a Butterworth Laboratories

WHITEPAPER

Determination of Ninhydrin-Positive Substances in PhEur Amino Acid Monographs

For decades before 2013, each of the 28 free amino acid (AA) monographs of the European Pharmacopoeia (PhEur) included a thin layer chromatography (TLC) test for the determination of ninhydrin-positive substances (NPS). Beginning in 2013, to address the shortcomings of the TLC test, it has been progressively replaced in monographs by a high-performance liquid chromatography (HPLC) procedure. AA sample components are separated using cation exchange HPLC. The resolved AAs have little UV activity and therefore the mobile phase containing resolved sample components exiting the HPLC column is mixed with ninhydrin reagent and passed through a heated reaction module where the AA react with the ninhydrin, giving coloured derivatives, which can be assessed using UV detection and hence the name NPS for the test.

In pharmacopeial terms, the revision of individual amino acid monographs to replace the TLC NPS test with HPLC has been carried out extremely quickly. To date, 21 AA monographs now prescribe HPLC. Where HPLC has been included in individual AA monographs, these contain all the instructions for preparing the required analytical solutions. All monographs have the same explicit requirement for the resolution of iso-Leucine and Leucine (NLT1.5) to demonstrate system suitability. However, the universal HPLC system suitability requirements addressing sensitivity and peak symmetry stipulated in the Chromatographic Separation Techniques general monograph 2.2.46 must also be fulfilled. Neither the AA Monographs nor the general monograph 2.2.56 AA Analysis contain any details about the chromatographic system or conditions other than that cation exchange HPLC chromatography with post-column ninhydrin derivatisation should be used.

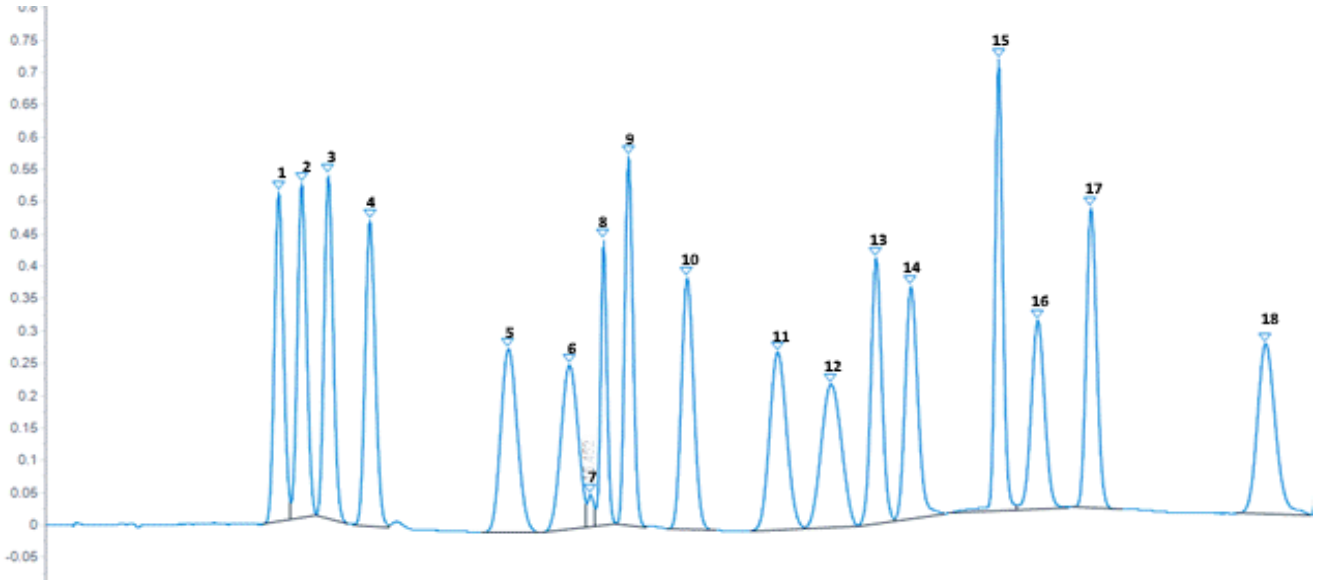
As per best practice, Butterworth Laboratories Ltd. (BLL) conduct verification exercises for the HPLC NPS test for individual AAs before any routine analysis requested is undertaken. The verification performed for each AA covers accuracy, precision, linearity, specificity (including demonstration of resolution, detection and quantitation limits and demonstrates BLL's ability to deliver valid analytical results for clients. As shown in the table below, BLL has already completed the verification for 13 of the 21 AA monographs requiring HPLC, with a further three scheduled for early 2024.

PhEur Free AA Monographs Requiring NPS Testing with BLL Verification Status

Monograph Title	Version Date	Monograph No.	BLL Verified
Alanine	01/2017	0752	Yes
Arginine*	01/2017	0806	Yes
Arginine hydrochloride	08/2019	0805	Yes
Aspartic acid	01/2018	0797	Yes
Cysteine hydrochloride monohydrate	08/2019	0895	Q4 2024
Cystine	01/2019	0998	Yes
Glycine	02/2024	0614	Yes
Histidine *	01/2017	0911	Yes
Histidine hydrochloride monohydrate	08/2019	0910	Yes
Isoleucine	01/2017	0770	Yes
Leucine	01/2017	0771	Yes
Lysine acetate	07/2023	2114	No
Lysine hydrochloride	01/2017	0930	Yes
Magnesium aspartate dihydrate	08/2019	1445	No
Phenylalanine	01/2017	0782	Yes
Proline	01/2017	0785	Yes
Serine	01/2017	0788	No
Threonine	01/2017	1049	Yes
Tryptophan	01/2017	1272	Yes
Tyrosine	01/2017	1161	No
Valine	01/2017	0796	No

*Note that verification has been performed on the salt/hydrated forms but is inferred on the base/anhydrous forms due to dissociation in solution

Historically, BLL had configured an existing HPLC system with a post-column reaction unit for the analysis; however, in January 2020, a Hitachi LA8080 High-Speed Amino Acid Analyser (AAA) was commissioned. This dedicated system has delivered many benefits, including simplicity of use, enabling an increased number of staff trained in its operation and reducing instrument downtime. BLL now performs routine testing for many clients, and all generated data complies with UK Good Manufacturing Regulations (GMP).



A typical mixed primary amine standard chromatogram produced by BLL. Note: Resolution of of iso-Leucine(11) & Leucine(12) is 1.5

Peak No.	Peak Identity	Peak No.	Peak Identity	Peak No.	Peak Identity
1	Aspartic Acid	7	Unknown	13	Tyrosine
2	Threonine	8	Cystine	14	Phenylalanine
3	Serine	9	Valine	15	Lysine
4	Glutamic Acid	10	Methionine	16	Ammonium
5	Glycine	11	iso-Leucine	17	Histidine
6	Alanine	12	Leucine	18	Arginine

The development of large numbers of peptide-based pharmaceuticals has spurred a renewed appreciation for the value of this analytical technique. As shown below, the PhEur already includes 12 peptide monographs.

Ph.Eur Peptide Monographs Requiring NPS Testing					
Monograph Title	**Version Date**	**Monograph No.**	**Monograph Title**	**Version Date**	**Monograph No.**
Buserelin	01/2016	1077	Octreotide	04/2023	2414
Desmopressin	07/2009	0712	Oxytocin	07/2023	0780
Felypressin	01/2008	1634	Oxytocin concentrated solution	01/2008	0779
Gonadorelin acetate	01/2013	0827	Smatostatin	07/2021	0949
Goserelin	01/2013	1636	Terlipressin	07/2017	2646
Leuprorelin	01/2008	1442	Tetracosactide	04/2010	0644

The analysis of these peptides requires initial sample hydrolysis to break the peptide links and release free AAs, which are then quantified in accordance with PhEur 2.2.56, AA Analysis Method 1. However, this will be the subject of a future Whitepaper when Butterworth has verified the sample preparation procedures. While not verified by BLL, our instrument manufacturer has published a procedure that resolves all 42 physiological AAs, including all proteins constituting AAs.

In theory, when coupled with the sample preparation methods described in the PhEur, it may be possible to develop procedures that would provide fully quantitative determinations of the AA composition of all AAs, peptides and proteins. BLL would be happy to discuss with clients the possibilities of the development of such analytical procedures applicable to all three classes of materials.

Note: The list of Verified Materials is at the time of writing. An up-to-date list can be found on the Applications page of our website under Ninhydrin-Positive Substances.

Author Biography

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Consultant Chemist, Chromatography

Frank started his career at Kings and Co. as a Senior Technician before joining Berridge Environmental Labs as an Organic Analysis Team Leader in 1990. After a short spell with Pharmaco LSR in its 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 and Making Pharmaceuticals exhibitions on various. chromatography subjects