*Journal of Chromatography*, 540 (1991) 177–185
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 973

# Amino acid analysis using derivatisation with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography

PAUL A. HAYNES*
*School of Chemistry, Macquarie University, North Ryde, N.S.W. 2109 (Australia)*
DAVID SHEUMACK
*Corbett Technology, Lidcombe, N.S.W. 2141 (Australia)*
JEFFREY KIBBY
*ICI Instruments, Dingley, Victoria 3172 (Australia)*
and
JOHN W. REDMOND
*School of Chemistry, Macquarie University, North Ryde, N.S.W. 2109 (Australia)*
(First received May 21st, 1990; revised manuscript received November 8th, 1990)

## ABSTRACT

A simple procedure for the precolumn derivatisation of amino acids with 9-fluorenylmethyl chloroformate and a liquid chromatographic method for the separation of the derivatives with fluorimetric detection in the picomole range are reported. The procedure does not involve a solvent extraction and gives single, stable derivatives of the common protein amino acids. The method has been demonstrated on hydrolysates of proteins and peptides.

## INTRODUCTION

The determination of amino acids has traditionally been based on ion-exchange chromatography followed by post-column derivatisation with ninhydrin [1]. In recent years rapid improvements in high-performance liquid chromatography (HPLC) equipment and high-efficiency chromatographic columns have led to a significant change in approach to amino acid analysis.

Precolumn derivatisation methods using HPLC which have been developed include those using phenyl isothiocyanate (PITC) [2–4], *o*-phthaldialdehyde (OPA) [5–8], 1-dimethylaminonaphthalene-5-sulphonyl (dansyl) chloride [9–12], and 9-fluorenylmethyl chloroformate (Fmoc-Cl) [13–18]. Despite reported shortcomings [19,20], these methods have become increasingly popular due to their speed, simplicity and sensitivity.

The PITC method has several disadvantages [20]: the derivatisation procedure is lengthy; a vacuum system is required to evaporate excess coupling reagent before

0021-9673/91/$03.50 

analysis; and while the limit of sensitivity is in the low picomole range [2,3,19], this is still higher than the femtomole sensitivity reported for current methods using detection of fluorescent derivatives [6,15,16,18,19,21].

The OPA method offers greater sensitivity, but is limited to primary amino acids and can not be used for proline, hydroxyproline or sarcosine. The instability of the OPA derivatives makes manual derivatisation difficult to reproduce, but satisfactory automated procedures have been developed [16,20,22]. The limits of sensitivity of detection for glycine and lysine are also noticeably higher than for other amino acids [19].

The quantitation of histidine is problematic in the dansylation method, as multiple derivatives are formed [20]. Furthermore, poor derivatisation is obtained for amino acids present at low levels in complex matrices [14]. The method is, however, the most suitable of those available for the quantitation of cystine [20].

Current procedures [14–18,23] using derivatisation with Fmoc-Cl are also troublesome. An excess of reagent must be used to provide effective derivatisation, and it is necessary to remove it before chromatography [19–21,24] because it interferes with the separation of the amino acid derivatives and is detrimental to column performance. Extraction into an organic solvent has been used [19–21,24], but this can lead to errors in quantitation of hydrophobic amino acids [16,18]. This problem has been addressed by the use of an excess of 1-aminoadamantane to react with the excess Fmoc-Cl, thus avoiding the extraction step [17,18]. Furthermore, histidine and tyrosine form mixtures of monosubstituted and disubstituted Fmoc derivatives. The complicates chromatography and makes quantitation of these amino acids unreliable [14,18–20].

A recent method [21,25] uses a combination of the Fmoc-Cl and OPA derivatives to allow for the derivatisation of both primary and secondary amino acids. The problem of the inherent instablity of the OPA derivatives is minimised by using an autosampler to carry out the derivatisation reaction and inject the sample without delay. The approach exploits the advantages of two different types of derivative but requires sophisticated detection.

We report here a method for amino acid derivatisation using Fmoc-Cl which produces the monosubstituted derivatives of tyrosine and histidine, and which does not require extraction to remove the excess Fmoc-Cl reagent.

## EXPERIMENTAL

### *Apparatus*

The chromatographic system consisted of two ICI Instruments LC 1100 HPLC pumps controlled by an ICI Instruments DP800 chromatography data station, an ICI Instruments TC 1900 column heater, and a Shimadzu RF535 fluorescence HPLC monitor (excitation wavelength 263 nm, emission wavelength 313 nm). Various columns were used, including a 100 × 4.6 mm I.D. Zorbax 5 μm RP-8, a 75 × 4.6 mm I.D. Ultratechsphere 3 μm ODS, a 150 × 4.6 mm I.D. Goldpak Hypersil 3 μm ODS-2 and a 150 × 4.6 mm I.D. Spherisorb 3 μm ODS-2. The columns were used in conjunction with a guard column, either a 15 × 3.2 mm I.D. Brownlee Newguard 7 μm ODS or a 50 × 4.6 mm I.D. Spherisorb 3 μm ODS-2. Sample volumes of 5 or 10 μl were introduced onto the column with an ICI Instruments LC1600 Autosampler. The data were collected using an ICI Instruments DP800 chromatography data station or an SIC Chromatocorder 12.

*Reagents and materials*

All aqueous solutions were prepared in Milli-Q water. 9-Fluorenylmethyl chloroformate (Sigma, St. Louis, MO, U.S.A.) was dissolved in acetonitrile (Mallinckrodt Australia, HPLC grade) as a 4.16 mg/ml solution, (16 m*M*). Borate buffer was prepared from 200 m*M* boric acid (Ajax Chemicals, Sydney, Australia) solution adjusted to pH 8.5 with 5 *M* sodium hydroxide solution prepared from sodium hydroxide pellets (BDH, Poole, U.K.). Phosphate buffer was prepared from 200 m*M* sodium dihydrogen orthophosphate (Ajax) adjusted to pH 7.7 with 200 m*M* disodium hydrogenorthophosphate (Ajax) and bicarbonate buffer was prepared from 100 m*M* sodium hydrogencarbonate (Ajax) adjusted to the required pH with 1 *M* sodium hydroxide solution (BDH). The alkaline cleavage reagent was prepared in 1-ml batches by mixing 680 μl of 850 m*M* sodium hydroxide solution with 300 μl of 500 m*M* hydroxylamine hydrochloride (Aldrich, WI, U.S.A.) solution and 20 μl of 2-(methylthio)-ethanol (Aldrich). The quenching reagent was acetonitrile–water–acetic acid (20:3:2).

Sodium acetate (4.0 *M*) and triethylammonium acetate (0.36 *M*) stock solutions, used for preparation of HPLC eluents, were adjusted to pH 6.5 with acetic acid. Sepramar amino acid calibration standard A was purchased from BDH, human angiotensin-II from Auspep (South Melbourne, Australia), chicken egg white lysozyme and 2-mercaptoethanol from Sigma, dithiothreitol from Boehringer Mannheim (Germany) and (3-methylthio)-1-hexanol from Aldrich.

*Hydrolysis procedure*

Samples were placed in glass autosampler vials (ICI Instruments), dried in a Speed Vac vacuum centrifuge (Savant Instruments, Hicksville, NY, U.S.A.) for 1 h and then placed in a hydrolysis vessel (Pierce, Rockford, IL, U.S.A.). A 500-μl volume of 6 *M* hydrochloric acid (Pierce) was placed in the bottom of the vessel, which was then flushed with helium and evacuated. The hydrolysis was carried out at 110°C for 24 h, and the samples were then dried in the vacuum centrifuge and redissolved in the derivatisation buffer.

*Derivatisation procedure*

The routine procedure for derivatisation involved placing the sample, dissolved in 50 μl of borate buffer, in an Eppendorf centrifuge tube and adding 50 μl of Fmoc-Cl solution. The tube was agitated and the derivatisation allowed to proceed for 90 s. A 30-μl volume of cleavage ragent was then added, and the solution mixed and allowed to stand for a further 3.5 min. The reaction was then stopped by the addition of 50 μl of quenching reagent and the mixture was used directly for analysis. For studies of the kinetics of the cleavage reaction, aliquots were removed at different times and added to an excess of quenching reagent.

*Separation*

Separation of the Fmoc-amino acid derivatives was carried out using a binary gradient. Two different eluents were used at different times for buffer A: 100 m*M* sodium acetate (pH 6.5) and 10% acetonitrile; and 50 m*M* sodium acetate, 7 m*M* triethylammonium acetate (pH 6.5) and 10% acetonitrile. Buffer B was acetonitrile–water (90:10). The flow-rate was constant at 1.0 ml/min and the column was

maintained at 43°C. The gradient profile was as follows: 0–1.5 min, 12%B; 1.5–2.0 min, 12–18%B; 2.0–9.0 min, 18%B; 9.0–9.5 min, 18–25%B; 9.5–12.5 min, 25%B; 12.5–13.0 min, 25–30%B; 13.0–16.0 min, 30%B; 16.0–17.0 min, 30–40%B; 17.0–20.0 min, 40%B; 20.0–22.0 min, 40–50%B; 22.0–23.0 min, 50%B; 23.0–24.0 min, 50–99%B.

## RESULTS AND DISCUSSION

The analysis of amino acids by HPLC of Fmoc derivatives was introduced by Einarsson *et al.* [14]. Later reports have described variations in the extraction solvent [23], the reaction cosolvent [16,26], the pH of the reaction buffer [16,23], and the use of amine addition rather than extraction to remove the excess Fmoc-Cl [17,18].

The method presented here includes modification which avoid major disadvantages of the approach. A solvent extraction, to remove excess Fmoc-Cl reagent, is unnecessary as it is sequestered by the addition of excess of alkaline hydroxylamine. The Fmoc-hydroxylamine product remains soluble in the reaction mixture and does not interfere with the chromatography of the amino acid derivatives. The treatment with alkaline hydroxylamine also serves to convert the disubstituted derivatives of histidine and tyrosine into the stable monosubstituted derivatives.

### *Optimisation of reaction conditions for derivatisation*

Acetonitrile was used as the reaction cosolvent in preference to acetone as it avoids the problem of precipitation of the derivatives of hydrophobic amino acids reported by Einarsson *et al.* [16]. In preliminary studies, borate, phosphate, and bicarbonate buffers were investigated for use in the derivatisation. Both borate and bicarbonate were satisfactory, but phosphate proved unacceptable as it produced a large interfering peak. Derivatisations of amino acid standards were carried out in borate buffer at pH 7.7 [14] for times of from 30 to 120 s. Leucine, used as a reference standard, gave a constant peak area over the range of times.

The derivatives of aspartic and glutamic acid were formed more slowly than those of other amino acids, and the reaction was incomplete after 120 s. The use of acetonitrile as cosolvent evidently causes derivatisation of these amino acids to proceed more slowly than in acetone, as previous reports have indicated that derivatisation is complete in 45 s [14,24]. Adjustment of the pH of the borate buffer to 8.5 [23] gave complete derivatisation of aspartic and glutamic acids in 90 s.

For short reaction times at pH 7.7, tyrosine gave mainly the early eluting N-Fmoc derivative (mono-Fmoc-Tyr), while longer reaction times favoured the more hydrophobic N,O-disubstituted derivative (di-Fmoc-Tyr). Histidine gave only the disubstituted Fmoc histidine derivative (di-Fmoc-His) over the range of times.

In a further study, tyrosine and histidine were derivatised for 60 s in hydrogencarbonate buffer of pH in the range 7.5 to 12.6. Varying amounts of mono-Fmoc-Tyr and di-Fmoc-Tyr were formed, with di-Fmoc-Tyr the major product in reactions carried out at pH between 10.0 and 12.0. At higher pH the porportion of mono-Fmoc-Tyr increased due to subsequent hydrolysis of the di-Fmoc-Tyr derivative. Histidine was detected only as di-Fmoc-His between pH 7.5 and pH 12.0, but some monosubstituted derivative (mono-Fmoc-His) was observed at higher pH values.

*Production of the monosubstituted tyrosine and histidine derivatives*

Alkaline treatment of di-Fmoc-His produces the monosubstituted N-Fmoc histidine derivative. Mono-Fmoc-His has an approximately 2.5 times greater peak area than di-Fmoc-His, and also gives an earlier eluting, more symmetrical chromatographic peak. Di-Fmoc-Tyr, on the other hand, has an approximately 2.3 times greater peak area than mono-Fmoc-Tyr.

Alkaline treatment of di-Fmoc-Tyr and di-Fmoc-His was investigated at room temperature. The conditions needed to convert all of the di-Fmoc-Tyr to mono-Fmoc-Tyr caused as much as 25% loss of the Fmoc derivatives of other amino acids. Di-Fmoc-His was more sensitive to alkali than di-Fmoc-Tyr, but it was not possible to convert it to mono-Fmoc-His without significant hydrolysis of di-Fmoc-Tyr.

A mixture of hydroxylamine and sodium hydroxide proved effective in converting both di-Fmoc-Tyr and di-Fmoc-His to the monosubstituted derivatives. The cleavage reagent adopted proved optimal, as it had little effect on normal N-Fmoc groups. This reagent had the added advantage of converting the unreacted Fmoc-Cl to the unreactive Fmoc-hydroxylamine product, thus making removal of the excess reagent unnecessary.

*Inclusion of a thioether as an antioxidant*

These results provided the basis for a method which gives single stable derivatives for tyrosine and histidine and complete derivatisation of aspartic and glutamic acids. When applied to a full amino acid standard, however, methionine was detected as a mixture of two artifacts, presumably the sulphone and sulphoxide derivatives resulting from oxidation by the alkaline hydroxylamine. The problem was overcome by the addition of a thioether, 2-(methylthio)ethanol, to the alkaline hydroxylamine reagent to act as an antioxidant. A less volatile thioether, 3-(methylthio)-1-hexanol, was unsuitable because of its insolubility in the reaction mixture. Thiols such as 2-mercaptoethanol and dithiothreitol were also unsuitable because of their reaction with Fmoc-Cl.

*Chromatography of the Fmoc amino acid derivatives*

There are a number of differences between the chromatographic separation shown in this paper and those previously reported [14,16,20,22,23]. The effects of variations in the organic modifier, type of buffer used, buffer pH and buffer ionic strength were all studied before the separation as shown was developed. A number of different reverse columns were investigated (see Experimental), with the Spherisorb and Goldpak columns the most satisfactory.

The use of acetonitrile as the only organic modifier in the mobile phase, combined with a pH of 6.5 for the eluent, ensures that Fmoc-OH elutes later in relation to the amino acid derivatives. A mobile phase of pH 6.5 also changes the elution order of the early eluting peaks and improves the resolution. A buffer of considerable ionic strength is necessary to resolve the arginine and monosubstituted histidine derivatives from other closely eluting peaks.

Our earlier work was carried out using 100 m*M* sodium acetate in buffer A, but the inclusion of triethylammonium acetate (7 m*M*) allowed a lower concentration of sodium acetate (50 m*M*) to be used while still achieving the desired separation. This change in the mobile phase resulted in an increase in column lifetime, and an

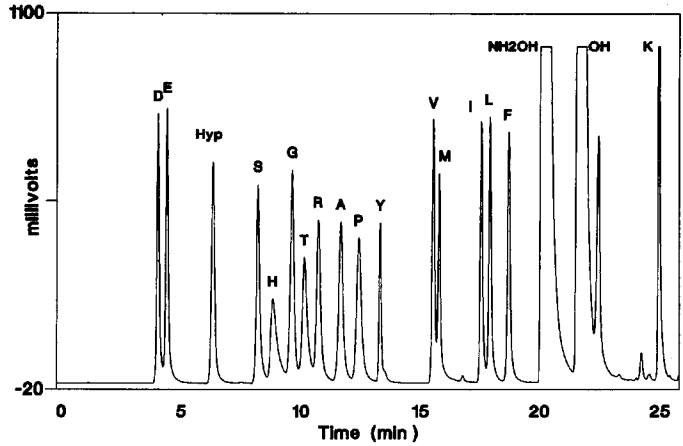


Fig. 1. Typical chromatogram of an amino acid standard derivatised with Fmoc. Peaks are labelled with one-letter abbreviations for protein amino acids, and: Hyp = hydroxyproline; NH2OH = Fmoc-hydroxylamine; OH = Fmoc-OH. Chromatographic conditions: column, 150 × 4.6 mm I.D. Spherisorb 3 μm ODS-2 with 15 × 3.2 mm I.D. Brownlee Newguard 7 μm ODS guard column; buffer A, 50 m*M* sodium acetate (pH 6.5), 7 m*M* triethylamine acetate (pH 6.5) and 10% acetonitrile; buffer B, acetonitrile–water (90:10); flow-rate 1.0 ml/min; column temperature 43°C; gradient as in Experimental; injection volume 10 μl; 280 pmol of each amino acid.

improvement in performance. This is illustrated by the difference in resolution between Fig. 1, a chromatogram of a typical amino acid standard, and Fig. 2, a chromatogram of a lysozyme hydrolysate.

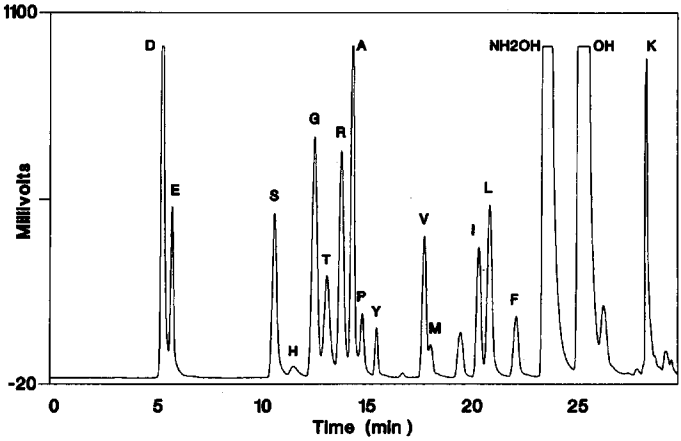


Fig. 2. Chromatogram of chicken egg white lysozyme hydrolysate derivatised with Fmoc. Peak labelling as in Fig. 1. Chromatographic conditions: column, 150 × 4.6 mm I.D. Goldpak Hypersil 3 μm ODS-2 with 50 × 4.6 mm I.D. Spherisorb 3 μm ODS-2 guard column; buffer A, 10% acetonitrile in 100 m*M* sodium acetate (pH 6.5); buffer B, acetonitrile–water (90:10); flow-rate 1.0 ml/min; column temperature 43°C; gradient as in Experimental; injection volume 10 μl.

TABLE I

RELATIVE STANDARD DEVIATIONS AND CORRELATION COEFFICIENTS FOR PEAK AREAS OF Fmoc AMINO ACIDS

Relative standard deviations (R.S.D.) based on 10 derivatisations of 500 pmol of amino acid standard. Correlation coefficients ($r$) calculated from 10 derivatisations of amino acid standards ranging from 2.6 pmol to 260 pmol.

| Amino acid | R.S.D. (%) ($n=10$) | $r$ ($n=10$) | Amino acid | RS.D. (%) ($n=10$) | $r$ ($n=10$) |
|---|---|---|---|---|---|
| Asp | 1.5 | 0.9976 | Pro | 0.3 | 0.9996 |
| Glu | 1.7 | 0.9984 | Tyr | 1.4 | 0.9997 |
| Ser | 1.5 | 0.9990 | Val | 1.0 | 0.9994 |
| His | 1.1 | 0.9991 | Met | 0.9 | 0.9995 |
| Gly | 0.5 | 0.9993 | Ile | 0.6 | 0.9994 |
| Thr | 1.5 | 0.9994 | Leu | 0.6 | 0.9986 |
| Arg | 1.6 | 0.9980 | Phe | 0.6 | 0.9996 |
| Ala | 0.8 | 0.9994 | Lys | 1.4 | 0.9986 |

*Reproducibility and linearity of the method*

Reproducibility was established by a series of ten derivatisations of a standard containing 500 pmol of each amino acid. Linearity was determined with duplicate derivatisations of five different concentrations of amino acid standard, over a one hundred-fold range. These standards gave on-column injection amounts ranging from 2.6 to 260 pmol, and all sixteen amino acids gave a linear response. The relative standard deviations of peak area ($n=10$) relative to a hydroxyproline internal standard, and the correlation coefficients for peak area over the range ($n=10$), are shown in Table I.

*Analysis of peptide and protein hydrolysates*

Human Angiotensin-II, an eight-amino acid synthetic peptide, was selected as a test for this method as it contains aspartic acid, tyrosine and histidine. The peptide has a molecular weight of 1047 and the full amino acid sequence is Asp–Arg–Val–Tyr–

TABLE II

AMINO ACID COMPOSITION OF HUMAN ANGIOTENSIN-II

| Amino acid | Molar ratio determined (normalized to Arg) | Expected value |
|---|---|---|
| Asp | 1.1 | 1.0 |
| His | 1.0 | 0.9 |
| Arg | 1.0 | 1.0 |
| Pro | 0.9 | 1.0 |
| Tyr | 1.0 | 0.9 |
| Val | 1.1 | 0.9 |
| Ile | 1.1 | 0.9 |
| Phe | 1.0 | 1.1 |

TABLE III

AMINO ACID COMPOSITION OF CHICKEN EGG WHITE LYSOZYME

N.D. = Not determined.

| Amino acid | Molar ratio determined (normalised to Phe) | Lit. [27] |
|---|---|---|
| Asp | 18.9 | 21 |
| Glu | 6.4 | 5 |
| Ser | 9.0 | 10 |
| His | 1.0 | 1 |
| Gly | 10.0 | 12 |
| Thr | 6.5 | 7 |
| Arg | 9.7 | 11 |
| Ala | 11.2 | 12 |
| Pro | 2.3 | 2 |
| Tyr | 3.1 | 3 |
| Val | 5.7 | 6 |
| Met | 2.3 | 2 |
| Ile | 6.0 | 6 |
| Leu | 7.6 | 8 |
| Phe | 3.0 | 3 |
| Lys | 6.1 | 6 |
| Cys | N.D. | 8 |
| Trp | N.D. | 6 |

Ile–His–Pro–Phe. The hydrolysis and derivatisation were carried out on 10 nmol of peptide, and a 300-pmol aliquot was analysed. The results (Table II) are in excellent agreement with the expected values.

Lysozyme, a protein with a molecular weight of 14 000, contains only one histidine residue and three tyrosine residues. A 1.4-nmol amount was hydrolysed and derivatised, and an 80-pmol aliquot of this was analysed. The results (Fig. 2, Table III) are in good agreement with the literature values [27], with the determination of histidine and tyrosine correct even though both are present at low levels.

The method has been applied to a number of other protein and peptide hydrolysates. All have given results in agreement with expected values, with no evidence of significant interference with either the derivatisation or alkaline cleavage steps. The method appears sufficiently robust for samples that contain high levels of protein or inorganic salt contamination.

## CONCLUSION

A method has been developed which enables the common protein amino acids to be accurately determined as the Fmoc derivatives. It is linear and reproducible within the stated range, and applicable to various sample matrices. The method is currently being extended to include analysis of physiological samples.

ACKNOWLEDGEMENTS

This work was carried out as a Teaching and Industry scheme project (awarded to J.W.R. and K.L. Williams) funded by ICI Instruments Australia and the N.S.W. Government. The HPLC equipment was supplied by ICI Instruments Australia. Thanks are due to Keith Williams and Andrew Gooley (Macquarie University) for practical support and Robin Greenwood-Smith (ICI Instruments) for administrative assistance.

REFERENCES

1 S. Moore and W. H. Stein, *J. Biol. Chem.*, 176 (1948) 367.
2 R. L. Heinrikson and S. C. Meredith, *Anal. Biochem.*, 136 (1984) 65.
3 B. A. Bidlingmeyer, S. A. Cohen and T. L. Tarvin, *J. Chromatogr.*, 336 (1984) 93.
4 G. E. Tarr, in J. E. Shively (Editor), *Methods of Protein Microcharacterisation*, Humana Press, Clifton, NJ, 1986, pp. 155–194.
5 M. Roth, *Anal. Chem.*, 43 (1971) 880.
6 P. Lindroth and K. Mopper, *Anal. Chem.*, 51 (1979) 1667.
7 D. W. Hill, F. H. Walters, T. D. Wilson and J. D. Stuart, *Anal. Chem.*, 51 (1979) 1338.
8 R. F. Chen, C. Scott and E. Trepman, *Biochim. Biophys. Acta*, 576 (1979) 440.
9 J. M. Wilkinson, *J. Chromatogr. Sci.*, 16 (1978) 547.
10 Y. Tapuhi, D. E. Schmidt, W. Lindner and B. L. Karger, *Anal. Biochem.*, 115 (1981) 123.
11 F. J. Marquez, A. R. Quesada, F Sanchez-Jiminez and I. Nunez de Castro, *J. Chromatogr.*, 380 (1986) 275.
12 M. Simmaco, D. De Biase, D. Barra and F. Bossa, *J. Chromatogr.*, 504 (1990) 129.
13 L. A. Carpino and G. Y Han, *J. Org. Chem.*, 37 (1972) 3404.
14 S. Einarsson, B. Josefsson and S. Lagerkvist, *J. Chromatogr.*, 282 (1983) 609.
15 S. Einarsson, *J. Chromatogr.*, 348 (1985) 213.
16 S. Einarsson, S. Folestad, B. Josefsson and S. Lagerkvist, *Anal. Chem.*, 58 (1986) 1638.
17 I. Betner and P. Foldi, *Chromatographia*, 22 (1986) 381.
18 B. Gustavsson and I. Betner, *J. Chromatogr.*, 507 (1990) 67.
19 S. A. Cohen and D. J. Strydom, *Anal. Biochem.*, 174 (1988) 1.
20 P. Furst, L. Pollack, T. A. Graser, H. Godel and P. Stehle, *J. Chromatogr.*, 499 (1990) 557.
21 R. Schuster, *J. Chromatogr.*, 431 (1988) 271.
22 H. G. Barth, W. E. Barber, C. H. Lochmuller, R. E. Majors and F. E. Regnier, *Anal. Chem.*, 60 (1988) 387r.
23 F. Barry, B. Cronin and S. Doyle, *J. Prot. Chem.*, 7 (1988) 193.
24 T. Nasholm, G. Sandberg and A. Ericsson, *J. Chromatogr.*, 396 (1987) 225.
25 D. T. Blankenship, M. A. Krivanek, B. L. Ackermann and A. D. Cardin, *Anal. Biochem.*, 178 (1989) 227.
26 Z. Harduf, T. Nir and B. J. Juven, *J. Chromatogr.*, 437 (1988) 379.
27 S. Chiou and K. Wang, *J. Chromatogr.*, 448 (1988) 404.