[APPLICATION NOTE]

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ANALYSIS OF PHYSIOLOGICAL AMINO ACIDS WITH THE MASSTRAK™ AMINO ACID ANALYSIS SOLUTION

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INTRODUCTION

The analysis of free amino acids in physiological fluids is an important tool in the study of physiological processes. Changes in the free amino acid concentrations can reflect altered states of metabolic pathways. These changes may be due to the influence of nutrition, environmental factors or genetic disturbances of metabolism. Expanding research in this field creates a demand for more amino acid analysis. A more robust method can offer the higher throughput that is required with this increasing sample load.

Improved throughput is a function of both reducing run time and improving method robustness. Current ion exchange methods are time consuming and frequently require lengthy method adjustment. Waters has developed a new solution for research use only. The Waters MassTrak™ AAA Solution combines a well-characterized amino acid derivatization, QC tested columns and eluents, a pre-configured UltraPerfomance LC® method, standards, and compliance-ready software.

Following the benchtop pre-column derivatization of the analytes, separation and detection are achieved with a reversed-phase UPLC® column and TUV detector, respectively. The analysis is complete in less than 32 minutes and allows for the identification and quantification of 42 common amino acids and related compounds in a single run. Full profile amino acid analyses are conducted using a defined analytical method. Electronic reports are generated using pre-defined software templates. These may be customized to suit specific reporting needs.

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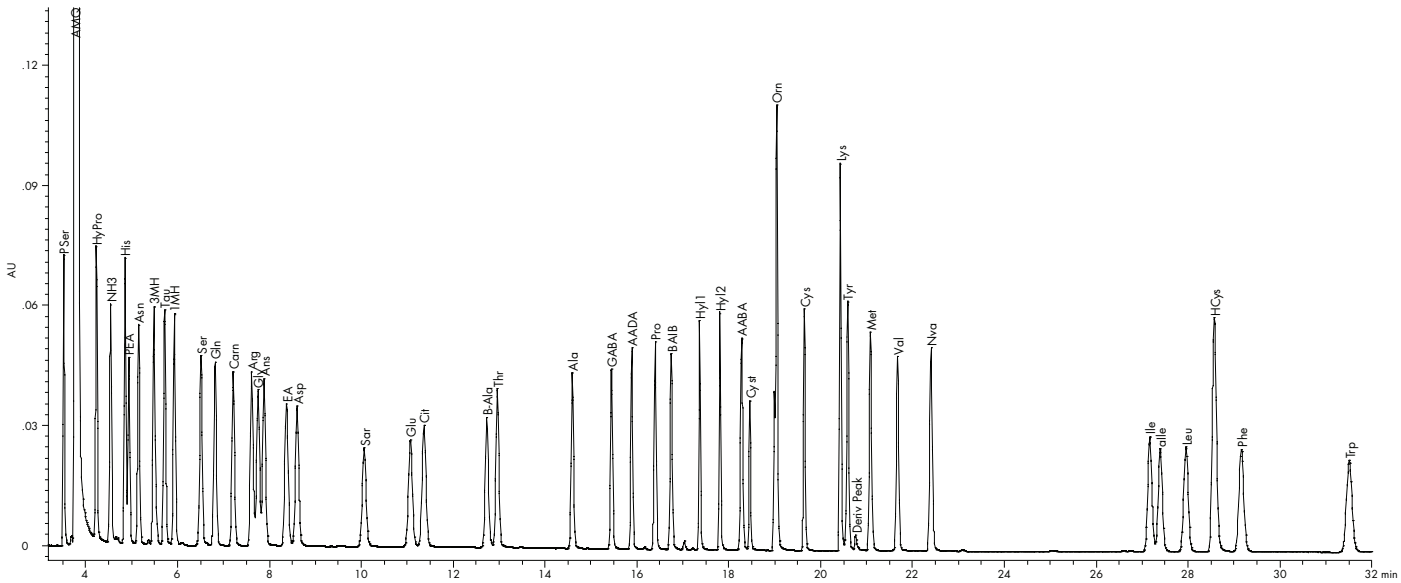

Figure 1. MassTrak AAA Solution Standard Chromatogram 250 µM

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EXPERIMENTAL

Sample Preparation

Sample preparation utilizes the MassTrak AAA derivatization kit and Total Recovery vials. The amino acids are derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (US Patent 5,296,599 and European Patent EP 0533 200 B1).

- Amino Acid Standards

  Amino acid standard mixtures are prepared as described in the users guide.

  - Acidics and Neutrals Standard Mixture
  - Basics Mixture
  - Glutamine
  - Allo-Isoleucine
  - Trytophan
  - Norvaline (Internal Standard)

- MassTrak AAA Derivatization Kit

  Derivatization followed the protocol as described in the System Guide.

  - MassTrak AAA Reagent
  - MassTrak AAA Borate Buffer
  - MassTrak AAA Reagent Diluent

- Total Recovery Vials

LC conditions

LC System:	Waters® ACQUITY UPLC® System with TUV Detector
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 (provided in solution)
Detection:	UV @ 260 nm
Injection Volume:	1 µL
Injection Mode:	Partial Loop with Needle Overfill (PLNO)

Physiological Sample Preparation

The sample preparation for the biological samples is dependent on the matrix. Plasma samples require deproteinization prior to analysis. Typical example:

1. Prepare 10% sulfosalicylic acid (Sigma-Aldrich P/N S7422) in water containing 250 µM norvaline (internal standard).
2. Plasma (250 µL) is mixed with an equal volume of sulfosalicylic acid/norvaline (250 µL).
3. The sample is centrifuged at 16,000 g for not more than 5 min.
4. A 20 µL aliquot of the deproteinized supernatant is subsequently derivatized using the standard protocol as described in the users guide.

RESULTS AND DISCUSSION

A reliable amino acid analysis method must meet specific requirements. The accurate identification of the individual amino acids is essential. Additionally, the amino acids need to be accurately quantified. This quantification must be linear over the range commonly observed in physiological samples.

The reliability and robustness of the analysis was measured to ensure accurate identification and quantification of all the amino acids included in the assay. The amino acids are identified by retention time relative to the supplied standard. Reliability of the identification of each amino acid is based on retention time and its the coefficient of variation expressed as a percentage (% CV) (Table 1).

Amino Acid	Average R_T	Std Dev R_T	% CV R_T	Amino Acid	Average R_T	Std Dev R_T	% CV R_T	Amino Acid	Average R_T	Std Dev R_T	% CV R_T
PSer	3.47	.006	.18	Asp	8.61	.011	.13	Om	19.06	.002	.011
HyPro	4.19	.006	.15	Sar	10.08	.009	.09	Cys	19.66	.002	.011
His	4.82	.007	.14	Glu	11.10	.012	.11	Lys	20.44	.002	.008
PEA	4.89	.007	.14	Cit	11.40	.012	.10	Tyr Deriv	20.61	.003	.016
Asn	5.12	.007	.14	B-Ala	12.75	.008	.06	Peak	20.75	.001	.007
3MH	5.45	.007	.13	Thr	12.98	.007	.05	Met	21.11	.003	.013
Tau	5.68	.008	.14	Ala	14.61	.005	.03	Val	21.70	.002	.011
1MH	5.90	.007	.12	GABA	15.46	.004	.03	Nva	22.44	.003	.011
Ser	6.49	.008	.13	AADA	15.90	.004	.03	Ile	27.23	.004	.015
Gln	6.80	.008	.12	Pro	16.41	.004	.02	alle	27.46	.004	.015
Carn	7.20	.008	.11	BAIB	16.76	.004	.02	Leu	28.02	.004	.016
Arg	7.62	.009	.12	Hyl1	17.37	.002	.01	HCys	28.65	.006	.022
Gly	7.75	.009	.11	Hyl2	17.81	.003	.01	Phe	29.25	.006	.020
Ans	7.88	.008	.10	AABA	18.29	.003	.02	Trp	31.63	.009	.028
EA	8.37	.008	.10	Cyst	18.46	.002	.01				

Table 1. Interrun precision of absolute retention times. MassTrak AAA Standard, 250 µM duplicate derivatizations, triplicate injections. Six injections total.

Retention time precision for a standard is typically less than 0.2% CV over a series of runs. This interrun variability of retention time is much less than the retention difference between adjacent peaks, thereby ensuring accurate and reliable identification. For example, His and PEA are the most closely spaced peaks. The retention time standard deviation for both His and PEA is .001. The difference in retention time between these peaks is .007, approximately three times the sum of the standard

Amino Acid	Mean Area	Std Dev Area	% CV Area	Amino Acid	Mean Area	Std Dev Area	% CV Area	Amino Acid	Mean Area	Std Dev Area	% CV Area
PSer	126285	1640	1.30	EA	152311	2434	1.60	AABA	151851	1284	.85
HyPro	149534	607	.41	Asp	192533	3578	1.86	Cyst	192719	1143	.59
His	159801	668	.42	Sar	130924	1274	.97	Om	259219	2358	.91
PEA	133246	1785	1.34	Glu	159742	1552	.97	Lys	119659	698	.58
Asn	100541	620	.62	Cit	151982	992	.65	Lys	236621	2105	.89
3MH	152821	701	.46	B-Ala	144877	2405	.66	Tyr	151004	613	.41
Tau	154723	941	.61	Thr	153485	2434	.59	Met	155275	899	.58
1MH	158081	882	.56	Ala	149080	1391	.93	Val	153932	1171	.76
Ser	156120	1517	.97	GABA	142138	1631	1.15	Nva	146594	1066	.73
Gln	141989	934	.66	AADA	131980	1316	1.00	Ile	156293	1258	.80
Carn	152286	929	.61	Pro	141609	998	.70	alle	160799	1072	.67
Arg	153531	859	.56	BAIB	150112	1387	.92	Leu	151032	1044	.69
Gly	155310	859	.55	Hyl1	131248	936	.71	HCys	133597	944	.71
Ans	79676	446	.56	Hyl2	114704	852	.74	Phe	160096	792	.49
								Trp	159576	821	.51

Table 2. Interrun precision. MassTrak AAA Standard, 250 µM duplicate derivatizations, triplicate injections. Six injections

deviations. This low variability in retention time allows for increased confidence in the identification of each amino acid. To further ensure that each peak is correctly identified the retention time is referenced to a well resolved peak in each sample.

Reliable quantification of the amino acids is dependent upon reproducibility, sensitivity and linearity. With the MassTrak AAA Solution, the interrun precision, as measured by the CV of peak area counts, is within 2 % for all the amino acids (Table 2). The variability in quantification is comparable, if not superior, to existing methods. The quantification can be more precise when an internal standard is incorporated.

Another important feature of any amino acid analysis is linearity of response. The MassTrak AAA solution exhibits a the linear response over the range 1-2µM to 500 µM with the complete amino acid standard. Each of the amino acids in the mixture has a linear response from 1-2 µM to 500 µM with a R2 ≥ 0.995 (e.g. Figure 2). For points at the low end of the calibration curve or less than 5µM, the deviation from calculated values is <20 %. Points at higher levels are within 10 % from the calculated concentrations. The % deviation for both the low and high end of the calibration curve is within generally accepted limits.

The Lower Limits of Quantification (LLOQ) and the Limits of Detection (LOD) are dependent upon the response seen for each amino acid and the related background contamination. The LLOQ is defined as the lowest level that can be reproducibly and accurately quantified within 10 % of a known value. With the MassTrak AAA solution the LLOQ is 1-2 µM for amino acids (Figure 3). The limit of detection (LOD) is defined as a signal-to-noise of 4 or greater. All of the amino acids have a LOD less than 0.5 µM.

The constraint in the upper limit of quantification (ULOQ) is the amount of excess reagent that is required for the complete derivatization of all of the amino acids in the sample. The derivatization reagent excess should be greater than four times the total amount of amino acids in a sample. Since a known amount of reagent is added to each sample, complete derivatization requires the total amino acid amount in a raw sample not exceed 21 mM. If the sum of all the amino acids exceeds this amount, the more slowly reacting amino acids are not completely derivatized. However, biological samples rarely exceed 21 mM total amino acid content. Single amino acids have been shown to provide linear response up to 10 mM.

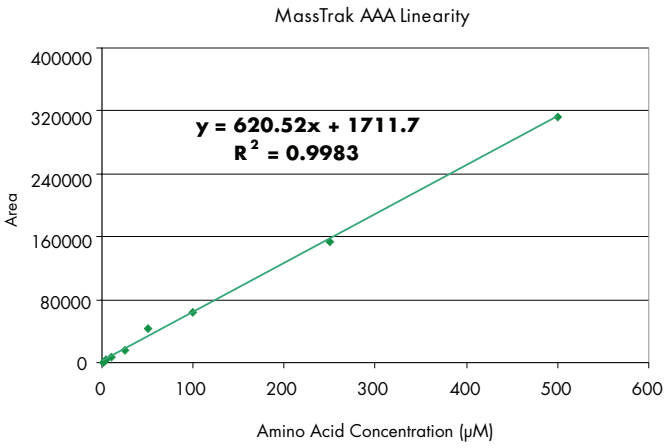

Figure 2. Linearity for Phenylalanine

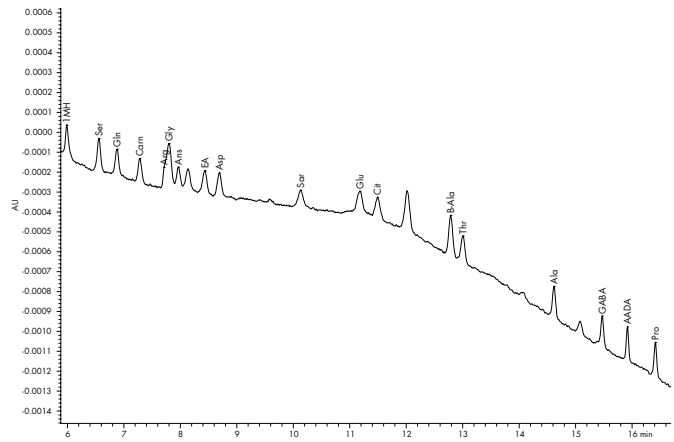

Figure 3. MassTrak AAA Solution Standard Chromatogram: Limit of Quantification 1 µM (selected region).

Analysis of a Plasma Sample

The MassTrak AAA Solution is designed for the analysis of physiological amino acids in a variety of matrices, including deproteinized plasma (Figure 4). Replicate analyses of a plasma sample demonstrate the retention time precision within run 0.2 % CV interrun (Table 3). The area precision for the deproteinized samples is typically within 2 % CV interrun. The low level of variability in a complex matrix demonstrates the robustness of the method even in the presence of possible interferences and provides added confidence, accurate identification and quantification.

Amino Acid	% CV R_T	% CV Area Ratio	Amino Acid	% CV R_T	% CV Area Ratio
HyPro	.11	.57	Thr	.09	.75
His	.14	.93	Ala	.05	.67
Asn	.14	.86	Pro	.03	.55
Tau	.16	.94	AABA	.02	.85
1MH	.16	1.58	Orn	.02	.72
Ser	.16	1.51	Lys	.02	1.15
Gln	.17	.83	Tyr	.02	1.29
Carn	.18	1.17	Met	.01	1.06
Arg	.19	.92	Val	.01	.60
Gly	.16	.88	Ile	.02	.73
EA	.16	.60	Leu	.02	.71
Asp	.18	1.53	Phe	.03	1.09
Glu	.05	.76	Trp	.05	1.49
Cit	.18	.79			

Table 3 . Interrun precision. Plasma sample, triplicate derivatizations, triplicate injections. Nine injections total.

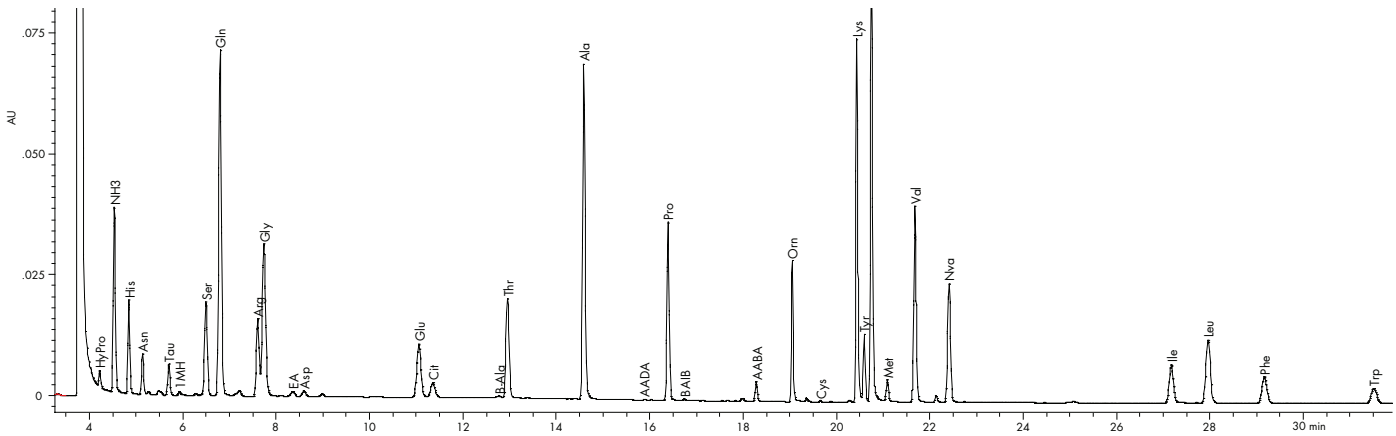

Figure 4. Chromatogram of a pooled human plasma sample

CONCLUSION

The MassTrak AAA Solution is a total system for the analysis of physiological amino acids for research use only. This solution includes columns, eluents, standards, derivatization reagents, instrumentation, software and support, all of the necessary components to provide continuous and reliable operation.

The MassTrak AAA Solution is based on a well-studied derivatization chemistry. The quantitative analysis is therefore robust and easy to troubleshoot should any problems should arise. The derivatives are separated using UltraPerfomance LC using an ACQUITY UPLC system with TUV detection. The separation chemistry uses columns and eluents that are tested during manufacture to assure consistent and successful analyses. The software includes system suitability tests that monitor the performance while running the series of samples. It also incorporates custom reports that are adapted to the analysts requirements.

This solution provides accurate and precise qualitative and quantitative amino acid analysis. Quantitative precision is better than 2 % CV and the demonstrated linearity exceeds the levels that are commonly observed in physiological samples. The LLOQ of 1 µM meets the generally desired quantifiable levels in physiological samples. In addition, the LOD of 0.5 µM provides greater sensitivity than comparable methods.

The MassTrak AAA Solution provides a robust, reliable, less time consuming tool allowing higher sample throughput for physiological amino acid analysis.

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