) TEA and 0.3% (v/v) THF. The THF was added to acetate buffer containing TEA after adjustment of the pH to 7.2 with 1% (v/v) acetic acid (Suprapur, Merck, Darmstadt, Germany). Solvent B was MeCN/MeOH/0.1 M NaOAc pH 7.2 in a ratio of 2/2/1 (v/v). Both solvents were filtered through an 0.45 μm Millex FH membrane (Millipore, Bedford, MA, U.S.A.). Helium degassing was used to avoid bubble formation in the solvent-delivery system and the flow cell of the detector. The column was kept at a constant temperature of 40 °C in the column compartment. The gradient elution started with 100% solvent A and increased in five linear steps to 100% B in 8.5 min with a flow rate of 1.4 ml/min. When the last amino acid derivative (FMOC-Pro) eluted at 8 min, the column was washed with 100% solvent B for 4 min, and reequilibrated to the initial conditions (Table 2). For detection of the OPA/3-MPA derivatives of primary amino acids, the excitation and emission wavelengths of the fluorescence detector were adjusted to 340 and 450 nm, respectively. After the elution of the primary amino acids, at 6.6 min the excitation and emission wavelengths were automatically switched to 264 and 313 nm, to detect the FMOC-Cl-derivatized secondary amino acids. All instrument parameters, including autoinjector, autosampler, solvent-delivery system, column compartment temperature and also fluorescence detector parameters, were accurately controlled by an HP 9000 Series 310 computer supplied with HP 79998A ChemStation software.

RESULTS AND DISCUSSION

The amino acids were derivatized automatically by means of the autoinjector and autosampler according to Schuster [8] with minor alterations. In order to ensure the stability of the FMOC-Cl reagent in the autosampler against its ready

TABLE 1

Injector Program for Two-Step Derivatization of Amino Acids

Line #	Function	Amount (µl)	Details	Substance
1	Draw	5	From vial 2	Borate buffer (0.4 N, pH 10.4)
2	Draw	1	From vial 0	OPA/3-MPA reagent
3	Draw	0	From vial 100	Water for needle wash
4	Draw	1	From sample	Primary and secondary amino acids
5	Draw	0	From vial 100	Water for needle wash
6	Draw	0	From vial 3	Anhydrous MeCN for needle wash
7	Mix	7	Seven cycles	Reactions of primary amino acids
8	Draw	1	From vial 1	FMOC-Cl reagent
9	Draw	0	From vial 4	Anhydrous MeCN for needle wash
10	Mix	8	Four cycles	Reactions of secondary amino acids
11	Inject	8		

TABLE 2

Time-table for Separation and Detection of Amino Acid Derivatives

Time	Gradient parameters (%)		Detection wavelengths (nm)	
(min)	Solvent A	Solvent B	Excitation	Emission
0	100	0	340	450
0.5	94	6		
3.0	80	20		
6.5	50	50		
6.8			264	313
8.0	25	75		
8.5	0	100		
12.5	0	100		
13.5	100	0	340	450

hydrolysis, two washing steps with anhydrous MeCN were applied to remove the water traces remaining on the outer surface of the injection needle. The complete autoinjector program in 11 steps, including drawing, mixing and needle rinsing cycles, required 5 min only. The time necessary to complete the derivatizations did not increase the total analysis time, because the columns were equilibrated with 100% solvent A during the injector program.

The high-speed separation of amino acid derivatives was performed by using a relatively short column (10 cm x 4 mm ID) filled with Hypersil ODS packing. To increase the lifetime of this separation column, use of a guard column is recommended, as otherwise a number of compounds in the crude extract, the reagents and their by-products can quickly destroy the separation column. However, the connection of a guard column to an analytical column usually requires a capillary, which adds an extra delay volume to the system, decreasing the resolution between compounds eluting close to each other (as in this case). Therefore, use of a cartridge-type column with a built-in guard column (no extra delay volume between columns) is the best choice. To separate the derivatized amino acids, a binary gradient in five linear steps was applied through the column. The NaOAc concentration (0.018 M) in solvent A was critical to obtain a good separation for Ala/Arg. When its concentration was raised to 0.02 M or above, the Ala/Arg resolution decreased. On the other hand, when the NaOAc concentration was lower than 0.018 M Gln, His and Gly did not separate well. The underivatized silanol groups in the column packing were blocked by pipetting a small amount of TEA (0.02% v/v) into solvent A. When TEA was omitted or its concentration decreased to 0.01%, His/Gly were not resolved. In solvent B, the mixture of MeCN and MeOH (2 parts each, mixed with 1 part of 0.1 M NaOAc) had a crucial role in separating Trp from Val/Met and Phe/Ile. When MeOH or MeCN was omitted or when they were mixed with NaOAc in other ratios (e.g. 1/3/1or 3/1/1), Trp eluted together with Val/Met or Phe/Ile.

Under the experimental conditions used, all common amino acid standards, including Nval as internal standard, were well separated, with a resolution of 1.64 (Phe/Ile) or better. This resolution resulted in a good separation of the free amino acids in potato tuber extracts as physiological samples (Figure 1). There was no interference with the reagent excess and reaction by-products, since OPA did not fluoresce and FMOC-Cl and its by-products eluted at least 1 min later than FMOC-Pro. The separation of a 50 pmol/µl amino acid standard mixture revealed that the isoindole derivatives eluted from the column in sharp peaks. Their widths

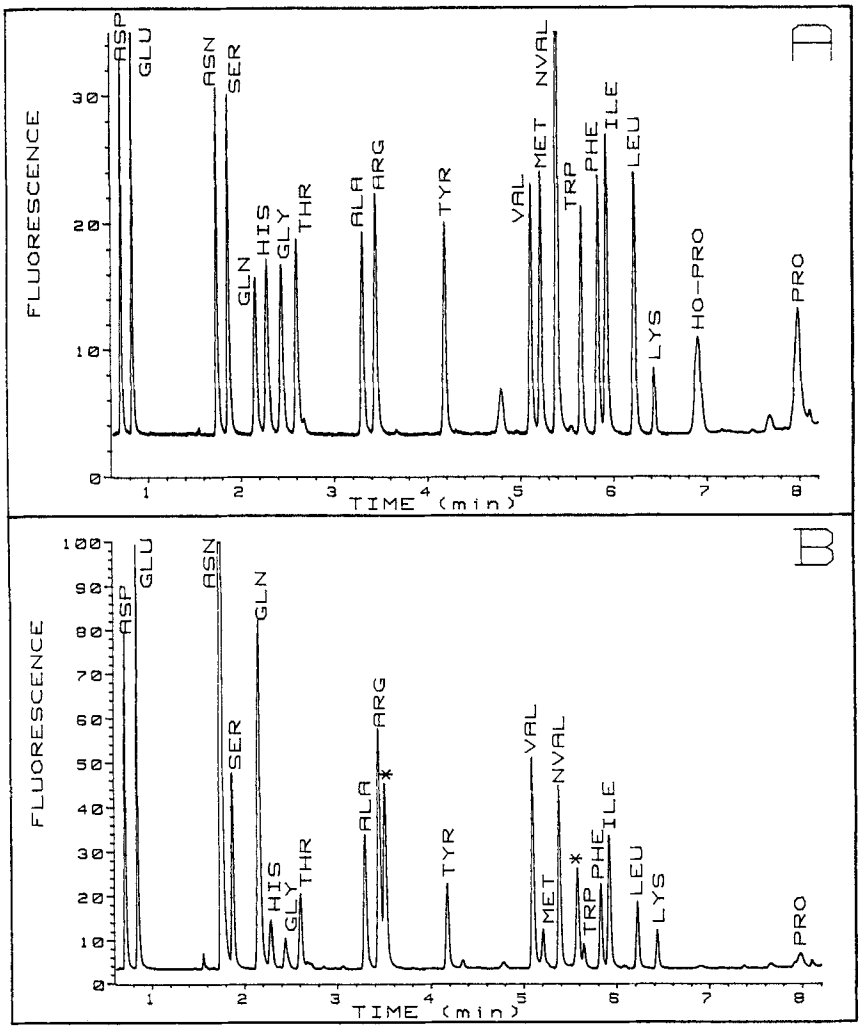

FIGURE 1. High-speed RP-HPLC analysis of derivatized amino acids. A) Separation of an amino acid standard mixture (50 pmol each) including Nval as internal standard (100 pmol). B) Chromatogram of the amino acids extracted from potato tubers including internal standard. The peaks labeled with an asterisk are unknown. Conditions: column, Hewlett-Packard 100x4.0 mm cartridge-type (with built-in guard column) filled with 3 μm Hypersil ODS packing; flow rate, 1.4 ml/min; column compartment temperature, 40 °C. For other conditions (gradient and detection parameters) see Table 2.

at half height and at the baseline ranged between 0.015 and 0.03 min and between 0.03 and 0.072 min, respectively. The excellent peak shape may be due to the limited chromatographic dispersion inside the column (packing with 3 μm particles) and the higher flow rate (1.4 ml/min) through the detector cell. The FMOC derivatives of HO-Pro and Pro eluted in peaks with slightly higher widths at half height (0.065-0.076 min) and at the baseline (0.111-0.125 min). The isoindole derivatives of Cys and Cys-Cys do not fluoresce, and they can therefore be detected with a less sensitive UV detector after formation of a mixed disulfide with 3,3'-dithiodipropionic acid following reaction with OPA/3-MPA to give an isoindole derivative [14,15].

The amino acids containing primary amino group(s) eluted within 6.5 min. The last separated amino acid was the FMOC-Pro, with a retention time of 8 min. The reproducibilities of the retention times and relative retention times were also calculated and are shown in Table 3. With this method, the lowest detectable amounts ranged between 1 and 5 pmol of amino acid at a signal to noise ratio of 5/1. Including the time necessary for derivatization, separation, column washing and reequilibration cycles, samples could be analysed at 18-min intervals, and the sample throughput is therefore up to 80 samples per day during unattended operation.

The calibration curves were linear within the examined concentration range. Due to the peak sharpness, the correlation coefficients were higher when calculated relative to the peak heights rather than to peak areas, except for HO-Pro and Pro derivatized with FMOC-Cl (Table 4).

CONCLUSIONS

This paper describes a rapid, sensitive and reproducible method for the RP-HPLC analysis of amino acids as demonstrated on the example of potato tuber extracts. The method involves an automated precolumn two-step derivatization as published by Schuster [8] and Blankenship et al. [9], a high-speed separation and fluorescence detection. Solvents and an appropriate gradient consisting of several linear steps are applied. Together with the lower cost cartridge-type ODS column this allows shortening of the analysis time considerably, while the resolution remains similar to or is even better than that reported so far. The problem of

TABLE 3

Precision Calculation for Retention Times of Amino Acid Derivatives (n=10)

Amino acid	Retention times (min)		Retention times (min) Relative to Nval ISTD	
	Mean	R.S.D. (%)	Mean	R.S.D. (%)
Aspartic acid	0.709	1.45	0.137	1.13
Glutamic acid	0.831	1.09	0.163	0.86
Asparagine	1.747	0.91	0.330	0.58
Serine	1.871	0.94	0.354	0.61
Glutamine	2.160	1.30	0.412	0.98
Histidine	2.286	1.22	0.435	0.90
Glycine	2.439	1.20	0.465	0.88
Threonine	2.602	0.93	0.494	0.60
Alanine	3.305	0.79	0.622	0.47
Arginine	3.446	0.77	0.649	0.45
Tyrosine	4.185	0.60	0.780	0.27
Valine	5.113	0.35	0.948	0.02
Methionine	5.221	0.39	0.968	0.06
Norvaline	5.394	0.33	1.000	
Tryptophan	5.657	0.36	1.048	0.05
Phenylalanine	5.835	0.32	1.080	0.02
Isoleucine	5.928	0.28	1.096	0.05
Leucine	6.224	0.28	1.150	0.04
Lysine	6.435	0.28	1.186	0.06
HO-Proline	6.903	0.26	1.278	0.06
Proline	7.982	0.17	1.473	0.17

TABLE 4

Comparison of Correlation Coefficients for Peak Areas r(a) and Heights r(h)

Amino acid	r (a)	r (h)	Amino acid	r (a)	r (h)
Aspartic acid	0.992	0.996	Tyrosine	0.996	0.997
Glutamic acid	0.990	0.995	Valine	0.992	0.997
Asparagine	0.993	0.996	Methionine	0.991	0.998
Serine	0.993	0.997	Tryptophan	0.995	0.998
Glutamine	0.990	0.996	Phenylalanine	0.993	0.997
Histidine	0.995	0.995	Isoleucine	0.990	0.996
Glycine	0.986	0.996	Leucine	0.989	0.996
Threonine	0.984	0.997	Lysine	0.990	0.998
Alanine	0.992	0.997	HO-Proline	0.994	0.990
Arginine	0.991	0.997	Proline	0.996	0.991

Correlation coefficients r(a) and r(h) were calculated from 5 analyses of a standard mixture of amino acids ranging from 20 pmol to 200 pmol.

limited stability of the isoindole derivatives can be avoided via the automated derivatization and quite rapid separation time.

ACKNOWLEDGEMENTS

The authors are grateful to É. V. Bogdán for technical assistance and to D. Durham for revision of the English text. This research was supported by Grant No. 242 from the National Scientific Research Foundation (OTKA), Hungary.

REFERENCES

1) Moore, S. and Stein, W.H., Chromatography of Amino Acids on Sulfonated Polystyrene Resins, J. Biol.Chem., 192, 663, 1951

2) Sherwood, R.A., Titheradge, A.C. and Richards, D.A., Measurement of Plasma and Urine Amino Acids by High-Performance Liquid Chromatography with Electrochemical Detection Using Phenylisothiocyanate Derivatization J. Chrom., 528, 293, 1990

3) Davey, J.F. and Ersser, R.S., Amino Acid Analysis of Physiological Fluids by High-Performance Liquid Chromatography with Phenylisothiocyanate Derivatization and Comparison with Ion-Exchange Chromatography, J. Chrom., 528, 9, 1990

4) Tapuhi, Y., Schmidt, D.E., Linder, W. and Karger, B.L., Dansylation of Amino Acids for High-Performance Liquid Chromatography Analysis, Anal. Biochem., 115, 123, 1981

5) Bayer, E., Grom, B., Kaltenegger, B. and Uhman, R., Separation of Amino Acids by High-Performance Liquid Chromatography, Anal. Chem.,48, 1106, 1976

6) Jansen, E.H.J.M., Van Den Berg, R.H., Both-Miedema, R. and Doorn, L., Advantages and Limitations of Pre-column Derivatization of Amino Acids with Dabsyl Chloride, J. Chrom., 553, 123, 1991

7) Stocchi, V., Piccoli, G., Magnoni, M., Palma, F., Biagiarelli, B. and Cucchiarini, L., Reversed-Phase High-Performance Liquid Chromatography Separation of Dimethylaminoazobenzene Sulfonyl- and Dimethylaminoazobenzene Thiohydantoin-Amino Acid Derivatives for Amino Acid Analysis, and Microsequencing Studies at the Picomole Level, Anal. Biochem.,178, 107, 1989

8) Schuster, R., Determination of Amino Acids in Biological, Pharmaceutical, Plant and Food Samples by Automated Precolumn Derivatization and High-Performance Liquid Chromatography, J. Chromatogr., 431, 271, 1988

9) Blankenship, D.T., Krivanek, M.A., Ackermann, B.L. and Cardin, A.D., High Sensitivity Amino Acid Analysis by Derivatization with o-Phthaldialdehyde and 9-Fluorenylmethyl Chloroformate Using Fluorescence Detection: Application in Protein Structure Determination, Anal. Biochem.,178, 227, 1989

10) Haginaka, J. and Wakai, J., Automated Precolumn Derivatization of Amino Acids with ortho-Phthalaldehyde Using a Hollow-Fibre Membrane Reactor, J. Chrom., 502, 317, 1990

11) Haynes, P.A., Sheumack, D., Kibby, J. and Redmond, J.W., Amino Acid Analysis Using Derivatization with 9-Fluorenylmethyl Chloroformate and Reversed-Phase High Performance Liquid Chromatography, J. Chrom., 540, 177, 1991

12) Einarsson, S., Josefsson, B. and Lagerkvist, S., Determination of Amino Acids with 9-Fluorenylmethyl Chloroformate and Reversed-Phase High Performance Liquid Chromatography, J. Chrom., 282, 609, 1983

13) Skaaden, T. and Greibrokk, T., Determination of Polyamines by Precolumn Derivatization with o-Phthalaldehyde and Ethanethiol in Combination with Reversed-Phase High Performance Liquid Chromatography, J. Chrom., 247, 111, 1982

14) Barkholt, V.and Jensen, A.L., Amino Acid Analysis: Determination of Cysteine Plus Half-Cystine in Proteins after Hydrochloric Acid Hydrolysis with a Disulfide Compound as Additive, Anal. Biochem., 177, 318, 1989

15) Seitz, P. and Godel, H., Quantification of Cysteine and Cystine, Hewlett-Packard Application Note, 1991, Publication Number 12-5091-0775E

Received: May 11, 1994
Accepted: May 24, 1994