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Fully automated amino acid analysis for protein and peptide hydrolysates by precolumn derivatization with 9-fluorenyl methylchloroformate and 1-aminoadamantane

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SUMMARY

A fully automated precolumn derivatization method for the determination of primary and secondary amino acids using reversed-phase chromatography is described. The derivatization reagent 9-fluorenyl methylchloroformate is used, together with 1-aminoadamantane for reaction with excess of reagent. The derivatization procedure is described in detail. Reproducibility data are presented and compared between the manual and the fully automated methods. Optimization strategies for separation, and differences in detection between fluorescence and UV methods are discussed. Analytical data for protein and peptide hydrolysates are compared with their sequenced data.

INTRODUCTION

9-Fluorenyl methylchloroformate (FMOC) was introduced as a precolumn derivatization reagent in high-performance liquid chromatographic (HPLC) amino acid analysis in 1983[1]. FMOC forms derivatives for both primary and secondary amino acids. The derivatives are highly fluorescent, and are rapidly formed within less than 1 min in a buffered aqueous solution. The derivatized amino acids and the reagent itself have almost identical excitation and emission spectra, and therefore the excess of reagent has to be removed before injection, so that the detection of some amino acids is not affected. This could be done by pentane extraction[1] or by derivatizing the excess of reagent with a hydrophobic amine[2]. In this paper, we describe a fully automated derivatization method based on the hydrophobic amine approach.

An advantage with the hydrophobic amine method, using 1-aminoadamantane (ADAM), is that the risk of partial extraction of the hydrophobic amino acids into the organic phase is eliminated. This problem has been discussed in several reports[1,3–5]. Our experience is that when derivatizing amino acids at a concentration of 20 pmol per amino acid or less, the pentane extraction transfers 50–75% of the more hydrophobic amino acids (histidine, ornithine, lysine) into the organic phase. This will adversely effect the reproducibility of the analysis for these amino acids. The

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objective of this study was to develop a reliable amino acid analysis method. To meet this objective, we developed a fully automated derivatization method with a few sample preparation steps as possible, in order to make it easy to use in routine amino acid analysis.

EXPERIMENTAL

HPLC system

The chromatographic system was a two-pump gradient system. AminoSys (Pharmacia–LKB, Bromma, Sweden) and consisted of Model 2248 HPLC pumps, a Model 2248-300 dynamic high-pressure mixer, a Model 2156 solvent conditioner, a Model 2146 HPLCmanager PC control system in combination with Nelson evaluation software on an IBM AT computer, a Model 2157-020 autosampler (the automated method with an experimental modification of the autosampler; the modification consisted of an external mixmotor and software changes), a Model 2144 fluorescence detector, a Model 2141 variable-wavelength monitor and a Model 2155 column oven.

Chemicals

Solvents were of HPLC grade and chemicals were of the highest purity available, all from E. Merck (Darmstadt, F.R.G.), except FMOC from Sigma (St. Louis, MO, U.S.A.), ADAM · HCl from Serva (Heidelberg, F.R.G.) and amino acid standards from Pharmacia LKB Biochrom Ltd. (Cambridge, U.K.) or Pierce (Rockford, IL, U.S.A.).

Mobile phase

A stock solution of 100 m*M* sodium acetate buffer was prepared by adding acetic acid to water and adjusting the pH to 4.4 with 30% sodium hydroxide solution. The buffer was filtered through a 0.45-μm filter. Eluent A was sodium acetate buffer (100 m*M*, pH 4.4)–tetrahydrofuran–acetonitrile (75:15:10) and eluent B was acetonitrile–tetrahydrofuran (85:15), with the following gradient profile: 0–2.5 min, 0% B; 2.5–6.6 min, 7% B; 6.6–8.3 min, 14% B; 8.3–8.4 min, 21% B; 8.4–10 min, 21% B; 10.0–10.1 min, 17% B; 10.1–20.0 min, 19% B; 20–29 min, 55% B; 29–30 min, 100% B. The flow-rate was 1.5 ml/min.

Column

The stationary phase was Kromasil C_8, 5 μm (EKA Nobel, Surte, Sweden) in a 250 × 4 mm I.D. column. The column temperature was 45°C.

Derivatization procedure

A 10-μl volume of sample was pipetted into a clean autosampler vial and 10 μl of borate buffer (0.5 *M* boric acid solution adjusted to pH 7.7 with 30% sodium hydroxide solution) were added. After vortexing, 20 μl of FMOC (1 m*M*) in acetone were added and vortex mixed immediately. After incubation for 45 s, 20 μl of ADAM (40 m*M*) in water–acetone (1:3, v/v) were added and the mixture was vortex mixed. After incubation for at least 45 s, 5–10 μl were injected for HPLC analysis. The procedure was used either in the manual mode by using ordinary pipettes or in the automated mode by utilizing the modified autosampler.

Hydrolysis procedure

The hydrolysis vessel was supplied by Ciba Corning Diagnostics (Essex, U.K.). Solutions of proteins and peptides were added to the sample tubes and were dried in a Speed Vac vacuum centrifuge (Savant Instrument, Farmingdale, NY, U.S.A.) for 20 min and then placed in the hydrolysis vessel. The vessel was flushed with nitrogen and 2 ml of 6 *M* hydrochloric acid were transferred into the bottom of the vessel. The vessel was evacuated and purged with nitrogen. The hydrolysis was carried out at 110°C for 23 h. After cooling, the vessel was opened and the samples were dried in a vacuum centrifuge for 20 min. The contents were redissolved in 0.01 *M* hydrochloric acid or water (HPLC grade).

RESULTS AND DISCUSSION

Derivatization buffer

As we have observed precipitations of borate and FMOC, we modified the acetone to water ratio given in the earlier published method[3]. The pH was optimized for this modified derivatization system; pH 7.7 was choosen so that the area of the FMOC hydrolysis product (FMOC-OH) was minimized, but still gave a good response for all amino acids (Fig. 1).

FMOC reaction

The FMOC stock solution concentration (1 m*M*) gave a linear response between 0.25 and 35 pmol per amino acid for the method with respect to fluorescence

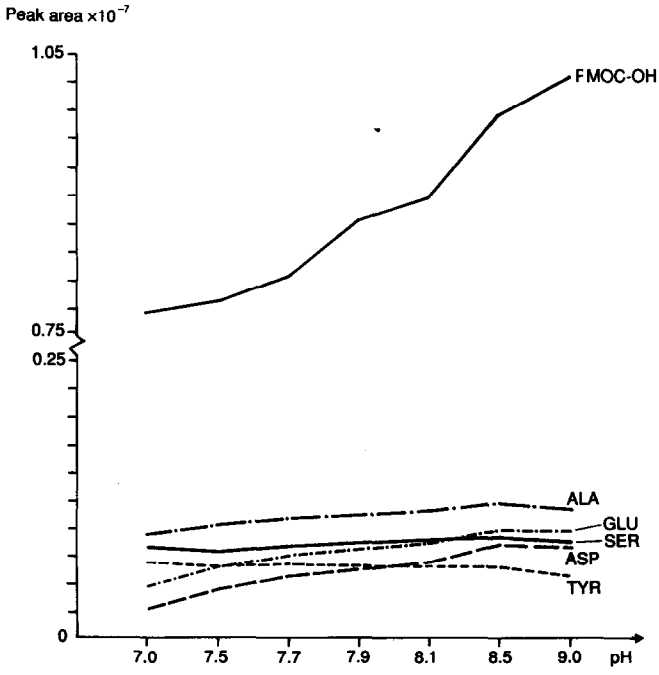

Fig. 1. Effect of derivatization pH on peak area.

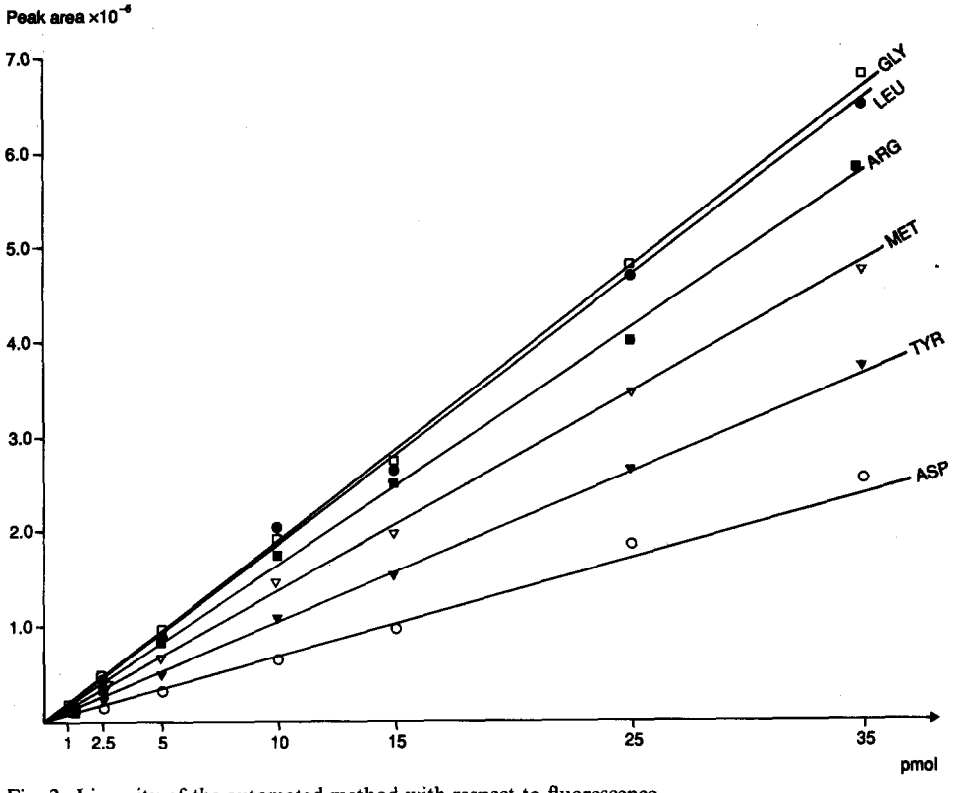

Fig. 2. Linearity of the automated method with respect to fluorescence.

(Fig. 2). This FMOC concentration gave an optimum response for all amino acids[3]. The optimum reaction time for the FMOC derivatization was 45 s for all amino acids. Most amino acids were fully derivatized after 30 s, but the acidic amino acids (aspartic acid, glutamic acid) needed a longer reaction time.

Stability of derivatives

Stability of the FMOC derivatives was measured at both 22°C (room temperature) and 6°C by injecting a constant volume from a single derivatization of a protein hydrolysate standard every hour for 24 h. One derivative that showed a significant breakdown at ambient temperature was di-derivatized histidine (49.4%), which was converted to the mono-derivatized form. Cysteine also showed a decrease in response (26.0%). These effects were reduced when thermostating the vials in the autosampler at 6°C. The relative standard deviation of the peak area for the different amino acids was between 1.7 and 5.1% except for di-derivatized histdine (20%). This shows that the stability of the derivatives is sufficient to be able to use the method in a manual derivatization mode, if the vials are stored at 6°C during analysis, except for unstable histidine.

Influence of ADAM

The concentration ratio of ADAM to FMOC in the reaction mixture was 40:1.

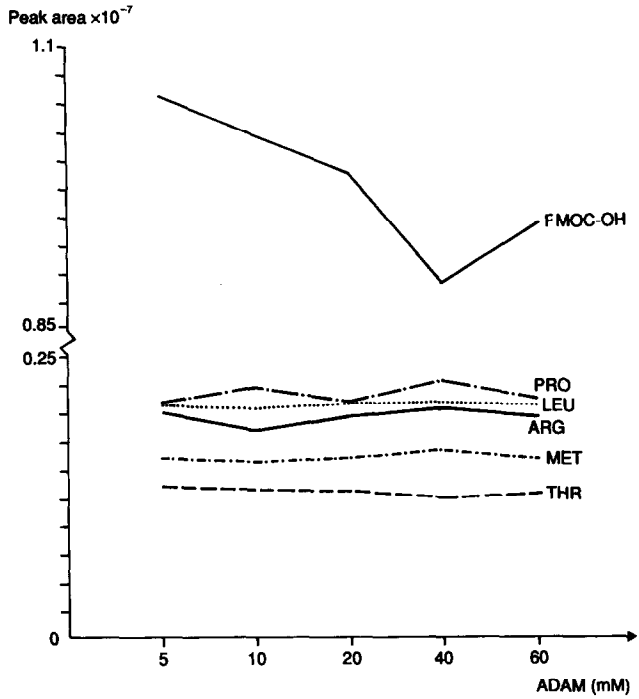

Fig. 3. Effect of ADAM concentration on peak area.

With this ratio, the interference with the remaining FMOC-OH peak and the more hydrophobic amino acids was kept to the minimum. The response of the amino acid derivatives was independent of the ADAM concentration within the tested concentration range (Fig. 3).

Reproducibility of manual and automated methods

The reproducibility of the manual and automated derivatization methods was measured by analysing ten different derivatizations from the same protein hydrolysate standard sample.

As we have observed the risks of introducing contaminants in the pipetting steps during a manual derivatiation, and introducing volume errors when pipetting small volumes with commercially available automatic pipettes, we aimed to make the derivatization steps fully automated by using an autosampler. The automation should make the method more reliable, as the number of manual pipetting steps is reduced. The instability of the histidine derivative will also not be a limiting step.

A comparison of the relative standard deviations of peak areas between the manual derivatization method and the fully automated method is shown in Table I. To be able to achieve reproducible results, all chemicals and reagents must be freshly prepared and the highest purity available[3].

Stationary phase

Instability of the silica matrix is still a common problem associated with re-

TABLE I

RELATIVE STANDARD DEVIATION FOR PEAK AREA: COMPARISON BETWEEN MANUAL AND AUTOMATIC DERIVATIZATION

Amino acid	*R.S.D. (%) (n = 10)*			*R.S.D. (%) (n = 10)*	
	Automatic	*Manual*		*Automatic*	*Manual*
Arginine (Arg)	2.04	4.76	Tyrosine (Tyr)	2.79	4.16
Serine (Ser)	1.72	4.01	Methionine (Met)	2.19	4.01
Aspartic acid (Asp)	4.69	4.81	Valine (Val)	4.11	5.19
Glutamic acid (Glu)	4.81	4.72	Phenylalanine (Phe)	2.91	5.26
Threonine (Thr)	2.59	4.39	Isoleucine (Ile)	2.45	4.17
Glycine (Gly)	3.18	3.32	Leucine (Leu)	2.07	4.38
Alanine (Ala)	2.36	4.26	Histidine (His)	4.71	8.35
Proline (Pro)	2.53	3.19	Lysine (Lys)	2.42	4.86

versed-phase chromatography. We have observed an increase in retention of arginine compared with other amino acids as the column ages[3]. This cannot be compensated for with the mobile phase used in routine analysis. Therefore, a new column matrix was choosen for this study. This column gave satisfactory results, especially for stability of the retention times of basic amino acids.

Mobile phase

The mobile phase system was optimized for a baseline separation of all the amino acids in a protein hydrolysate with the column matrix choosen in this study. For mobile phase optimization, pH, temperature, organic modifiers and ionic strength were investigated.

A decrease in pH increased the retention of all amino acids. Methionine was eluted together with the FMOC-OH peak and cystine together with leucine. The retention times of the FMOC-OH and ammonia peaks were only slightly affected by the pH of the eluent. When the pH was increased, serine and aspartic acid were not fully resolved, and valine eluted together with the FMOC-OH peak; pH 4.4 gave the best selectivity for this separation.

The use of an acetonitrile gradient was not sufficient to separate some of the hydrophilic amino acids. Therefore, another organic modifier, tetrahydrofuran (THF), was introduced into the mobile phase system. The optimized gradient profile was necessary in order to elute methionine before the FMOC-OH peak.

The ionic strength of the elution buffer mostly affected the peak shape of cystine. An increase in the ionic strength of the buffer reduced the interaction of cystine with the matrix; 100 m*M* acetate was sufficient to separate cystine as a sharp peak.

Reproducibility of the retention time for the optimized separation system was excellent; the relative standard deviation was between 0.1 and 0.7%. The column temperature was maintained at 45°C. A separation of a protein hydrolysate standard is shown in Fig. 4.

Detection and analysis

The FMOC derivatization gave highly fluorescent adducts which were detected

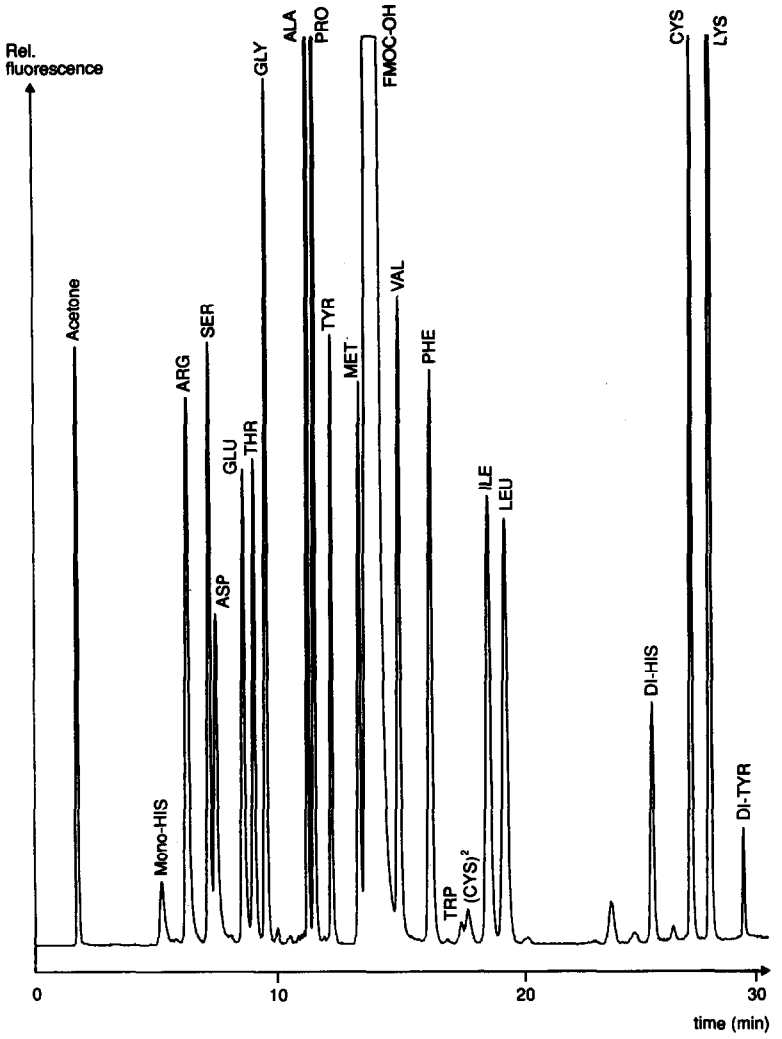

Fig. 4. Separation of protein hydrolysate standard; 10 pmol per amino acid. Fluorescence detection, λ_{ex} 263 nm, and λ_{em} 313 nm. Injection volume, 5 μl.

TABLE II

RELATIVE RESPONSE OF PEAK AREA RELATED TO Ala FOR FMOC DERIVATIVES, DETECTED BY UV AND FLUORESCENCE METHODS

Amino acid	*UV*	*Fluorescence*	*Amino acid*	*UV*	*Fluorescence*
Mono-His	0.44	0.66	Met	0.96	0.93
Arg	0.79	1.08	Val	1.10	1.27
Ser	0.69	0.88	Phe	1.14	1.39
Asp	0.11	0.31	Cys[2] (cystine)	0.63	N.D.
Glu	0.46	0.50	Trp	1.41	0.05
Thr	0.66	0.80	Ile	1.10	1.36
Gly	1.25	1.44	Leu	1.02	1.22
Ala	1.00	1.00	Di-His	0.67	0.19
Pro	1.28	1.43	Cys (cysteine)	1.49	1.51
Tyr	1.30	0.89	Lys	1.93	2.15

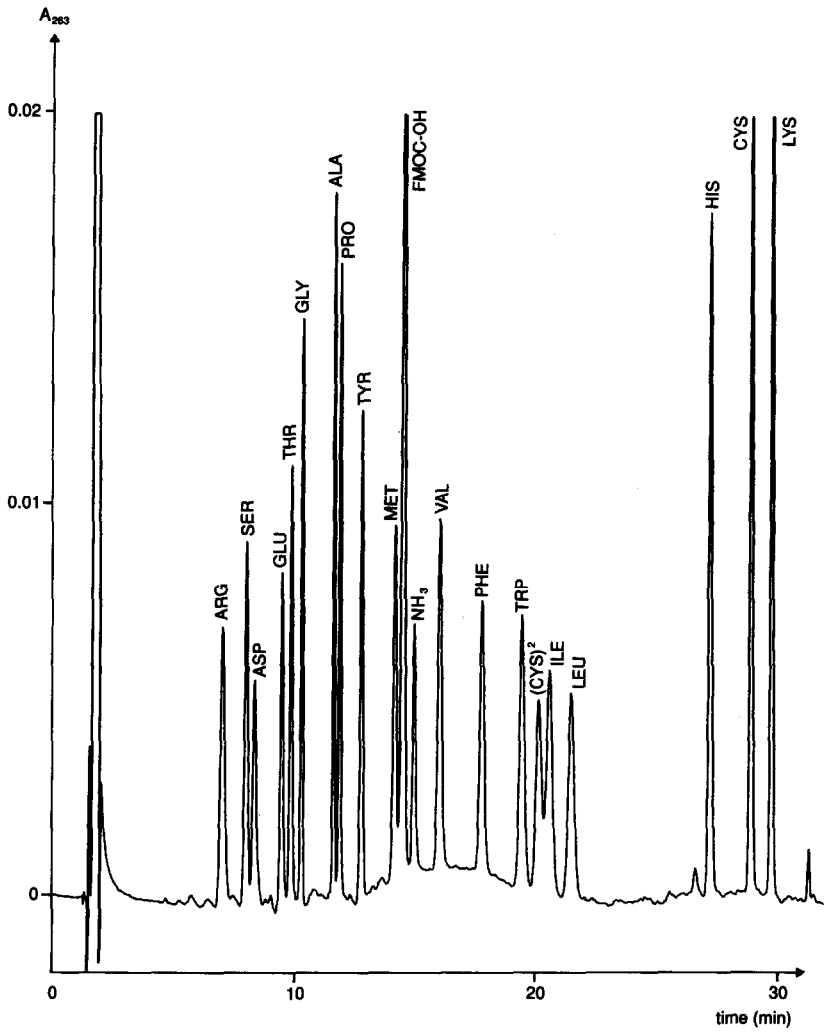

Fig. 5. Separation of protein hydrolysate standard; 200 pmol per amino acid. UV detection at 263 nm. Injection volume, 6 μl.

with excitation at 263 nm and emission at 313 nm[3]. In this study, we used a filter fluorescence detector with an interference filter at 265 nm, with a band-with of 8 nm. A Schott WG 305 cut-off filter was used in combination with a Schott UG11 block filter in order to obtain the highest possible signal-to-noise ratio (S/N). With this combination the detection limit (S/N = 3) was 40 fmol for alanine. However, the highest sensitivity routinely obtainable was about 100 fmol, because of the contamination of amino acids in a standard laboratory environment[3].

Owing to intramolecular quenching in fluorescence, the response for some amino acids, *e.g.*, cystine and tryptophan, was low. To investigate this fully, the response

TABLE III

AMINO ACID COMPOSITION OF LYSOZYME, RPI-1 AND SM-5

Comparison of sequenced data with FMOC analysis and dedicated amino acid analyser.

Amino acid	*Lysozyme*		*Synthetic peptide RPI-1*			*Synthetic peptide SM-5*		
	Seq.	*FMOC*	*Seq.*	*FMOC*	*Nin-hydrin*	*Seq.*	*FMOC*	*Nin-hydrin*
Arg	11	11.1	1	1.0	1.0			
Ser	10	8.7						
Asp[a]	21	18.3				7	7.2	6.4
Glu[b]	5	4.6	2	2.1	1.9	5	6.2	4.9
Thr	8	6.4	1	1.0	1.0	3	2.9	2.6
Gly	11	11.4				2	1.9	2.4
Ala	12	11.8	2	1.6	2.3	3	3.0	3.0
Pro	2	2.1	1	1.0	1.0	1	1.0	1.0
Tyr	3	2.8				1	0.7	0.6
Met	2	0.5						
Val	6	5.1				1	1.0	1.0
Phe	3	2.9	1	0.9	1.0			
Trp	6	N.D.						
Ile	6	5.9	1	1.0	1.0	1	1.0	0.9
Leu	8	8.2	1	1.0	1.0	5	4.9	4.6
His	1	0.6				1	1.0	1.0
Cys[c]	8	0.2						
Lys	6	5.6				4	3.6	3.6

[a] Aspartic acid + aspargine
[b] Glutamic acid + glutamine.
[c] Cysteine.

factors for these amino acids relative to alanine were compared with the same chromatogram with UV detection at 263 nm, using a variable-wavelength monitor (Table II and Fig. 5).

A hydrolysate of lysozyme was analysed using the automated FMOC method, and a comparison with its sequenced data is shown in Table III.

Hydrolysates from the synthetic ten-residue peptide Retroviral Protease Inhibitor, RPI-1 (Fig. 6), and a 35-residue peptide SM-5 were also analysed using the automated FMOC method. The results were compared both with their sequenced data and with the results obtained with a Model 4151 Alpha Plus ninhydrin post-column derivatization dedicated amino acid analyser (Pharmacia LKB Biochrom Ltd., Cambridge, U.K. (Table III).

The results for cysteine, tryptophan and methionine were low, owing to degradation in the hydrolysis method used in this analysis[6]. No precautions for the protection of labile amino acids were made in this hydrolysis method. Oxidation of the sample prior to hydrolysis would be an alternative[7]. With this gradient, cysteic acid and methionine sulphone are eluted at 3.2 and 7.5 min, respectively.

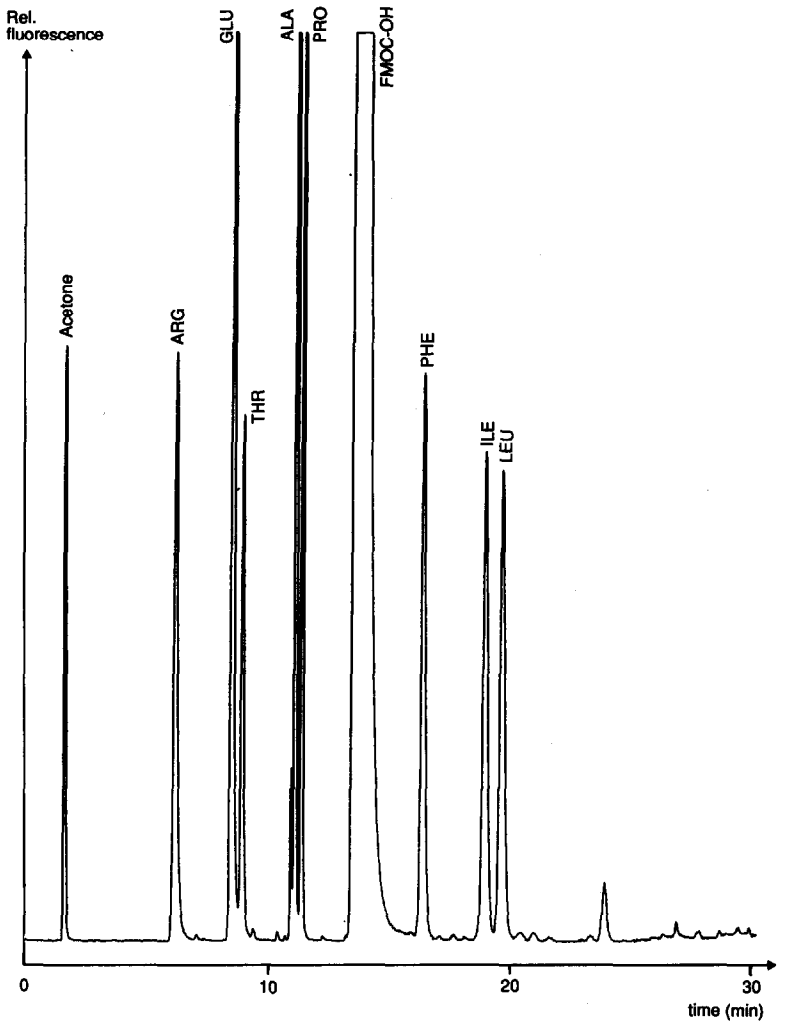

Fig. 6. Chromatogram of the synthetic ten-residue peptide Retroviral Protease Inhibitor (RPI-1) hydrolysate. Fluorescence detection, λ_{ex} 263 nm, λ_{em} 313 nm. Injection volume, 6 μl.

CONCLUSIONS

The fully automated FMOC–ADAM approach fulfils the need for a reliable method for the routine determination of protein and peptide hydrolysates at levels of less than 10 pmol per amino acid. Absolute control over the different steps in the derivatization procedure is necessary in order to achieve reproducible results.

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REFERENCES

1 S. Einarsson, B. Josefsson and S. Lagerkvist, *J. Chromatogr.*, 282 (1983) 609–618.
2 I. Betnér and P. Földi, *Chromatographia*, 22 (1986) 381–387.
3 I. Betnér and P. Földi, *LC · GC Mag. Liq. Gas Chromatogr.*, 6 (1988) 832–840.
4 S. Einarsson, *J. Chromatogr.*, 348 (1985) 213–220.
5 H. Godel, *Thesis*, University of Hohenheim, Stuttgart, 1986.
6 A. S. Inglis, *Methods Enzymol.*, 91 (1983) 26–36.
7 V. C. Mason, M. Rudemo and S. Bech-Anderson, *Z. Tierphysiol. Tierernähr. Futtermittelkd.*, 43 (1980) 35.