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アプリケーションノート

Linearity of Quantitation of the MassTrak AAA Solution

Paula Hong, Kendon S. Graham, Alexandre Paccou, Thomas E. Wheat, Diane M. Diehl

日本ウォーターズ株式会社

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Abstract

This application note demonstrates the ability of the MassTrak AAA Solution to quantify the entire range of amino acids from micro to millimolar levels.

Introduction

Amino acid analysis of physiological fluids is an important tool used in the study of metabolic pathways. Disturbances to these pathways may give rise to very high levels of one or more amino acids in any given sample while other amino acids in the same sample remain at the low micromolar level or below. It is therefore necessary that the method of analysis offers an analytical measurement range (AMR) that is compatible with the wide range distribution of expected concentrations of amino acids in physiological samples.[1]

The linear range of current ion exchange methods for amino acid analysis have been previously examined.[2,3] However, the reported upper limit of quantification is often equivalent to the concentration of the commercially available physiological standards. While these levels meet the needs of most laboratories, accurate quantification at even higher concentrations must be assured.

In this work, the linear range of the MassTrak AAA Solution (representative chromatogram – Figure 1) is shown to span 1 µM to 10 mM. Accurate quantification of elevated amino acid levels in biological matrices is also demonstrated. These studies show the analytical measurement range of the MassTrak AAA Solution – a range that is comparable to existing methods for amino acid analysis.

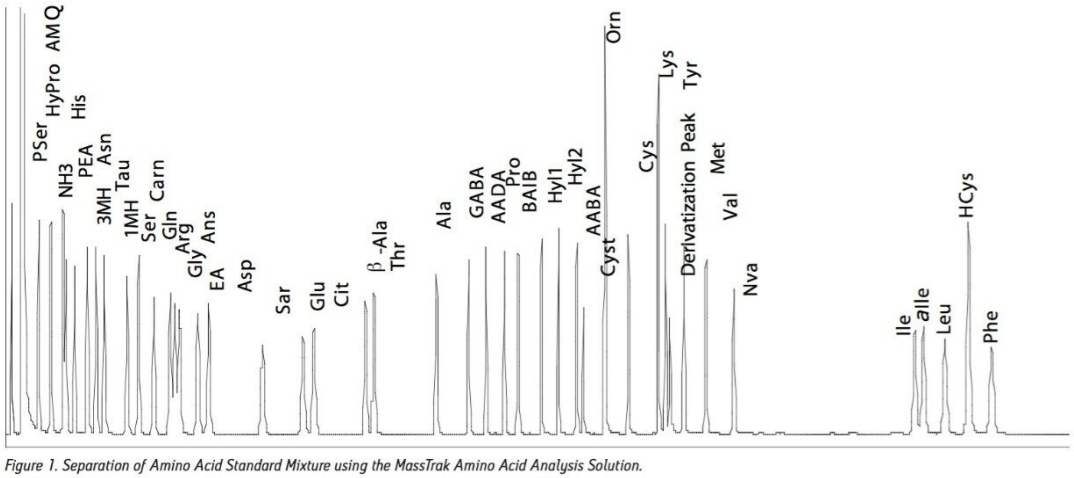

Figure 1. Separation of Amino Acid Standard Mixture using the MassTrak Amino Acid Analysis Solution.

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Experimental

Experimental Design

Several factors may contribute to imposing an upper limit of quantification in any analytical method. These may include detector linearity, solubility of the analyte, saturating amounts of other sample components, and so on. In any derivatization method, the analysis of elevated levels is limited by the amount of derivatization reagent. MassTrak AAA Reagent must be present in approximately four-fold molar excess for complete derivatization of all amino acids. Therefore the total molar amount of amino acids per derivatization must be considered in defining the AMR. These experiments test both the total amounts of all amino acids, as well as the presence of one amino acid at extremely elevated levels.

Preparation/Derivatization of Standards

Amino acids were prepared at concentrations of 1 µM to 10 mM. At concentrations less than 2.5 mM, amino acids were tested in sets of four. For concentrations above 2.5 mM, amino acids were prepared in smaller subsets. Amino acid samples were derivatized with the MassTrak AAA kit. The amino acids react with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC, US Patent 5,296,599. and European Patent EP 0533 200 B1). The protocol followed that described in the MassTrak AAA Solution User’ s Guide. Norvaline was used as an internal standard.

1. A single amino acid (A) was solubilized in 0.1 N HCl to form a 10 mM standard solution (Standard 1A).
2. Step 1 was repeated for amino acids 1B, 1C and 1D (Standards 1B, 1C and 1D).
3. In a new vial, 500 µL of Standard 1A and 500 µL of Standard 1B were combined to form Standard 1A/B, containing 5 mM of each amino acid: 1A and 1B.
4. Step 3 was repeated for Standards 1C and 1D. Standard 1C/D contained 5 mM of each amino acid: 1C and 1D.
5. In a new vial, 500 µL of Standard 1A/B and 500 µL of Standard 1C/D were combined. Standard 1A/B/C/D contained 2.5 mM of each amino acid: 1A, 1B, 1C, and 1D.
6. Standard 1A/B/C/D was diluted by factors of 2, 10, 100, and 1000 (Figure 2).
7. Standards from Step 6 were diluted to form remainder of standards (Table 1).

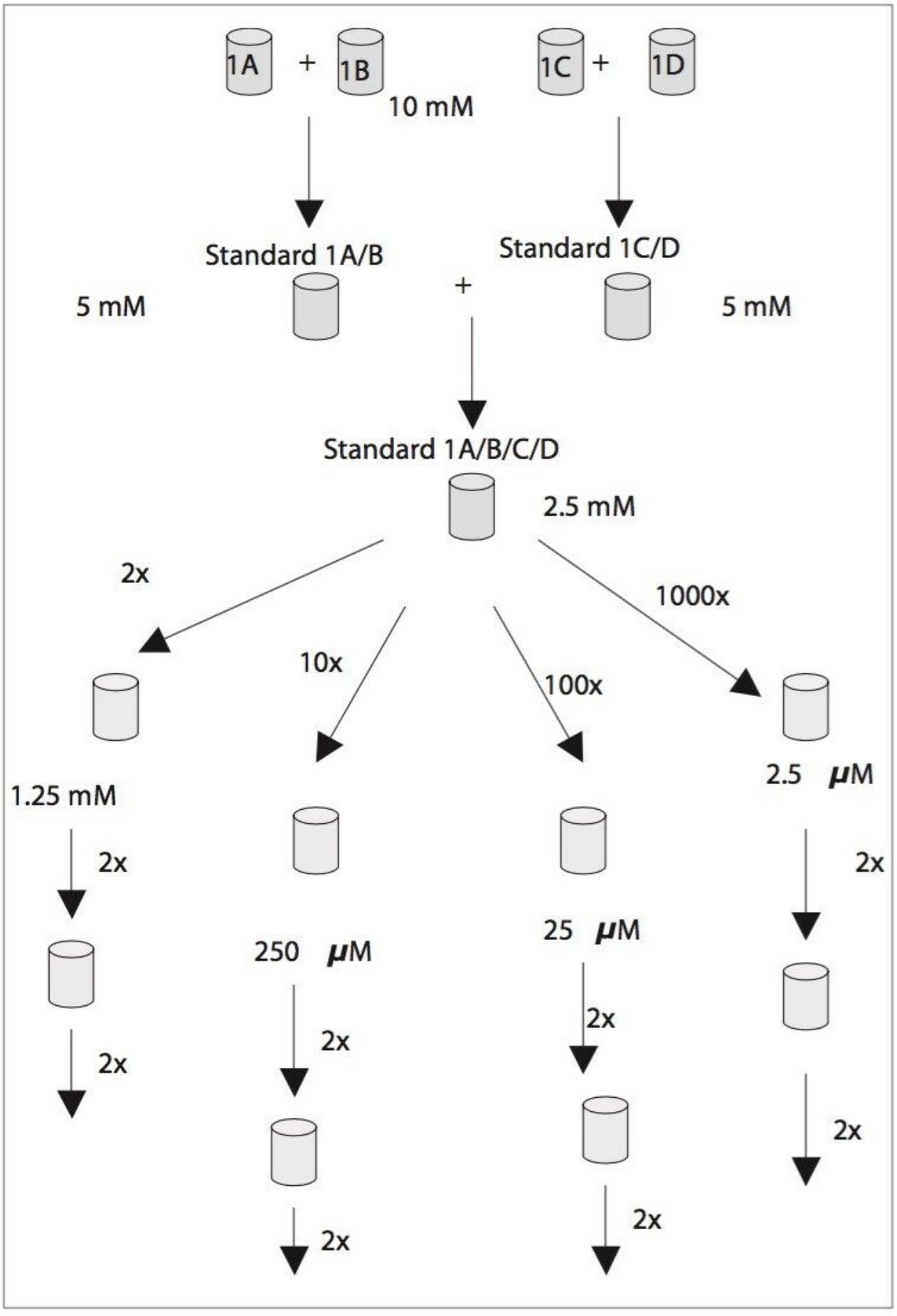

Figure 2. Preparation of dilution series one set of four amino acids.

Physiological Sample Preparation

2. 10 µL of phenylalanine standard from Step 1 was combined with 90 µL of plasma.
3. A 10% sulfosalicylic acid (Sigma-Aldrich P/N S7422) solution containing 250 µM norvaline was prepared.
4. An equal volume of spiked plasma (Step 2) and sulfosalicylic acid/norvaline (Step 3) were combined. Typical volumes were 100 µL.
5. The sample was centrifuged at 16,000 g at room temperature for 5 min.
6. A 20 µL aliquot of the deproteinized supernatant (Step 5) was derivatized following the standard protocol as described in the MassTrak AAA Solution User’ s Guide.

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7. The final concentration of phenylalanine in plasma ranged from 40 to 2000 µM - dependent on the standard used from Step 1.

LC Conditions

LC system:	Waters ACQUITY UPLC System with TUV
Column:	MassTrak AAA Column, 2.1 x 150 mm, 1.7 µm
Column temp.:	43 °C
Flow rate:	400 µL/min.
Mobile phase A:	MassTrak AAA Eluent A Concentrate, diluted 1:10
Mobile phase B:	MassTrak AAA Eluent B
Weak needle wash:	5/95 Acetonitrile/Water
Strong needle wash:	95/5 Acetonitrile/Water
Gradient:	MassTrak AAA Standard Gradient

Detection:	UV @ 260 nm
Injection volume:	1 µL
Injection mode:	Partial Loop with Needle Overfill (PLNO)

Results and Discussion

In previous work, the MassTrak AAA solution has been shown to exhibit a linear response from 1–2 to 500 µM for the complete amino acid standard.[4] However, for each amino acid a linear response over the whole analytical range is required.[5] For biological fluids, this corresponds to levels above 500 µM.

In order to accurately evaluate the entire linear range for the MassTrak AAA Solution, certain criteria were set. These included:

- A coefficient of determination (R^2) $\geqslant$0.998
- % deviation between the spiked concentration and the calculated value <20% for points below 10 µM
- % deviation between the spiked concentration and the calculated value <10% for all other points

All amino acids in the MassTrak AAA standard were analyzed for linear responses over four orders of magnitude: from Lower Limit of Quantification (LLOQ) of 1 µM to an Upper Limit of Quantification (ULOQ) of 10 mM.

For a subset of amino acids, overlaid chromatograms of the complete set of calibration standards are shown in Figure 3. There is no significant distortion of peak shape or retention time that compromises identification of any of the amino acids over the tested range of concentrations. In addition, the detection levels are within the linearity specifications for the ACQUITY TUV Detector. These characteristics indicate that the qualitative properties of the assays are maintained, even over this very wide concentration range.

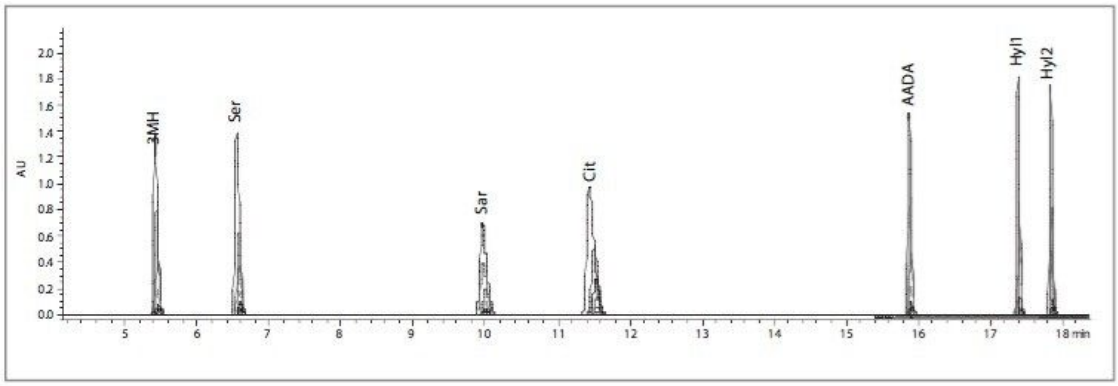

Figure 3. Overlay chromatograms of selected amino acids.

Empower 2 was used to plot response curves for each of the amino acids. A software generated curve is shown for a representative amino acid (Cit) (Figure 4). The calibration curve is over 4 orders of magnitude with R^2 = 0.999, and y intercept = 0.000012. The individual data points in the calibration curve are within 4% of the expected value (Table 2), meeting previously established criteria for linear responses.

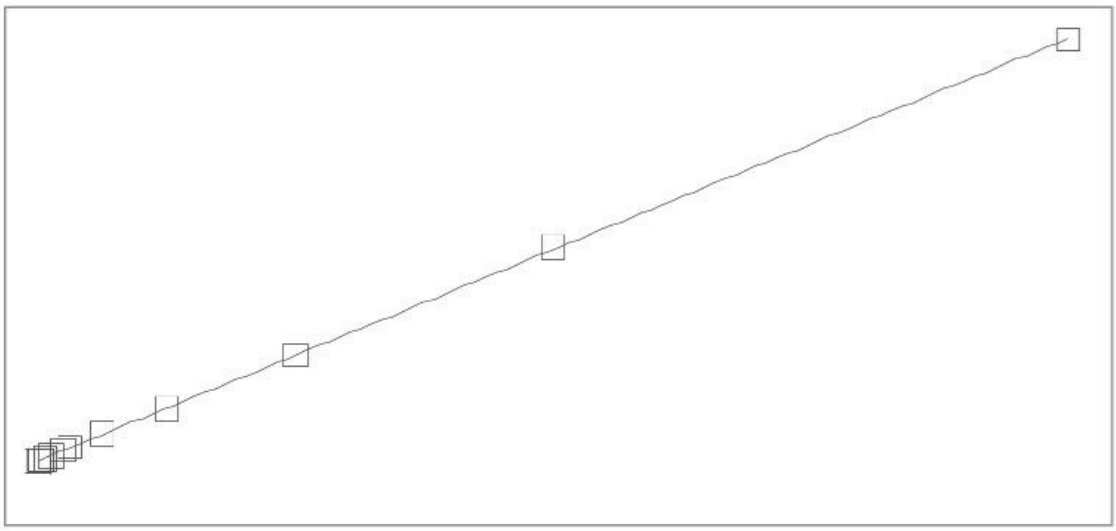

Figure 4. Calibration curve for cit: generated using Empower.

Concentration of Standard (µM)	Calculated Value (µM)*	% Deviation*
1.25	1.29	3.57
2.50	2.52	0.84
3.13	3.08	-1.40
6.25	6.46	3.36
12.5	12.7	1.83
25.0	25.3	1.19
31.3	30.3	-3.06
62.5	61.6	-1.44
125	123	-1.73
250	245	-1.84
313	306	-2.16
625	635	1.67
1250	1233	-1.39
2500	2492	-0.31
5000	5059	1.18
10000	9970	-0.30

*Table 2. Calibration curve data for cit *Calculated using Empower.*

Similar results were observed for all of the amino acids in the physiological standard as listed in Table 3. For the majority of amino acids the lower limit of quantification is demonstrated to be 1 µM. However, due to background contamination of some ubiquitous amino acids, such as Gly, an LLOQ of 2 µM is achieved for select analytes.

Amino Acid	LLOQ	ULOQ
Pser	2 µM	10 mM
HyPro	1 µM	10 mM
His	1 µM	10 mM
PEA	2 µM	10 mM
Asn	1 µM	10 mM
3MH	1 µM	10 mM
Tau	1 µM	10 mM
1MH	1 µM	10 mM
Ser	1 µM	10 mM
Gln	1 µM	10 mM
Carn	1 µM	10 mM
Arg	1 µM	10 mM
Gly	1 µM	10 mM
Ans	2 µM	10 mM
EA	1 µM	10 mM
Asp	1 µM	10 mM
Sar	1 µM	10 mM
Glu	1 µM	10 mM
Cit	1 µM	10 mM
B-Ala	1 µM	10 mM
Thr	1 µM	10 mM

Amino Acid	LLOQ	ULOQ
Ala	1 µM	10 mM
GABA	1 µM	10 mM
AADA	1 µM	10 mM
Pro	1 µM	10 mM
BAIB	1 µM	10 mM
Hyl1/2	1 µM	10 mM
AABA	1 µM	10 mM
Cyst	1 µM	10 mM
Orn	1 µM	10 mM
Cys	1 µM	10 mM
Lys	1 µM	10 mM
Tyr	1 µM	10 mM
Met	1 µM	10 mM
Val	1 µM	10 mM
Ile	1 µM	10 mM
Alle	1 µM	10 mM
Leu	1 µM	10 mM
HCys	1 µM	10 mM
Phe	1 µM	10 mM
Trp	1 µM	10 mM

Table 3. LLOQ and ULOQ of individual amino acids.

Quantifying Elevated Levels in Biological Fluids

Various disturbances to amino acid metabolism may give rise to exceptionally high levels of one or more amino acids in physiological fluids. Phenylalanine, for example, has been observed at very high concentrations in biological samples.[1] It is important, therefore, to accurately quantify high concentrations of particular amino acids in biological samples.

In this set of experiments, phenylalanine was spiked into pooled human plasma prior to deproteinization. The chromatograms containing increasing levels of spiked phenylalanine are shown in Figure 5. The concentration of phenylalanine in the unspiked or blank plasma was measured and subtracted from the spiked sample analyses. The resulting sample concentrations were within 6% of expected values (Table 4), demonstrating a wide linear range comparable to existing ion-exchange methods.[1]

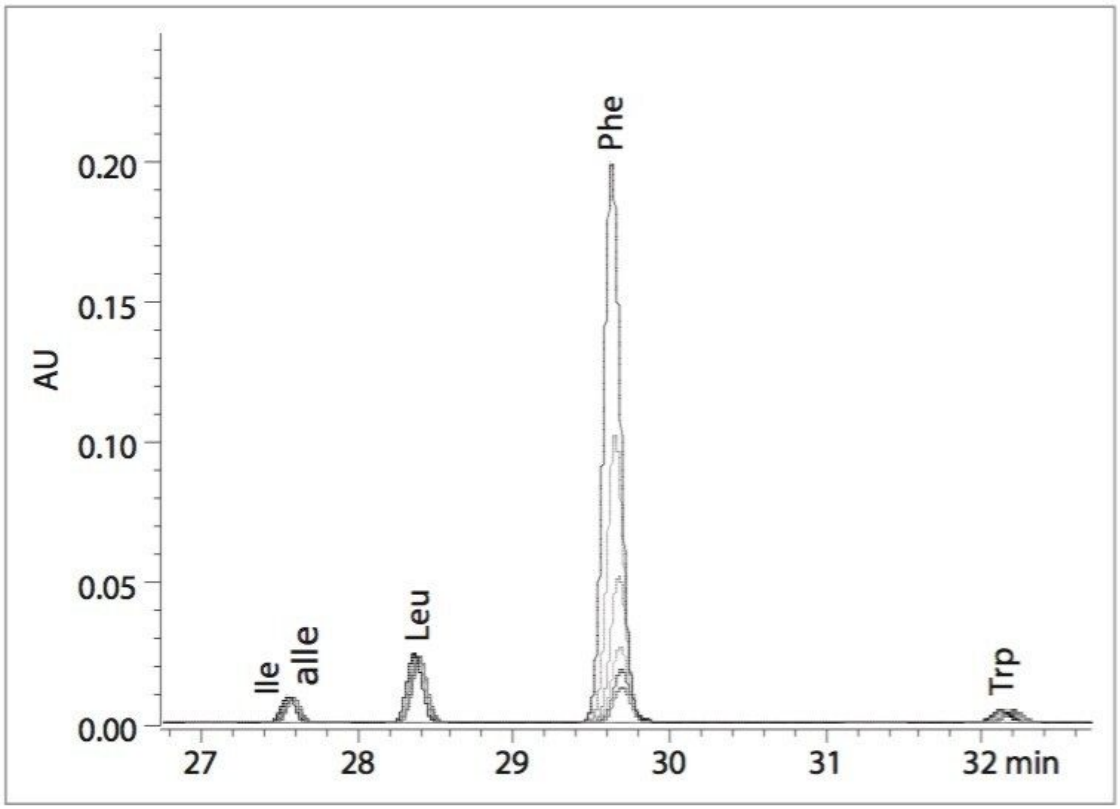

Figure 5. Overlay chromatograms of plasma sample spiked with increasing amounts of phenylalanine.

Spiked Concentration (µM)	Sample Concentration - Blank (µM)	% Deviation
2000	2024	1.2
1000	1018	1.8
500	507	1.5
200	205	2.5
100	100	0.2
40	42	5.7

Table 4. Quantitative analysis of plasma sample spiked with increasing amounts of phenylalanine.

In any pre- column derivatization technique, a limiting factor on the upper limit of quantification is the

amount of reagent available. A report method was therefore developed to ensure that the MassTrak AAA Solution consistently yields accurate results, even for biological samples containing highly elevated amounts of more than one amino acid.

The MassTrak AAA Solution contains a custom report (Figure 6) in which total amino acid content is measured and compared to the amount of derivatization reagent. If this ratio is sufficient for complete derivatization, the results are acceptable and the sample is “within linear range.” If the levels of total amino acid content are too high for complete derivatization, the report flags the sample for “review” and dilution may be required.

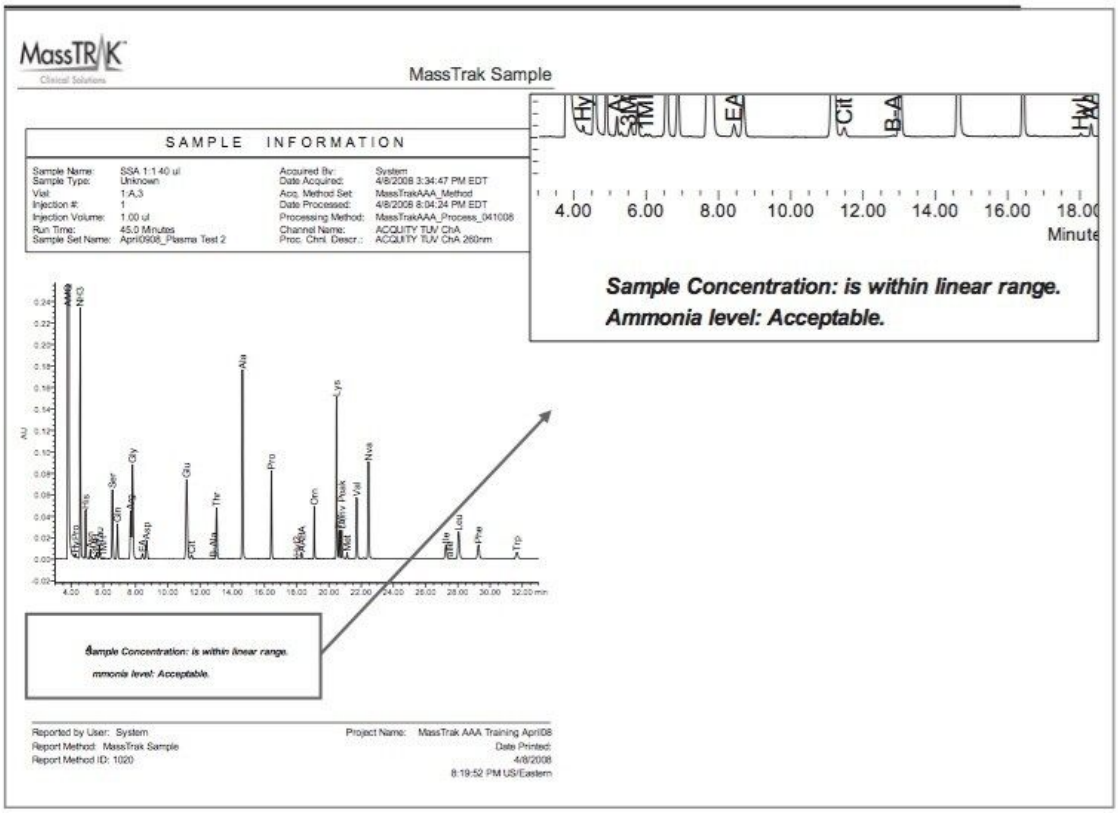

Figure 6. Example MassTrak AAA Solution sample report.

Conclusion

Amino acid analysis is used to study and monitor various metabolic pathways. In a number of disorders, the amino acid concentration can vary widely depending on the physiological state. There is therefore a need to quantify unusually high levels of amino acids in biological samples.

The MassTrak AAA Solution meets these needs, demonstrating a linear response from 1-2 µM up to 10 mM for all the amino acids in the common physiological standard. This method has also been shown to accurately quantify elevated amino acids in biological matrices. However, if a sample’ s total amino acid content were to be outside of the method’ s linear range, immediate determination can be made with the MassTrak AAA Solution reporting methods.

These results successfully demonstrate the ability of the MassTrak AAA Solution to quantify the entire range of amino acids from micro to millimolar levels. The linear range is comparable to if not greater than that shown for ion exchange methods. More importantly, the linear ranges of all amino acids meet the general guidelines in which linearity must be demonstrated to the highest expected levels.

References

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4. P. Hong, T. E. Wheat, D. M. Diehl, Analysis of Physiological Amino Acids with the MassTrak Amino Acid Analysis Solution. 2009, Waters Corp., Application Note, 720002903EN < https://www.waters.com/nextgen/us/en/library/application-notes/2009/analysis-physiological-amino-acids-masstrak-amino-acid-analysis-solution.html> .

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