BUTTERWORTH LABORATORIES
science on demand

Amino Acid Analysis By HPLC

Amino Acid Analysis by High Performance Liquid Chromatography (HPLC) using Post-Column Derivatisation with Ninhydrin and Dual Wavelength Detection

The European Pharmacopeia (Ph Eur) moved away from the historical Thin Layer Chromatography technique (TLC) for the determination of Ninhydrin-positive substances, to more specific and sensitive methods utilising Liquid Chromatography (LC) or Amino Acid Analysers in 2015. This paper will explore the history of the test, the benefits of the change and considerations for validation of the LC approach to analysis.

The Ph Eur has to date published monographs for 17 amino acids (Table 1) adopting method 1 with post-column Ninhydrin derivatisation as the analytical procedure required for the determination of the Ninhydrin-positive substances, in place of the previous TLC procedure. Whilst the focus has been on the 17 monographs in Table 1, there are a further 11 monographs employing the 2.2.56 General Monograph for Amino Acid Analysis (Table 2), which do not involve Ninhydrin derivatisation but other Pre- and Post-column techniques. Dependent upon client interest, these would be subject to future validation/verification by Butterworth Laboratories Ltd.

Ninhydrin is a chemical used to detect Ammonia or primary and secondary amines. When reacting with primary amines, a deep blue or purple colour known as Ruhemann's purple is produced, whilst secondary amines produce a yellow colour. Most amino acids and amines are hydrolyzed and react with Ninhydrin in this way.

The TLC method uses Ninhydrin as the spray reagent to allow visualisation of the amino acids. Identification of the amino acid is confirmed by running a certified reference standard against the test sample with the Retardation factor (Rf) being used for comparison. A reference solution equivalent to 0.5% of the quantity of amino acid on test is also run alongside and used as a limit of any impurities present in the form of Ninhydrin-positive substances. Whilst this method is adequate for Identification and Limit Test purposes, the unreliability for related substances detection and the subjective nature of visual spot intensity comparison can be overcome by using LC and Amino Acid Analysers which are able to introduce increased accuracy and specificity to the analysis.

To accommodate the change in the Ph Eur requirements at Butterworth Laboratories Limited, the three approaches available for amino acid analysis were: the purchase of a dedicated Amino Acid Analyser, analysis by Ion Chromatography (IC) with Pulsed Amperometric Detection (PAD) or adaption of an existing HPLC system with the necessary post- column derivatisation pump and reactor.

Given the investment costs and timescale requirements of purchasing, qualifying and validating a new dedicated amino acid analyser, this option was ruled out fairly early on. Whilst the necessary equipment for using IC with PAD was already available, this option was ruled out due to the requirement of method 1 in Ph Eur 2.2.56 for post-column Ninhydrin derivatisation. This was considered essential in maintaining continuity and comparability between results obtained using TLC and HPLC. It was therefore decided to purchase a high temperature post-column derivatisation system to use in conjunction with an existing HPLC system with Diode Array Detection (DAD). This also significantly reduced requirements for training on the system and software validation, speeding up the commercial availability of the method.

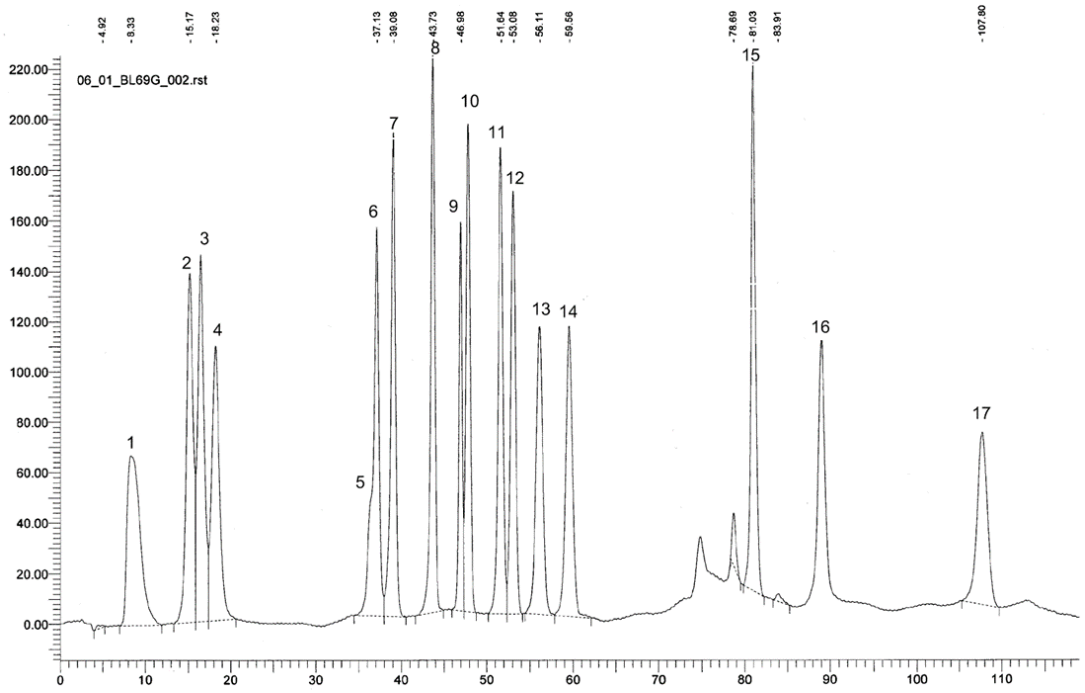
Having decided on the HPLC option the next stage was to select which system best met the requirements. Metrohm already had an application note for the analysis of amino acids using post-column derivatisation with Ninhydrin reagent. Unlike other published methods this uses a gradient with two buffers (some gradients involve four or more different buffers and a column flush with Lithium Hydroxide). Lithium Hydroxide is corrosive to injection valve seals and other materials used in the construction of a standard HPLC system, and it was not considered essential for the Ninhydrin-positive substances test. The Metrohm system enables preparation of the necessary buffers from standard laboratory chemicals, whereas other commercial systems employ proprietary buffers. Ninhydrin reagent is notoriously unstable so it was decided to purchase the pre-prepared Biochrom EZ Nin reagent which had recently become commercially available.

Having installed and validated the instrumentation, validation of the published application was undertaken. It soon became clear that there were going to be issues with the sensitivity of the responses in meeting the monograph specifications. Literature searches indicated that whilst the application uses a reaction temperature of 120°C, most others use a temperature of 130°C. To make this change meant investing in a further reactor, which brought about the necessary sensitivity, without the need to increase injection volumes causing potential column overload.

During the early stages of the validation we were also aware that the 10cm column proposed by the instrument manufacturer, was not producing the necessary separation of all the amino acids and in particular Isoleucine and Leucine. Our solution to this problem has been to have a slightly longer column custom made using the same stationary phase.

Having established a working HPLC method, work began verifying the method for specific test items. Working with clients the following list of substances were prioritised, based on TLC work previously undertaken and recent enquiries. Arginine HCl; Histidine; Lysine HCl; Phenylalanine; Serine; Threonine; Tryptophan; Isoleucine and Valine. If requests for other substances are received from clients, then verification work will be carried out as and when required.

The verification for each substance covers Accuracy, Precision and Linearity together with Detection and Quantitation Limits as well as ensuring that the Ph Eur 2.2.56 General Monograph system suitability requirements for method 1 can be met. An example chromatogram is shown below



1 Aspartic acid
2 Serine
3 Threonine
4 Glutamic acid
5 Proline
6 Glycine
7 Alanine
8 Valine
9 Cystine
10 Methionine
11 Isoleucine
12 Leucine
13 Tyrosine
14 Phenylalanine
15 Lysine
16 Histidine
17 Arginine

Table 1

Monograph Title	Version Date	Monograph No.
Alanine	07/2014	0752
Arginine	07/2014	0806
Arginine hydrochloride	07/2014	0805
Cysteine hydrochloride monohydrate	01/2014	0895
Glycine	07/2015	0614
Histidine	07/2014	0911
Histidine hydrochloride monohydrate	07/2014	0910
Isoleucine	07/2013	0770
Leucine	07/2013	0771
Lysine hydrochloride	07/2013	0930
Phenylalanine	07/2014	0782
Proline	01/2014	0785
Serine	01/2014	0788
Threonine	01/2014	1049
Tryptophan	01/2015	1272
Tyrosine	07/2014	1161
Valine	01/2014	0796

Table 2

Monograph Title	Version Date	Monograph No.
Buserelin	07/2011	1077
Calcitonin salmon	01/2008	0471
3-O-Desacyl-4 - monophosphoryl lipid A	07/2011	2537
Desmopressin	07/2009	0712
Felypressin	01/2008	1634
Goserelin	01/2013	1636
Leuprorelin	01/2008	1442
Oxytocin	01/2008	0780
Oxytocin concentrated solution	01/2008	0779
Somatostatin	04/2014	0949
Tetracosactide	04/2010	0644

Author Biographies

Frank Judge BSc (Hons) CChem MRSC MChromSoc CSci
Consultant Chemist Chromatography

Frank started his career at Kings and Co Ltd as a Senior Technician before joining Berridge Environmental Labs as Organic Analysis Team Leader in 1990. After a short spell with Pharmaco LSR in their Department of Aquatic Toxicology Studies, he joined the Chromatography Department of Butterworth Laboratories in 1994 and has progressed through various roles to his current position.

Frank has spoken at JPAG meetings on Organic Volatile Impurities analysis and has been trained in preparing expert witness statements.

Derek Sharman CChem MRSC
Senior Analytical Chemist

Derek began his career at BP Chemicals as a Laboratory Assistant moving via Beecham Pharmaceuticals and Rentokil Laboratories before becoming Senior Scientist at SmithKline Beecham Consumer Healthcare for 17 years. He joined Butterworth Laboratories Ltd in 1996 and has continued to develop the HPLC capabilities in Method Development and Validation.

We welcome discussion and feedback. If you wish to get in touch with us, just contact info@butterworth-labs.co.uk or click the Enquiry button on our website.

If you wish to discuss your contract analytical chemistry requirements, just drop us a line at marketing@butterworth-labs.co.uk