HPLC application note

Amino Acid Analysis by Precolumn Derivatisation using a New FMOC Procedure

'...the resultant derivatives are very stable and highly fluorescent, offering assay sensitivity in the fmol range...'

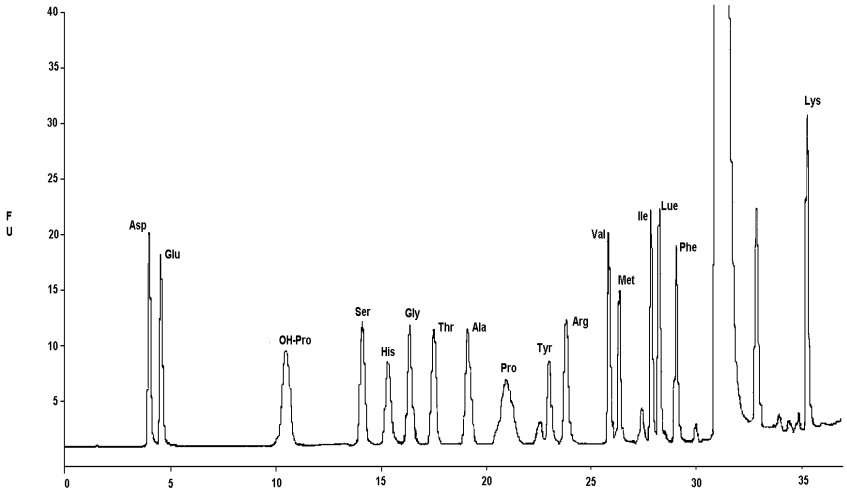

Figure 1 *Separation of Hydrolysate Amino Acid Standard with Fluorescence Detection*

Abstract

A rapid, sensitive method for the determination of primary and secondary common protein amino acids is described. This is based on a newly developed, simple and effective procedure for precolumn derivatisation using FMOC-Cl (9-fluorenylmethyl chloroformate). The method is applicable to both manual and automated protocols. It does not require any solvent extraction or incubation and gives stable single adducts including histidine and tyrosine. The HPLC conditions have been developed to deliver high assay accuracy and precision. Application of the technique has been demonstrated on hydrolysate samples and procedures for the execution of the application is described in detail.

Keywords:
Amino Acid, AMINOMATE, Automated Precolumn Derivatisation, FMOC

Traditionally, the determination of amino acids has been conducted by ion-exchange chromatography, followed by postcolumn derivatisation with ninhydrin or o-phthalaldehyde (OPA). In recent years, with developments in LC instrumentation and methodology, precolumn derivatisation and reversed-phase HPLC have been used as an alternate method for amino acid analysis.

The FMOC precolumn derivatisation method is attractive as it is applicable to both primary and secondary amino acids. The derivatisation is rapid and is conducted at ambient temperature. It is also not susceptible to any major matrix interferences. The resultant derivatives are very stable and highly fluorescent, offering assay sensitivity in the fmol range.

Our newly developed FMOC method has incorporated a modified procedure[1,2] which enhances the effectiveness of the FMOC chemistry by removal of the inherent setbacks of the traditional technique.

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The new derivatisation protocol and HPLC conditions allow:

1. Elimination of any requirement for solvent extraction, thus simplifying derivatisation and providing accurate quantitation of the hydrophobic amino acids.
2. Generation of single, stable adducts, allowing reliable quantitation of all amino acids including histidine and tyrosine.
3. Elimination of interference from reagent by-products.

'...a comparison of experimental results and literature values for angiotensin II, neurotensin, chymotrysinogen A and pepsin is presented...'

Several different peptide and protein hydrolysates have been chosen as examples to confirm the viability of this new FMOC method, especially its accuracy in the quantitation of the histidine and tyrosine residues. A comparison of experimental results and literature values for the analysis of two synthetic peptides, angiotensin II and neurotensin, and three proteins, lysozyme, chymotrysinogen A and pepsin is presented in the following table (Table 1).

Each derivatisation was performed on 10 pmol of hydrolysate and 5 pmol of the derivatised samples were analysed. The results agree very well with the expected values, including the determination of histidine and tyrosine at low levels of complex amino acid mixtures.

Reproducibility and Linearity of Automated Derivatisation

In an experiment using the recommended hardware configuration, the reproducibility of the automated procedure was established by analysing a series of 10 and 20 consecutive amino acid standards at a concentration of 5 pmol and 100 pmol on column (i.e., 20 and 40 µM respectively) with hydroxyproline as an internal standard. The reproducibility of the retention time was less than or equal to 0.5. The RSD for peak area (corrected to OH-Pro) is in the range of 0.5 – 2.2 at the 100 pmol level and 1.5 – 4.1 at 5 pmol level.

The linearity of the automated procedure was established over a 200-fold concentration range between 1 µM and 200 µM. All of the amino acids were found to give linear derivatisation over this range, with correlation coefficients.

Limit of Detection

The FMOC derivatives of amino acids are highly fluorescent and can be detected at very low levels. The detection limit for hydroxyproline, chosen for this study as it was not present in reagent blank derivatisation, was 50 fmol at a signal to noise ration of 3:1.

Table 1: Amino Acid Composition of Angiotensin II, Neurotensin, Lysozyme, Chymotrysinogen A and Pepsin

			*Molar Ratio**		
Acid	***Angiotensin***	***Neurotensin***	***Lysozyme***	***Chymotrysinogen***	***Pepsin***
Asn**	1.0 (1.2)	1.0 (1.1)	21.4 (21)	23.1 (23)	44.1 (42)
Clx***		2.0 (2.0)	5.2 (5)	14.4 (15)	26.9 (26)
Ser			9.3 (10)	23.2 (28)	40.7 (44)
His	0.9 (0.8)		0.8 (1)	2.0 (2)	1.0 (1)
Gly			11.8 (12)	21.7 (23)	34.9 (35)
Thr			7.0 (7)	21.9 (22)	26.4 (26)
Ala			12.6 (13)	22.3 (22)	17.2 (16)
Pro	1.0 (1.1)	2.0 (2.0)	2.1 (2)	8.9 (9)	14.8 (15)
Tyr	0.9 (1.0)	2.0 (2.0)	2.9 (3)	4.0 (4)	13.7 (16)
Arg	1.0 (1.0)	2.0 (2.0)	11.0 (11)	4.3 (4)	2.3 (2)
Val	0.9 (0.9)		5.6 (6)	20.4 (23)	19.9 (22)
Ile	0.9 (0.9)	1.0 (1.0)	5.5 (6)	9.0 (10)	21.9 (26)
Leu		2.0 (2.0)	8.0 (8)	19.8 (19)	25.8 (26)
Phe	1.1 (1.0)		3.1 (3)	6.4 (6)	13.8 (14)

* Expected Values given in parentheses (peptide sequences from suppliers data and Protein compositions from Swiss protein data bank)

** Asp + Asn

*** Glu + Gln

In routine analysis of protein hydrosylate samples, lower µM concentrations are achievable with fluorescence detection. UV detection is typically 25 times less sensitive than fluorescence, but is useful for analysis in the higher µM concentration range and the determination of tryptophan and cystine which form non-fluorescent FMOC derivatives.

Summary

The new FMOC method enables common protein amino acids to be accurately determined in lower µM concentrations. This methodology is easy to execute and is applicable to both manual and automated derivatisation. The method delivers stable adducts, is linear and reproducible within the stated concentration range. Its viability has been confirmed by the analysis of various hydrolysate samples.

'...the reproducibility of the retention time was less than or equal to 0.5%...'

GBC HPLC Instrumentation

LC1150 Quaternary Gradient Pump
LC1150 Oven Option
LC1250 Fluorescence Detector
LC1650 ACSIS Advanced Autosampler
WinChrom 1-2 Data Management System*

(*plus compatible 486 PC & accessories)

Conditions

Column : Hypersil Column for Amino Acid Analysis, 150 x 4.6 mm ID

Fluorescence Detector:
Ex: 270 nm
Em: 316 nm

Mobile Phase A: 30 mM Ammonium Phosphate (pH 6.5) in 15% Methanol/85% Water

Mobile Phase B: 15% Methanol / 85% Water

Mobile Phase C: 90% Acetonitrile / 10% Water

Equilibration: 5 minutes

Flow Rate: 1.00 ml/min

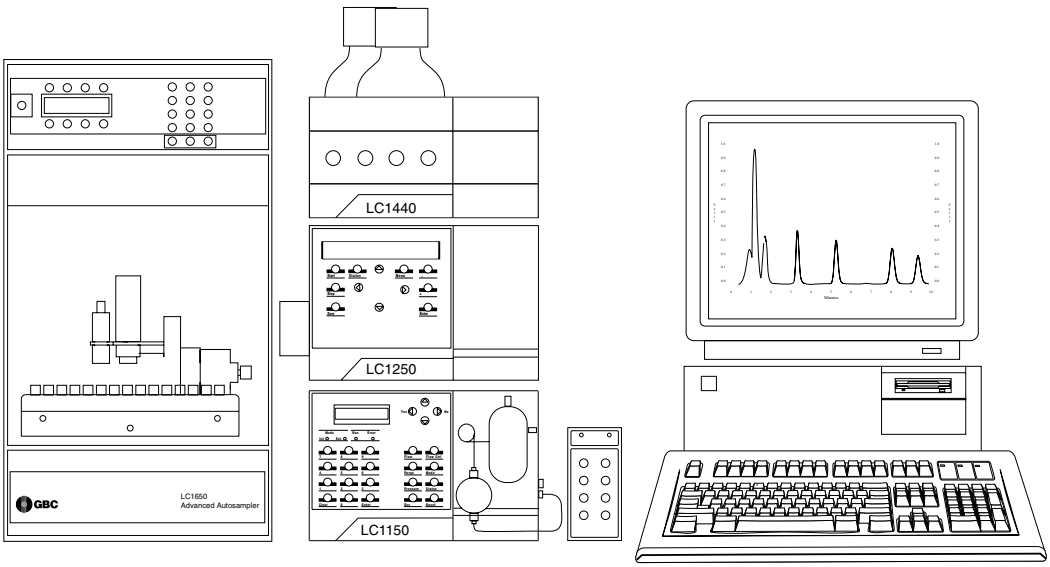
Temperature: 38ºC

Injection Vol. : 5 ul

References

1. P.A. Haynes, D. Sheumack, J. Kibby and J.W. Redmond, J. Chromatogr., 499 (1990) 557.

2. P.A. Haynes, D.Sheumack, L.G. Greig, J. Kibby and J.W. Redmond, J. Chromatogr., 588 (1991) 107.



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GBC publication number 01-0344-00
September, 1995