<u>Amino Acids? What have I got? -</u> Simultaneous, quantitative analysis of amino acids (AA) and untargeted metabolomics using hydrophilic interaction chromatography (HILIC) with electrospray ionisation (ESI) quadrupole time-of-flight (QTOF) detection

AA	RT (min)	Quantifier (Qualifiers) in *m/z*
L-Phenylalanine	3.51	166.0863 (120.0799;103.0542)
L-Leucine	3.84	132.1019 (44.0495;86.0964)
L-Tryptophan	4.02	205.0972 (146.06;118.0651)
L-Isoleucine	4.16	132.1019 (69.0699;86.0964)
L-Methionine	4.67	150.0583 (61.0106;56.0495)
L-Proline	5.43	116.0706 (70.0651;68.0495)
L-Valine	5.45	118.0863 (55.0542;72.0808)
L-Tyrosine	5.52	182.0812 (136.0757;91.0542)
L-Alanine	6.91	90.0549 (44.0495;44.9971)

AA	RT (min)	Quantifier (Qualifiers) in *m/z*
L-Threonine	7.05	120.0655 (102.055;74.06)
L-Glutamine	7.59	147.0764 (84.0444;56.0495)
L-Serine	7.63	106.0499 (60.0444;88.0393)
L-Asparagine	7.68	133.0608 (74.0236;46.0287)
L-Glutamic acid	8.05	148.0604 (84.0444;56.0495)
L-Aspartic acid	8.73	134.0448 (74.0237;88.0393)
L-Histidine	9.50	156.0768 (110.0713;93.0447)
L-Arginine	10.00	175.119 (70.0651;60.057)
L-Lysine	10.58	147.1128 (84.0808;67.0542)
L-Ornithine	10.72	133.0971 (70.0651;116.0706)

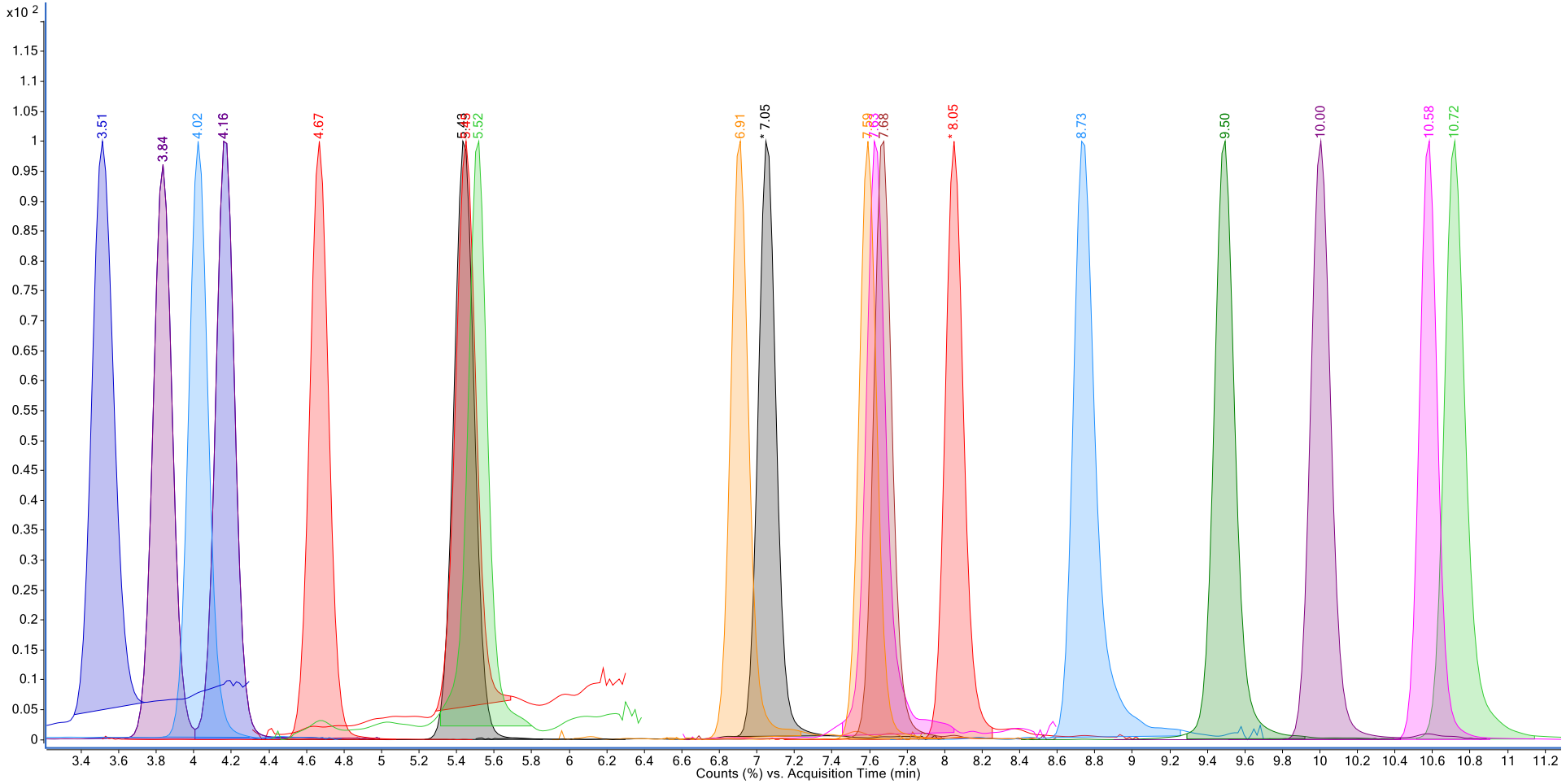
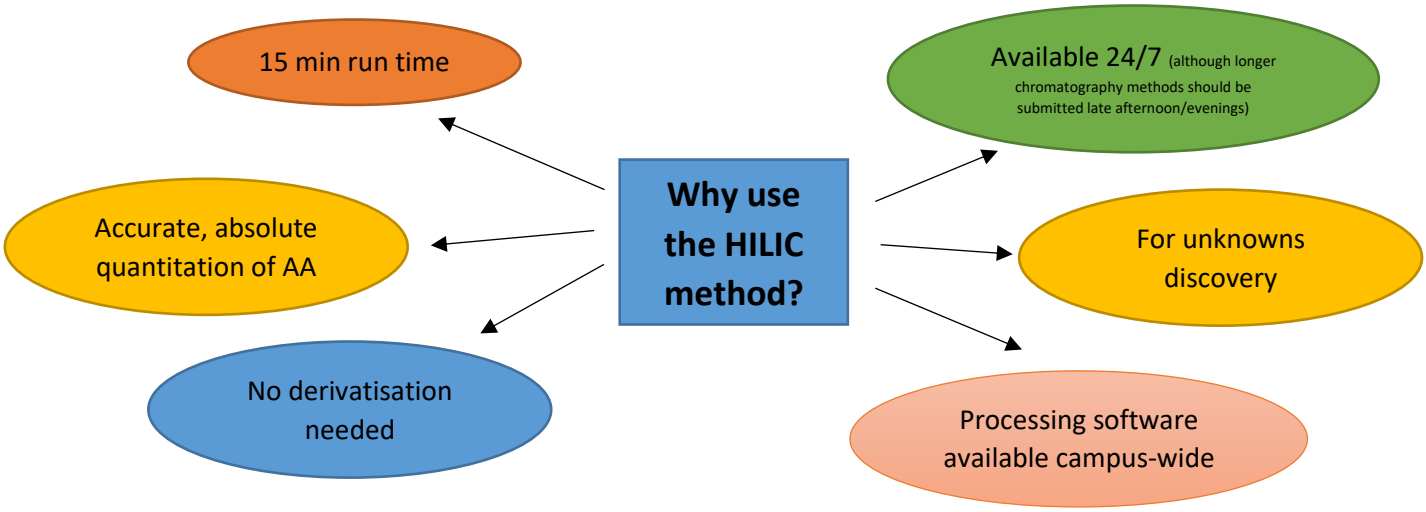


Figure 1: Elution profile of a 500 ng/mL AA standard separated by HILIC chromatography (peak heights are normalised).

A walk-up (ESI-QTOF) method for the **quantitative analysis of underivatised amino acids** (AA) was developed. Nineteen AAs were separated using hydrophilic interaction chromatography (HILIC), with **baseline resolved peaks for leucine and isoleucine isomers.** The workflow consists of **a combined MS and MS/MS acquisition method to ensure reliable identification**; each AA is confirmed with accurate mass (quantifier ion), and MS/MS fragments (qualifier ions). Data processing ensured correlation of qualifier co-elution with the correct precursor ion. The QTOF data generated can be searched retrospectively for other polar analytes (Metlin or the Human Metabolome Database) with MS/MS providing confirmatory ions to enable confident metabolite identification.



Results

Nineteen AAs were separated using HILIC chromatography with a 15-minute run time. Isobars leucine (retention time (RT); 3.84 min) and isoleucine (RT 4.16 min) could be separated with baseline resolution (Fig. 1). No derivatisation was required, reducing typical variability associated with sample preparation procedures. **Excellent linear responses for the AA were demonstrated across the calibration ranges** (Fig. 2, Fig. 3 and Table 1). Quantitation of each AA relies on accurate mass matching of the precursor (quantifier) ion, and the co-elution of product (qualifier) ions, as demonstrated for L-glutamic acid in Fig 4. This ensures **unambiguous identification of each AA based on curated fragment mass data**. The method detection limit (MDL, Table 1) for all of the AA, apart from tyrosine, was below 100 ng/mL. Traditionally triple quadrupole MS (QQQ) instruments are use where detection of very low analyte concentrations is required, and indeed this QTOF method was less sensitive than reported QQQ methods[1]. However, **the major advantage of utilising a QTOF instrument is the possibility for retrospective untargeted analysis and compound discovery**, as shown below.

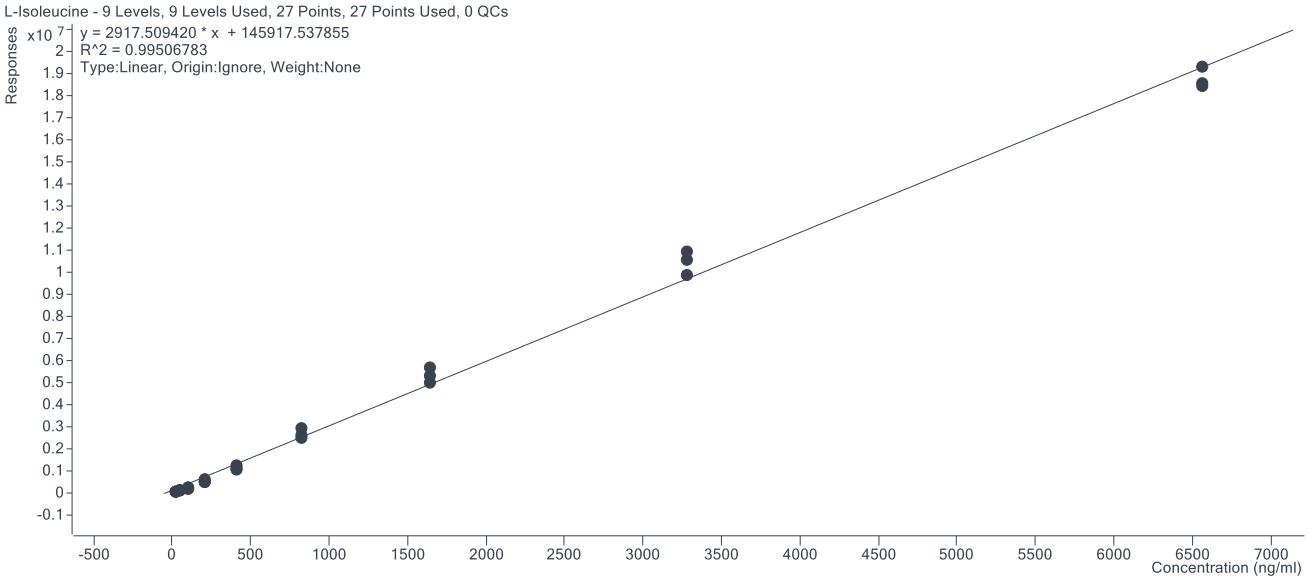


Figure 2. Calibration curve for L-isoleucine.

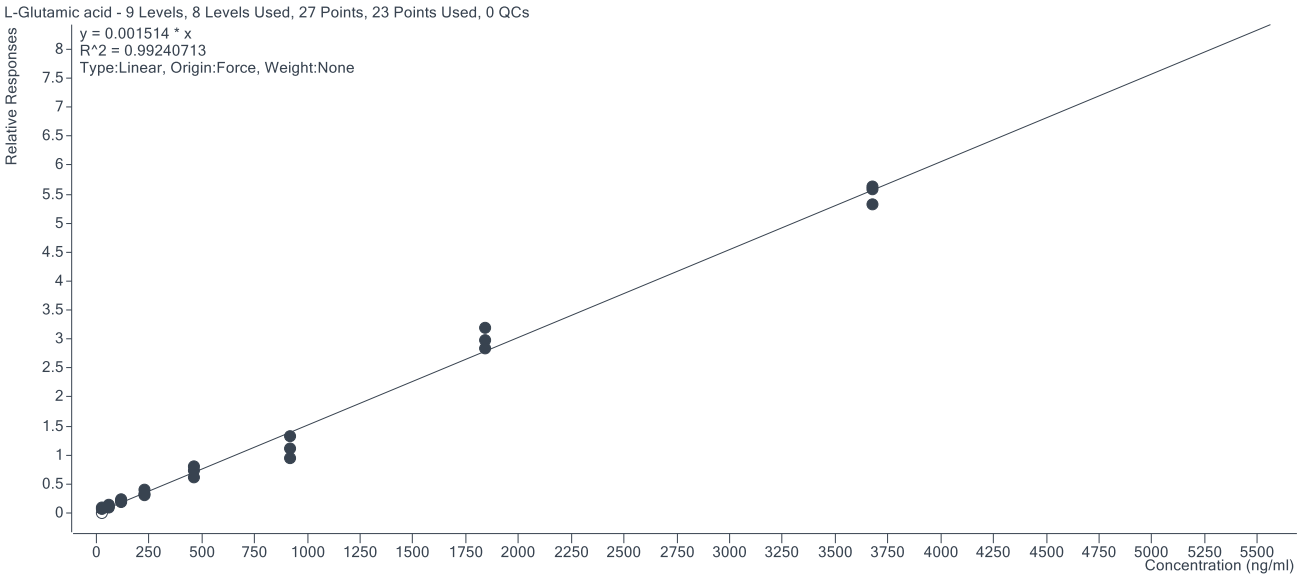


Figure 3: Calibration curves for L-glutamic acid with area ratio response relative to the internal standard (L-^{15}N-Glutamic acid).

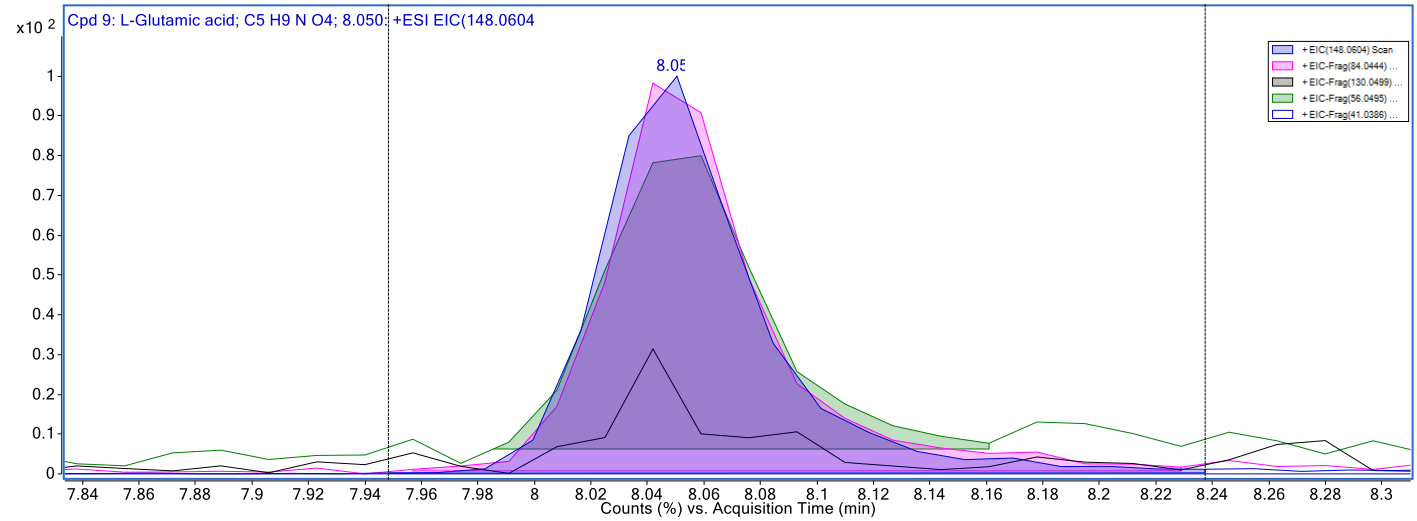


Figure 4: Extracted ion chromatogram (EIC) for intact L-glutamic acid at 500 ng/mL (blue trace 148.0604 *m/z*), with qualifier ions at 84.0444, 130.0499, 56.0495 and 41.0386 *m/z*.

Table 1: Calibration table for HILIC AA analysis

AA (internal standard)	Linear calibration range		R^2 from calibration curve	S/N at ~250 ng/mL	Method detection limit (MDL in ng/mL)
	µM	corresponding ng/mL			
L-Phenylalanine	0.2 - 25.0	32.2 - 4130	0.9922	88.9	31.2
L-Leucine	0.2 - 50.0	25.6 - 6560	0.9967	74.0	19.5
L-Tryptophan	0.2 - 9.8	31.3 - 2000	0.9908	94.8	1.7
L-Isoleucine	0.2 – 50.0	25.6 - 6560	0.9950	55.6	17.0
L-Methionine	0.2 – 50.0	29.1 - 7460	0.9971	148.3	18.5
L-Valine	0.8 – 12.5	91.6 - 1465	0.9824	6.68	32.1
L-Tyrosine	1.5 – 50.0	283.1 - 9060	0.9854	11.1	148.2
L-Proline	0.2 - 12.5	22.5 -1438	0.9919	75.9	18.5
L-Alanine	0.8 – 50.0	69.6 - 4450	0.9972	27.8	52.0
L-Threonine	0.2 - 50.0	23.3 - 5955	0.9934	18.5	12.3
L-Asparagine	0.5 - 33.7	62.5 - 4000	0.9978	32.5	18.8
L-Glutamine	0.3 – 40.7	31.2 - 4000	0.9931	82.0	5.6
L-Serine	0.8 – 25.0	82.1 - 2628	0.9929	14.4	14.1
L-Glutamic acid (L-^{15}N-Glutamic acid)	0.2 – 25.0	28.7 - 3678	0.9924	30.7	53.8
L-Aspartic acid	0.4 – 25.0	52.0 - 3328	0.9931	10.9	43.9
L-Histidine	0.4 – 25.0	60.3- 3880	0.9968	173.3	5.1
L-Arginine	0.2 - 6.25	34.0- 1088	0.9922	221.3	42.6
L-Lysine	0.4 – 50.0	57.1- 7310	0.9942	78.5	37.1
L-Ornithine	0.2 – 30.3	31.2 - 4000	0.9970	51.6	2.1

The method detection limit (MDL, the minimum amount of analyte required to produce a signal that is statistically distinguishable from the background noise level within 99% confidence level) was calculated using n = 3 with $t_{(n-1,\ 1-\alpha\ -\ 0.99)}$ = 6.965 and the following formula; MDL = t × (RSD / 100) × amount measured. MDLs are matrix and analyte specific.

Application of the method

The developed quantitation method was utilised to **monitor fluctuations of AAs in nitrogen-limited media** inoculated with a non-*Saccharomyces* wine yeast, sampled at defined intervals (Fig. 4). For preparation, the samples were diluted 1/40 in mobile phase B (as added benefit this ensured protein precipitation), thereafter fortified with the ^{15}N-Glutamic acid internal standard (ISTD) and submitted for analysis. The trends suggest changes in amino acid metabolism over time, however the large amino acid concentration variation within samples at the same time point suggests non-homogeneous sampling from the bioreactors, and would need to be addressed before more robust conclusions could be drawn.

Mass spectrometry - Material and Chemical Characterisation (MC^2)
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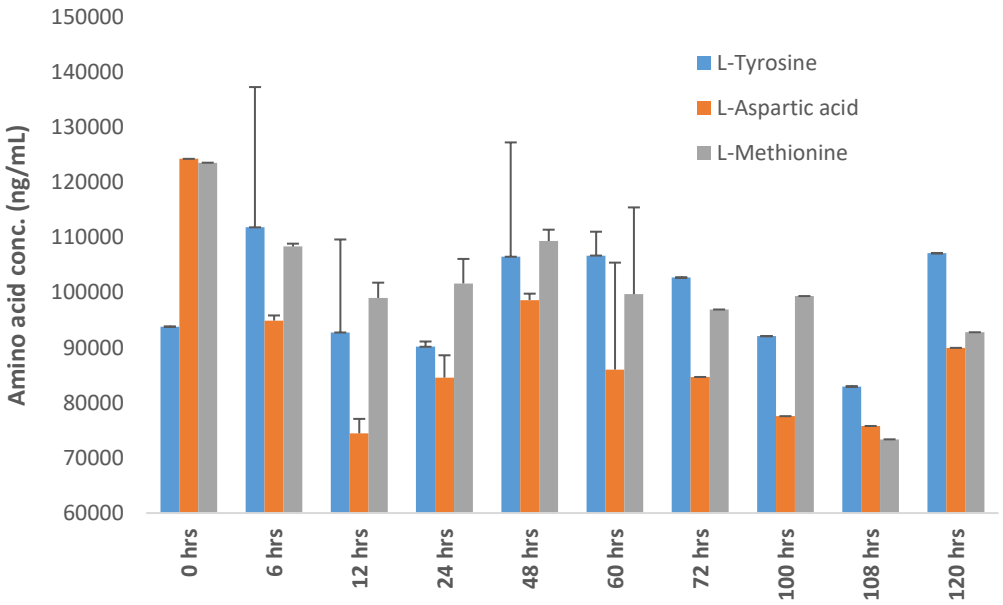

Figure 4: Monitoring of L-tyrosine, L-aspartic acid and L-methionine in broth samples sampled at specific time points. Levels are reported as ng/mL accounting for sample dilution, error bars represent standard deviation of replicate samples (n=2).

Additional **untargeted analysis was performed by screening the unknown ions against the Metlin MassHunter Personal Compound Databases and Libraries (PCDL)**. The nucleosides, adenosine and guanosine, with nucleobases adenine and guanine were confirmed with accurate mass quantifier and qualifier ions, and relative peak areas were determined in all samples (Fig. 5 and 6). The dynamic trends observed for the identified metabolites underscores their importance, and this preliminary finding now requires unequivocal identification and quantification by the inclusion of certified standards within the method.

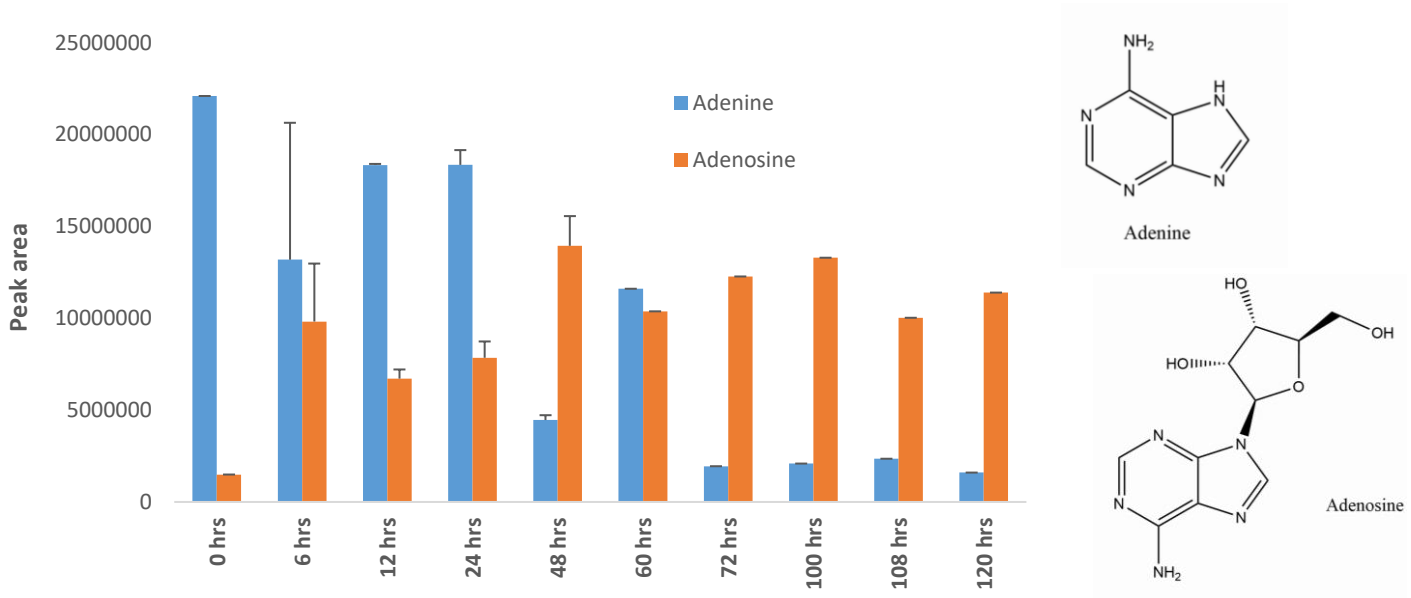

Figure 5: Peak areas of adenine and adenosine ions in broth samples during the time course. Peak areas are shown with error bars representing the standard deviation of replicate samples (n=2).

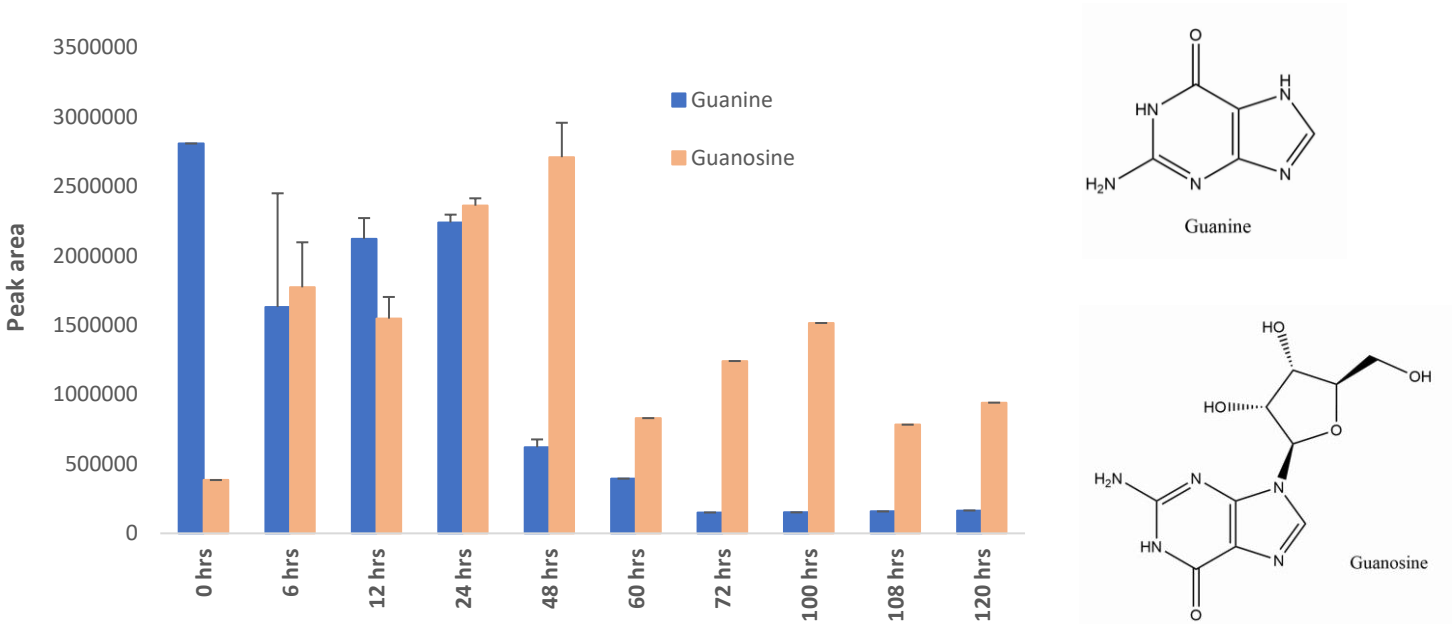

Figure 6: Peak areas of guanine and guanosine ions in broth samples during the time course. Peak areas are shown with error bars representing the standard deviation of replicate samples (n=2).

Conclusions

A simple and robust walk-up method has been created to enable absolute quantitation of AAs in liquid samples. Capabilities of the QTOF-MS, and further information in the data acquired, allows for untargeted screening and discovery of unexpected chemically relevant features as an added benefit. The hydrophilic interaction chromatography was also shown to be suitable for the separation of other biologically important molecules (including carbohydrates, small peptides), such as nucleobases and nucleosides.

Considerations when submitting samples for the Walk-up HILIC MS method

Although external calibration curves can be used to determine sample AA concentrations, to account for ionization differences in vastly different matrices, we recommend a matrix-matched calibration curve should be included initially. In the study illustrated above we have limited costs of the quantification by not including a complete range of stable isotope labelled internal standards (SILs). However, to account for sample preparation errors we recommend that additional SILs for each AA should be included (see https://shop.isotope.com/productdetails.aspx?itemno=MSK-A2-1.2). Samples should also be diluted to have target analyte concentrations within the linear part of the calibration ranges for each AA. MDLs are matrix and analyte specific.

Reagents and chemicals

All reagents were purchased from Sigma Aldrich unless otherwise stated. MS grade H_2O was purchased from VWR. The combination AA Standard (17 component mixture, AAS18, Sigma Aldrich) with the following individual AAs were utilised; L-tryptophan (Prod. No. T0254), L-glutamine (Prod. No. 49419), L-asparagine (Prod. No. A0884) and L-ornithine (Prod. No. 57197). L-glutamic acid - ^{15}N (Prod. No. 332143) was utilised as the internal standard. Primary stock solutions of the individual AAs were prepared at 2 mg/mL in H_2O, thereafter combined into a secondary stock of 0.2 mg/mL. The working standard solution was prepared at an individual AA concentration of 4 µg/mL, with 50 µM of the AAS18 standard (approx. 4 µg/mL) in 90% ACN containing 20 mM ammonium formate (pH 3).

Instrument parameters

LC-MS analyses were performed using an Agilent QTOF 6545 with Jetstream ESI spray source coupled to an Agilent 1260 Infinity II Quat pump HPLC with 1260 autosampler, column oven compartment and variable wavelength detector (VWD). The MS was operated in positive ionization mode with the gas temperature at 300°C, drying gas flow at 13 L/min and nebulizer gas flow at 30 psi (2.06 bar). The sheath gas temperature and flow were set to 350°C and 12 L/min, respectively. The MS was calibrated using reference calibrant introduced from the independent ESI reference sprayer. The VCap, Fragmentor and Skimmer voltages were set to 1500, 100 and 45 V respectively. For All ions MS/MS the three scan segments were set with collision energies of 0, 20 and 40 eV. The chromatographic separation (5 µL sample injection) was performed on an Agilent InfinityLab Poroshell 120 HILIC-Z 2.1 x 100 mm, 2.7 µm column at 0.5 mL/min. Mobile phase A was H_2O with 0.1% formic acid, and mobile phase B consisted of 20 mM ammonium formate (pH 3) in 90% acetonitrile. Gradient elution started at 100% B, progressing to 70% A at 11.5 min, returned to 100% B at 12 min, and held for 3 min for re-equilibration in a total 15 min run. Data analyses were performed in MassHunter Quantitative analysis B0.10 (available via virtual server platform).

References

1. Werneth M., Koellensperger G., Hann S. (2019) Analysis of Underivatized AAs: Zwitterionic Hydrophilic Interaction Chromatography Combined with Triple Quadrupole Tandem Mass Spectrometry. In: Alterman M. (eds) AA Analysis. Methods in Molecular Biology, vol. 2030. Humana, New York, NY, Print ISBN 978-1-4939-9638-4, Online ISBN 978-1-4939-9639-1

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