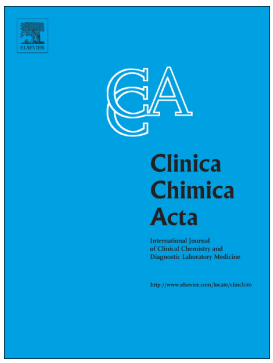

Rapid amino acid quantitation with pre-column derivatization; ultra-performance reverse phase liquid chromatography and single quadrupole mass spectrometry

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1. Title

Rapid amino acid quantitation with pre-column derivatization; ultra-performance reverse phase liquid chromatography and single quadrupole mass spectrometry.

2. Running head:

Rapid amino acid quantitation with mass spectrometry.

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8. Abbreviations:

UPLC: ultra-performance liquid chromatography

AQC: 6-aminoquinolyl-N-hydroxysuccinimidyl carbonate

nva: norvaline

UV:	ultraviolet
MS:	mass spectrometry
lis:	labeled internal standards ($^{13}C^{15}N$ isotopes)
Sd:	standard deviation
csf:	cerebrospinal fluid
PEA:	phosphoethanolamine

Abstract

BACKGROUND: We optimized a quantitative amino acid method with pre-column derivatization, norvaline (nva) internal standard and reverse phase ultra-performance liquid chromatography by replacing the ultraviolet detector with a single quadrupole mass spectrometer (MS_{nva}).

METHOD: We used $^{13}C^{15}N$ isotopically labeled amino acid internal standards and a C18 column with 1.6 μm particles to optimize the chromatography and to confirm separation of isobaric compounds (MS_{lis}). We compared the analytical performance of MS_{nva} with MS_{lis} and the original method (UV_{nva}) with clinical samples.

RESULTS: The chromatography time per sample of MS_{nva} was 8 minutes, detection capabilities were <1 μmol/L for most components, intermediate imprecisions at low concentrations were <10% and there was negligible carryover. MS_{nva} was linear up to a total amino acid concentration in a sample of approximately 9 500 μmol/L. The agreements between most individual amino acids were satisfactory compared to UV_{nva} with the latter prone to outliers and suboptimal quantitation of urinary arginine, aspartate, glutamate and methionine. MS_{nva} reliably detected argininosuccinate, β-alanine, citrulline and cysteine-s-sulfate.

CONCLUSION: MS_{nva} resulted in a more than fivefold increase in operational efficiency with accurate detection of amino acids and metabolic intermediates in clinical samples.

1. Introduction.

Quantitative amino acid analysis in plasma, urine and cerebrospinal fluid is central to the diagnosis and therapeutic management of inherited disorders of amino acid transport and metabolism. The reproducible ninhydrin post column derivatization method with spectrophotometric quantitation described by Stein and Moore (1, 2) has been the foundation of this analysis for nearly seven decades. Separation of the amino acids and related components was typically achieved with ion exchange liquid chromatography.

The relatively recent introduction of reverse phase ultra-performance liquid chromatography (UPLC) and 6-aminoquinolyl-N-hydroxysuccinimidyl carbonate (AQC) (3) pre-column derivatization with norvaline (nva) as internal standard and ultraviolet (UV) detection has reduced the chromatography time from approximately 3 hours to 45 minutes per sample (4). Analysis of multiple amino acids in a single chromatography run remains a challenge due to their diverse chemical characteristics and the wide concentration ranges encountered in health and disease. Unpredictable interference can occur in some patients due to co-eluting exogenous compounds that also absorb in the UV spectrum (e.g. gabapentin and histidine; aminoglycosides and phenylalanine; aminocaproic acid (6)). The incomplete resolution of rarely occurring endogenous metabolites such as argininosuccinate with ethanolamine and 1-methyl histidine with cysteine-s-sulfate can also be problematical and repeat analyses with altered chromatographic conditions are required to resolve them (5). The throughput of a UV based method is therefore limited by the long chromatography times and the occasional need to repeat the analysis.

Tandem mass spectrometry (MS/MS) with its inherent analytical specificity has the potential to avoid the deficiencies of UV based methods. MS/MS methods with or without a derivatization step and using either a single unlabeled or multiple isotopically labeled internal standards (lis) have been described (7, 8, 9). Isobaric compounds (leucine; isoleucine;

alloisoleucine; proline, β-alanine; alanine; sarcosine, 1 and 2-methylhistidines and α-, β- and γ-butyrates) must be resolved either chromatographically or by a unique ion fragmentation pattern.

Single quadrupole mass spectrometry (MS) detectors are more affordable, robust, and relatively simple to operate and is a promising detection technique with analytical properties intermediate between UV and MS/MS (10). The accurate mass detection may provide superior resolution to UV for co-eluting compounds with a different molecular mass, but the absence of ion fragments in this type of detector makes chromatographic separation of isobaric components paramount. A potential obstacle to MS detection is the lack of readily available isotopically labeled internal standards for each amino acid and related metabolite of interest in clinical specimens.

Our aim was to optimize an established quantitative amino acid method by using MS detection, streamlining sample preparation and optimizing the chromatographic conditions. We retained the AQC derivatization procedure and also confirmed the suitability of nva as a single isotopically unlabeled internal standard.

2. Materials and Methods

As a proof of concept we swapped the UV detector of a commercial amino acid method (UV_{nva}) with a MS detector while keeping the sample preparation and chromatographic conditions unchanged. In a stepwise manner we optimized the sample preparation and the chromatographic conditions while using isotopically labeled amino acids as internal standards (MS_{lis}). To assess the suitability of nva as internal standard we compared this combination (MS_{nva}) to MS_{lis} as well as the original method with UV detection (UV_{nva}).

2.1 INSTRUMENTATION AND REAGENTS

The following items were sourced from Waters (Waters Corporation, Milford, USA): MassTrak AAA Solution kit, an Acquity UPLC system with a binary solvent manager,

sample organizer and Empower data manager to perform UV_{nva} (4). An Acquity QDa single quadrupole mass detector, a Cortecs UPLC BEH C18 column (1.6 μm particles; 2.1 x 150 mm), the neutral, acidic and basic amino acid standard solutions as well as allo-isoleucine, glutamine, nva and AQC reagents of the MassTrak AAA Solution kit were used for MS_{lis} and MS_{nva}.

We expanded the range of amino acids in the commercial calibrators by adding argininosuccinate, cysteine-s-sulfate and homocitrulline (all obtained from Sigma Aldrich, St Louis, USA) to the mixture. Working calibrators were prepared at concentrations of 100; 200 and 400 μmol/L for all the components with the exception of cystine and cystathionine which were present at 50; 100 and 200 μmol/L respectively. ^{13}C and ^{15}N isotopically labeled amino acids (Metabolomics Amino Acid Mix Standard) were obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). The formic acid (Sigma Aldrich) and acetonitrile (Thermo Fisher, Waltham, USA) were MS-grade.

Two levels of ClinChek amino acid quality control material (Recipe, Munich, Germany) were used to investigate imprecision. External quality proficiency material was obtained from the European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM, Netherlands) and the Australasian Society for Inborn Errors of Metabolism and the Human Genetics Society of Australasia (Sydney, Australia) education program.

2.2 SAMPLE PREPERATION

With UV_{nva} the samples, calibrators and controls were prepared according to the manufacturer's instructions (4). To improve workflow we investigated whether we could perform the penultimate heating step of the original UV_{nva} after the final addition of 900 μL water. In the optimized procedure we added 25 μL of either the calibrator, quality control material or sample and 25 μL water to 50 μL of 200 μmol/L norvaline in 10% 5-

sulfosalicylic acid. Ten (10) μL of supernatant obtained after centrifugation for 5 minutes at 10000g was added into 70 μL borate buffer in 0.2 mol/L NaOH. This was followed by adding 20 μL of the derivatization reagent and vortexing for 10 seconds. The volume was then made up to 1000 μL with water, vortexed for 10 seconds after which the vials were placed in a 55^{o}C heating block for 10 min.

2.3 OPTIMIZED CHROMATOGRAPHY AND MASS SPECTROMETRY CONDITIONS

The column temperature was 55^{o}C, flow rate 0.5 mL/min with the initial conditions of 99% mobile phase A (0.1% formic acid in water) and 1% mobile phase B (0.1% formic acid in acetonitrile). After 0.5 μL of sample was injected a linear gradient (curve 6) was applied to 87% A at 2 min, followed by 85% A at 4 min and 5% A at 5 min before returning to initial conditions from 7.6 to 8 min for re-equilibration. The ion source was a heated electrospray interface operated in the positive mode with the detector programmed to sample single ion reactions of selected atomic masses during timed intervals as depicted in Figure 1. The probe temperature was 600^{o}C, the cone voltage 0.8 kV and the sampling rate 5 points/second.

2.4 METHOD VALIDATION

We characterized the detection capability, imprecision, carryover and linearity of the MS_{nva} method. The detection capability was estimated by analyzing samples with concentrations of 0, 1, 5 and 10 μmol/L ten times each in one run, plotting the standard deviation (Sd) against concentration and then estimating by extrapolation the Sd_{blank} and concentration where the imprecision was 20% ($LOQ_{20\%}$). Intermediate imprecision was determined over 20 days at two control levels. Carryover between samples was assessed by analyzing a blank immediately after a sample with high concentrations. Grossly elevated total amino acid concentrations in clinical samples may potentially exhaust the derivatizing reagent and result in non-linearity. We investigated this by analyzing 3 separate post mortem plasma samples, with extremely high total amino acid concentrations at several dilutions and then sequentially

discarding the highest value before repeating the statistical analysis. We tested the linearity of the high calibrator and also for selected amino acids that were anticipated to occur at higher levels in clinical samples (glycine, histidine, threonine and serine).

We compared MS_{nva} to the MS_{lis} with 139 plasma, 100 urine and 97 cerebrospinal fluid (csf) samples submitted for routine amino acid analysis. After the optimization process was complete we compared MS_{nva} with UV_{nva} in another set of 80 plasma and 114 urine samples in order to standardize the time between sample preparation and analysis so that any artefactual conversion of glutamine to glutamate and arginine to ornithine was minimized. In our routine service delivery we use the UV_{nva} as originally described (4) and if interference is suspected in a sample we follow this up with a second analysis under alternative chromatographic conditions (5). In the scatterplots and comparisons we only present results obtained with the original UV_{nva} and not the final value obtained with the further analysis. We tested the ability of MS_{nva} to deal with problematical samples by analyzing QAP and stored patient samples. The statistical analysis and non-parametric Passing Bablok regressions were performed with Analyse-it version 2.30 (Leeds, UK). Pearson's parametric correlation was used to assess the relative strengths of the associations.

3. Results

The addition of water before the heating step resulted in a more efficient process and did not significantly change the median values for the 17 amino acids with labeled internal standards measured in 37 plasma and 28 urine samples ($p > 0.05$ in all instances). The total chromatographic run time of the optimized MS_{nva} method was 8.0 minutes with resolution of isobaric components (Figure 1). Carryover between successive runs varied between 0.1 and 0.5% for all amino acids tested and did not materially affect subsequent samples. The analytical performance characteristics of the MS_{nva} method are presented in table 1. The detection limits for the individual amino acids were acceptable and only the Sd_{blank} of serine

was above 1 µmol/L which did not materially impact on the intended clinical utility. The intermediate imprecision was also appropriate for clinical purposes. The precision data is presented as the mean and Sd in Table 1 to allow an appreciation of the concentration ranges of the individual components in the quality control material. In summary the coefficient of variation was between 5% and 10% for the majority of individual amino acids. The MS_{nva} linear range extended up to a total amino acid concentration of approximately 9500 µmol/L (Supplementary Figure 1), which compared favorably with the 3500 µmol/L reported for UV_{nva} (4). We also confirmed the linearity of the high calibrator that contained 33 components with individual concentrations of 400 µmol/L and two with 200 µmol/L each. We confirmed the individual linearity in pure solutions of glycine up to 5000 µmol/L and of histidine, threonine and serine each up to 2000 µmol/L.

The scatter plots comparisons of plasma and urine samples assayed with MS_{nva}, MS_{lis} and UV_{nva} for amino acids with an available isotopically labeled internal standard are depicted in Figures 2 and 3 as well as in Supplementary Figure 2. The comparisons between MS_{nva} and UV_{nva} for those without a labeled internal standard are shown in supplementary Figure 3. The regression statistics are summarized in Supplementary Table 1. With some notable exceptions the agreements between the methods were good with MS_{nva} results acceptable for clinical use. In the group with labeled internal standards the agreement between the MS_{lis} and MS_{nva} was generally better than between MS_{nva} and UV_{nva} with R^2 values and regression slopes closer to 1 and offsets closer to 0. There were suboptimal agreements between MS_{nva} and UV_{nva} with arginine, aspartate, and glutamate (Supplementary Figure 2) and with methionine (Figure 2) in urine samples. Due to concerns about the stability of asparagine, arginine and glutamine in stored samples we repeated the comparisons between MS_{nva} and UV_{nva} in a second sample set with the sample preparations and analyses performed on the same day – we present only the data of this second comparison in this

manuscript, although the comparisons were not materially different for any individual component. The external proficiency samples demonstrated differences in plasma glutamine and glutamate concentrations due to spontaneous conversion during storage, but the qualitative interpretation of the results were not affected (data not shown).

In the group of amino acids without labeled internal standards poor agreements were noted between MS_{nva} and UV_{nva} with plasma alloisoleucine, β-alanine and urine citrulline. A striking feature in this group was the number of fliers with an apparent very high UV_{nva} result and a much lower MS_{nva} result as shown in Figure 3 with urine isoleucine. We found the opposite in only one plasma β-alanine sample where a low UV_{nva} result occurred with an relatively elevated MS_{nva} (Supplementary Figure 3). The comparisons of all the amino acids measured in csf samples are not shown, but the results for methionine and isoleucine that are illustrated in Figures 2 and 3 were representative for this sample type. The comparisons in csf were complicated by the low concentrations of most amino acids, but the same general pattern observed with plasma and urine samples was present, namely better agreement between MS_{nva} and MS_{lis} than between MS_{nva} and UV_{nva}.

Argininosuccinate, β-alanine, 1-methylhistidine and cysteine-s-sulfate were accurately detected according to their atomic masses and the isobaric amino acids were chromatographically resolved with accurate quantitation by MS_{nva} (Figure 1, Supplementary Figures 4 and 5). With UV_{nva} argininosuccinate co-elutes with ethanolamine and a modification of with chromatographic conditions is required to separate them (5). In instances where co-elution occurred with MS_{nva}, i.e. with arginine, asparagine and phosphoethanolamine, the mass selectivity allowed unambiguous detection (Figure 1). Interferences that affected UV_{nva} did not impact on MS_{nva} (Supplementary Figures 5 and 6).

4. Discussion

We describe a modified amino acid method with pre-column AQC derivatization, norvaline as a single unlabeled internal standard, reverse phase UPLC and single quadrupole mass spectrometry. The short chromatography time was a function of a C18-reverse phase column with smaller particles and of the MS detector with orthogonal atomic mass separation of co-eluting non-isobaric components. The streamlined sample processing, short chromatography time and confident identification of multiple components in complex sample matrices resulted in a more than five-fold improvement in operational efficiency. Our routine UV_{nva} service was nearing its maximum capacity and this motivated us to explore alternative options, such as MS, rather than purchasing a second dedicated amino acid analyzer system. The cost of a single quadrupole mass detector is approximately half that of a complete UPLC system with an UV detector, but instead of simply doubling the capacity MS_{nva} has resulted in much larger gains and at a lesser cost. This was also achieved without increasing the instrument footprint, equipment maintenance or additional staff.

The comparisons between MS_{nva} and UV_{nva} were suboptimal for urinary arginine, aspartate, glutamate and methionine (Supplementary Figure 2 and Figure 2). In each instance the scatter around the regression lines were greater than could be explained by their respective analytical imprecisions. With this group of amino acids the good agreement with the $^{13}C^{15}N$ isotopically labelled MS_{lis} results led us to conclude that MS_{nva} was more accurate than UV_{nva}. This finding was expected due to the inherent analytical properties of mass spectrometry compared to UV detection. We could not identify a cause(s) for the outliers illustrated in Figure 3 and they persisted in selected cases even when we repeated the UV_{nva} under alternative chromatographic conditions. The clinical impact of the poor agreement, which we attribute to the inherent limitations of UV_{nva}, should however not be overstated as the affected amino acids are not pathognomonic and inaccurate measurement has not been a major issue to date with UV_{nva}. To put this into context one should appreciate that only two

cases of a putative methionine malabsorption syndrome (OMIM % 250900) have been reported. The isolated instances of proportional bias (Supplementary Table 1) could be attributed to the small analytical range (<10 μmol/L) and were mostly confined to csf with the exception of u-methionine and p-/u-glutamate. The effect on clinical interpretation and reference intervals are expected to be minimal, but this will be monitored prospectively.

Our decision to persist with pre-column AQC derivatization and reverse phase chromatography was influenced by the proven efficiency of this modality to separate amino acids that range from acidic to basic and it also avoided the use of more complex column switching or MS unfriendly ion-paring chromatography. Derivatization to butyl esters was reported to destroy asparagine (7), but from our data this does not appear to occur with AQC. Although the use of a labeled internal standard for each analyte in the chromatogram have theoretical advantages and would be required for a reference method, we propose that a nva internal standard provides a pragmatic alternative. Isotopically labeled internal standards for each amino acid and related metabolites are not freely available, will result in added cost and may complicate the data analyses. Furthermore the sampling frequency of peaks will decrease and these closely eluting labeled components may contribute to ion suppression.

The ability to rapidly and confidently identify clinically important metabolites such as cysteine-s-sulfate and argininosuccinate may potentially lead to improved clinical outcomes. With UV_{nva} this required an additional set of chromatographic conditions (4, 5) and a second analysis which resulted in complex workflows and reliance on experienced analysts. With UV_{nva} tyrosine elutes close to the N-hydroxy-succimide derivatization byproduct that absorbs in the UV-spectrum and can thus negatively affect accurate quantitation. Since MS_{nva} relies on atomic mass it was not affected by this interference. The detection capability and extended linear range makes MS_{nva} suitable for use with all types of biological samples and the

analytical sensitivity can be further improved, albeit at the expense of linear range, when one considers that only 0.5 μL is injected onto the column.

There MS_{nva} chromatographic peak sizes of the calibrator, with all the components essentially at the same concentration, varied considerably (Figure 1). The ion counts of citrulline, ethanolamine, homocitrulline, phosphoethanolamine and taurine were markedly lower than those of ornithine, lysine and phenylalanine. In comparison with UV_{nva} and the same calibrator, the peaks of ornithine and lysine were prominent, but those of the other components were average. This suggested to us that the derivatization efficiencies of ornithine and lysine were higher than average and that this variability may contribute to range of peak sizes, but could not fully explain it. The chromatograms of a MS/MS method without derivatization (9) had an even more variation in the ion count peaks and we speculated that variable ionization efficiencies of individual amino acids and other components may partly contribute to this peak size phenomenon. We did not detect a pattern in the physical properties such as pI, molecular size or presence of side chains that could have suggested what the underlying mechanism may be. Ion suppression can also theoretically contribute to variations of peak sizes, but we would have expected that at least one of the 17 labeled amino acids would have had a poor comparison between MS_{nva} and MS_{lis} if this was a common occurrence. Notably taurine and lysine were on opposite sides of the peak size spectrum and both had good agreements between MS_{nva} and MS_{lis}.

The absence of performance and comparative data for homocystine is a potential limitation. Homocystine is present in the calibrators (Figure 1), but not in the commercial quality control materials and we therefore did not investigate the analytical performances for this analyte. The highest MS_{nva} homocystine signals detected corresponded to concentrations of approximately 0.3 and 0.1 μmol/L in plasma and urine samples respectively. We did not detect a homocystine signal in any sample with UV_{nva}. Our historic practice is not to report

UV_{nva} homocystine levels, but to monitor plasma methionine levels as a surrogate marker for homocystinuria (OMIM # 236200) and to measure total homocyst(e)ine as confirmatory test (11). Similarly serum vitamin B12 and urine methylmalonic acid would be measured in cases with suspected metabolic disorders (OMIM # 277400, # 275350) or vitamin deficiency states.

In our opinion MS_{nva} is a superior analytical technique with improved analytical specificity, a short analysis time, and is well suited for routine clinical use. The introduction of MS_{nva} has resulted in a marked operational efficiency improvement for a relatively modest capital investment.

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Table1. Analytical performance characteristics of the UPLC single quadrupole mass spectrometry method with pre-column AQC derivatization and with norvaline as internal standard (MS_{nva}).

Amino acid	[a]Retention	[b]m/z	[c]Sd_{blank}	[d]$LoQ_{20\%}$	[e]$QC1_{Mean}$	[e]$QC1_{Sd}$	[e]$QC2_{Mean}$	[e]$QC2_{Sd}$
Histidine	2.169	163.6	0.31	0.99	72	5.7	291	16.7
3-methylhistidine	2.217	170.6[f]	0.41	1.82	5.4	0.6	9.9	0.6
1-methylhistidine	2.238	170.6[f]	0.32	1.01	4.8	0.9	7.3	1.2
Hydroxyproline	2.223	302.1[g]	0.16	0.50	15	1.8	35	1.9
Arginine	2.282	173.1	0.30	1.04	52	6.0	247	20.9
Asparagine	2.278	303.1	0.29	0.90	51	3.7	246	11.4
Taurine	2.344	296.1	0.15	0.42	33	2.7	118	8.8
Argininosuccinate	2.350	231.2	0.32	1.84	na	na	na	na
Glutamine	2.361	317.1	0.13	0.35	289	14.1	640	28.5
Serine	2.375	276.1	1.12	4.70	88	4.8	250	8.1
Glycine	2.439	246.1	0.82	3.91	168	14.1	573	27.7
Aspartate	2.475	304.1	0.29	0.67	10	0.4	81	3.4
Citrulline	2.490	346.1	0.32	0.89	23	2.0	204	7.6
Glutamate	2.536	318.1	0.24	0.60	159	13.8	263	22.9
Threonine	2.612	290.1	0.34	0.91	107	5.5	195	9.4
Alanine	2.713	260.1	0.81	2.12	356	18.9	611	24.4
Ornithine	2.829	237.1	0.57	1.74	71	5.3	302	10.9
Proline	2.846	286.1	0.18	0.46	205	10.9	425	15.6
Cystine	3.009	291.2	0.08	0.32	14	1.62	20	1.9
Lysine	3.031	244.1	0.20	0.84	152	9.2	231	9.8
α-aminobutyrate	3.067	274.1	0.07	0.20	15	0.9	61	2.8
Tyrosine	3.465	352.1	0.05	0.14	58	5.5	232	7.8
Methionine	3.718	320.1	0.08	0.23	34	2.2	58	3.5
Valine	2.850	288.1	0.24	0.69	203	9.7	381	10.4
Isoleucine	5.683	302.1[g]	0.10	0.31	68	4.2	116	3.4
alloisoleucine	5.761	302.1[g]	0.10	0.28	na	na	na	na
Leucine	5.956	302.1[g]	0.24	0.92	186	9.0	294	11.1
Phenylalanine	6.369	336.1	0.27	0.75	81	5.1	402	16.2
Tryptophan	6.420	375.1	0.14	0.41	52	5.6	210	11.1

[a] retention time in minutes, [b] Atomic mass (Dalton) to charge ratio, [c] Standard deviation of the blank (µmol/L), [d] Limit where a 20% coefficient of variation was achieved (µmol/L), [e] Intermediate precision data collected over 20 days with two levels of quality control material are presented as the mean value and standard deviation in µmol/L, [f] isobaric with m/z 170.6, [g] isobaric with m/z 302.1, na: not available. Please note that histidine, 1- and 2-methylhistidine, arginine and argininosuccinate are doubly charged

molecules. In source fragmentation may possibly explain the apparent loss of atomic mass of ornithine, cystine and lysine.

Figure legends.

Figure 1. Total ion count chromatograms of the optimized MS_{nva} method.

The ion count intensities of each amino acid in the 200 μmol/L calibrator (cystine and cystathionine 100μmol/L) are depicted. After the addition of water and the internal standard the concentrations were diluted fourfold to 50 μmol/L while the 200 μmol/L norvaline is diluted twofold to 100 μmol/L. The ion counts were obtained in single ion reaction monitoring mode with the atomic mass charge ratios of the derivatized amino acids indicated. The isobaric components are highlighted with the same color text boxes. In the bottom part of the figure the window between 2.1 and 3.1 minutes is expanded to facilitate peak identification. Cystathionine is separated into the L(+) and L(-) allocystathionine stereoisomers. PEA phosphoethanolamine.

Figure 2. Comparison of plasma, urine and cerebrospinal fluid methionine measured with MS_{lis} MS_{nva} and UV_{nva}

MS_{nva} single quadrupole mass spectrometry with norvaline as internal standard.

MS_{lis} single quadrupole mass spectrometry with $^{13}C^{15}N$ labeled internal standard.

UV_{nva} UV detction with norvaline as internal standard.

a: Passing Bablok regression offset,

b: Passing Bablok regression slope

R^2: Pearson correlation coefficient.

Solid symbols indicate the comparison between MS_{lis} and $MS_{nva.}$

Open symbols indicate the comparison between UV_{nva} and $MS_{nva.}$

Green indicates plasma, blue urine and black cerebrospinal fluid.

Figure 3. Comparison of isoleucine measured with MS_{lis} MS_{nva} and UV_{nva} in plasma, urine and cerebrospinal fluid.

MS_{nva} single quadrupole mass spectrometry with norvaline as internal standard.

MS_{lis} single quadrupole mass spectrometry with $^{13}C^{15}N$ labeled internal standard.

UV_{nva} UV detction with norvaline as internal standard.

a: Passing Bablok regression offset,

b: Passing Bablok regression slope

R^2: Pearson correlation coefficient.

Solid symbols indicate the comparison between MS_{lis} and $MS_{nva.}$

Open symbols indicate the comparison between UV_{nva} and $MS_{nva.}$

Green indicates plasma, blue urine and black cerebrospinal fluid.

We modified a quantitative amino acid method with a norvaline internal standard (nva), pre-column derivatization, reverse phase ultra-performance liquid chromatography and ultraviolet detection. The modified method retained the nva and derivatization steps but with a smaller particle C18 column and detection by single quadrupole mass spectrometry amino acids were accurately quantitated within 8 minutes. The new method increased capacity by more than fivefold and accurately detected problematical metabolites such as argininosuccinate.

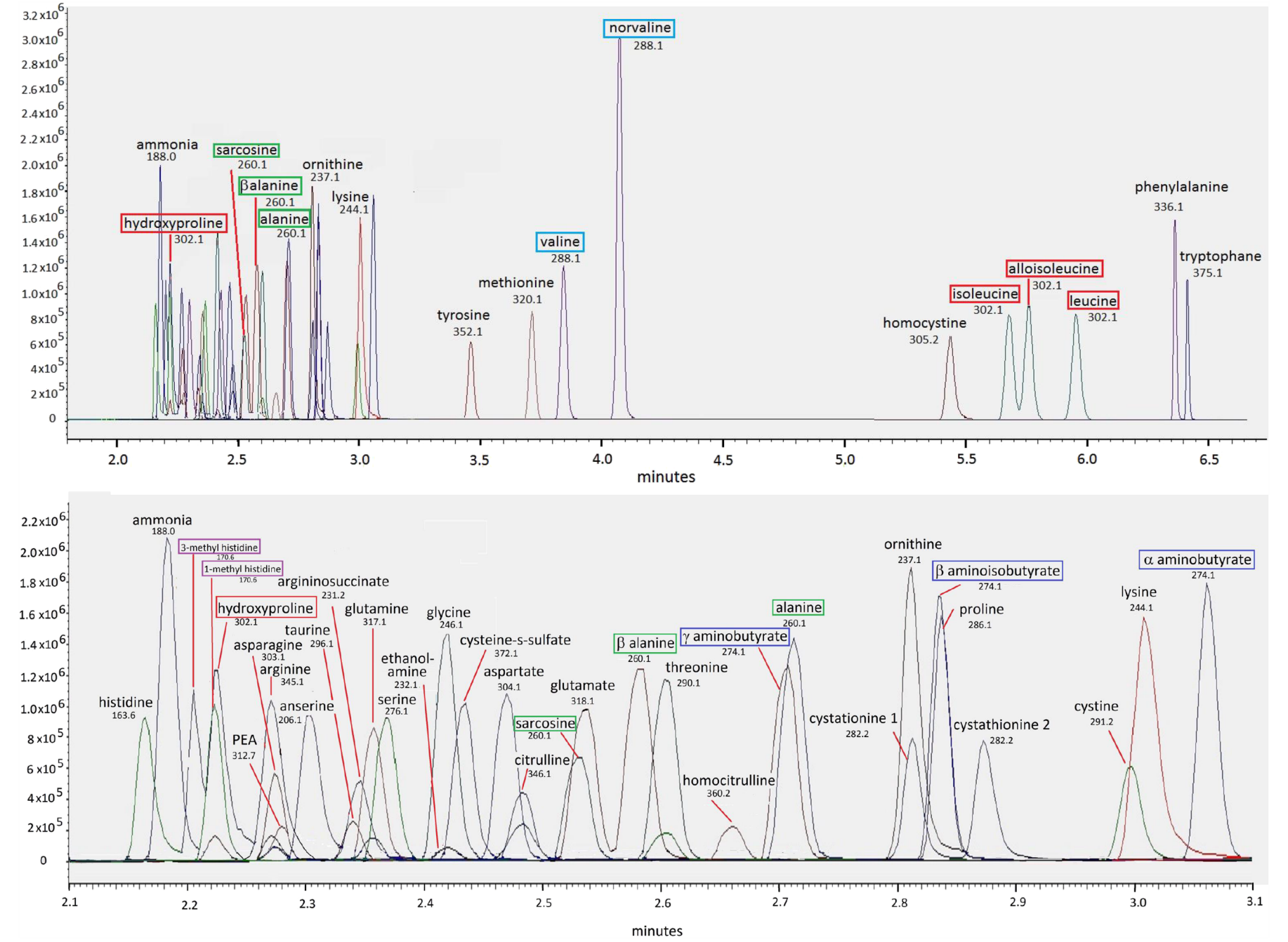
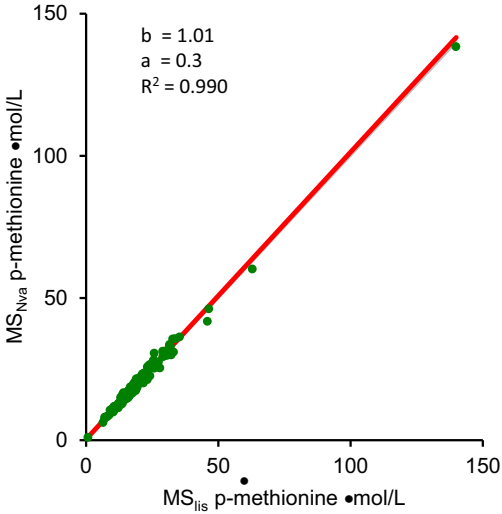
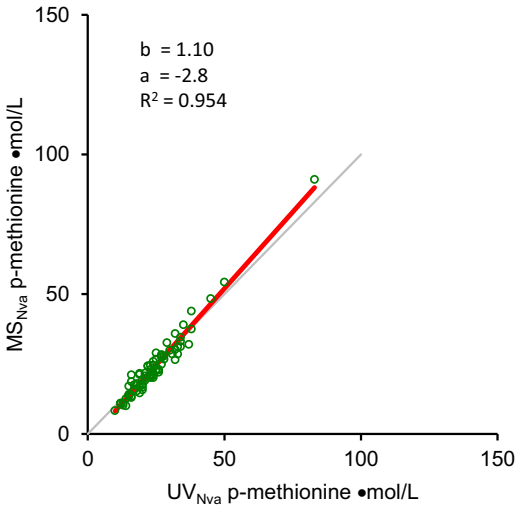
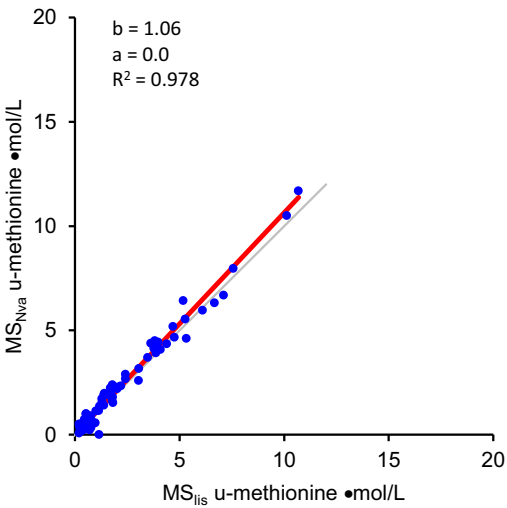
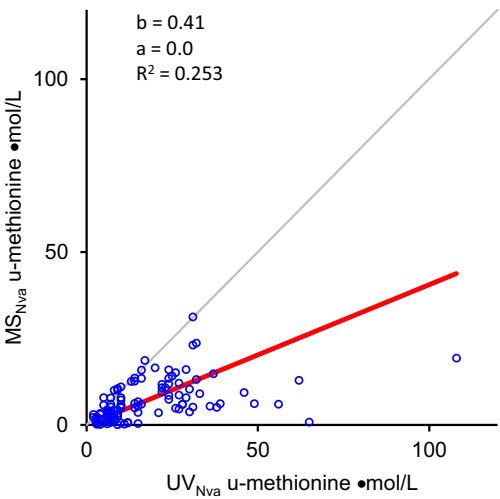

norvaline
288.1
ammonia
188.0
sarcosine
260.1
ornithine
237.1
βalanine
260.1
lysine
244.1
hydroxyproline
302.1
alanine
260.1
valine
288.1
phenylalanine
336.1
tryptophane
375.1
alloisoleucine
302.1
isoleucine
302.1
leucine
302.1
methionine
320.1
tyrosine
352.1
homocystine
305.2
minutes
ammonia
188.0
3-methyl histidine
170.6
1-methyl histidine
170.6
argininosuccinate
231.2
hydroxyproline
302.1
glutamine
317.1
glycine
246.1
taurine
296.1
asparagine
303.1
cysteine-s-sulfate
372.1
β alanine
260.1
γ aminobutyrate
274.1
alanine
260.1
ornithine
237.1
β aminoisobutyrate
274.1
α aminobutyrate
274.1
lysine
244.1
proline
286.1
arginine
345.1
ethanol-
amine
232.1
aspartate
304.1
threonine
290.1
glutamate
318.1
histidine
163.6
anserine
206.1
serine
276.1
sarcosine
260.1
cystationine 1
282.2
cystathionine 2
282.2
cystine
291.2
PEA
312.7
citrulline
346.1
homocitrulline
360.2
minutes

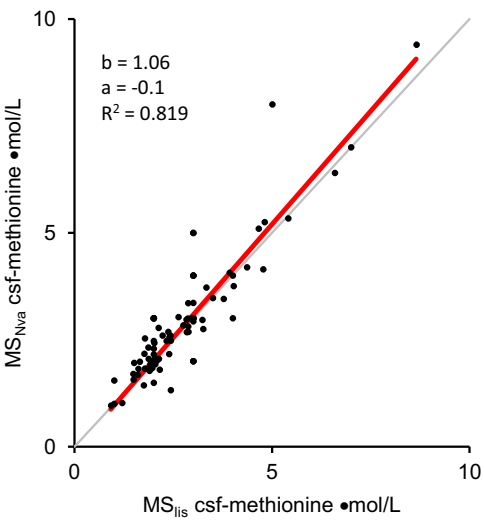
Figure 1


b = 1.01
a = 0.3
R² = 0.990
MSNva p-methionine •mol/L
MSlis p-methionine •mol/L
0
50
100
150


b = 1.10
a = -2.8
R² = 0.954
MSNva p-methionine •mol/L
UVNva p-methionine •mol/L
0
50
100
150


b = 1.06
a = 0.0
R² = 0.978
MSNva u-methionine •mol/L
MSlis u-methionine •mol/L
0
5
10
15
20


b = 0.41
a = 0.0
R² = 0.253
MSNva u-methionine •mol/L
UVNva u-methionine •mol/L
0
50
100


b = 1.06
a = -0.1
R² = 0.819
MSNva csf-methionine •mol/L
MSlis csf-methionine •mol/L
0
5
10

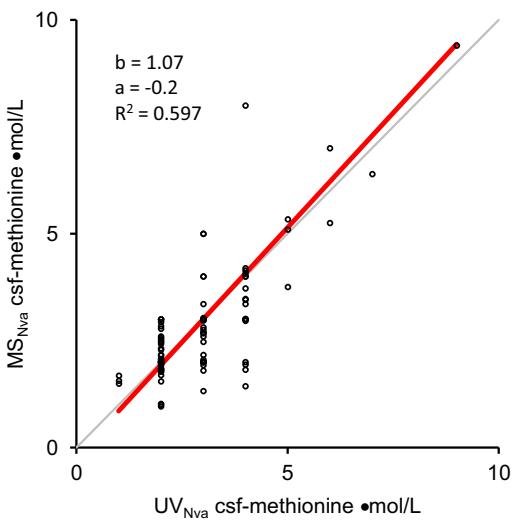
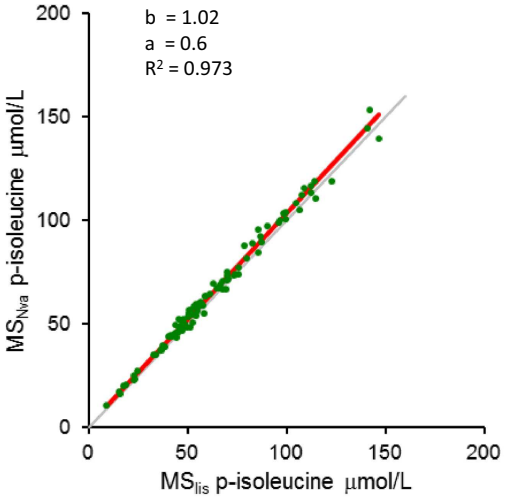
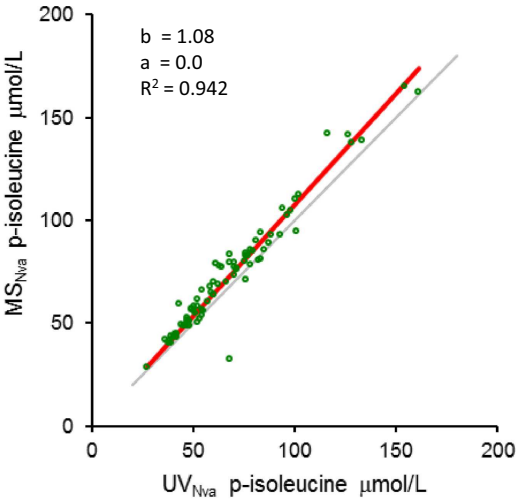
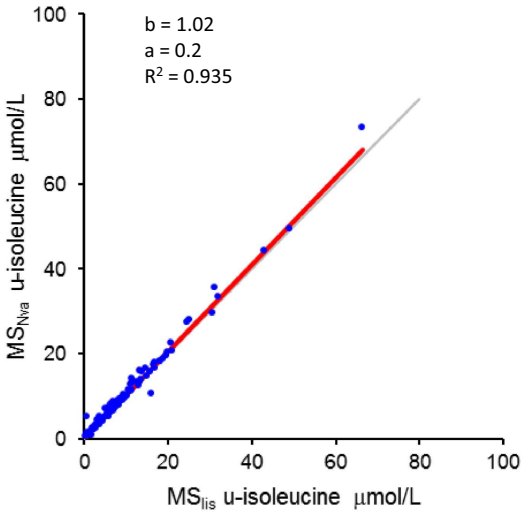
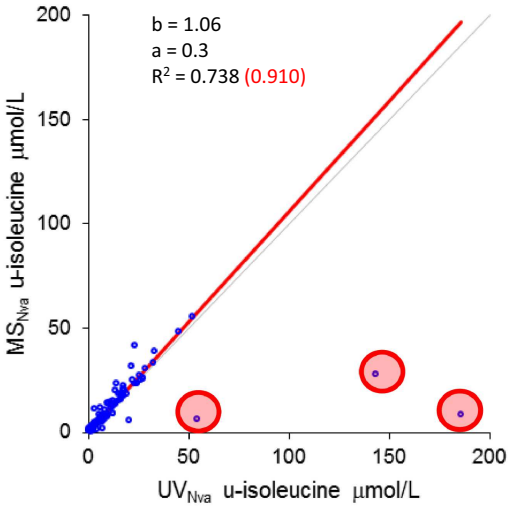

b = 1.07
a = -0.2
R² = 0.597
MSNva csf-methionine •mol/L
UVNva csf-methionine •mol/L
0
5
10

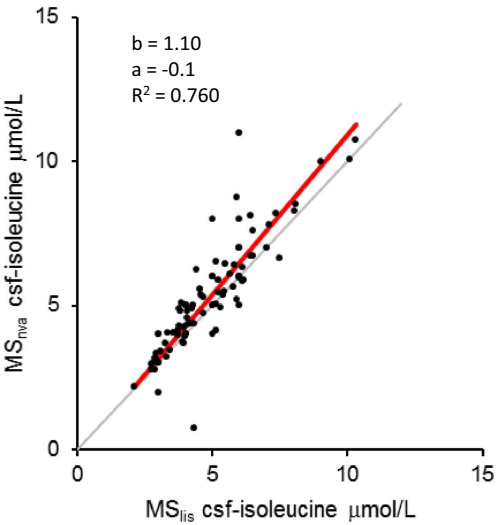
Figure 2


b = 1.02
a = 0.6
R² = 0.973
MSNva p-isoleucine µmol/L
MSlis p-isoleucine µmol/L


b = 1.08
a = 0.0
R² = 0.942
MSNva p-isoleucine µmol/L
UVNva p-isoleucine µmol/L


b = 1.02
a = 0.2
R² = 0.935
MSNva u-isoleucine µmol/L
MSlis u-isoleucine µmol/L


b = 1.06
a = 0.3
R² = 0.738 (0.910)
MSNva u-isoleucine µmol/L
UVNva u-isoleucine µmol/L


b = 1.10
a = -0.1
R² = 0.760
MSnva csf-isoleucine µmol/L
MSlis csf-isoleucine µmol/L

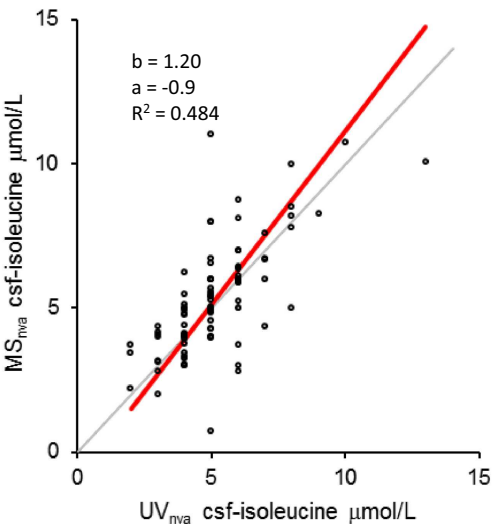

b = 1.20
a = -0.9
R² = 0.484
MSnva csf-isoleucine µmol/L
UVnva csf-isoleucine µmol/L

Figure 3