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Plasma Amino Acid Analysis by LC-MS/MS

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@rachblacklabel

Clinical requirements of plasma amino acid analysis?

"Always interested in timely result, especially same day in acutely unwell patient. Needs to be accurate. Cost is secondary"

"TAT and a rapid screen for treatable amino acidopathies"

"Clear, unambiguous interpretation of the results"

Analytical requirements for Amino Acid Analysis?

- Accurate
- Precise
- Traceable
- Robust method
- Capable of rapid TAT
- UKAS accredited
- UKCA

METBIONET GUIDELINES FOR AMINO ACID ANALYSIS.

ACMG TECHNICAL STANDARD | Genetics inMedicine

Laboratory analysis of amino acids, 2018 revision: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

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Disclaimer This laboratory standard is designed primarily as an educational resource for clinical laboratory geneticists to help them provide quality clinical laboratory genetic services. Adherence to this standard is voluntary and does not necessarily assure a successful medical outcome. This standard should not be considered inclusive of all proper procedures and tests or exclusive of other procedures and tests that are reasonably directed to obtaining the same results. In determining the propriety of any specific procedure or test, the clinical laboratory geneticist should apply his or her own professional judgment to the specific circumstances presented by the patient or specimen.

Clinical laboratory geneticists are encouraged to document in the patient's record the rationale for the use of a particular procedure or test, whether or not it is in conformance with this standard. They also are advised to take notice of the date any particular standard was adopted, and to consider other relevant medical and scientific information that becomes available after that date. It also would be prudent to consider whether intellectual property interests may restrict the performance of certain tests and other procedures.

Amino acid abnormalities are observed in a broad spectrum of inherited metabolic diseases, such as disorders of amino acid metabolism and transport, organic acidemias, and ureagenesis defects. Comprehensive analysis of physiologic amino acids in blood, urine, and cerebrospinal fluid is typically performed in the following clinical settings: evaluation of symptomatic patients in whom a diagnosis is not known; evaluation of previously diagnosed patients to monitor treatment efficacy; evaluation of asymptomatic or presymptomatic (at-risk) relatives of known patients; follow-up testing for an abnormal newborn screen; and assessment of dietary protein adequacy or renal function in general patient populations. Currently, the most common analytical method to quantify amino acids is based on ion exchange chromatography using post-column derivatization with ninhydrin and spectrophotometric detection. Newer methodologies are based on liquid chromatographic separation with detection by mass spectrometry or spectrophotometry. Amino acid analysis by nonseparation methods, such as the flow injection–tandem mass spectrometric (MS/MS) method used for newborn screening, is considered inadequate for the diagnosis of at-risk patients. The purpose of this document is to provide a technical standard for amino acid analysis as applied to the diagnosis and management of inborn errors of metabolism.

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Keywords: clinical genetic testing; technical standards; amino acidopathies; amino acids

BACKGROUND

Amino acids and proteins

Amino acids serve as protein building blocks, metabolic intermediates, and substrates for energy production. By definition, amino acids contain an amino group and a carboxyl group, and often contain another functional group (e.g., sulfhydryl, hydroxyl, or secondary amino- or carboxyl-group). Proteins consist of 20 different amino acids, half of which are synthesized endogenously (nonessential), while the remaining amino acids are obtained from dietary sources (essential). For almost a century, the detection of amino acids depended on ninhydrin, a chemical that reacts specifically with primary and secondary amines to produce a purple color that can be measured spectrophotometrically. The development of the amino acid analyzer (based on ion exchange chromatographic separation of amino acids coupled with post-column ninhydrin derivatization) in the 1950s was an important advance that started large-scale investigations into

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The Board of Directors of the American College of Medical Genetics and Genomics approved this technical laboratory standard on 21 May 2018.

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Published online: 19 October 2018

Methods for plasma amino acid analysis

- GC-MS
- FIA-MS/MS
- HPLC (UV detection)
- LC-MS
- LC-MS/MS
- Ion exchange chromatography (UV detection)

Plasma amino acid methods

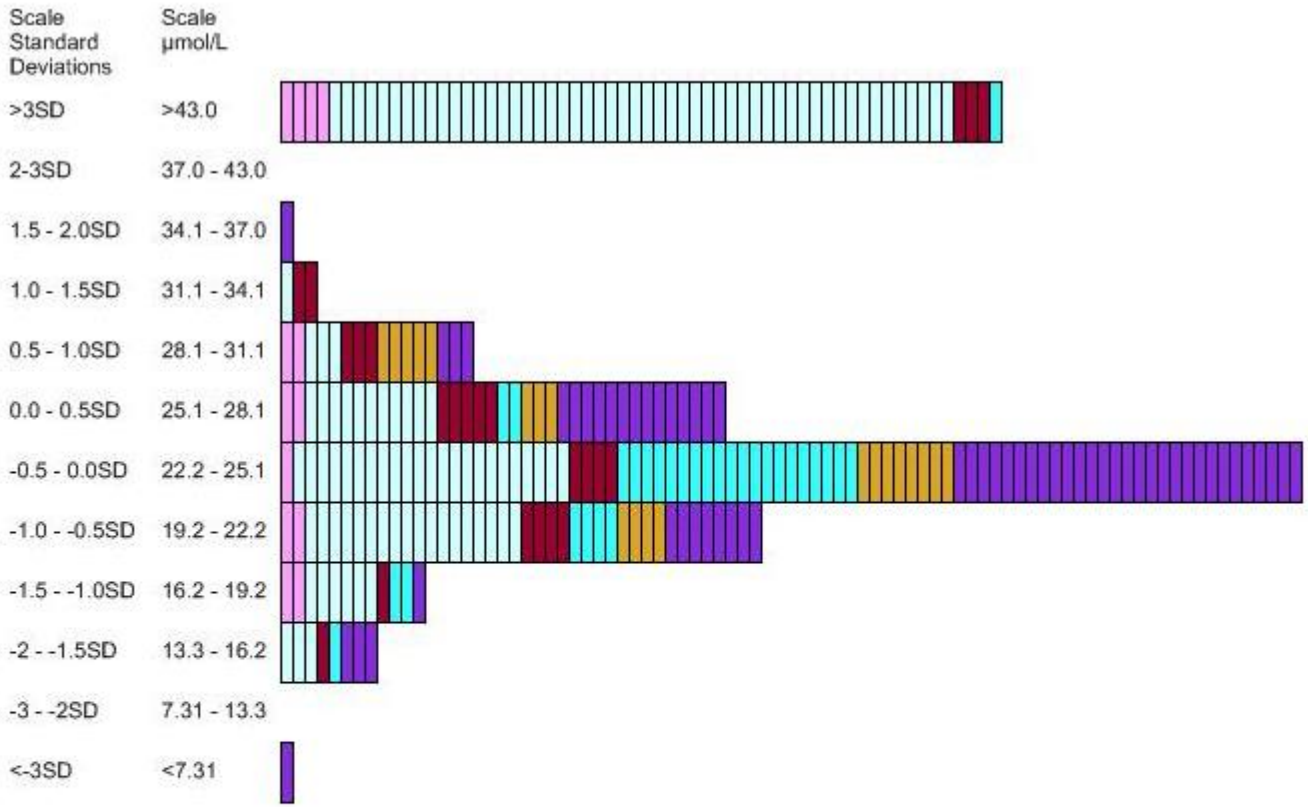
- Tried & tested method
- Stable, precise and sensitive
- Wide linear range
- Identifies all analytes of interest including atypical amino acids
- Minimal sample prep required - no derivatisation step
- Commercial kit (reagent rental)
- Suitable for analysis of AA in plasma, urine, CSF and DBS

Disadvantages of Ion Exchange Chromatography

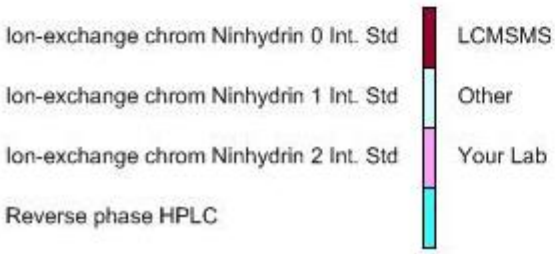
- Legacy method; essentially unchanged in 40 years
- Long analysis time ~ 120 min
- Dedicated instruments, typically running at capacity
- Operator expertise required

- Calibration is minimal, infrequent, typically single point
- Likewise IQC
- Structural analogue internal standard only
- Lacks selectivity
    - identification based on retention time alone
    - co-eluting substances interfere as do drugs/ninhydrin positive compounds
    - poor resolution of some analytes e.g. sulphocysteine

ERNDIM Phenylalanine Return

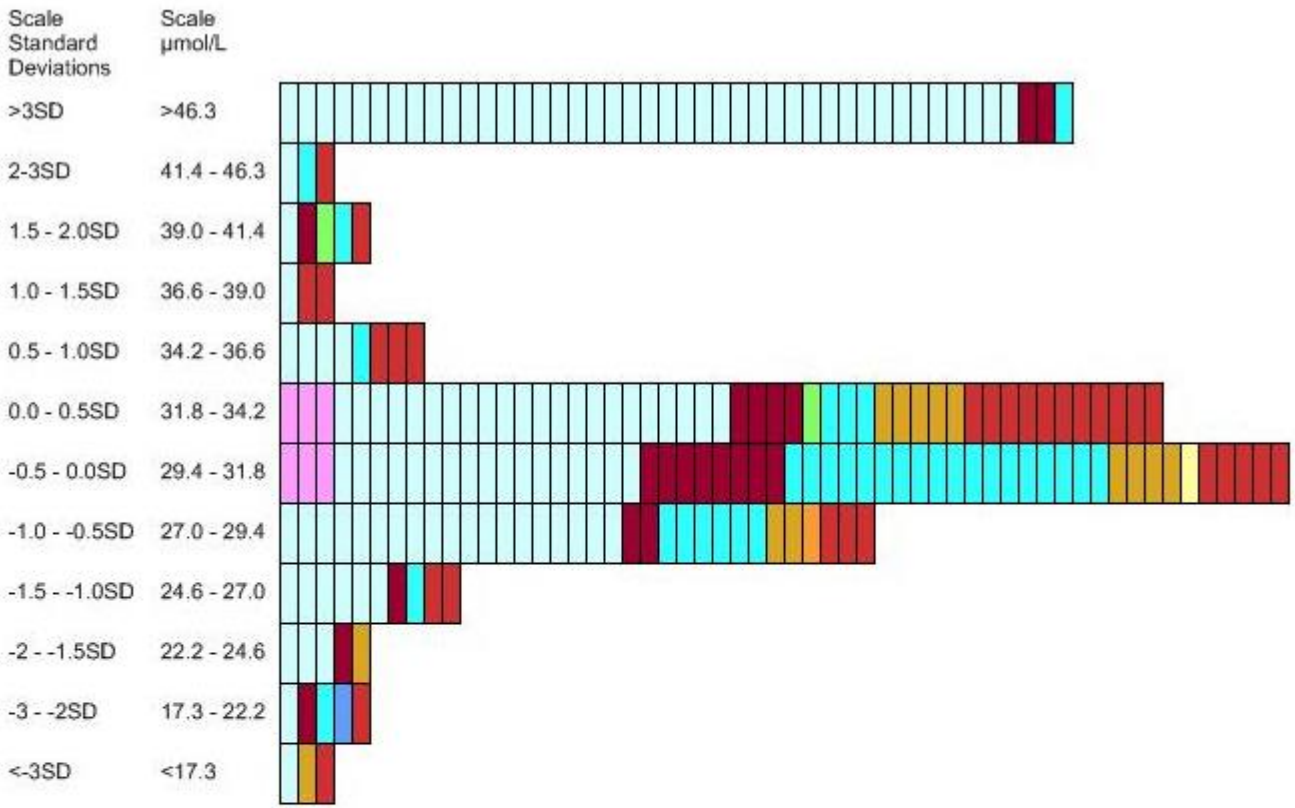


Co-elution of Phenylalanine & 5-ALA

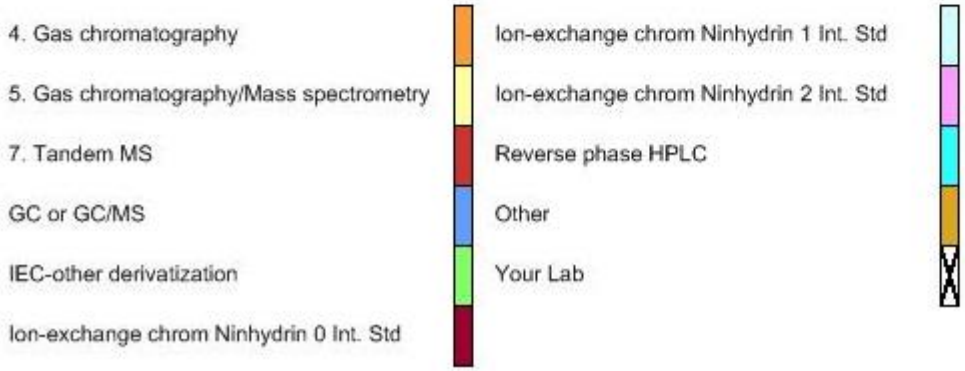


All Labs results	
n:	263
Mean:	25.1
Median:	25.0
SD:	5.95

ERNDIM Methionine Return



Co-eluting Compounds: Methionine & Homocitrulline



All Labs results	
n:	224
Mean:	31.8
Median:	31.8
SD:	4.82

Status Quo: Routinely Used Methods for Quantitative Plasma Amino Acid Analysis

Data courtesy of ERNDIM Quantitative Amino Acid Scheme

Method	2001	2007	2015	2016	2017	2019	2020	2021
LC-MS/MS	0	2.8	9.9	12.0	23.4	25.4	25.6	27.8
RP-HPLC	12.7	13.5	14.0	13.7	14.0	12.5	12.4	11.9
IEC	85.8	82.3	70.8	66.8	54.0	54.0	53.1	52.6
LC-MS					5.7	2.9	4.4	4.1
Other	2.3	1.1	5.3	6.6	3.0	5.2	4.4	3.7
Participants	*134*	*178*	*243*	*241*	*265*	*272*	*273*	*270*

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Limitations of early LC-MS/MS methods

- Relied upon derivatisation and/or use of ion pair reagent
- Interference from isobaric compounds (iso/leu/allo/hydroxyproline)
- Lacked sensitivity - limited number of AA in profile
- Challenging to optimise the method; sacrifice sensitivity and specificity of some analytes in preference for other others
- Operator expertise required
- Post-analytical data processing was complex
- Sourcing stable isotopes

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LC-MS/MS methods today

- Technology has improved in last 5 - 10 years
- Instruments have faster scan speeds/shorter dwell times/improved pos-neg switching
- A given experiment can contain more SRMs and still produce quantitative data
- Analysis of full amino acid profile is possible **without** sample derivatisation and/or use of an ion pair reagent
- Stable isotope IS readily available
- Commercial kits have been developed – don’t need to rely on LDT

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Advantages of LC-MS/MS

- Rapid analysis time e.g. 15-20 mins per sample
- Quantifies 45+ amino acids in single injection
- Superior selectivity; combination of SRM & chromatographic separation enables identification of most isobaric compounds
- Sensitivity comparable to IEC
- Utilises stable isotope internal standards

Cost of LC-MS/MS

- Generally considered to be more expensive than IEC
- Biochrom £60K vs LC-MS/MS £180K
- Analysis time is 20 minutes vs 125 minutes
- Analyse 4 times as many samples – easier to manage workload
- LC-MS/MS is open access – other assays can be performed

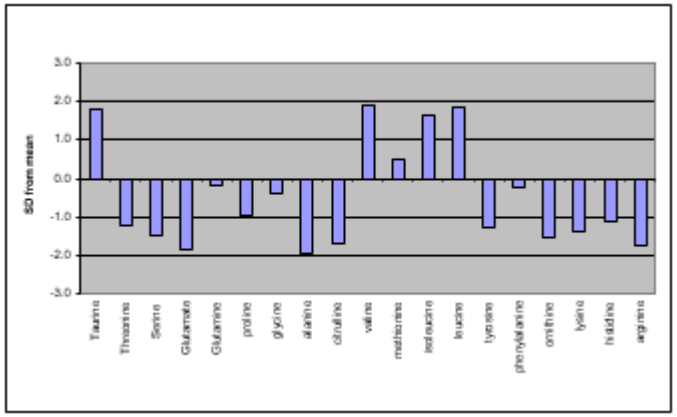
Viapath LC-MS/MS Amino Acid method

- Waters Xevo TQs with Acquity UPLC
- SpotOn reagents (IS, calibrators, column)
- 15-minute analysis time
- 6 time functions, 5 positive ionisation, one negative ionisation
- 37 analytes and 26 stable isotope internal standards
- Simple sample prep, no derivatization required
- Chirobiotic column with 50% Acetonitrile, 0.025% formic acid mobile phase
- 2 plus 1 calibration curve

- Sample prep; 10μL sample, 150μL methanolic IS. Vortex mix, centrifuge 10 min, transfer supernatant and inject

Challenges with LC-MS/MS

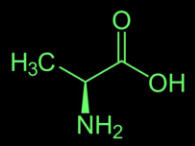
- Reagent volumes impractical e.g. 10uL IS
- Not all compounds have stable isotope IS e.g. ASA
- Sensitivity of smaller molecules e.g. glycine, alanine, proline
- Linearity is analyte dependent, some analytes non-linear at 600μM e.g. phe, arg, his (necessitates detune and/or alternative product ion
- Isobaric compounds not separated – iso/allo, ala/sarcosine
- Post analytical processing 'clunky'
- Operator expertise required
- Mindset change – numerical profile not chromatogram



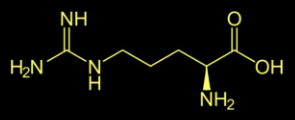
Conclusions

- Plasma amino acid analysis is challenging
- Multiple analytes, concentrations spanning 3 orders magnitude
- Important that labs understand the limitations of their method
- LC-MS/MS supersedes IEC as the gold standard method of plasma amino acid analysis

How amino acids got their names

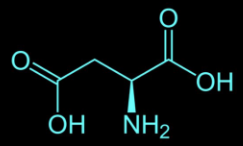


ALANINE
Al- is a shortening of *aldehyde*. The infix *-an-* was added to make it easier to pronounce.

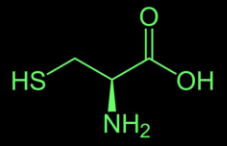


ARGININE
From the Greek word *arginóeis*, which meant "silver" due to the appearance of arginine nitrate.

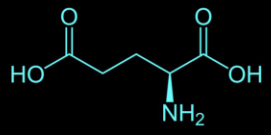
ASPARAGINE
First extrafted in 1806 from a sample of asparagus juice, after which it was named.



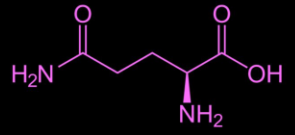
ASPARTIC ACID
Named after *asparagine*, because it was first isolated from it by hydrolysis in 1827.



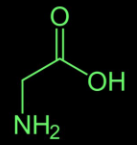
CYSTEINE
Had an earlier spelling of *cystine*. That comes from the Ancient Greek word for "bladder", *kústis*.



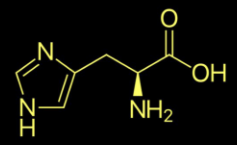
GLUTAMIC ACID
Glut- refers to how the compound was first isolated from gluten in 1866 by chemist Karl Ritthausen.



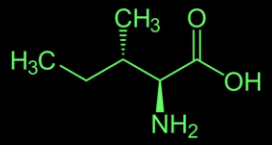
GLUTAMINE
Named before it was isolated, because it was hypothesized to be similar to *glutamic acid*.



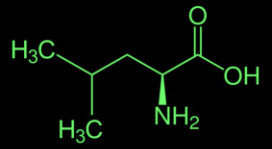
GLYCINE
From the Greek word *glukús*, meaning "sweet", because it was first isolated from gelatin.



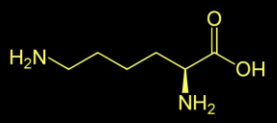
HISTIDINE
From Greek *histós*, meaning "tissue", because it was thought to be important to tissue function.



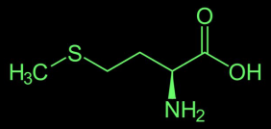
ISOLEUCINE
Named in 1904 by Felix Ehrlich, who observed that it was similar but not identical to leucine.



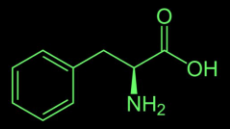
LEUCINE
First used in 1826 by chemist William Henry. Comes from the Greek word *leukós*, "white".



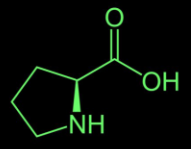
LYSINE
Named in 1889 from the Ancient Greek word *lúsis*, meaning "loosening".



METHIONINE
Coined in 1926 by Barger and Coyne as a contraction of *γ-methiol-α-aminobutyric acid*.



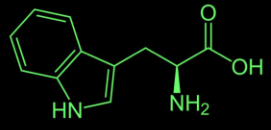
PHENYLALANINE
Named by Erlenmeyer and Lipp in 1883 because it looks like alanine with a phenyl group.



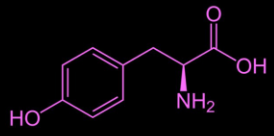
PROLINE
The name is a contraction of *pyrrolidine*, which makes up a side chain of the compound.

SERINE
From the Latin word *sericum*, meaning "silk", because it was first obtained from silk protein.

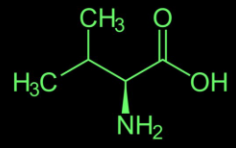
THREONINE
Named in 1936 after *threose*, a type of monosaccharide that it was thought to resemble.



TRYPTOPHAN
Traces to the Greek roots *tripsis*, meaning "rubbing", and *phainein*, meaning "to show".



TYROSINE
From Greek *tyrós*, meaning "cheese", because it was obtained from old cheese.



VALINE
Named in 1906 after a type of acid that occurs in the roots of the *valerian* plant.

Sources: etymonline.com, wiktionary.org, merriam-webster.com, oed.com, chemtymology.co.uk, bioetymology.blogspot.com

Basic polar
Acidic polar
Nonpolar
Polar

TWITTER: @etymology_nerd
INSTAGRAM: @etymologynerd

Thank you for listening

Any Questions?

Credit for schematic to:

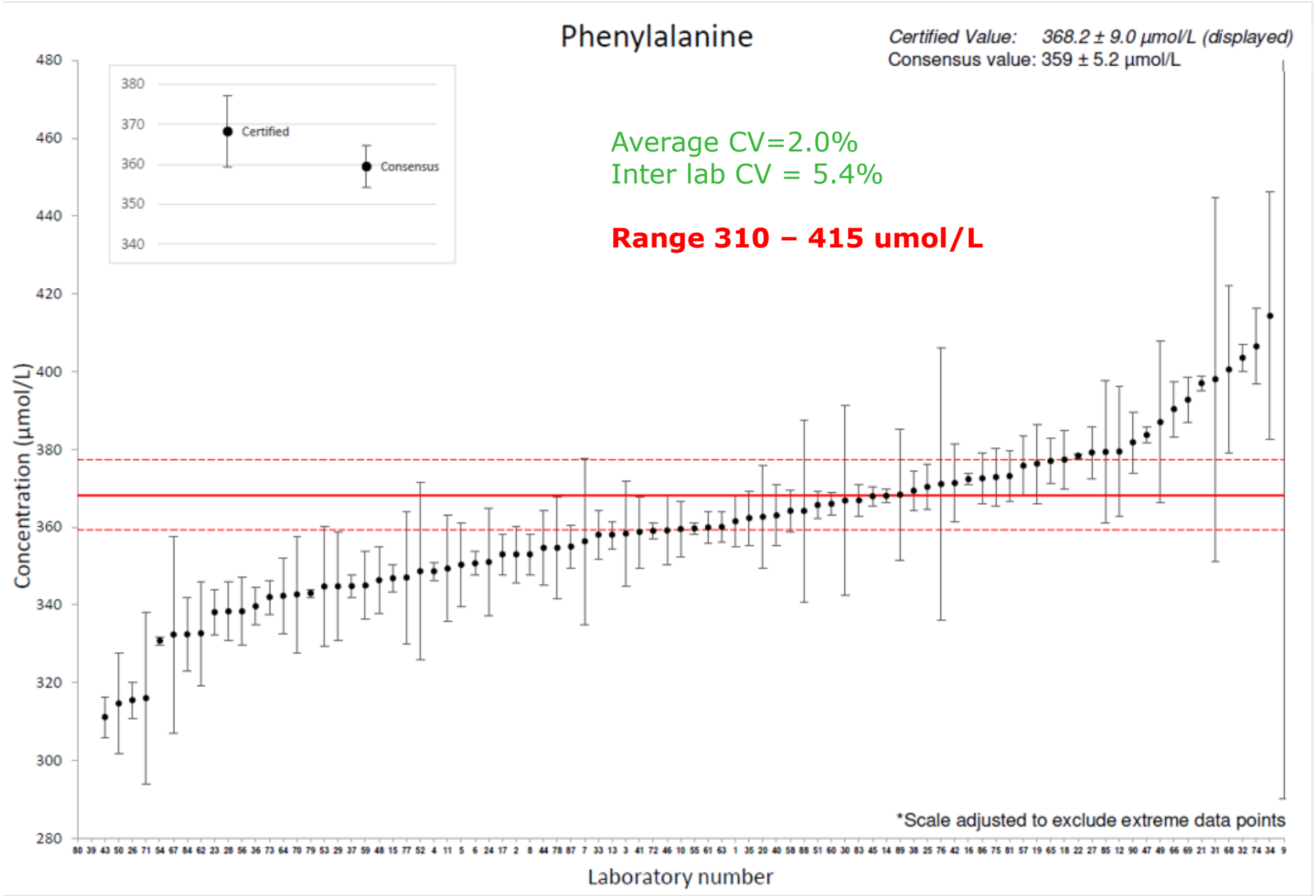
The Etymology Nerd
@etymology_nerd

Nathaniel Martin
@NatMart_Chembio

Standardisation of plasma amino acids

- No matrix matched certified reference material (CRM) for AA in plasma
- Aqueous CRM available (SigmaTrace Cert)
- This is commonly used to calibrate plasma AA methods
- Accuracy of plasma phenylalanine methods?
- Erndim scheme reports against derivation of ALMT not spiked value

- Collaborative project between Erndim, NML & Viapath
- Produce a CRM for plasma phenylalanine
- Sample distributed to n=89 Erndim laboratories
- Results compared with certified value


Phenylalanine
Certified Value: 368.2 ± 9.0 µmol/L (displayed)
Consensus value: 359 ± 5.2 µmol/L
Certified
Consensus
Average CV=2.0%
Inter lab CV = 5.4%
Range 310 – 415 umol/L
*Scale adjusted to exclude extreme data points
Concentration (µmol/L)
Laboratory number