Journal of Chromatography, 476 (1989) 83–92
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1617

LIQUID CHROMATOGRAPHIC ANALYSIS OF AMINO AND IMINO ACIDS IN PROTEIN HYDROLYSATES BY POST-COLUMN DERIVATIZATION WITH *o*-PHTHALALDEHYDE AND 3-MERCAPTOPROPIONIC ACID

ANTONIO FIORINO*, GIANCARLO FRIGO and EUGENIO CUCCHETTI
Ellem Research Centre, Viale dell'Industria 17, 20094 Corsico (Milan) (Italy)

SUMMARY

A method for the simultaneous determination of imino and amino acids is reported. The method, based on the derivatization of amino acids, separated by ion-exchange chromatography, by reaction with alkaline hypochlorite and *o*-phthalaldehyde–3-mercaptopropionic acid, increases the sensitivity towards imino acids to the picomole level. The use of 3-mercaptopropionic acid gives intensely fluorescent derivatives and improves the stability of primary and secondary amino acid fluorophores towards oxidation.

The accuracy and reproducibility of the method are increased by using three internal standards. The determination of all amino and imino acids present in protein hydrolysates, including tryptophan, in a single run is demonstrated.

INTRODUCTION

A great deal of interest has been focused on the development of methods for amino acid determination since the pioneering work of Moore and co-workers in the 1950s[1,2]. The original method, based on the post-column derivatization of separated amino acids with ninhydrin, was significantly modified in the 1970s with the advent of reversed-phase adsorbents. The new methods, involving pre-column derivatization and reversed-phase high-performance liquid chromatographic (HPLC) separation of amino acid derivatives, have dramatically improved the speed and sensitivity of analysis. Problems associated with the instability of derivatives have led many analysts to adopt the classical ion-exchange chromatography with post-column derivatization, because this approach offers the advantage of non-destructive separation of amino acids.

Most of the new ion-exchange analysers for HPLC instruments have improved the performance of amino acid analysis by reducing the time of analysis and the detection limits. In these systems, *o*-phthalaldehyde (OPA) has replaced the classical ninhydrin reagent, which suffers from the limitations of a slow reaction rate, which contributes to band broadening[3], and the need for dual-wavelength detectors for complete amino and imino acid monitoring.

Roth[4] first showed that OPA readily reacts with amino acids in the presence of 2-mercaptoethanol to give fluorescent compounds. As imino acids do not react with

0021-9673/89/$03.50

these reagents, oxidation steps have been developed in order to convert imino compounds to primary amines[5–8]. The related adducts showed poor fluorescence compared with the other amino acid derivatives.

Various thiols have been used in the derivatization reactions to improve the stability of fluorescent compounds [9,10]. Recently, Fujiwara *et al.*[11] reported the use of N-acetyl-L-cysteine (AcCys) as a thiol agent to increase the stability of adducts toward hypochlorite oxidation. The AcCys thiol showed excellent sensitivity in the detection of proline.

We have studied the amino acid composition of protein and peptide hydrolysates according to the Fujiwara method. Although AcCys gave quantitative, sensitive and reproducible results, it was often necessary to load different amounts of the sample in order to obtain greater accuracy for methionine and tyrosine, present in relatively small amounts in some hydrolysates. Therefore, we found this method to be insufficiently sensitive for these amino acids.

In order to obtain adducts of methionine and tyrosine with higher fluorescence and, at the same time, good sensitivity in the determination of secondary amino acids, we investigated other thiol compounds. The use of 3-mercaptopropionic acid (3-MPA), first investigated by Kucera and Umagat[12], yielded the desired results and allowed the simultaneous determination of primary and secondary amino acids.

In this work, the use of OPA–3-MPA as an alternative reagent for the determination of amino and imino acids in acidic hydrolysates of proteins and peptides is described. The use of three internal standards for monitoring variations in the analysis is also reported.

EXPERIMENTAL

Chemicals

Analytical-reagent grade chemicals and 3-MPA were purchased from Merck (Darmstadt, F.R.G.) and HPLC-grade water and OPA from Carlo Erba (Milan, Italy). Brij-35 (30%, w/v), chymotrypsinogen A (Type II, from bovine pancreas), the amino acid calibration mixture (AA-S-18) and individual amino acids were purchased from Sigma (St. Louis, MO, U.S.A.). The ultrafiltered milk protein sample (85% peptide content) (Ultimate, Saronno, Italy) and sodium hypochlorite (chlorine concentration 5–6%) (Procter & Gamble, Pomezia, Italy) were of commercial grade.

Apparatus

The analyses were performed with a Series 4 liquid chromatograph (Perkin-Elmer, Monza, Italy), equipped with a 650-10S fluorescence detector with an 18-μl flow cell (Perkin-Elmer), and data were quantitated by a Model 3600 computing integrator (Perkin-Elmer). Samples were injected into the mobile-phase stream via a Rheodyne (Cotati, CA, U.S.A.) 7125S valve with 25-, 50- and 100 μl filling loops. The stainless-steel column (170 × 4.6 mm I.D.), the reaction coils, the column oven and the two fluid metering pumps for post-column reactions were supplied by Jasco (Barzanò, Italy). The column packing consisted of 7–8-μm particles of MCI CK 10F gel (Mitsubishi, Tokyo, Japan), a strong cation-exchange resin composed of sulphonic acid groups attached to a styrene–divinylbenzene copolymer (cross-linkage, 10%).

The hypochlorite oxidation step was carried out in a 300 × 0.5 mm I.D. stain-

less-steel tube. The reaction coil for the derivatization step was made of the same tubing but with a length of 2.5 m.

Chromatographic conditions

The mobile phases consisted of (A) 0.2 *M* Na^+ and (B) 0.25 *M* Na^+. Eluent A contained sodium citrate dihydrate (15.78 g/l), sodium nitrate (3.4 g/l), phenol (1 g/l), 2-propanol (30 ml/l) and Brij-35 (0.2 ml/l). Eluent B contained the same amounts of sodium citrate, sodium nitrate and Brij-35 as in A, plus 9.54 g/l of sodium tetraborate decahydrate and 15 ml/l of 2-propanol. The buffers were adjusted to (A) pH 3.05 and (B) 10.45 with 20% nitric acid and 5 *M* sodium hydroxide. Citrate buffers are stable for 1 month if stored at room temperature.

The OPA reagent[4] was prepared by dissolving 3.2 g of OPA in 40 ml of anhydrous ethanol and mixing this solution with 1 l of degassed and filtered 0.35 *M* potassium borate buffer (pH 10.4). Amounts of 2.4 ml of 3-MPA and 2.0 ml of Brij-35 were then added. The sodium hypochlorite reagent, 0.2% (v/v) in borate buffer containing 1.5% (w/v) potassium sulphate, was stabilized overnight at 4°C before use. This solution, which is not stable, was not kept longer than 48 h. The OPA reagent kept in a nitrogen atmosphere for 1 week without appreciable change in response.

The column and reaction coils were thermostated in the column oven at 68°C. The mobile-phase flow-rate was set at 0.5 ml/min. The post-column reagents were delivered on-line by pumping the solutions into the reaction coils via two tee-valves, as described by Cunico and Schlabach[8]. The final flow-rate of each reactant was set at 0.35 ml/min. Peaks were detected by measurement at 360 nm excitation wavelength and 450 nm emission wavelength. The gradient conditions are summarized in Table I. The typical cycle time between two injections was 60 min.

TABLE I

GRADIENT PROGRAMME FOR CHROMATOGRAPHIC AMINO ACID ANALYSIS

Step	*Time (min)*	*Eluent A (%)*	*Eluent B (%)*	*Gradient profile*
Equilibration	14	100	–	–
1	13	80	20	Concave
2	15	65	35	Linear
3	14	–	100	Isocratic
Regeneration[a]	4	–	–	–

[a] With 0.2 *M* sodium hydroxide.

Amino acid standards

The amino acid calibration mixture and the individual amino acids were diluted in eluent A, containing the three internal standards (I.S. buffer). Each internal standard had a concentration of 10 μM. Standard solutions were stored refrigerated for 2 weeks. Stock solutions of individual amino acids in water were kept at −20°C before dilution.

Sample preparation

Chymotrypsinogen A was hydrolysed *in vacuo* with 4 *M* methanesulphonic acid containing tryptamine (2 mg/ml). Cysteines were converted to S-sulphocysteine (Cys-SO_3H) according to the method of Inglis *et al.*[13]. The same amount of protein was hydrolysed in 5.7 *M* hydrochloric acid (0.2% phenol) at 110°C for 22 h. Both hydrolysates were diluted with the I.S. buffer before conversion of the cysteines to Cys-SO_3H.

The ultrafiltered milk protein sample (5 mg) was hydrolysed with 1 ml of 4 *M* methanesulphonic acid (0.2% tryptamine) and diluted to 50 ml with the I.S. citrate buffer.

Leucotrofina, a therapeutic formulation of the calf thymus derivative Thymomodulin (Ellem, Milan, Italy)[14] in 35% (w/v) sorbitol was diluted with four volumes of I.S. buffer and injected (50 μl) into the HPLC system for free amino acid determination.

Calculations

Quantitative estimates of the amino acid content were based on internal standardization with homoserine (I.S.$_1$), norleucine (I.S.$_2$) and β-aminoisobutyric acid (I.S.$_3$).

RESULTS AND DISCUSSION

A typical HPLC separation of standard amino acids at the 250- and 40-pmol levels is shown in Fig. 1. Critical pairs of amino acids, Hyp–Asp, Thr–Ser, Ala–(Cys_2) and Leu–Nleu, are well separated with the gradient programme in Table I. Shorter programmes can affect the resolution of the Hyp–Asp pair. A 57-min gradient programme may be used for the analysis of hydrolysates lacking Hyp (Fig. 2) by shortening the equilibration time to 11 min. In this instance, the gradient slope of the first step must be lowered in order to maintain the performance of the separation.

The volume of the samples can seriously affect the resolution of the pairs Met (O)–Hyp and Hyp–Asp (Table II). Larger injection volumes are observed to contribute to the peak broadening in the acidic and hydroxyl amino acid zone. Very diluted peptide hydrolysates lacking Hyp can be injected in larger volumes without affecting the performance of the analysis. The internal standards (a) I.S.$_1$, (b) I.S.$_2$ and (c) I.S.$_3$ were used for the following three groups of amino acids, respectively: (a) $CysSO_3H$, Cya, Met(O), Hyp, Asp, Thr, Ser, Glu, Pro, Gly, Ala and $(Cys)_2$; (b) Val, Met, Ile, Leu, Tyr and Phe; (c) His, Trp, Lys and Arg.

The relative standard deviation (R.S.D.) of the retention time relative to that of the internal standards ranged between 0.2 and 1.0% for six replicate injections. The R.S.D. of the peak-area ratios ranged between 0.6 and 3.3 (Table III). The linearity of the assay was evaluated in the 0.1–2.5 nmole range. The linear correlation coefficients were higher than 0.985 ($n = 6$) for all amino acids except $(Cys)_2$ ($r = 0.955$), Met(O) ($r = 0.972$) and Hyp ($r = 0.982$).

The lowest limit of detection in this chromatographic procedure was 10 pmol for each amino acid (Fig. 1), but at this level of sensitivity the precision was low. The R.S.D. of the peak-area ratios at 40 pmol ranged between 3 and 7% for all of the amino acids except Hyp (8%), Cys (12%) and Trp (10%). The large variation of the

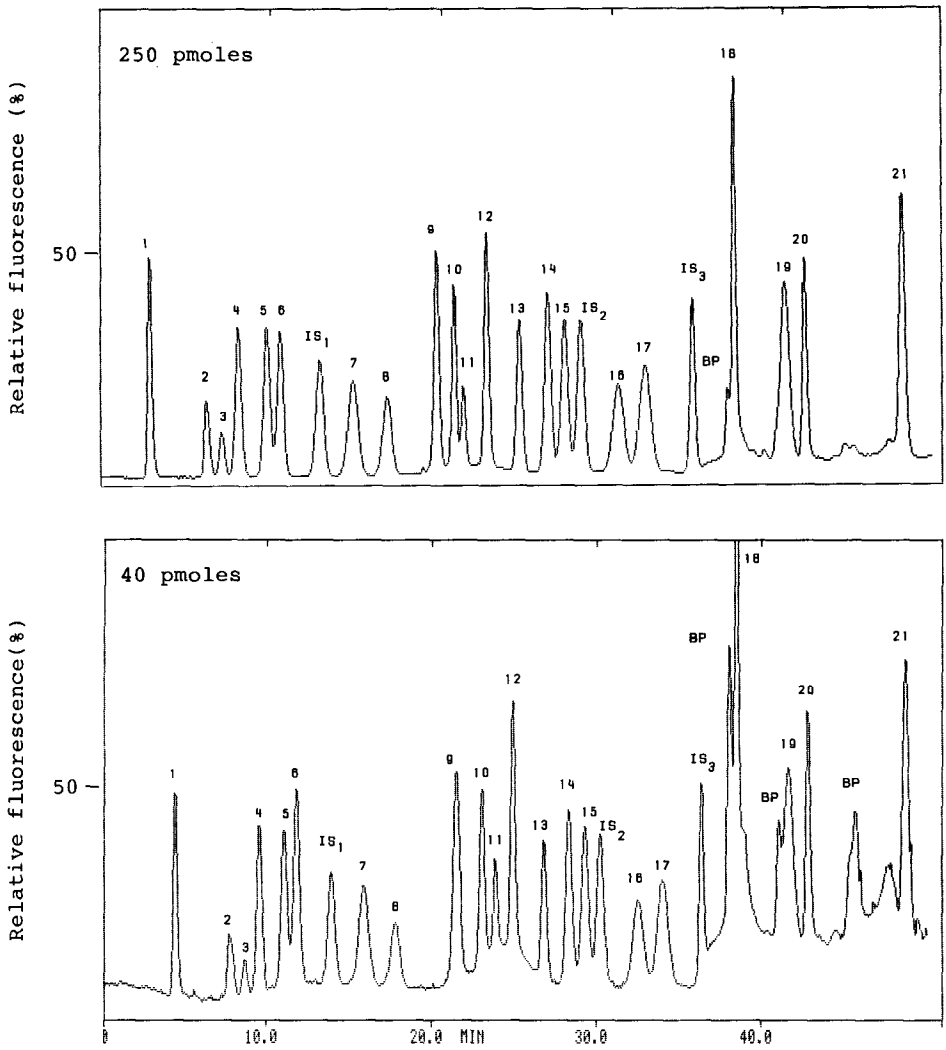

Fig. 1. HPLC and post-column detection of a standard mixture of amino acids. Conditions are summarized under Experimental. Gradient conditions of amino acids are given in Table I. Peaks: 1 = cysteic acid; 2 = Met(O); 3 = Hyp; 4 = Asp; 5 = Thr; 6 = Ser; 7 = Glu; 8 = Pro; 9 = Gly; 10 = Ala; 11 = $(Cys)_2$; 12 = Val; 13 = Met; 14 = Ile; 15 = Leu; 16 = Tyr; 17 = Phe; 18 = His; 19 = Trp; 20 = Lys; 21 = Arg. Amounts of Met(O) and Hyp were 125 pmol (above) and 20 pmol (below), and of $I.S._i$ was 250 pmol (above) and 40 pmol (below). BP = blank impurity peaks.

Trp peak-area ratio is caused by a blank impurity which is eluted in the Trp zone (Fig. 1). In the blank run this impurity ranged between 5 and 12 pmol, if evaluated as Trp. As its contribution is small, it can be subtracted from the Trp peak area.

The chromatograms show an impurity peak (BP) which is eluted in the His zone. This peak does not interfere in the His determination in the picomole range, but

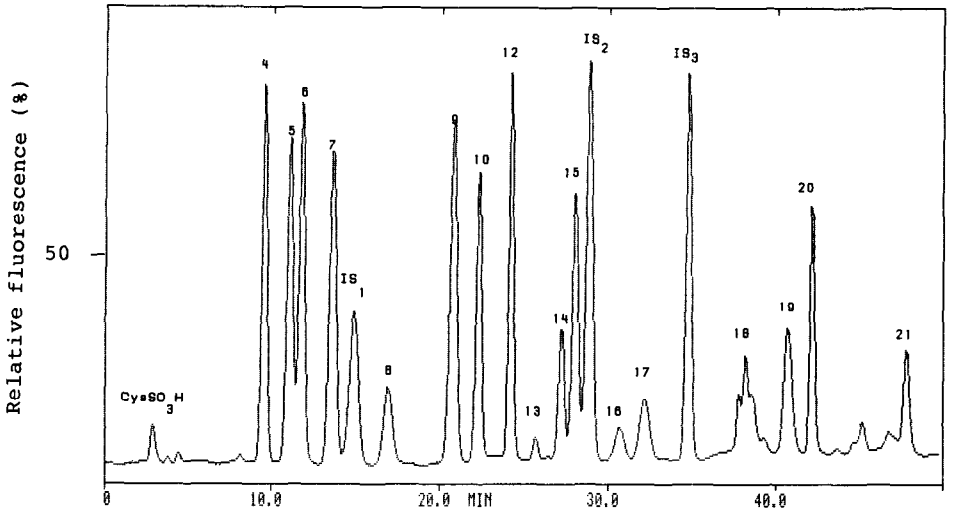

Fig. 2. Amino acid analysis of bovine chymotrypsinogen A hydrolysate performed with the methanesulphonic acid procedure. Peaks and conditions as in Fig. 1. $CysSO_3H$ = S-sulphocysteine. Amount of I.S.$_i$, 500 pmol.

TABLE II

INFLUENCE OF INJECTION VOLUME ON RESOLUTION FACTOR

Critical pair	*Injection volume (µl)*		
	25	*50*	*100*
Met(O)–Hyp	>1.4	>1.3	>1.0
Hyp–Asp	>1.3	>1.2	<1.0
Thr–Ser	>1.1	>1.1	>1.0

TABLE III

REPRODUCIBILITY OF PEAK-AREA RATIOS OF AMINO ACIDS

The peak-area ratios (R) are average values of six replicate injections. Each value corresponds to 500 pmol.

Amino acid	*R*	*R.S.D. (%)*	*Amino acid*	*R*	*R.S.D. (%)*
Cysteic acid	1.046	1.4	Val	1.034	1.5
Met(O)	0.848	1.3	Met	0.751	1.4
Hyp	0.488	1.6	Ile	1.045	0.9
Asp	1.123	2.5	Leu	0.970	1.6
Thr	1.113	0.6	Tyr	0.848	0.9
Ser	1.165	2.6	Phe	1.067	0.7
Glu	1.108	0.8	His	1.620	3.3
Pro	0.768	1.8	Trp	1.782	2.6
Gly	1.410	0.8	Lys	1.074	2.6
Ala	0.975	1.8	Arg	1.721	1.2
$\frac{1}{2}(Cys)_2$	0.407	3.6			

it is not separated from His at the nanomole level. Impurity and buffer-change peaks were also reported for dedicated commercial buffers[15].

The two reaction steps, oxidation and derivatization, were carried out at 68°C, because the best ion-exchange separation was obtained at this temperature. The length of the two reaction coils was chosen in order to maintain the oxidation and derivatization steps in the ranges 4–5 and 20–30 s, respectively. The highest Pro, Met and Tyr responses were found with 0.2% hypochlorite and 0.32% OPA. The molar ratio of 3-MPA to OPA in the derivatizing solution was kept at 1:1, as suggested by Fujiwara *et al.*[11].

The influence of reaction time on the fluorescence response of Pro, Met and Tyr was studied at different flow-rates of the reactants (Table IV). Although the best results were obtained with prolonged reaction times, we delivered each post-column reagent at 0.35 ml/min, because at lower flow-rates the excessive decrease in resolution of critical pairs can interfere with their determination. Lower flow-rates also affect the reproducibility of amino acid determinations.

TABLE IV

INFLUENCE OF REACTION TIME ON FLUORESCENCE RESPONSE

The fluorescence response was monitored as the mean of the peak areas of two 500-pmol samples of amino acids. The data were obtained by delivering the reagents (sodium hypochlorite and OPA–3-MPA) at 0.25, 0.30, 0.35 and 0.40 ml/min, respectively. The two reactions were delivered at the same flow-rate in each trial.

Amino acid	*Reaction time (s)*			
	30	*27*	*25*	*23*
Pro	36 220	34 500	30 413	24 608
Met	32 913	31 035	28 950	27 576
Tyr	37 086	35 610	32 909	32 584

The good recovery shown by the fluorescence responses of Pro, Met and Tyr suggests that 3-MPA is a suitable reagent for the reliable simultaneous determination of amino and imino acids.

The method was extended to the determination of tryptophan in protein, hydrolysed with 4 *M* methanesulphonic acid (MSA)[13] containing 0.2% tryptamine. Fig. 2 shows the chromatogram of the MSA hydrolysate of bovine chymotrypsinogen A. Table V compares the results of determinations using the 5.7 *M* hydrochloric acid and the 4 *M* MSA hydrolysis methods. The 90% tryptophan recovery indicates that MSA hydrolysis, coupled with the two-step post-column derivatization method, is satisfactory for the determination of Trp in purified peptides. In our experiments, the MSA hydrolysis seemed to improve the recovery of Ser, whereas Ile and Val were more resistant to the cleavage. In spite of various attempts, low recoveries of S-sulphocysteine was obtained with both the hydrochloric acid and the MSA hydrolysis methods. Although the composition of chymotrypsinogen is unbalanced (low contents of Met, Tyr and Hys), the analysis demonstrated that the OPA–3-MPA method

TABLE V

RECOVERY OF AMINO ACIDS OF CHYMOTRYPSINOGEN A BY HYDROLYSIS WITH 5.7 *M* HCl AND 4 *M* MSA

Amino acid	*HCl*		*MSA*	*Amino acid*	*HCl*		*MSA*
$CysSO_3H$	7.68[a]	(10)[b]	6.70	Met	1.82	(2)	1.90
Asx	22.40	(23)	23.06	Ile	8.82	(10)	8.09
Thr	22.01	(23)	22.31	Leu	18.60	(19)	18.37
Ser	24.75	(28)	26.91	Tyr	3.94	(4)	3.62
Glx	15.69	(15)	15.56	Phe	5.86	(6)	5.89
Pro	9.34	(9)	9.29	His	2.30	(2)	1.70
Gly	22.82	(23)	23.41	Trp	–	(8)	7.22
Ala	22.20	(22)	22.00	Lys	13.72	(14)	14.07
Val	20.49	(23)	18.58	Arg	4.02	(4)	4.14

[a] The number of residues was normalized to the number of Ala residues found. The absolute recoveries of Ala were 82% (HCl) and 81% (MSA), assuming 100% purity of the reference protein sample. Experimental values are means of two determinations of each hydrolysate.

[b] The numbers in parentheses are the expected values based on the protein sequence analysis.

is suitable for the determination of each amino acid. A representative chromatogram of an MSA hydrolysate of ultrafiltered milk proteins (85% protein) is shown in Fig. 3.

Several workers have recommended restricting the use of sulphonic acids to protein samples that do not contain carbohydrates, because Trp is destroyed during acid hydrolysis in the presence of carbohydrates[13].

The OPA–3-MPA post-column method also improved the determination of proline and hydroxyproline. Fig. 1 shows a good fluorescence response of Hyp and Pro at the picomole level. As these amino acids usually indicate the presence of

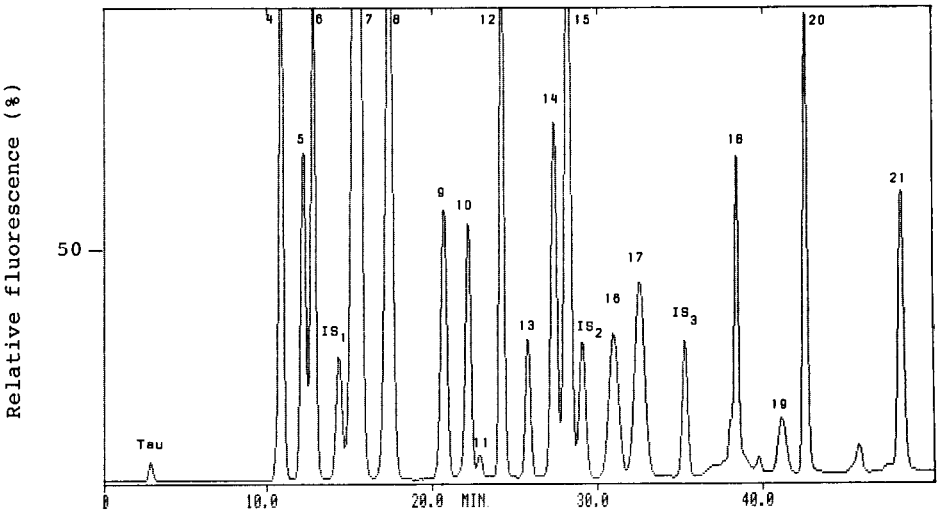

Fig. 3. Chromatogram of ultrafiltered milk protein after hydrolysis with 4 *M* MSA. Peaks and conditions as in Fig. 1. Tau = taurine. Amount of I.S.$_i$, 500 pmol.

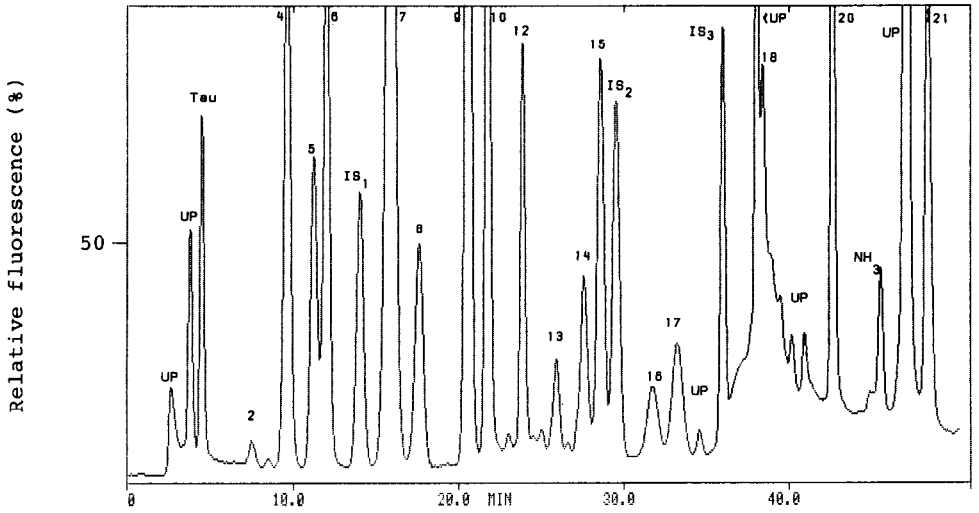

Fig. 4. Free amino acid analysis of a therapeutic formulation of the calf thymus derivative Thymomodulin. The sample syrup was diluted with I.S. buffer (see Experimental) and injected directly into the HPLC system. Peaks and conditions as in Fig. 1. UP = unknown peaks. Amount of I.S.$_i$, 500 pmol.

collagen in biological materials, the accurate determination of imino acids gives an estimate of the collagen content of the sample[16].

The chromatographic separation of free amino acids in the therapeutic formulation, Leucotrofina syrup, without previous purification is illustrated in Fig. 4. In this sample, the total free amino acids usually amount to 0.15–0.25 mg/ml. A complete assay is obtained simply by injecting the diluted sample directly into the HPLC system. The sorbitol in the sample (7%, w/v), does not interfere with the separation performance.

In conclusion, it has been shown that the use of OPA–3-MPA as the derivatizing agent, coupled with hypochlorite oxidation, in the HPLC and post-column detection of amino and imino acids is a useful tool for their quantitative determination.

ACKNOWLEDGEMENTS

We thank Dr. Giorgio Mazzola for suggesting the use of 3-MPA. We are also greatly indebted to Dr. Brunetto Brunetti for his support.

REFERENCES

1 S. Moore and W. H. Stein, *J. Biol. Chem.*, 211 (1954) 907.
2 S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, 30 (1958) 1185.
3 R. F. Pfeifer and D. W. Hill, *Adv. Chromatogr.*, 22 (1982) 1.
4 M. Roth, *Anal Chem.*, 43 (1971) 880.
5 M. Weigele, S. De Bernardo and W. Leingruber, *Biochem. Biophys. Res. Commun.*, 50 (1973) 352.
6 D. G. Dresker and K. S. Lee, *Anal. Biochem.*, 84 (1978) 559.
7 P. Böhlen and M. Mellet, *Anal. Biochem.*, 94 (1979) 313.
8 R. L. Cunico and J. Schlabach, *J. Chromatogr.*, 266 (1983) 461.

9 S. S. Simons and D. F. Johnson, *Anal. Biochem.*, 82 (1977) 250.
10 L. A. Allison, G. S. Mayer and R. E. Shoup, *Anal. Chem.*, 56 (1984) 1089.
11 M. Fujiwara, Y. Ishida, N. Nimura, A. Toyama and T. Kinoshita, *Anal Biochem.*, 166 (1987) 72.
12 D. Kucera and H. Umagat, *J. Chromatogr.*, 255 (1983) 563.
13 A. S. Inglis, D. T. W. McMahon, C. M. Roxburgh and H. Takayanagi, *Anal. Biochem.*, 72 (1976) 86.
14 A. Fiocchi, E. Borella, E. Riva, D. Arensi, P. Travaglini, P. Cazzola and M. Giovannini, *Thymus*, 8 (1986) 331.
15 G. R. Barbarash and R. H. Quarles, *Anal. Biochem.*, 119 (1982) 177.
16 K. Yaegaki, J. Tonzetich and A. S. K. Ng, *J. Chromatogr.*, 356 (1986) 163.