PICKERING LABORATORIES
CATALYST FOR SUCCESS

AMINO ACID ANALYSIS ACCORDING TO EUROPEAN PHARMACOPOEIA 8.0.

The **European Pharmacopoeia** (**Ph. Eur.**) defines requirements for the qualitative and quantitative composition of medicines, as well as the tests to be carried out on medicines and on substances and materials used in their production.

It covers active substances, excipients and preparations of chemical, animal, human or herbal origin, homoeopathic preparations and homoeopathic stocks, antibiotics, as well as dosage forms and containers. It also includes tests on biologicals, blood and plasma derivatives, vaccines and radiopharmaceutical preparations. The European Pharmacopoeia and its requirements are legally binding in the member states of the European Pharmacopoeia Convention and the European Union.

All manufacturers of medicines or substances for pharmaceutical use therefore must apply the Ph. Eur. quality standards in order to be able to market and use these products in Europe.

Amino Acids Analysis can be used for:

- Identification tests on biopharmaceutical active ingredients (e.g. peptides, proteins) by means of amino acids composition analysis;
- Impurities and related substances determination on APIs (Active Pharmaceutical Ingredients, e.g. free amino acids) and intermediates;
- Single or total amino acids quantification in drug products, including markers determination in complex matrixes (e. g. phytopharmaceuticals).

The following Ph. Eur. monographs have already officially introduced the Amino Acid Analysis method with post-column Ninhydrin derivatization as the analytical procedure required for the determination of the Ninhydrin-positive substances, and additional papers are expected to be published in upcoming months:

- Cysteine HCl Monohydrate 01/2014:0895
- Isoleucine 07/2013:0770
- Leucine 07/2013:0771
- Lysine HCl 07/2013:0930
- Serine 01/2014:0788
- Proline 01/2014:0785
- Threonine 01/2014:1049
- Valine 01/2014:0796
- Arginine 07/2014:0806

Pickering Laboratories, Inc. offers a complete solution for Amino Acids Analysis according to European Pharmacopoeia 8.0. This includes the Pinnacle PCX post-column derivatization instrument, analytical columns and GARDs, buffers and Trione® Ninhydrin reagent. The Pinnacle PCX is capable of performing column temperature gradients that allow easily modified conditions and improved run times and amino acids separations. The methods presented in this application note were optimized to comply with system suitability requirements of Pharmacopoeia 8.0 methods.

Each Pharmacopoeia monograph describes the preparation of the test and reference solutions specific for each amino acid. The solutions are used for calculations of percentage contents, impurity levels as well as parameters of system suitability. Resolution of 1.5 is required between Leucine and Isoleucine peaks.

Table 1 summarizes the solutions used in each monograph.

Table 1. Reference guide for Pharmacopoeia 8.0 methods

		REFERENCE SOLUTIONS, UG/ML												
Amino acids	**Test solutions ug/mL**	**Cys**	**CSSC**	**Lys**	**Ser**	**Thr**	**Val**	**Ala**	**Arg**	**Leu**	**Ile**	**Ile and Leu**	**Pro**	**NH4**
Valine	600						1.2				1.2	3 each	1.2	0.12
Proline	600							0.6				3 each	1.2	0.12
Leucine	600 and 24									1.2	0.12	3 each	1.2	0.12
Threonine	600					1.2						3 each	1.2	0.12
Serine	600				1.2							3 each	1.2	0.12
Lysine	600			1.2								3 each	1.2	0.12
Isoleucine	600						1.2			1.2	1.2	3 each	1.2	0.12
Arginine	600							1.2	1.2			3 each	1.2	0.12
Cysteine	600	1.2	1.2									3 each	1.2	0.12

Cys = Cysteine, CSSC = Cystine, Lys = Lysine, Ser = Serine, Thr = Threonine, Val = Valine, Ala = Alanine, Arg = Arginine, Ile = Isoleucine, Leu = Leucine, Pro = Proline, NH4 = ammonia

For all amino acids, except Cysteine, Sodium-based and Lithium-based methods are available. For Cysteine analysis, only Lithium-based methods are suitable. Sodium-based methods have shorter run times and are preferable for all amino acids except Cysteine.

Methods using High-efficiency Sodium column for analysis of following Amino Acids:

Valine, Proline, Leucine, Isoleucine, Serine, Threonine, Lysine, Arginine

Analytical Conditions

Column: High-efficiency Sodium cation-exchange column, 4.6 x 110 mm, Catalog Number 1154110T

Flow Rate: 0.6 mL/min

Mobile Phase: See method in Table 2

Injection Volume: 50 uL

Post-column Conditions

Post-Column System: Pinnacle PCX

Reactor Volume: 0.5 mL

Reagent: Trione®

Reagent Temperature: 130 °C

Column Temperature: See method in Table 3

Flow Rate: 0.3 mL/min

Detection: UV/VIS 570 nm for primary amino acids, 440 nm for secondary amino acids

Table 2. HPLC program for column 115411T

TIME	NA315	NA425	NA640	RG011
0	100	0	0	0
4	100	0	0	0
23	10	90	0	0
24	0	0	100	0
42	0	0	100	0
42.1	0	0	0	100
45	0	0	0	100
45.1	100	0	0	0
55	100	0	0	0

Table 3. Column oven program

TIME	TEMP, C
0	42
4	42
12	60
23	60
35	70
43	70
44	42

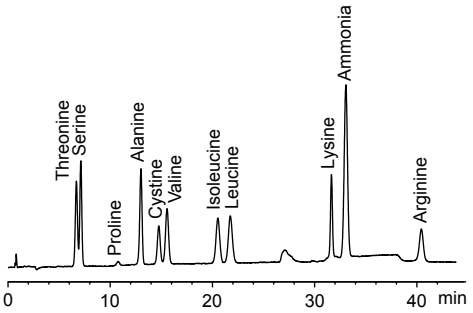

Fig. 1: Sodium chromatogram of amino acids analyzed using Pharmacopeia 8.0 methods (3 ug/mL each, 50 uL injection).

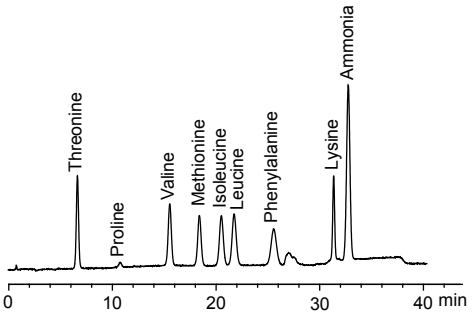

Fig. 2: Sodium chromatogram of alternative amino acids analyzed using Pharmacopeia 8.0 methods (3 ug/mL each, 50 uL injection).

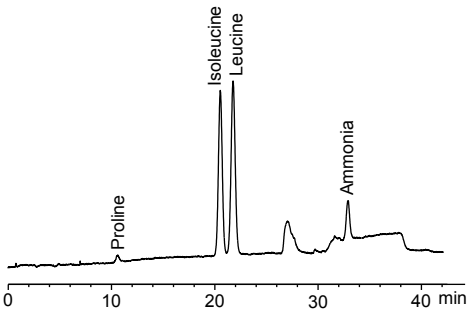

Fig. 3: Sodium chromatogram of amino acids used for calculations and system suitability check in Pharmacopeia 8.0 methods (refer to Table 1). Proline – 1.2 ug/mL; Isoleucine – 3 ug/mL; Leucine – 3 ug/mL; Ammonia – 0.12 ug/mL. Injection volume – 50 uL.

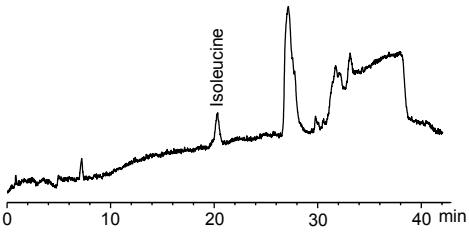

Fig. 4: Sodium chromatogram of low level Isoleucine reference solution – 0.12 ug/mL. Injection volume 50 uL

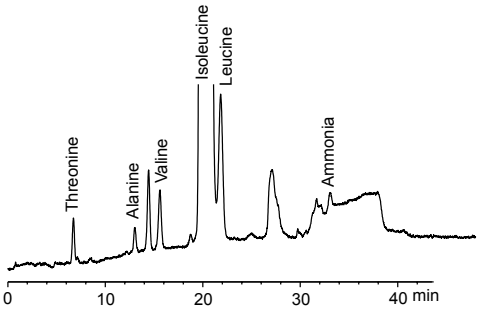

Fig. 5: Sodium chromatogram of Isoleucine test solution 600 ug/mL (zoomed). Injection volume 50 uL

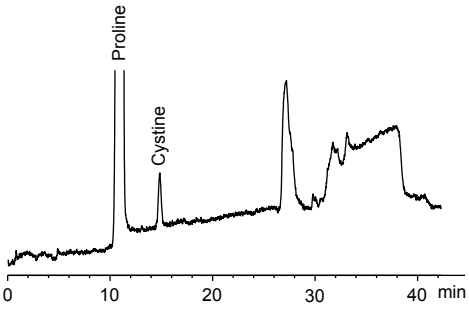

Fig. 6: Sodium chromatogram of Proline test solution 600 ug/mL (zoomed). Injection volume 50 uL

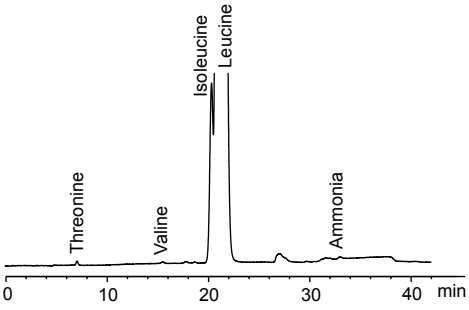

Fig. 7: Sodium chromatogram of Leucine test solution 600 ug/mL (zoomed). Injection volume 50 uL

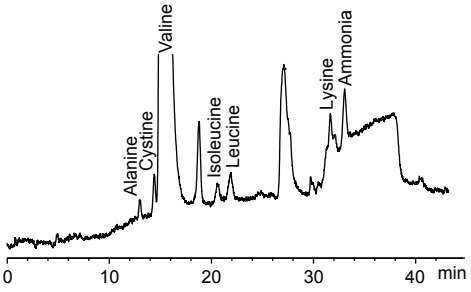

Fig. 8: Sodium chromatogram of Valine test solution 600 ug/mL (zoomed). Injection volume 50 uL

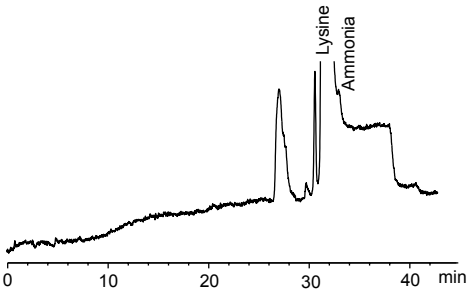

Fig. 9: Sodium chromatogram of Lysine test solution 600 ug/mL (zoomed). Injection volume 50 uL

Due to the poor separation of Cysteine and Proline peaks with the Sodium-based method, the Lithium High-efficiency column needs to be used for Cysteine analysis according to Pharmacopeia 8.0. The same Lithium method can be used for all other amino acids, though analysis time is longer than with the Sodium method.

Methods using High-efficiency Lithium column for analysis of following amino acids:

Cysteine, Valine, Proline, Leucine, Isoleucine, Serine, Threonine, Lysine, Arginine

Analytical Conditions

Column: High-efficiency Lithium cation-exchange column, 4.6 x 75 mm, Catalog Number 0354675T

Flow Rate: 0.55 mL/min

Mobile Phase: See method in Table 4

Injection Volume: 50 uL

Post-column Conditions

Post-Column System: Pinnacle PCX

Reactor Volume: 0.5 mL

Reagent: Trione®

Reagent Temperature: 130 ºC

Column Temperature: See method in Table 5

Flow Rate: 0.3 mL/min

Detection: UV/VIS 570 nm for primary amino acids, 440 nm for secondary amino acids

Table 4. HPLC Program for column 0354675T

TIME	1700-1125	LI365	LI375	RG003
0	100	0	0	0
15	100	0	0	0
35	40	60	0	0
38	0	100	0	0
43	0	100	0	0
43.1	0	0	100	0
57	0	0	100	0
57.1	0	0	0	100
60	0	0	0	100
60.1	100	0	0	0
72	100	0	0	0

Table 5. Column oven program

TIME	TEMP, C
0	34
6	34
30	45
32	70
59	70
60	34

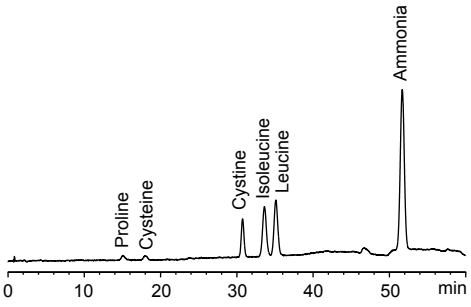

Fig. 10: Lithium chromatogram of amino acids used as reference solutions for Cysteine analysis (3 ug/mL each, 50 uL injection).

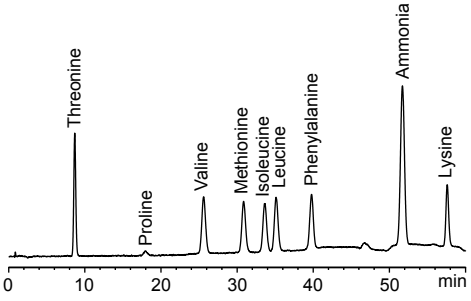

Fig. 11: Lithium chromatogram of amino acids analyzed using Pharmacopeia 8.0 methods (3 ug/mL each, 50 uL injection).

To make it easier to start using Pickering Laboratories methods, we offer chemistry kits that include: analytical column, GARD, buffers and reagents for Amino Acids Analysis. All parts of the kit could be ordered individually if needed. Please contact Pickering Laboratories if you have any questions regarding this application.

PART NUMBER	DESCRIPTION
0352-0057	**30-Minute High Efficiency Protein Hydrolysate Kit (for Sodium methods):**
1154110T	30-Minute Sodium Cation-exchange Column 4.6 x 110 mm & 1700-0070 amino acid test mixture
1700-3102	Cation-exchange GARD™ assembly: Holder w/ 2 replaceable GARD™
Na220	Sodium Diluent, pH 2.20, 4 x 250 mL
Na315	Sodium Eluant, pH 3.15, 4 x 950 mL
Na425	Sodium Eluant, pH 4.25
Na640	Sodium Eluant, pH 6.40
RG011	Sodium Column Regenerant, 950 mL
T100C	TRIONE® Ninhydrin Reagent, 4 x 950 mL (4-month shelf life)
012506H	Sodium Calibration Standard, for protein hydrolysate, 0.25 µmole/mL, 5 mL
0352-0058	**Kit Identical to 0352-0057 with T200 replacing T100C:**
T200	TRIONE® Two-part Ninhydrin Reagent, prepares 4 x 900 mL (12-month shelf life)
0352-0006	**70-minute Physiologic Fluid/Native Sample Kit (for Lithium methods):**
0354675T	Lithium Ion-exchange Column, 4.6 x 75 mm (with test mixture 1700-0070)
1700-3102	Cation-exchange GARD™ assembly: Holder w/ 2 replaceable GARD™
1700-1125	Lithium Eluant, pH 2.80, 4 x 950 mL
Li220	Lithium Diluent, pH 2.20, 4 x 250 mL
Li365	Lithium Diluent, pH 3.65, 4 x 950 mL
Li375	Lithium Diluent, pH 3.75, 4 x 950 mL
RG003	Lithium Column Regenerant, 950 mL
1700-0170	Lithium Calibration Standard, without Norleucine and AGPA, 0.25 µmole/mL, 5 mL
SP100	SERAPREP™, 250 mL
UP100	URIPREP™, 250 mL
T100C	TRIONE® Ninhydrin Reagent, 4 x 950 mL (3-month shelf life)
0352-0007	**Kit Identical to 0352-0006 with T200 replacing T100C:**
T200	TRIONE® Two-part Ninhydrin Reagent, prepares 4 x 900 mL (12-month shelf life)

Pickering Laboratories will keep updating its Pharmacopeia 8.0 methods as new monographs are released. Please contact support@pickeringlabs.com for the latest methods and chromatograms.

PICKERING LABORATORIES
1280 Space Park Way / Mountain View, CA 94043
sales@pickeringlabs.com / support@pickeringlabs.com
800-654-3330 / 650-694-6700 / Fax: 650-968-0749