CHROMSYSTEMS

STOFFWECHSELERKRANKUNGEN
METABOLIC DISEASES
MALADIES MÉTABOLIQUES
MALATTIE METABOLICHE
ENFERMEDADES METABÓLICAS

Instruction Manual for LC-MS/MS Analysis

MassChrom®

Amino Acid Analysis in plasma/serum

Order No. 75111

CE 0123 IVD

Incident reporting:
Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

Chromsystems Instruments & Chemicals GmbH is certified according to ISO 13485 (including MDSAP). Products are produced and put into circulation according to directive 98/79/EC on in vitro diagnostic medical devices.

You can download the declaration of conformity according to directive 98/79/EC from the download centre of our website.

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1 Ordering information

Order no.	Product	
75111	**LC-MS/MS Reagent Kit** ***MassChrom*® Amino Acid Analysis in plasma/serum**	
	Contents for 3 x 96 analyses:	
	Mobile Phase A	4 x 950 ml
	Mobile Phase B	1 x 950 ml
	Rinsing Solution	1 x 500 ml
	Internal Standard Set, consisting of:	
	Internal Standard Mix	3 x 5.0 ml (lyoph.)
	Reconstitution Buffer	3 x 5.5 ml
	Precipitation Reagent	3 x 40 ml
	Reaction Vials, 1.5 ml	3 x 100 pcs.
75111/DWP	**LC-MS/MS Reagent Kit** ***MassChrom*® Amino Acid Analysis in plasma/serum** with 96 Deep Well Plates	
	Contents for 3 x 96 analyses:	
	Mobile Phase A	4 x 950 ml
	Mobile Phase B	1 x 950 ml
	Rinsing Solution	1 x 500 ml
	Internal Standard Set, consisting of:	
	Internal Standard Mix	3 x 5.0 ml (lyoph.)
	Reconstitution Buffer	3 x 5.5 ml
	Precipitation Reagent	3 x 40 ml
	96 Deep Well Plates	4 pcs.
	Collection Plates	4 pcs.
	Pierceable Heat Seals, for 96 well plates	5 pcs.
	Components available separately	
75001	Mobile Phase A ***MassChrom*® Amino Acid Analysis**	950 ml
75002	Mobile Phase B ***MassChrom*® Amino Acid Analysis**	950 ml
75009	Rinsing Solution ***MassChrom*® Amino Acid Analysis**	500 ml
75146	Internal Standard Set ***MassChrom*® Amino Acid Analysis in plasma/serum, consisting of:**	
	Internal Standard Mix	3 x 5.0 ml (lyoph.)
	Reconstitution Buffer	3 x 5.5 ml
75105	Precipitation Reagent ***MassChrom*® Amino Acid Analysis in plasma/serum**	40 ml

	Accessories	
75100	Analytical Column (equilibrated, with test chromatogram)	
	MassChrom® Amino Acid Analysis	1 pc.
15010	PEEK Prefilter Housing	1 pc.
55033	PEEK Prefilter, 2 µm	5 pcs.
15080	Back Pressure Regulator Valve	1 pc.
J0601	Autosampler Vials, screw neck, amber glass, 1.5 ml	100 pcs.
J0504	PE Screw-on Caps, rubber/PTFE septa, 9 mm	100 pcs.
J0410	PP Screw-on Caps, pierceable silicone/PTFE septa, 1.0 mm	100 pcs.
J0505	Micro-Inserts for Autosampler Vials, clear glass, flat bottom	100 pcs.
33006	Reaction Vials, 1.5 ml	100 pcs.
75058	Collection Plates	
	MassChrom® Amino Acid Analysis	4 pcs.
75060	Pierceable Heat Seals, for 96 well plates	
	MassChrom® Amino Acid Analysis	5 pcs.
75156	96 Deep Well Plates	
	MassChrom® Amino Acid Analysis in plasma/serum	4 pcs.
42740	Heat Sealer for 96 Well Plates and Collection Plates (suitable insert included)	1 pc.
	System Check Solution and Tuning Mix for *MassChrom*® Amino Acid Analysis:	
75010	System Check Solution	1 x 1 ml
75015	Tuning Mix 1, Analytes and Internal Standards	1 x 1 ml
75016	Tuning Mix 2, Analytes and Internal Standards	1 x 1 ml
75017	Tuning Mix 3, Analytes and Internal Standards	1 x 1 ml
75018	Tuning Mix 4, Analytes and Internal Standards	1 x 1 ml
75019	Tuning Mix 5, Analytes and Internal Standards	1 x 1 ml
	Chromsystems multilevel calibrator and *MassCheck*® controls	
75128	**3PLUS1**® Multilevel Plasma Calibrator Set	
	MassChrom® Amino Acid Analysis in plasma/serum	4 x 0.5 ml (lyoph.)
0471	***MassCheck***® Amino Acid Analysis Plasma Control Level I	5 x 1.0 ml (lyoph.)
0472	***MassCheck***® Amino Acid Analysis Plasma Control Level II	5 x 1.0 ml (lyoph.)
0473	***MassCheck***® Amino Acid Analysis Plasma Control Level III	5 x 1.0 ml (lyoph.)

2 Introduction

2.1 Background information

Inherited metabolic disorders are extremely rare, taken individually; together, however, they affect one in every 500 newborns [15]. The cause in most cases is an inherited defect in an enzyme that reduces its activity or suppresses it entirely. As a consequence, the unreacted substrate of the enzymatic reaction accumulates in the body, while the product of the reaction is produced in deficient amounts or not at all, depending on the severity. People with enzyme deficiency are at risk of serious acute metabolic crisis by poisoning or hypoglycemia. The accumulation of toxic metabolites can lead to organ damage or multisystem diseases. Left undiagnosed, metabolic disorders may cause severe irreversible harm to newborns within the first few days after birth. Yet most of these diseases can be controlled if detected soon enough.

A large group of inherited metabolic disorders involve errors of amino acid conversion or breakdown. Acute symptoms of these conditions become manifest in particular in catabolic states, when proteins are broken down into amino acids and toxic metabolites accumulate as a result. Some disorders do not manifest in the form of acute crisis but nonetheless cause chronic organ damage, especially to the brain.

The first treatable genetic metabolic disorder was phenylketonuria (PKU). It was also the first disorder to be detected preventively by the implementation of universal newborn screening [15]. It is inherited in an autosomal recessive pattern. The affected enzyme is phenylalanine hydroxylase in classical PKU and the cofactor tetrahydrobiopterin in atypical PKU. The result in both cases is that phenylalanine is not, or less efficiently, converted to tyrosine and accumulates in the body. Phenylalanine is toxic to the brain and causes intellectual disability in early childhood. Adherence from infancy to a diet low in phenylalanine can suppress the symptoms entirely. The much rarer atypical form of PKU is treated by administering the cofactor tetrahydrobiopterin.

Maple syrup urine disease (MSUD) is due to a defect of the BCKDH complex (branched-chain alpha-ketoacid dehydrogenase complex), which catalyses oxidative decarboxylation of the branched-chain amino acids leucine, isoleucine and valine. These amino acids and the corresponding alpha ketoacids accumulate in the body. Severe forms cause severe encephalopathy as early as 3 to 5 days after birth. Mild forms cause developmental delays, neurological disorders and recurrent metabolic crises. Acute management consists in stopping protein intake and administration of glucose and insulin to create an anabolic state. Long-term management involves keeping to a diet low in protein. Again, the prognosis is better the earlier the disease is detected and treated.

Many more disorders of amino acid metabolism have been identified. The main treatable conditions from this group of disorders can be detected with this kit. Appendix IV contains an overview of medical conditions and their specific markers. Accurate interpretation of the results of an amino acid test requires sound background knowledge and experience, and it is essential to take other clinical findings into account before reaching a judgement. In all cases, a positive result in screening for multiple conditions requires confirmatory diagnostics. Once a disease has been confirmed and documented and treatment has begun, for example in the form of a diet, response needs to be monitored by assay of the amino acids involved. This kit enables rapid analysis in only 9 minutes to monitor PKU and MSUD.

Amino acid screening can provide important information in many areas, not just in the diagnosis of inherited metabolic disorders. Plasma-free amino acid profiles may also be abnormal in type 2 diabetes and some cancers, for instance [20]. Amino acid screening also delivers valuable information on nutrition status and in making sense of nonspecific symptoms.

2.2 Intended use

Chromsystems Reagent Kit ***MassChrom***® Amino Acid Analysis in plasma/serum is an *in-vitro* diagnostic product for use in clinical laboratories for the quantitative determination of the following amino acids and metabolic products in human plasma or serum samples using LC-MS/MS (liquid chromatography combined with mass spectrometry) as the analytical chemistry technique:

Acetyltyrosine, cystathionine, hydroxylysine, α-aminobutyric acid, cystine, methionine, -aminobutyric acid, homocystine, ornithine, γ-aminobutyric acid, cysteine sulphate, phenylalanine, 4-hydroxyproline, adenosylhomocysteine, phosphoethanolamine, alanine, ethanolamine, phosphoserine, α-aminoadipic acid, glutamine, pipecolic acid, anserine, glutamic acid, proline, arginine, glycine, saccharopine, argininosuccinic acid, histidine, sarcosine, asparagine, 1-methylhistidine, serine, aspartic acid, 3-methylhistidine, taurine, ß-alanine, leucine, threonine, carnosine, isoleucine, tryptophan, citrulline, allo-isoleucine, tyrosine, homocitrulline, lysine, valine

The kit is intended for amino acid metabolic screening, confirmation of a tentative diagnosis, and monitoring of treatment in individuals diagnosed with a metabolic disorder.

Diagnostic decisions should not be made solely based on results obtained with this device, but in conjunction with other accepted methods of clinical assessment.

2.3 Principle of the reagent kit

This Chromsystems Reagent Kit ***MassChrom***® Amino Acid Analysis in plasma/serum enables reliable routine quantitative assay of 48 amino acids and metabolic products in plasma/serum. The kit produces a complete amino acid profile in 20 minutes (see Table 1) which is sufficient to identify many metabolic disorders. Analyte quantification may be used for confirmatory analysis, treatment monitoring and various other applications. Analysis with a much shorter run time of less than 9 minutes is possible for diagnosis and therapeutic monitoring of PKU and MSUD using a modified gradient (PKU/MSUD panel, see Table 2).

Sample preparation consists of a very fast and easy protein precipitation step. The assay is available with reaction tubes or 96 deep well plates. The method does not require derivatisation, making it robust and time-saving. Isobaric compounds are separated chromatographically. A separate internal standard is used for each analyte, ensuring reproducible and reliable results. The **3PLUS1**® Multilevel Calibrator Set and ***MassCheck***® controls are obtained from human plasma to ensure that their composition approximates a patient sample as closely as possible, facilitating reliable review of the method.

Table 1: Parameter overview, complete amino acid profile (Full Panel)

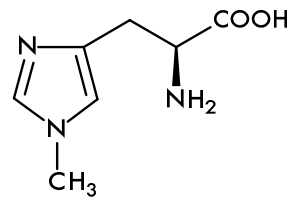
Analysable amino acids		
Acetyltyrosine	Cystathionine	Hydroxylysine
α-Aminobutyric acid	Cystine	Methionine
β-Aminoisobutyric acid	Homocystine	Ornithine
γ-Aminobutyric acid	Cysteine sulphate	Phenylalanine
4-Hydroxyproline	Adenosylhomocysteine	Phosphoethanolamine
Alanine	Ethanolamine	Phosphoserine
α-Aminoadipic acid	Glutamine	Pipecolic acid
Anserine	Glutamic acid	Proline

Analysable amino acids		
Arginine	Glycine	Saccharopine
Argininosuccinic acid	Histidine	Sarcosine
Asparagine	1-Methylhistidine	Serine
Aspartic acid	3-Methylhistidine	Taurine
ß-Alanine	Leucine	Threonine
Carnosine	Isoleucine	Tryptophan
Citrulline	Allo-isoleucine	Tyrosine
Homocitrulline	Lysine	Valine

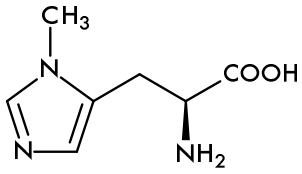
Table 2: Parameter overview, PKU/MSUD diagnostics (PKU/MSUD panel)

Maple syrup urine disease	Phenylketonuria	Others
Valine	Phenylalanine	Methionine
Leucine	Tyrosine	
Isoleucine		
Allo-isoleucine		

Please note:
The nomenclature for 1-methylhistidine and 3-methylhistidine was standardised by IUPAC several years ago. Based on usage in organic chemistry, it was decided that the *tau* form of methylhistidine would be known in future as 1-methylhistidine and the π form would be called 3-methylhistidine. The convention in biochemistry has been the precise opposite to date. This instruction manual uses the IUPAC names (see figure).



1-Methylhistidine (CAS 332-80-9)



3-Methylhistidine (CAS 368-16-1)

Figure 1: IUPAC nomenclature for methylhistidine isomers

3 LC-MS/MS system

Caution: When using the reagents comply with the hazard information in Appendix I.

3.1 Equipment and instrument parameters

Amino Acid Analysis requires a triple quadrupole tandem mass spectrometer with sufficient sensitivity and a gradient HPLC system (operating pressure limit of at least 300 bar). A 2-position, 6-way selector valve, column oven and autosampler with cooling are optional.
Keep the mobile phases closed or covered even when in use. The substances are separated on an analytical column (order no. 75100).

Instrument settings:

Autosampler:	Racks protected from light, optional refrigeration feature
Injection volume:	≤ 5 µl (mass spectrometer dependent)
Run time:	20.5 min (Full Panel) 9.0 min (PKU/MSUD Panel)
Flow rate:	0.3–1.8 ml/min
Column temperature:	+25 °C
Needle rinsing solution for the injector:	Rinsing Solution (order no. 75009)

To establish the optimum injection volume, inject increasing volumes of a prepared calibrator 1 (REF 75128/1), up to a maximum of 5 µl, until the required peak size and an appropriate signal-to-noise ratio has been established. Then use the calibration curves to verify that all the analytes are linear throughout the working range.

Gradient profile:
The gradient profile shown is intended as a basis for optimisation. Because of the different void volumes of individual HPLC systems the following gradient profile may have to be modified.

Table 3: Gradient profile, Full Panel

Time	Mobile Phase A	Mobile Phase B	Flow rate
0.00 min	100 %	0 %	0.8 ml/min
0.50 min	100 %	0 %	0.8 ml/min
1.00 min	89 %	11 %	0.8 ml/min
6.30 min	89 %	11 %	0.8 ml/min
12.30 min	83 %	17 %	0.8 ml/min
14.80 min	68 %	32 %	0.8 ml/min
14.90 min	0 %	100 %	0.8 ml/min
16.4 min	0 %	100%	0.8 ml/min
16.5 min*	0 %	100 %	0.3 ml/min
17.40 min	0 %	100 %	0.3 ml/min
17.50 min	0 %	100 %	0.8 ml/min
17.60 min	0 %	100 %	0.8 ml/min

Time	Mobile Phase A	Mobile Phase B	Flow rate
17.7 min	100 %	0 %	0.8 ml/min
19.3 min**	100 %	0 %	0.8 ml/min
19.8 min	100 %	0 %	1.8 ml/min
20.4 min	100 %	0 %	1.8 ml/min
20.5 min	100 %	0 %	0.8 ml/min

* The analytes homocystine, phosphoethanolamine, argininosuccinic acid, cystathionine, cystine and saccharopine should elute in the retention time window between 16.5 and 17.4 min (at a reduced flow rate of 0.3 mL/min) (see also retention times in chapter 4.2). These two times and the subsequent steps must be adjusted if necessary. (Please refer to section I Appendix VI for detailed instructions regarding gradient optimisation.)

** All analytes should have eluted by the time the flow rate is increased to 1.8 mL/min (cysteine sulphate and phosphoserine elute last). This time and the subsequent steps must be adjusted if necessary. (Please refer to section II Appendix VI for detailed instructions regarding gradient optimisation.)

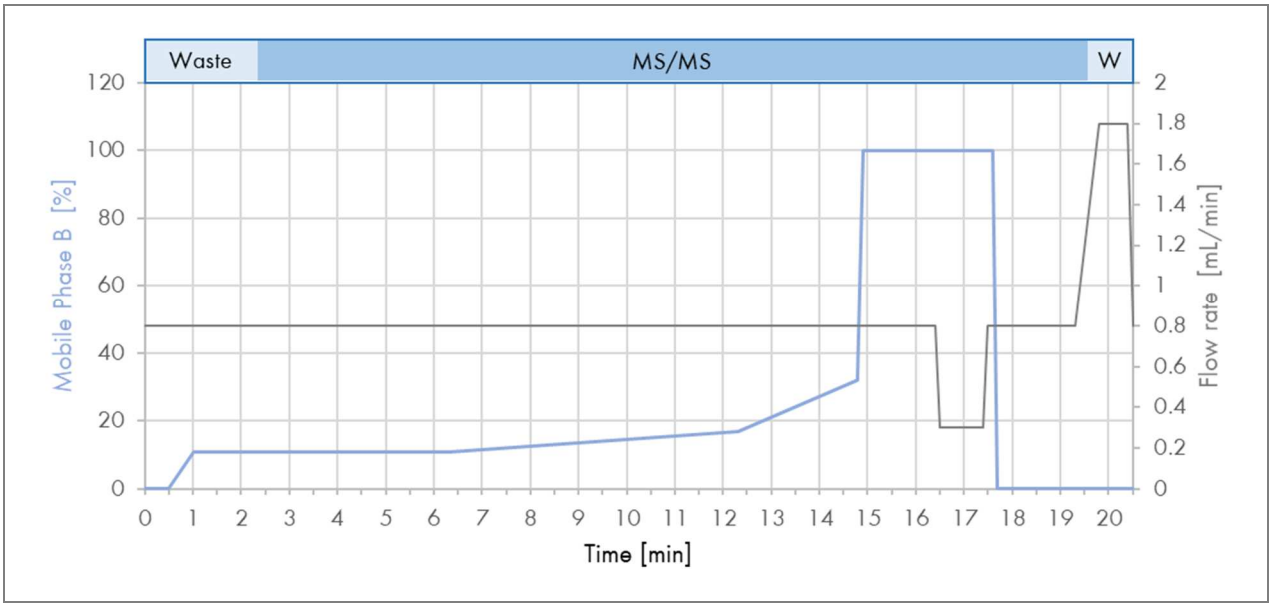


Figure 2: Diagram of the gradient profile

Table 4: Gradient profile, PKU/MSUD panel

Mobile Phase A	Mobile Phase B	Flow rate
89 %	11 %	0.8 ml/min

MS/Waste switching profile:

An MS/waste switch is an optional feature which transports the solvent for waste disposal after it passes the analytical column. The valve position is changed prior to analyte elution (MS/MS).

Table 5: MS/Waste- switching profile, Full Panel

Time	Valve position
0–2.5 min	Waste
2.5–19.3 min	MS/MS*
19.3–20.5 min	Waste

*The switch from MS/MS to waste at 19.3 min should correspond to the time of the flow increase to 1.8 mL/min in the gradient and must be adjusted if necessary.

Table 6: MS/Waste- switching profile, PKU/MSUD Panel

Time	Valve position
0–3.0 min	Waste
3.1–7.3 min	MS/MS
7.4–9.0 min	Waste

3.2 MS/MS operation

Principle of operation:
Mass spectrometers measure molecules according to their mass to charge ratio (m/z). The analytes first have to be ionised and transferred to the gas phase. Electrospray ionisation (ESI) has proven to be a highly versatile and user-friendly ionisation method for this purpose.

The triple quadrupole tandem MS method uses a variety of measurement modes. The only one used for the assay presented here is multiple reaction monitoring.

Multiple reaction monitoring (MRM):
In MRM mode, both the first and second mass filter are set statically to a particular mass-charge ratio (m/z). In MS 1, the molecular ion of the analyte is selected. Ions with a different mass-charge ratio are not shown. The molecular ion undergoes fragmentation in the collision cell and MS 2 detects the characteristic fragment ion. MRM mode enables exceptionally sensitive and selective quantification.

3.3 Optimising the MRMs (tuning)

It is highly recommended to check the accuracy and the mass resolution of the MS/MS system. If accuracy and mass resolution are outside the specifications of the manufacturer of the instrument, a re-calibration of the mass spectrometer is recommended. Following this the analyte MRM transitions should be tuned as follows:

1. Dilute the Tuning Mix (order no. 75015, 75016, 75017, 75018, 75019) separately with Mobile Phase A (order no. 75001) as appropriate for the specific device
2. Inject diluted Tuning Mix or infuse by syringe pump through a T-piece into the solvent stream (50 % Mobile Phase B at a flow rate of 0.5 ml/min)
3. Use Q1 Scan (MS Scan) to determine the exact positions of the signal maxima of MS1 masses (precursor/parent ions) (to at least one decimal)
4. Determine the exact positions of the signal maxima of MS2 masses (product/daughter ion) by product ion scan (to at least one decimal)
5. Optimise the individual parameters for each MRM transition (e. g. collision energy)

6. Use the optimised MRM transitions to optimise the ion source, especially the capillary voltage, the temperature and the gas flows

It is recommended to read the operating manual of the LC-MS/MS system. For any questions contact the instrument manufacturer. Training by the manufacturer may be required as necessary.

3.4 System start-up

Before starting a sequence prepare the LC-MS/MS system as follows:

1. Before installing the column, rinse the system for at least 15 min with 50% acetonitrile (the system must be free of other solvents, especially methanol)
2. Then rinse both pumps with the respective mobile phase and rinse the system with this for about 5 minutes. Flush the autosampler solvent line for external needle wash with Rinsing Solution
3. After this, install the column and equilibrate the system for 10 minutes under the initial conditions of the method
4. Homogenize System Check Solution (order no. 75010) as indicated in the instructions and then repeatedly inject until the retention times and signal intensities of the analytes are consistent
5. Start the sequence

For proper use of your LC-MS/MS system, read the instruction manual of your LC-MS/MS system. If you have any questions, ask the device manufacturer. Training from the device manufacturer may be required.

3.5 Precautions for analysis

Contamination risks during routine activities

> **Caution:**
> The mobile phases, especially Mobile Phase A, are sensitive to contamination by amino acids. Avoid skin contact with capillaries and other equipment parts that may come into contact with the mobile phases. Clean all vessels thoroughly with distilled water prior to use. Always wear fresh gloves when changing the mobile phases. Make sure the gloves do not come into contact with body fluids beforehand, in particular sweat. If this should happen, change the gloves.

Avoid pressure spikes
The HPLC Analytical Column ***MassChrom***® Amino Acid Analysis Column (order no. 75100) is sensitive to excessively high pressure.

> **Caution:**
> Take care to avoid pressure spikes > 280 bar which might irreversibly damage the column bed. Set an upper pressure limit of 280 bar in the measurement method for this purpose.

A regulator valve is available for pressure regulation (Back Pressure Regulator Valve, order no. 15080) which automatically protects the analytical column in the event of pressure peaks and thus increases column life.

Column storage

> **Caution:**
> To ensure the stability of retention times, the HPLC Analytical Column ***MassChrom***® Amino Acid Analysis (order no. 75100) must be stored in 100% Mobile Phase B (order no. 75002) for non-use of more than 3 days.

If the column is used in continuous/routine operation, rinsing of the column in 100 % Mobile Phase B is not necessary, but not disadvantageous either.
The column should only be rinsed with the solutions specified in this instruction manual. Other solvents could irreversibly damage the column.

Carry-over effects

Caution:
When injecting a blank sample immediately after a highly concentrated sample, analyte concentrations may be determined, in particular of phosphoethanolamine and phosphoserine. The analyte carry-over rates depend on the HPLC system you are using and the injection needle wash settings.

Therefore, be sure to determine the carry-over rate for the system used and take suitable countermeasures.

Changes in retention times

Caution:
After change of mobile phases or column lots, retention times might vary. We therefore recommend to check the retention times with lot changes to ensure that the retention times of the analytes lie centrally in individual measuring time windows.

3.6 System shut-down

To pause operation, switch off the HPLC pump and leave the mobile phase in the HPLC system. Salt crystals are unlikely to build up on the piston seals of the HPLC pumps. To protect the ion source and multiplier, switch the MS/MS system to standby mode. Leave the vacuum pumps of the LC-MS/MS system on. For non-use of more than 3 days, store the column in 100 % Mobile Phase B (see section 3.5: Precautions for analysis).

4 MRM transitions and retention time windows

4.1 MRM transitions

The following table includes all MRM transitions for the analytes and their isotopically labelled internal standards. Measure the analytes and the internal standards in positive ionisation mode (ESI).

All mentioned MRM transitions (MRM 1 = quantifier, MRM 2 = qualifier) are validated. It is recommended to use MRM 1 for quantification.

Table 7: Recommended MRM transitions Full Panel

Substance/internal standard (ISTD)	MRM 1	MRM 2
1-Methylhistidine	170 → 81	170 → 83
1-Methylhistidine ISTD	173 → 81	
α-Aminobutyric acid	104 → 58	104 → 41

Substance/internal standard (ISTD)	MRM 1	MRM 2
α-Aminobutyric acid ISTD	107 → 61	
β-Aminoisobutyric acid	104 → 86	104 → 30
β-Aminoisobutyric acid ISTD	107 → 89	
3-Methylhistidine	171 → 110	171 → 97
3-Methylhistidine ISTD	173 → 112	
4-Hydroxyproline	132 → 86	132 → 68
4-Hydroxyproline ISTD	135 → 89	
Acetyltyrosine	224 → 136	224 → 178
Acetyltyrosine ISTD	227 → 137	
Adenosylhomocysteine	385 → 136	385 → 88
Adenosylhomocysteine ISTD	389 → 138	
Alanine	91 → 45	90 → 29
Alanine ISTD	94 → 48	
Allo-isoleucine	132 → 69	132 → 44
Allo-isoleucine ISTD	142 → 78	
α-Aminoadipic acid	162 → 98	162 → 116
α-Aminoadipic acid ISTD	165 → 101	
Anserine	241 → 109	241 → 96
Anserine ISTD	245 → 109	
Arginine	176 → 71 176 → 117*	176 → 60
Arginine ISTD	181 → 74	
Argininosuccinic acid	291 → 70	291 → 116
Argininosuccinic acid ISTD	301 > 75	
Asparagine	133 → 74	133 → 87
Asparagine ISTD	138 → 76	
Aspartic acid	134 → 74	134 → 88
Aspartic acid ISTD	137 → 75	
β-Alanine	90 → 30	90 → 72
β-Alanine ISTD	94 → 32	
Carnosine	227 → 156	227 → 210
Carnosine ISTD	231 → 156	
Citrulline	176 → 70	177 → 160
Citrulline ISTD	180 → 74	
Cystathionine	223 → 88	223 → 134
Cystathionine ISTD	227 → 92	
Cysteine sulphate	202 → 120	202 → 74
Cysteine sulphate ISTD	205 → 123	
Cystine	241 → 152	241 → 120
Cystine ISTD	245 → 154	

Substance/internal standard (ISTD)	MRM 1	MRM 2
Ethanolamine	62 → 44	
Ethanolamine ISTD	66 → 48	
γ-Aminobutyric acid	104 → 87	104 → 69
γ-Aminobutyric acid ISTD	110 → 93	
Glutamine	148 → 131	148 → 85
Glutamine ISTD	154 → 136	
Glutamic acid	149 → 131	149 → 85
Glutamic acid ISTD	151 → 133	
Glycine	76 → 30	76 → 48
Glycine ISTD	79 → 32	
Histidine	157 → 84	157 → 94
Histidine ISTD	159 → 85	
Homocitrulline	190 → 173	190 → 84
Homocitrulline ISTD	193 → 176	
Homocystine	269 → 136	269 → 88
Homocystine ISTD	277 → 140	
Hydroxylysine	163 → 82	163 → 128
Hydroxylysine ISTD	168 → 87	
Isoleucine	132 →69	132 → 41
Isoleucine ISTD	139 → 74	
Leucine	132 → 43	132 → 44
Leucine ISTD	135 →46	
Lysine	147 → 56	148 → 85
Lysine ISTD	150 →59	
Methionine	150 → 61	150 → 56
Methionine ISTD	153 → 64	
Ornithine	134 → 71	134 → 117
Ornithine ISTD	139 → 76	
Phenylalanine	167 → 77	167 → 104
Phenylalanine ISTD	171 → 81	
Phosphoethanolamine	142 → 44	
Phosphoethanolamine ISTD	146 → 48	
Phosphoserine	186 → 88	186 → 70
Phosphoserine ISTD	190 → 92	
Pipecolic acid	130 → 56	131 → 85
Pipecolic acid ISTD	139 → 61	
Proline	117 → 71	116 → 43
Proline ISTD	123 → 77	
Saccharopine	277 → 213	
Saccharopine ISTD	281 → 88	

Substance/internal standard (ISTD)	MRM 1	MRM 2
Sarcosine	90 → 44	
Sarcosine ISTD	93 → 47	
Serine	106 → 60	106 → 42
Serine ISTD	109 → 63	
Taurine	126 → 44	126 → 30
Taurine ISTD	130 → 48	
Threonine	120 → 102	120 → 56
Threonine ISTD	125 → 107	
Tryptophan	206 →119	206 → 147
Tryptophan ISTD	210 →122	
Tyrosine	183 →137	183 → 166
Tyrosine ISTD	186 → 140	
Valine	119 → 73	119 → 55
Valine ISTD	126 → 80	

*optional transition if using Waters® equipment

Table 8: Recommended MRM transitions PKU/MSUD Panel

Substance/internal standard (ISTD)	MRM 1	MRM 2
Allo-isoleucine	132 → 69	132 → 44
Allo-isoleucine ISTD	142 → 78	
Isoleucine	132 →69	132 → 41
Isoleucine ISTD	139 → 74	
Leucine	132 → 43	132 → 44
Leucine ISTD	135 →46	
Methionine	150 → 61	150 → 56
Methionine ISTD	153 → 64	
Phenylalanine	167 → 77	167 → 104
Phenylalanine ISTD	171 → 81	
Tyrosine	183 →137	183 → 166
Tyrosine ISTD	186 → 140	
Valine	119 → 73	119 → 55
Valine ISTD	126 → 80	

The specified masses are starting points for optimisation. The precise position of the exact masses may vary slightly from MS system to MS system and needs to be determined precisely during method tuning. To set up the method, we recommend specifying mass position to at least one decimal place. Use a Tuning Mix for this purpose (order no. 75015, 75016, 75017, 75018, 75019, see section 3.3 on optimising MRM transitions).

4.2 Retention time windows

For Full Panel analysis, the method needs to be set up with measurement time segments or retention time windows (e.g. SCIEX scheduled MRM). Measurement of the analytes in a small time window ensures that sufficient measuring time and data points for reliable analysis can be generated per analyte. The use of measurement time segments for the PKU/MSUD Panel is optional.

We recommend a time window of 120 seconds (retention time ± 60 s) for all analytes and internal standards.

Table 9: Retention times Full Panel

Substance	Retention time
1-Methylhistidine	15.6 min
α-Aminobutyric acid	9.2 min
β-Aminoisobutyric acid	4.7 min
3-Methylhistidine	15.8 min
4-Hydroxyproline	13.7 min
Acetyltyrosine	3.4 min
Adenosylhomocysteine	15.2 min
Alanine	10.8 min
Allo-isoleucine	6.6 min
α-Aminoadipic acid	11.1 min
Anserine	16.0 min
Arginine	14.6 min
Argininosuccinic acid	16.8 min
Asparagine	14.4 min
Aspartic acid	15.8 min
β-Alanine	5.4 min
Carnosine	15.6 min
Citrulline	15.8 min
Cystathionine	16.9 min
Cysteine sulphate	18.2 min
Cystine	17.0 min
Ethanolamine	4.4 min
γ-Aminobutyric acid	4.0 min
Glutamine	14.6 min
Glutamic acid	12.9 min
Glycine	12.6 min
Histidine	15.0 min
Homocitrulline	15.3 min
Homocystine	16.6 min
Hydroxylysine	15.9 min

Substance	Retention time
Isoleucine	6.2 min
Leucine	6.1 min
Lysine	15.6 min
Methionine	7.2 min
Ornithine	15.7 min
Phenylalanine	6.1 min
Phosphoethanolamine	16.9 min
Phosphoserine	19.1 min
Pipecolic acid	10.3 min
Proline	12.1 min
Saccharopine	17.0 min
Sarcosine	12.7 min
Serine	13.5 min
Taurine	8.8 min
Threonine	12.0 min
Tryptophan	5.6 min
Tyrosine	7.4 min
Valine	7.7 min

Table 10: Retention times PKU/MSUD Panel

Substance	Retention time
Allo-isoleucine	4.7 min
Isoleucine	4.3 min
Leucine	4.15 min
Methionine	5.3 min
Phenylalanine	4.3 min
Tyrosine	5.5 min
Valine	5.5 min

If you require more information about setting up the method on your LC-MS/MS system, please contact our Chromsystems support staff by calling our hotline +49 89 18930-111 or by e-mail at support@chromsystems.com.

5 Sample preparation

Caution:
When using the reagents comply with the hazard information in Appendix I.

5.1 Collection and storage of patient specimens

Use plasma or serum for analysis.

There are indications that different matrices/blood collection systems may affect specific amino acid results [2].

Due to the enzyme-inhibiting and hence stabilizing effects, we recommend using EDTA plasma.

The blood sample should be collected in the morning before the person has eaten; for toddlers and babies, before feeding [2]. Fasting blood samples are essential for glycine, serine, methionine, proline and citrulline tests in order to avoid misinterpretation [17].

An exception applies when screening for hyperammonaemia, where postprandial blood is more suitable if blood ammonia levels are to be determined [2].

The use of medicinal products (e.g. acetylcysteine or penicillamine) capable of breaking disulphide bridges of amino acids should be avoided because of the risk of producing falsely low concentrations of the amino acids homocystine, cystine and cysteine sulphate.

Avoid artificial hemolysis! For more information, consult section 15 "Clinical limitations".

Separation of blood cells from plasma/serum is essential and must be done quickly in order to inhibit enzyme activities (arginine converts to ornithine, for example) [2, 17].

Centrifuge the whole blood as quickly as possible.

Due to enzymatic transformation processes and the instabilities of some analytes, immediate analysis is advisable. Otherwise, amino acids containing sulphate (e.g. cystine, homocystine) may bind irreversibly to plasma proteins and thus reduce the analyte levels obtained. Therefore, storage or shipment of plasma/serum samples at room temperature is not recommended [2, 17]. This is consistent with our own studies, which are presented in the "Patient sample stability" section in Appendix III: Analytical Performance data.

Plasma/serum samples for amino acid analysis should be transported frozen.

Analyse the samples as soon as possible.

Otherwise freeze the plasma/serum samples at –20 °C, but please note that some amino acids are unstable even at these storage temperatures (conversion of glutamine to γ-aminobutyric acid for instance cannot be completely prevented even when stored at –20°C) [17].

Note:
It is the responsibility of the individual laboratory to use all available references and/or its own studies to determine specific stability criteria for its laboratory.

5.2 Reconstitution of the Internal Standard Mix

Prior to sample preparation, reconstitute the Internal Standard Mix (REF 75104) as follows:

1. Pipette 5.0 ml Reconstitution Buffer (REF 75106, component of order no. 75146) into the original vial of the Internal Standard Mix (REF 75104, component of order no. 75146)
2. Reconstitute for approx. 5 min at +20 to +25 °C, swirl repeatedly

Check that the vial contents are homogeneous. If undissolved substances are still visible, extend the reconstitution time.

Caution
The same internal standard solution must be used for all samples within a sequence. If working with large batches, pool a sufficient quantity of reconstituted internal standard prior to each series.

Storage life of the internal standards after reconstitution:
The internal standards dissolved in Reconstitution Buffer have the following storage lives:

Table 11: Stability of the internal standards after reconstitution

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	1 week	Light protection, tightly closed, glass vial
+2 to +8 °C	2 weeks	Light protection, tightly closed, glass vial
below –18 °C	3 months	Light protection, tightly closed, glass vial

5.3 Reconstitution of the calibrators

Caution:
Always use fresh distilled water from previously rinsed glass beakers for reconstitution of the calibrators. Be sure to avoid skin contact with the water or transport container.

Prior to sample preparation, reconstitute the plasma calibrators (order no. 75128) as follows:

1. Pipette 0.5 ml distilled water into the original vial
2. Reconstitute for 10 to 15 min at +20 to +25 °C, swirl repeatedly

Check that the vial contents are homogeneous. If undissolved substances are still visible, extend the reconstitution time.

The calibrator levels are traceable to initial weights of pure substances. The analyte concentrations in the calibrator are batch-dependent. Individual levels are given in the calibrator leaflet. You can also download the values as an excel table in the download centre of our website.

Caution:
This product is manufactured from pooled human plasma which has been tested by the manufacturer and found negative for infections by the human immunodeficiency virus (HIV), the hepatitis B virus (HBV), the hepatitis C virus (HCV) and the bacterium *Treponema pallidum*. Nevertheless, a potential risk of infection cannot be entirely excluded. Consider all products containing human source material as potentially infectious and exercise the same care in the handling of this product as in the handling of potentially infectious patient samples.

Storage life of the calibrators after reconstitution:
The calibrators dissolved in water have the following storage lives:

Table 12: Stability of the calibrators after reconstitution

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	6 hours	Light protection, tightly closed
+2 to +8 °C	1 day	Light protection, tightly closed
below –18 °C	Asparagine, methionine, homocystine: 6 weeks All other analytes: 3 months	Light protection, tightly closed
below –70 °C	3 months	Light protection, tightly closed

5.4 Reconstitution of the controls

Caution:
Always use fresh distilled water from prerinsed glass beakers for reconstitution of the calibrators.
Be sure to avoid skin contact with the water or transport container.

Prior to sample preparation, reconstitute the plasma controls (order no. 0471, 0472, 0473) as follows:

1. Pipette 1.0 ml distilled water into the original vial
2. Reconstitute for 10 to 15 min at +20 to +25 °C, swirl repeatedly

Check that the vial contents are homogeneous. If undissolved substances are still visible, extend the reconstitution time.

The analyte concentrations in the controls are batch-dependent. Individual levels are given in the leaflet accompanying each control. You can also download the values as an excel table in the download centre of our website.

Caution:
This product is manufactured from pooled human plasma which has been tested by the manufacturer and found negative for infections by the human immunodeficiency virus (HIV), the hepatitis B virus (HBV), the hepatitis C virus (HCV) and the bacterium *Treponema pallidum*. Nevertheless, a potential risk of infection cannot be entirely excluded. Consider all products containing human source material as potentially infectious and exercise the same care in the handling of this product as in the handling of potentially infectious patient samples.

Storage life of the controls after reconstitution:
The controls dissolved in water have the following storage lives:

Table 13: Stability of the controls after reconstitution

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	6 hours	Light protection, tightly closed
+2 to +8 °C	1 day	Light protection, tightly closed
below –18 °C	Asparagine, methionine, homocystine: 6 weeks All other analytes: 3 months	Light protection, tightly closed
below –70 °C	3 months	Light protection, tightly closed

5.5 Sample preparation

5.5.1 Manual sample preparation

To prepare patient samples, controls and calibrators for analysis, work through the following steps in the order given:

1. Pipette 25 µl sample/calibrator/control into a reaction vial (order no. 33006)
2. Add 50 µl reconstituted Internal Standard Mix (order no. 75146, see chap. 5.2)
3. Add 400 µl Precipitation Reagent (order no. 75105)
4. Mix for 30 s (vortex)
5. Centrifuge for 5 min at 16000 x g
6. Transfer 200 µl supernatant into an autosampler vial
7. Inject up to 5 µl of the prepared sample into the LC-MS/MS system

Information on determining the optimum injection volume is given in section 3.1.

5.5.2 Sample preparation with 96 Deep Well Plate

To prepare patient samples, controls and calibrators for analysis, work through the following steps in the order given:

1. Pipette 25 µl sample/calibrator/control into a well of a 96 Deep Well Plate (order no. 75156)
2. Add 50 µl of the reconstituted Internal Standard Mix (order no. 75146, see chap. 5.2)
3. Add 400 µl Precipitation Reagent (order no. 75105)
4. Mix 2 min at 1200 rpm
5. Centrifuge 5 min at 2000 x g
6. Transfer 200 µl of the supernatant into a Collection Plate (order no. 75058)
7. Seal plate with a heat seal (order no. 75060, recommendation: 170 °C, 3 s)
8. Inject up to 5 µl of the prepared sample into the LC-MS/MS system

Information on determining the optimum injection volume is given in section 3.1.

5.6 Storage life of prepared samples

Prepared samples are stable for 7 days in the collection plate at +2 to +8°C if sealed with a Pierceable Heat Seal (order no. 75060). If this is not an option, the sample extracts need to be transferred to tightly closed glass vials (e.g. autosampler vials).

Samples prepared for analysis as indicated in section 5.5 have the following storage life:

Table 14: Storage life of the prepared samples

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	1 week	Light protection, tightly closed, glass vials
+2 to +8 °C	1 week	Light protection, tightly closed, glass vials
below –18 °C	2 weeks	Light protection, tightly closed, glass vials

5.7 Handling samples outside the linear range

The following analytes may show concentrations outside the linear range if samples are pathological: acetyltyrosine, allo-isoleucine, arginine, argininosuccinic acid, citrulline, homocystine, isoleucine, methionine, ornithine, phenylalanine, pipecolic acid and sarcosine.

Patient samples with concentrations of these analytes above the linear range are to be treated as follows:

Prior to sample preparation, dilute the starting samples with isotonic saline (9.0 g/l), not exceeding a ratio of 1:10, so that the test results are within the linear range of the method without taking the dilution factor into account. Then conduct sample preparation as described in section 5.5.

Take the respective dilution factor into consideration when calculating the analyte concentrations of the diluted samples.

6 Additionally required equipment

The Amino Acid Analysis using LC-MS/MS requires the following additional materials not supplied in the reagent kit:

- Triple quadrupole tandem mass spectrometer with ESI source (sufficient sensitivity provided)
- Gradient HPLC system

For manual sample preparation, you will also need:
- Centrifuge for 1.5 ml reaction vials
- Vortex mixer

For preparation using 96 deep well plates, you will also need:

- Heat Sealer for 96 well plates (Heat Sealer, suitable insert included: order no. 42740)
- Centrifuge for 96 well filter plates
- Shaker for 96 well plates

Optional/recommended equipment:

- 2-position 6-way selector valve for MS waste switching
- Autosampler with cooling
- Column oven
- Multichannel pipette to transfer the supernatants to a collection plate

7 Data acquisition and evaluation

Run a full calibration of the analysis system for each series of measurements. Use **3PLUS1**® Multilevel Calibrators (order no. 75128) for this purpose. The concentrations of the various analytes in the calibrators are batch-dependent. Exact levels are given in the package leaflet. Calibration curves are constructed by calculating the analyte to internal standard (ISTD) peak area ratio on the y axis against calibrator concentrations on the x axis. Then plot a calibration curve for all analytes using linear regression and 1/x weighting.

To make sure the LC-MS/MS conditions within the measuring sequence have not changed, inject the prepared ***MassCheck***® controls at least once during and at the end of a sample series.

Check the mass precision and resolution of the mass spectrometer periodically and recalibrate the mass spectrometer if you notice deviations. Read the information in the instruction manual of your mass spectrometer before doing so and contact the device manufacturer if you require further information.

8 Quality control

Monitor precision and accuracy of the analyses by including additional controls (***MassCheck***® controls, order no. 0471, 0472, 0473) in each analytical run. If the analysis of these controls yields values outside the range given on the accompanying information leaflet, check the system. If the discrepancy continues to exist, re-calibrate the system.

Monitor the quality of chromatographic separation by comparison of the retention times and chromatographic peak shapes of the analytes and internal standards with the chromatogram of the column certificate or with an example chromatogram (chapter 13, for retention times see chapter 4.2). In case of a column in use, compare to preceding analytical runs of the same assay (e.g. in the course of system start-up, chapter 3.4). Significant deviations might be due to decreasing performance of the analytical column. Typical indicators would be broadening of the peaks and deterioration of the chromatographic resolution.

For more information, see chapter 16 Troubleshooting.

9 Reference ranges

The stated reference ranges are guides based on the literature. They may differ from other published data. As the levels vary depending on patient population and measurement method, determine specific reference ranges for your laboratory. When determining ranges, make sure that you comply with local national requirements. Please also note that some levels of healthy subjects may be below the LLOQ.

Table 15: Reference ranges

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
1-Methylhistidine*	premature babies	plasma	HPLC	5–33	[1]
	0–1 month		HPLC	0–5	
	1–24 months		HPLC	0–5	
	2–18 years		HPLC	0–5	
	>18 years		HPLC	0	
α-Aminobutyric acid	premature babies	plasma	HPLC	14–52	[1]
	0–1 month		HPLC	8–24	
	1–24 months		HPLC	3–26	
	2–18 years		HPLC	4–31	
	>18 years		HPLC	5–41	
	<3 months		n.s.	3–24	[2]
	children	plasma	HPLC	12–43	
	adolescents		HPLC	8–36	
	adults ♀		HPLC	7–35	
	adults ♂		HPLC	15–35	
β-Aminoisobutyric acid	premature babies	plasma	HPLC	0	[1]
	0–1 month		HPLC	0	
	1–24 months		HPLC	0	
	2–18 years		HPLC	0	
	>18 years		HPLC	0	
	newborns	plasma	HPLC	0	[5]
	children		HPLC	0	
	adults		HPLC	0	
3-Methylhistidine*	premature babies	plasma	HPLC	4–28	[1]
	0–1 month		HPLC	0–43	
	1–24 months		HPLC	0–44	
	2–18 years		HPLC	0–42	
	>18 years		HPLC	72–124	
4-Hydroxyproline	premature babies	plasma	HPLC	0–80	[1]
	0–1 month		HPLC	0–91	
	1–24 months		HPLC	0–63	

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
	2–18 years		HPLC	3–45	
	>18 years		HPLC	0–53	
Acetyltyrosine	no data available.				
Adenosylhomocysteine	n.s.	plasma	n.s.	0.009–0.045	[10]
	n.s.	plasma	n.s.	0.015–0.045	[11]
Alanine	6 months	plasma	HPLC	182–396	[9]
	2 years		HPLC	173–349	
	6 years		HPLC	182–319	
	16 years		HPLC	240–482	
	premature babies	plasma	HPLC	212–504	[1]
	0–1 month		HPLC	131–710	
	1–24 months		HPLC	143–439	
	2–18 years		HPLC	152–547	
	>18 years		HPLC	177–583	
	<3 months	plasma	n.s.	142–421	[2]
	children		HPLC	120–600	
	adolescents		HPLC	242–594	
	adults ♀		HPLC	218–474	
	adults ♂		HPLC	146–494	
	<28 days	plasma	n.s.	185–645	[7]
	4 months		n.s.	110–550	
	1–12 months		n.s.	100–310	
	adults ♀		n.s.	200–550	
	adults ♂		n.s.	240–600	
Allo-isoleucine	n.s.	plasma	HPLC	0–5	[12]
	<3 years	plasma	HPLC	0.5–2.6	[13]
	3–11 years		HPLC	0.7–2.5	
	adults		HPLC	0.7–3.4	
	premature babies (<6 weeks)	plasma	HPLC	0	[4]
	0–1 month		HPLC	0	
	1–24 months		HPLC	0	
	2–18 years		HPLC	0	
	adults		HPLC	0	
α-Aminoadipic acid	premature babies	plasma	HPLC	0	[1]
	0–1 month		HPLC	0	
	1–24 months		HPLC	0	
	2–18 years		HPLC	0	
	>18 years		HPLC	0–6	
	n.s.	plasma	n.s.	<5	[8]

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
Anserine	premature babies	plasma	HPLC	–	[1]
	0–1 month		HPLC	0	
	1–24 months		HPLC	0	
	2–18 years		HPLC	0	
	>18 years		HPLC	0	
Arginine	6 months	plasma	HPLC	43–120	[9]
	2 years		HPLC	46–90	
	6 years		HPLC	50–99	
	16 years		HPLC	68–128	
	premature babies	plasma	HPLC	34–96	[1]
	0–1 month		HPLC	6–140	
	1–24 months		HPLC	12–133	
	2–18 years		HPLC	10–140	
	>18 years		HPLC	15–128	
	<3 months	plasma	n.s.	7–128	[2]
	children		HPLC	12–112	
	adolescents		HPLC	1–81	
	adults ♀		HPLC	28–108	
	adults ♂		HPLC	28–96	
	<28 days	plasma	n.s.	65–200	[7]
	4 months		n.s.	41–190	
	1–12 months		n.s.	10–65	
	adults ♀		n.s.	25–125	
	adults ♂		n.s.	35–140	
Argininosuccinic acid	<28d	plasma	n.s.	<2	[7]
Asparagine	6 months	plasma	HPLC	31–56	[9]
	2 years		HPLC	29–56	
	6 years		HPLC	31–67	
	16 years		HPLC	37–81	
	premature babies	plasma	HPLC	90–295	[1]
	0–1 month		HPLC	29–132	
	1–24 months		HPLC	21–95	
	2–18 years		HPLC	23–112	
	>18 years		HPLC	35–74	
	<3 months	plasma	n.s.	38–121	[2]
	children		HPLC	15–83	
	adolescents		HPLC	34–94	
	adults ♀		HPLC	26–74	
	adults ♂		HPLC	32–92	
Aspartic acid	6 months	plasma	HPLC	4–18	[9]

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
	2 years		HPLC	3–8	
	6 years		HPLC	3–6	
	16 years		HPLC	2–5	
	premature babies		HPLC	24–50	
	0–1 month		HPLC	20–129	
	1–24 months	plasma	HPLC	0–23	[1]
	2–18 years		HPLC	1–24	
	>18 years		HPLC	1–25	
	<3 months		n.s.	0–31	
	children		HPLC	1–17	
	adolescents	plasma	HPLC	3–15	[2]
	adults ♀		HPLC	3–6	
	adults ♂		HPLC	2–9	
β-Alanine	premature babies		HPLC	0	
	0–1 month		HPLC	0–10	
	1–24 months	plasma	HPLC	0–7	[1]
	2–18 years		HPLC	0–7	
	>18 years		HPLC	0–12	
	newborns		HPLC	0–10	
	children	plasma	HPLC	0–7	[5]
	adults		HPLC	0–12	
Carnosine	premature babies		HPLC	-	
	0–1 month		HPLC	0-19	
	1–24 months	plasma	HPLC	0	[1]
	2–18 years		HPLC	0	
	>18 years		HPLC	0	
Citrulline	6 months		HPLC	14–32	
	2 years	plasma	HPLC	17–35	[9]
	6 years		HPLC	23–37	
	16 years		HPLC	23–39	
	premature babies		HPLC	20–87	
	0–1 month		HPLC	10–45	
	1–24 months	plasma	HPLC	3–35	[1]
	2–18 years		HPLC	1–46	
	>18 years		HPLC	12–55	
	<3 months		n.s.	8–36	
	children		HPLC	8–47	
	adolescents	plasma	HPLC	19–52	[2]
	adults ♀		HPLC	10–58	
	adults ♂		HPLC	19–47	

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
	<28 days	plasma	n.s.	13–45	[7]
	4 months		n.s.	8–36	
	1–12 months		n.s.	10–30	
	adults ♀		n.s.	15–55	
	adults ♂		n.s.	20–55	
Cystathionine	premature babies	plasma	HPLC	5–10	[1]
	0–1 month		HPLC	0–3	
	1–24 months		HPLC	0–5	
	2–18 years		HPLC	0–3	
	>18 years		HPLC	0–3	
	n.s.	plasma/ serum	n.s.	<1	[6]
Cysteine sulphate	n.s.	plasma/ serum	n.s.	<1	[6]
Cystine	6 months	plasma	HPLC	21–53	[9]
	2 years		HPLC	27–52	
	6 years		HPLC	33–54	
	16 years		HPLC	36–61	
	premature babies	plasma	HPLC	15–70	[1]
	0–1 month		HPLC	17–98	
	1–24 months		HPLC	16–84	
	2–18 years		HPLC	5–45	
	>18 years		HPLC	5–82	
	<3 months	plasma	n.s.	6–43	[2]
	children		HPLC	23–68	
	adolescents		HPLC	36–58	
	adults ♀		HPLC	31–49	
	adults ♂		HPLC	24–54	
Ethanolamine	0–1 month	plasma	HPLC	0–115	[1]
	1–24 months		HPLC	0–4	
	2–18 years		HPLC	0–7	
	>18 years		HPLC	0–153	
γ-Aminobutyric acid	premature babies	plasma	HPLC	0	[1]
	0–1 month		HPLC	0–2	
	1–24 months		HPLC	0	
	2–18 years		HPLC	0	
	>18 years		HPLC	0	
	<1 year	serum	HPLC	0.12–0.5	[3]
	>1 year	serum	HPLC	0.12–0.5	
Glutamine	6 months	plasma	HPLC	474–737	[9]

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
	2 years		HPLC	473–692	
	6 years		HPLC	493–724	
	16 years		HPLC	551–797	
	premature babies		HPLC	248–850	
	0–1 month		HPLC	376–709	
	1–24 months	plasma	HPLC	246–1182	[1]
	2–18 years		HPLC	254–823	
	>18 years		HPLC	205–756	
	<3 months		n.s.	402–776	
	children		HPLC	333–809	
	adolescents	plasma	HPLC	457–857	[2]
	adults ♀		HPLC	340–696	
	adults ♂		HPLC	466–798	
	<28 days		n.s.	380–710	
	4 months		n.s.	200–720	
	1–12 months	plasma	n.s.	60–470	[7]
	adults ♀		n.s.	440–810	
	adults ♂		n.s.	550–830	
Glutamic acid	6 months		HPLC	31–113	
	2 years	plasma	HPLC	25–81	[9]
	6 years		HPLC	13–65	
	16 years		HPLC	11–46	
	premature babies		HPLC	107–276	
	0–1 month		HPLC	62–620	
	1–24 months	plasma	HPLC	10–133	[1]
	2–18 years		HPLC	5–150	
	>18 years		HPLC	10–131	
	<3 months		n.s.	8–179	
	children		HPLC	14–78	
	adolescents	plasma	HPLC	17–69	[2]
	adults ♀		HPLC	6–38	
	adults ♂		HPLC	6–62	
Glycine	6 months		HPLC	138–276	
	2 years	plasma	HPLC	138–276	[9]
	6 years		HPLC	144–282	
	16 years		HPLC	183–322	
	premature babies		HPLC	298–602	
	0–1 month	plasma	HPLC	232–740	[1]
	1–24 months		HPLC	81–436	
	2–18 years		HPLC	127–341	

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
	>18 years		HPLC	151–490	
	<3 months		n.s.	154–338	
	children		HPLC	107–343	
	adolescents	plasma	HPLC	166–330	[2]
	adults ♀		HPLC	100–384	
	adults ♂		HPLC	147–299	
	newborns		HPLC	220–500	
	children	plasma	HPLC	100–400	[5]
	adults		HPLC	120–550	
Histidine	6 months	plasma	HPLC	61–91	[9]
	2 years		HPLC	61–91	
	6 years		HPLC	63–93	
	16 years		HPLC	77–107	
	premature babies	plasma	HPLC	72–134	[1]
	0–1 month		HPLC	30–138	
	1–24 months		HPLC	41–101	
	2–18 years		HPLC	41–125	
	>18 years		HPLC	0–8	
	<3 months	plasma	n.s.	37–83	[2]
	children		HPLC	47–135	
	adolescents		HPLC	68–108	
	adults ♀		HPLC	68–104	
	adults ♂		HPLC	72–108	
Homocitrulline	no data available.				
Homocystine	premature babies	plasma	HPLC	3–20	[1]
	0–1 month		HPLC	0	
	1–24 months		HPLC	0	
	2–18 years		HPLC	0–5	
	>18 years		HPLC	0	
	n.s.	plasma	n.s.	<1	[6]
Hydroxylysine	premature babies	plasma	HPLC	0	[1]
	0–1 month		HPLC	0–7	
	1–24 months		HPLC	0–7	
	2–18 years		HPLC	0–2	
	>18 years		HPLC	0	
	n.s.	plasma	n.s.	indeterminable	[8]
Isoleucine	6 months	plasma	HPLC	39–76	[9]
	2 years		HPLC	4–78	
	6 years		HPLC	40–69	
	16 years		HPLC	47–74	

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
	premature babies	plasma	HPLC	23–85	[1]
	0–1 month		HPLC	26–91	
	1–24 months		HPLC	31–86	
	2–18 years		HPLC	22–107	
	>18 years		HPLC	30–108	
	<3 months	plasma	n.s.	12–77	[2]
	children		HPLC	6–122	
	adolescents		HPLC	34–106	
	adults ♀		HPLC	39–67	
	adults ♂		HPLC	46–90	
	premature babies (<6 weeks)	plasma	HPLC	23–85	[4]
	0–1 month		HPLC	26–91	
	1–24 months		HPLC	31–86	
	2–18 years		HPLC	22–107	
	adults		HPLC	30–108	
Leucine	6 months	plasma	HPLC	77–153	[9]
	2 years		HPLC	79–147	
	6 years		HPLC	86–136	
	16 years		HPLC	101–159	
	premature babies	plasma	HPLC	151–220	[1]
	0–1 month		HPLC	48–160	
	1–24 months		HPLC	47–155	
	2–18 years		HPLC	49–216	
	>18 years		HPLC	72–201	
	<3 months	plasma	n.s.	46–147	[2]
	children		HPLC	30–246	
	adolescents		HPLC	86–206	
	adults ♀		HPLC	98–142	
	adults ♂		HPLC	113–205	
	premature babies (<6 weeks)	plasma	HPLC	151–200	[4]
	0–1 month		HPLC	48–160	
	1–24 months		HPLC	47–155	
	2–18 years		HPLC	49–216	
	adults		HPLC	72–201	
Lysine	6 months	plasma	HPLC	87–171	[9]
	2 years		HPLC	88–172	
	6 years		HPLC	96–181	
	16 years		HPLC	157–242	

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
	premature babies	plasma	HPLC	128–255	[1]
	0–1 month		HPLC	92–325	
	1–24 months		HPLC	52–196	
	2–18 years		HPLC	48–284	
	>18 years		HPLC	0–39	
	<3 months	plasma	n.s.	69–200	[2]
	children		HPLC	66–270	
	adolescents		HPLC	116–276	
	adults ♀		HPLC	119–203	
	adults ♂		HPLC	135–243	
	<28d	plasma	n.s.	110–330	[7]
	4 months		n.s.	60–275	
	1–12 months		n.s.	45–145	
	adults ♀		n.s.	115–250	
	adults ♂		n.s.	135–260	
Methionine	6 months	plasma	HPLC	14–38	[9]
	2 years		HPLC	13–22	
	6 years		HPLC	14–25	
	16 years		HPLC	20–34	
	premature babies	plasma	HPLC	37–91	[1]
	0–1 month		HPLC	10–60	
	1–24 months		HPLC	9–42	
	2–18 years		HPLC	7–47	
	>18 years		HPLC	10–42	
	<3 months	plasma	n.s.	9–44	[2]
	children		HPLC	3–43	
	adolescents		HPLC	13–41	
	adults ♀		HPLC	14–30	
	adults ♂		HPLC	13–37	
	newborns	plasma	HPLC	1–400	[5]
	children		HPLC	2–59	
	adults		HPLC	6–40	
Ornithine	6 months	plasma	HPLC	25–103	[9]
	2 years		HPLC	24–60	
	6 years		HPLC	25–50	
	16 years		HPLC	37–62	
	premature babies	plasma	HPLC	77–212	[1]
	0–1 month		HPLC	48–211	
	1–24 months		HPLC	22–103	
	2–18 years		HPLC	10–163	

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
	>18 years		HPLC	48–195	
	<3 months		n.s.	41–129	
	children		HPLC	20–136	
	adolescents	plasma	HPLC	47–195	[2]
	adults ♀		HPLC	36–96	
	adults ♂		HPLC	55–135	
	<28d		n.s.	55–420	
	4 months		n.s.	28–150	
	1–12 months	plasma	n.s.	10–110	[7]
	adults ♀		n.s.	20–90	
	adults ♂		n.s.	30–100	
Phenylalanine	6 months		HPLC	38–78	
	2 years	plasma	HPLC	39–65	[9]
	6 years		HPLC	40–61	
	16 years		HPLC	47–74	
	premature babies		HPLC	98–231	
	0–1 month		HPLC	38–137	
	1–24 months	plasma	HPLC	31–75	[1]
	2–18 years		HPLC	26–91	
	>18 years		HPLC	35–85	
	<3 months		n.s.	25–74	
	children		HPLC	26–98	
	adolescents	plasma	HPLC	34–86	[2]
	adults ♀		HPLC	42–62	
	adults ♂		HPLC	46–74	
Phosphoethanolamine	premature babies		HPLC	5–35	
	0–1 month		HPLC	3–27	
	1–24 months	plasma	HPLC	0–6	[1]
	2–18 years		HPLC	0–69	
	>18 years		HPLC	0–40	
Phosphoserine	premature babies		HPLC	10–45	
	0–1 month		HPLC	7–47	
	1–24 months	plasma	HPLC	1–20	[1]
	2–18 years		HPLC	1–30	
	>18 years		HPLC	2–14	
Pipecolic acid	<1 week	plasma	GC-MS	3.75–10.8	[14]
	>1 week			0.70–2.46	
	newborns	plasma	n.s.	6.4–17.6	[8]
	adults		n.s.	0.5–3.7	
Proline	6 months	plasma	HPLC	93–265	[9]

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
	2 years		HPLC	93–220	
	6 years		HPLC	93–201	
	16 years		HPLC	113–271	
	premature babies		HPLC	92–310	
	0–1 month		HPLC	110–417	
	1–24 months	plasma	HPLC	52–298	[1]
	2–18 years		HPLC	59–369	
	>18 years		HPLC	97–329	
	<3 months		n.s.	97–254	
	children		HPLC	40–332	
	adolescents	plasma	HPLC	58–324	[2]
	adults ♀		HPLC	112–220	
	adults ♂		HPLC	97–297	
	<28d		n.s.	120–310	
	4 months		n.s.	64–272	
	1–12 months	plasma	n.s.	50–190	[7]
	adults ♀		n.s.	70–270	
	adults ♂		n.s.	100–380	
Saccharopine	n.s.	plasma	n.s.	indeterminable	[8]
	premature babies		HPLC	0	
	0–1 month		HPLC	0–625	
Sarcosine	1–24 months	plasma	HPLC	0	[1]
	2–18 years		HPLC	0–9	
	>18 years		HPLC	0	
	6 months		HPLC	98–160	
	2 years	plasma	HPLC	97–154	[9]
	6 years		HPLC	96–155	
	16 years		HPLC	101–177	
	premature babies		HPLC	127–248	
	0–1 month		HPLC	99–395	
Serine	1–24 months	plasma	HPLC	71–186	[1]
	2–18 years		HPLC	69–187	
	>18 years		HPLC	58–181	
	<3 months		n.s.	92–178	
	children		HPLC	70–194	
	adolescents	plasma	HPLC	92–196	[2]
	adults ♀		HPLC	78–166	
	adults ♂		HPLC	89–165	
Taurine	6 months	plasma	HPLC	39–111	[9]
	2 years		HPLC	39–80	

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
	6 years		HPLC	41–69	
	16 years		HPLC	41–66	
	premature babies		HPLC	151–411	
	0–1 month		HPLC	46–492	
	1–24 months	plasma	HPLC	15–143	[1]
	2–18 years		HPLC	10–170	
	>18 years		HPLC	54–210	
	<3 months		n.s.	10–167	
	children		HPLC	20–120	
	adolescents	plasma	HPLC	2–90	[2]
	adults ♀		HPLC	18–66	
	adults ♂		HPLC	27–95	
Threonine	6 months	plasma	HPLC	61–162	[9]
	2 years		HPLC	61–115	
	6 years		HPLC	65–125	
	16 years		HPLC	104–188	
	premature babies		HPLC	150–330	
	0–1 month		HPLC	90–329	
	1–24 months	plasma	HPLC	24–174	[1]
	2–18 years		HPLC	35–226	
	>18 years		HPLC	60–225	
	<3 months		n.s.	46–222	
	children		HPLC	40–204	
	adolescents	plasma	HPLC	102–246	[2]
	adults ♀		HPLC	93–197	
	adults ♂		HPLC	92–180	
Tryptophan	6 months	plasma	HPLC	34–73	[9]
	2 years		HPLC	35–73	
	6 years		HPLC	37–76	
	16 years		HPLC	54–93	
	premature babies		HPLC	28–136	
	0–1 month		HPLC	0–60	
	1–24 months	plasma	HPLC	23–71	[1]
	2–18 years		HPLC	0–79	
	>18 years		HPLC	10–140	
	<3 months		n.s.	21–75	
	children	plasma	HPLC	12–69	[2]
	adolescents		HPLC	–	
	adults ♀		HPLC	17–53	

Substance	Patient collective/age	Matrix	Method	Reference range in µmol/l	Literature
	adults ♂		HPLC	25–65	
Tyrosine	6 months	plasma	HPLC	43–108	[9]
	2 years		HPLC	40–77	
	6 years		HPLC	39–65	
	16 years		HPLC	46–87	
	premature babies	plasma	HPLC	147–420	[1]
	0–1 month		HPLC	55–147	
	1–24 months		HPLC	22–108	
	2–18 years		HPLC	24–115	
	>18 years		HPLC	34–112	
	<3 months	plasma	n.s.	13–91	[2]
	children		HPLC	19–119	
	adolescents		HPLC	35–107	
	adults ♀		HPLC	26–78	
	adults ♂		HPLC	37–77	
Valine	6 months	plasma	HPLC	135–260	[9]
	2 years		HPLC	147–255	
	6 years		HPLC	165–234	
	16 years		HPLC	178–275	
	premature babies	plasma	HPLC	99–220	[1]
	0–1 month		HPLC	86–190	
	1–24 months		HPLC	64–294	
	2–18 years		HPLC	74–321	
	>18 years		HPLC	119–336	
	<3 months	plasma	n.s.	79–217	[2]
	children		HPLC	132–480	
	adolescents		HPLC	155–343	
	adults ♀		HPLC	172–248	
	adults ♂		HPLC	179–335	
	premature babies (<6 weeks)	plasma	HPLC	99–220	[4]
	0–1 month		HPLC	86–190	
	1–24 months		HPLC	64–294	
	2–18 years		HPLC	74–321	
	adults		HPLC	119–336	

***Please take note of the information on methylhistidine nomenclature in section 2.3.**

Table 16: German PKU treatment guidelines [15]

Substance	Target value [µmol/l]			
	1st to 10th year of life	11th to 16th year of life	from 16th year of life	pregnancy
Phenylalanine	40–240	40–900	< 1200	120–360

Table 17: Therapeutic target values in MSUD [15]

Substance	Target value [µmol/l]
Leucine	100–250
Isoleucine	50–150
Valine	150–250

10 Conversion factors

The following table lists conversion factors between mass and molar concentrations and conversely.

Table 18: Conversion factors

Substance	mg/l to µmol/l	µmol/l to mg/l
1-Methylhistidine	5.9108	0.1692
α-Aminobutyric acid	9.6974	0.1031
β-Aminoisobutyric acid	9.6974	0.1031
3-Methylhistidine	5.9108	0.1692
4-Hydroxyproline	7.6260	0.1311
Acetyltyrosine	4.4799	0.2232
Adenosylhomocysteine	2.6014	0.3844
Alanine	11.225	0.0891
Allo-isoleucine	7.6237	0.1312
α-Aminoadipic acid	6.2052	0.1612
Anserine	4.1622	0.2403
Arginine	5.7405	0.1742
Argininosuccinic acid	3.4450	0.2903
Asparagine	7.5700	0.1321
Aspartic acid	7.5126	0.1331
β-Alanine	11.225	0.0891
Carnosine	4.4203	0.2262
Citrulline	5.7081	0.1752

Substance	mg/l to µmol/l	µmol/l to mg/l
Cystathionine	4.4992	0.2223
Cysteine sulphate	4.9697	0.2012
Cystine	4.1615	0.2403
Ethanolamine	16.372	0.0611
γ-Aminobutyric acid	9.6974	0.1031
Glutamine	6.8428	0.1461
Glutamic acid	6.7967	0.1471
Glycine	13.321	0.0751
Histidine	6.4452	0.1552
Homocitrulline	5.2851	0.1892
Homocystine	3.7265	0.2684
Hydroxylysine	6.1657	0.1622
Isoleucine	7.6237	0.1312
Leucine	7.6237	0.1312
Lysine	6.8404	0.1462
Methionine	6.7020	0.1492
Ornithine	7.5666	0.1322
Phenylalanine	6.0536	0.1652
Phosphoethanolamine	7.0890	0.1411
Phosphoserine	5.4034	0.1851
Pipecolic acid	7.7425	0.1292
Proline	8.6858	0.1151
Saccharopine	3.6194	0.2763
Sarcosine	11.224	0.0891
Serine	9.5157	0.1051
Taurine	7.9911	0.1251
Threonine	8.3950	0.1191
Tryptophan	4.8964	0.2042
Tyrosine	5.5191	0.1812
Valine	8.5360	0.1172

11 Storage and lifetime of the reagents

Unopened, and provided that transport and storage conditions are met, the reagents are stable until the expiry date stated on the label. Transport and store the reagents under the following conditions:

Table 19: Transport conditions for the reagent kit

Product	Transport temperature
Reagent kit (order no. 75111, 75111/DWP)	+18 to +30 °C

Immediately unpack reagents after transport and store individually as stated below:

Table 20: Storage conditions for the reagents

Product	Storage temperature
Mobile Phase A (order no. 75001)	+18 to +30 °C
Mobile Phase B (order no. 75002)	+18 to +30 °C
Rinsing Solution (order no. 75009)	+18 to +30 °C
Internal Standard Set (order no. 75146)	below –18 °C
Internal Standard Mix (REF 75104)	below –18 °C
Reconstitution Buffer (REF 75106)	below –18 °C
Precipitation Reagent (order no. 75105)	+18 to +30 °C
Plasma calibrator (order no. 75128)	below –18 °C
Plasma controls (order no. 0471, 0472, 0473)	below –18 °C
System Check Solution (order no. 75010)	below –18 °C
Tuning Mix (order no. 75015, 75016, 75017, 75018, 75019)	below –18 °C

Close the reagents immediately after use and store them at the specified temperature. The in-use shelf-life is one year but does not extend beyond the stated expiry date. Details of the stability of the reconstituted internal standards, calibrators and controls are given in sections 5.2, 5.3 and 5.4.

12 Waste disposal

Hazardous waste
Mobile Phase A (order no. 75001), Precipitation Reagent (order no. 75105) and System Check Solution (order no. 75010) contain organic solvents. Dispose product residues into a collection container for organic halogen-free solvents.

Tuning Mix 1 to 5 (order nos. 75015, 75016, 75017, 75018, 75019) and Reconstitution Buffer (REF 75106) contain a skin sensitising substance and must be collected and disposed of as hazardous waste. The reconstituted Internal Standard Mix has to be classified just as the Reconstitution Buffer and discarded accordingly.

Residues of patient samples, prepared samples, controls (order nos. 0471, 0472, 0473) and calibrators (order no. 75128) as well as laboratory consumables contaminated with human material must be collected and disposed of as potentially infectious waste.

Hazardous waste must not be disposed of with domestic waste. Do not circulate into the main water supply. Dispose of in compliance with Directive 2008/98/EC on Waste and national and local requirements. The waste containers must be stored appropriately and access must be restricted to authorised personnel.

Non-hazardous waste
Mobile Phase B (order no. 75002), Rinsing Solution (order no. 75009) as well as non-contaminated laboratory consumables are not classified as hazardous. Dispose of in compliance with Directive 2008/98/EC on Waste and national and local requirements.

13 Examples of chromatograms

The following graphs provide several examples of chromatograms created using this method.

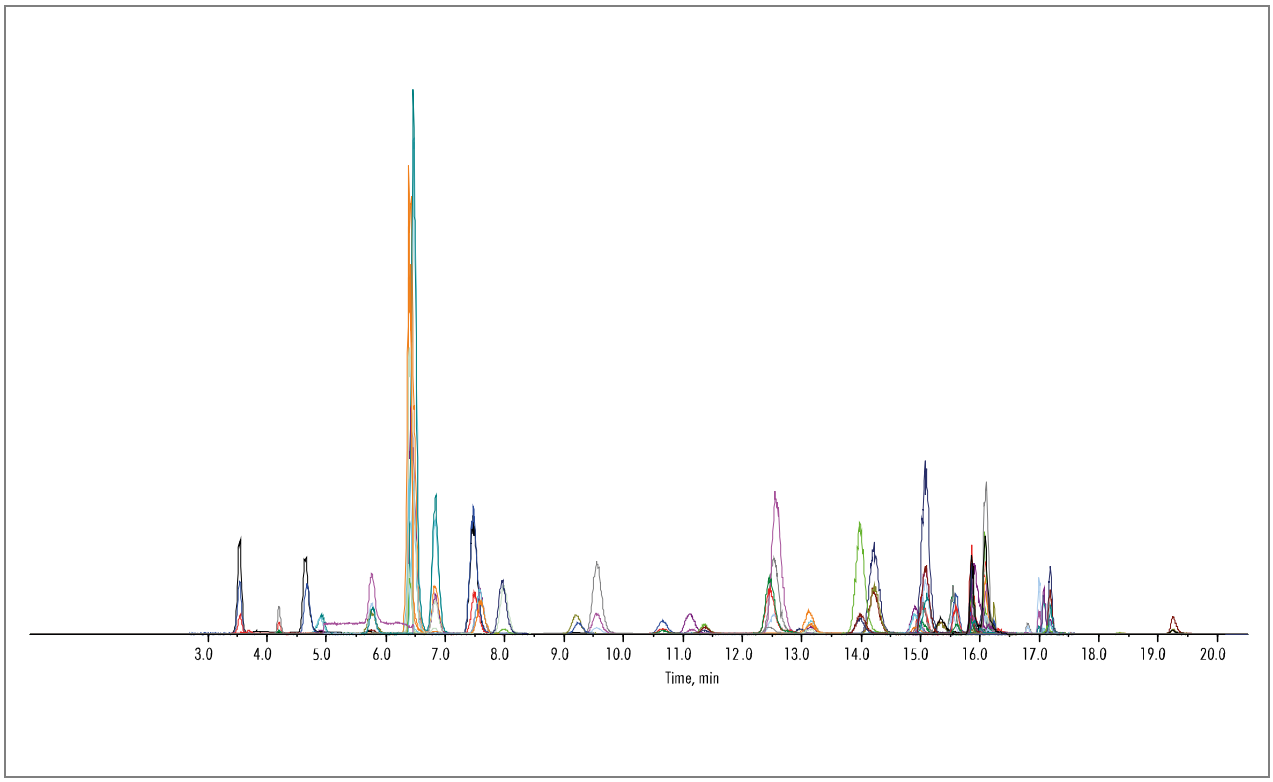


Figure 3: Chromatogram Full Panel of a calibrator 2 level

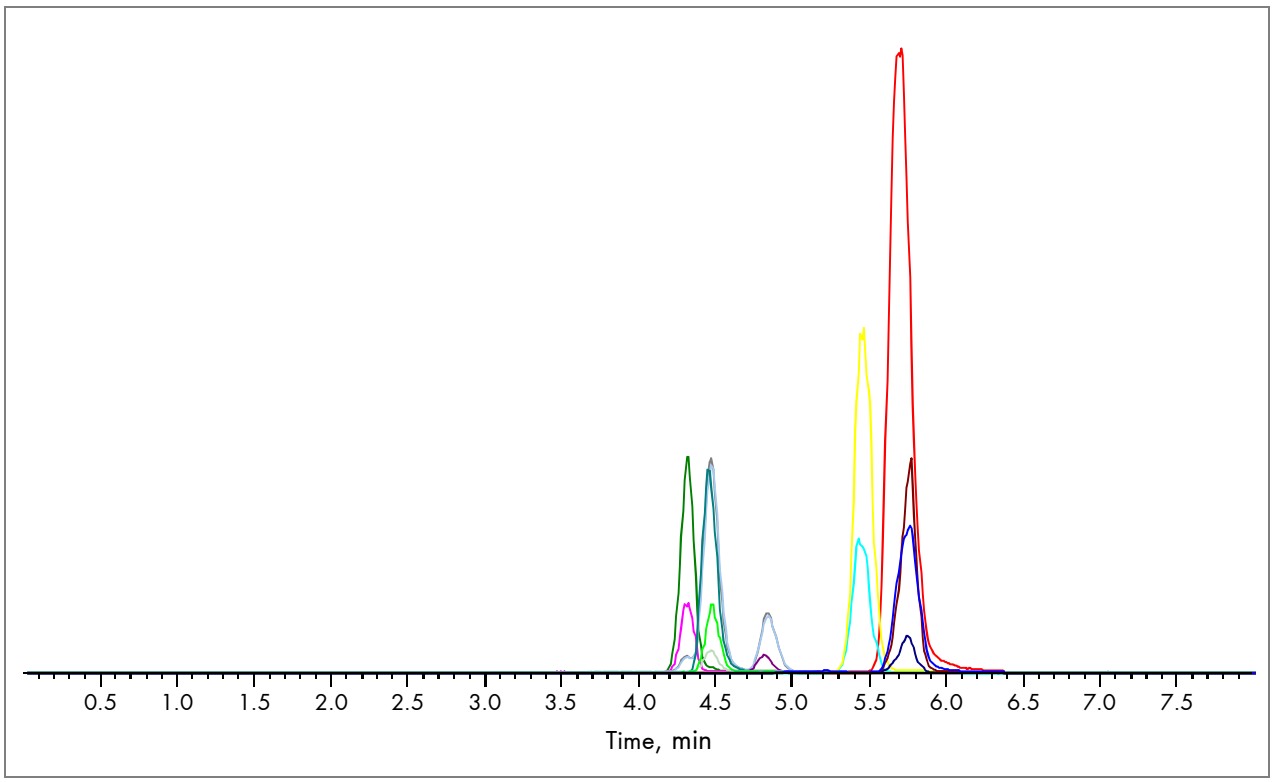


Figure 4: Chromatogram PKU/MSUD Panel

14 Interference testing

Plasma samples were spiked with isobaric compounds, metabolites and drugs in the highest likely concentrations and tested for interferences with an AB SCIEX Triple Quad™ 4500 mass spectrometer.

Some blood collection systems for EDTA plasma show contamination with sarcosine. This phenomenon is described in the literature [16] and consistent with our own studies.

The following substances were tested without any appreciable interferences being detected. Quantitative results were not affected (deviation ≤ 15%):

Metabolites and isobaric compounds
Abacavir, acetazolamide, acetylsalicylic acid, aciclovir, allopurinol, aminocaproic acid, malic acid, ascorbic acid, atropine, benzoic acid, succinic acid, biotin, carbamoyl glutamic acid, cathinone, chlordiazepoxide, clavulanic acid, creatine, desmethylflunitrazepam, dextromethorphan, ethambutol, etilefrine, flucytosine, flurbiprofen, fumaric acid, gabapentin, gentisic acid, guaifenesin, hydralazine, hydrochlorothiazide, isocitrate, isoniazid, isosorbide mononitrate, ketoglutaric acid, lamivudine, levodopa, mannitol, mercaptopurine, meropenem, methionine sulfone, methionine sulfoxide, methocarbamol, 4-methoxyamphetamine, metoclopramide, metronidazole, misoprostol, moxonidine, sodium phenylbutyrate, nicotine, nitisinone, norepinephrine, oxaloacetic acid, oxazepam, oxipurinol, pantoprazole, paracetamol, prazosin, pregabalin, pseudoephedrine, pyrazinamide, pyridostigmine, pyridoxine, salbutamol, salicylamide, sapropterin, sulfisoxazole, tapentadol, terazosin, terbutaline, thiamazole, tranylcypromine, trichloroethanol, urapidil, valproic acid.

Drugs
Acetazolamide, amikacin, amlodipine, amoxicillin, amphotericin B, ampicillin, azathioprine, azithromycin, betaine, bisoprolol, captopril, carbamazepine, carbamazepine-10,11-epoxide, cefaclor, cefixime, cefotaxime, ceftriaxone, cefuroxime, cephradine, chloral hydrate, chloramphenicol, cimetidine, ciprofloxacin, clarithromycin, clemastine, clindamycin, clobazam, clonazepam, dexamethasone, diazepam, diclofenac, digitoxin, digoxin, dihydrocodeine, dimenhydrinate, dimetindene, disopyramide, doxycycline, enalaprilat, erythromycin, ethosuximide, flucloxacillin, furosemide, ganciclovir, gentamicin, granisetron, hydrochlorothiazide, ibuprofen, isosorbide dinitrate, itraconazole, □-hydroxybutyric acid, ketamine, ketoconazole, lacosamide, lamotrigine, levetiracetam, levofloxacin, levothyroxine, lidocaine, lorazepam, metamizole, methicillin, methylprednisolone, metoprolol, metronidazole, midazolam, mycophenolic acid, mycophenolic acid glucuronide, N-acetylprocainamide, nadolol, sodium fluoride, N-desmethyldiazepam, neomycin, nifedipine, norverapamil, omeprazole, oxcarbazepine, penicillin G, penicillin V, phenobarbital, phenytoin, piperacillin, prednisone, procainamide, propofol, propranolol, ramipril, ranitidine, rifampicin, risperidone, salicylic acid, spironolactone, streptomycin, sulbactam, sulfamethoxazole, sultiame, tacrolimus, tazobactam, teicoplanin, tobramycin, tramadol, triamterene, trimethoprim, verapamil.

Interferences have been observed in the presence of the following substances:
Vigabatrin, an anti-epileptic medicine, produces false-positive γ-aminobutyric acid results. Both MRMs are affected.

Vancomycin, a glycopeptide antibiotic, produces false-positive cystine results. Both MRMs are affected.

If you have any questions concerning interferences, contact your local Chromsystems representative or our Chromsystems support staff directly by calling our hotline +49 89 18930-111 or by e-mail at support@chromsystems.com.

15 Clinical limitations

There are no universally applicable reference ranges for the analytes covered in the LC-MS/MS reagent kit ***MassChrom***® Amino Acid Analysis in plasma/serum. Results obtained using different test methods cannot be compared. Laboratories should indicate the method used for analysis to enable accurate interpretation of the results.

Users must specify their own reference ranges based on clinical assessment. Conversion factors between different methods of analysis should not be used to predict results for a specific patient.

Hemolytic samples may affect readings for many amino acids and thus falsify the results of analysis. Therefore, hemolytic samples may not be used for amino acid analysis.

16 Troubleshooting

Table 21: Troubleshooting

Problem	Possible cause	Remedy
Interfering signals	Injection system contaminated	Inject mobile phase B 10 x
	Autosampler vials contaminated	Use new vials
	Vial seals	Use other seals
	Mobile phases or columns contaminated	Replace mobile phases / columns and purge system
	Mass resolution too low	Optimise mass resolution
Analytical abnormalities accompanied by retention time shifts and high coefficients of variation	Traces of other solvents in the system, especially methanol	Rinse system for at least 25 min with 50% acetonitrile If the column has been severely damaged by other solvents, especially methanol, it may need to be replaced
	Changed retention times due to lot change of column or mobile phases	Optimise retention times according to chapter 3.1
No signal	Defective injector	Check injector
	Defective pump	Check pump
	Transfer capillary not connected to ion source	Connect capillary to ion source
	MS/MS-System not ready	Check MS/MS system
	MS/Waste switching	Check positions and switching times
	The retention time window is not properly adjusted for the analyte in question	Check whether the cause of the missing signal is due to shifted retention times or if the windows are set incorrectly or too narrowly. If so, adjust retention times in the acquisition method

Problem	Possible cause	Remedy
Poor sensitivity	Ion source contaminated	Clean ion source
	Mass spectrometer contaminated	Clean mass spectrometer
	Injection valve leaking	Check injector
	Detector aged	Replace detector or increase multiplier voltage
Poor precision of analytes homocystine, phosphoethanolamine, argininosuccinic acid, cystathionine, cystine and saccharopine	Number of acquired data points is too small	Ensure that elution occurs in 0.3 mL/min flow rate step
Unstable signal	Ionisation unstable	Check ion source; optimise voltage and gas flow, if necessary
	Flow unstable	Check pumps
	Gas unstable	Check gas flow and gas supply
No vacuum	Defective vacuum pump	Check all vacuum pumps
	Vacuum system leaks	Check vacuum tubes and vacuum connections
No gas supply	Defective nitrogen generator	Check nitrogen generator
	Defective compressor	Check compressor
	Empty gas cylinder	Replace gas cylinder
	Gas pressure out of rated value	Adjust gas pressure
Leucine/Isoleucine resolution not sufficient	Injection volume too high for used instrumention	Reduce injection volume (please also note information in chap. 3.1)
Phenylacetylglutamine, Ethanolamine and γ-Aminobutyric acid not visible and/or Leucine/Isoleucine resolution not sufficient	Injection volume too high for used instrumention	Reduce injection volume (please also note information in chap. 3.1)

If you have any questions concerning troubleshooting, contact your local Chromsystems representative or our Chromsystems support staff directly by calling our hotline +49 89 18930-111 or by e-mail at support@chromsystems.com.

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Appendix I: Hazardous substance information

When using the reagents, note the following hazard information and take the relevant safety measures. More information can be gathered from our safety data sheets. These can be downloaded from our website www.chromsystems.com or are available upon request.

Table 22: Hazard and precautionary statements

Pictograms	Hazard and precautionary statements
Mobile Phase A (order no. 75001)	
	**Danger** H225 Highly flammable liquid and vapour. H302+H312+H332 Harmful if swallowed, in contact with skin or if inhaled. H319 Causes serious eye irritation. P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P241 Use explosion-proof electrical/ventilating/lighting equipment. P243 Take action to prevent static discharges. P280 Wear protective gloves/protective clothing/eye protection/face protection.
Precipitation Reagent (order no. 75105)	
	**Danger** H225 Highly flammable liquid and vapour. H302+H312+H332 Harmful if swallowed, in contact with skin or if inhaled. H319 Causes serious eye irritation. P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P241 Use explosion-proof electrical/ventilating/lighting equipment. P243 Take action to prevent static discharges. P280 Wear protective gloves/protective clothing/eye protection/face protection.
System Check Solution (order no. 75010)	
	**Danger** H225 Highly flammable liquid and vapour. H302+H312+H332 Harmful if swallowed, in contact with skin or if inhaled. H319 Causes serious eye irritation. P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P241 Use explosion-proof electrical/ventilating/lighting equipment. P243 Take action to prevent static discharges. P280 Wear protective gloves/protective clothing/eye protection/face protection. Contains 2-methyl-2H-isothiazol-3-one. May produce an allergic reaction.

Pictograms	Hazard and precautionary statements
Tuning Mix 1 to 5 (order nos. 75015, 75016, 75017, 75018, 75019)	
	**Warning** H317 May cause an allergic skin reaction.
Internal Standard Set (order no. 75146), consisting of:	
Internal Standard Mix (REF 75104)	
Not classified as dangerous according to European Union legislation.	
Reconstitution Buffer (REF 75106)	
	**Warning** H317 May cause an allergic skin reaction.
The reconstituted Internal Standard Mix has to be classified just as the Reconstitution Buffer and the appropriate precautionary measures are to be adhered to	
3PLUS1® Multilevel Plasma Calibrator Set (order no. 75128) and *MassCheck®* Amino Acid Analysis Plasma Controls (order nos. 0471, 0472, 0473)	
	**Warning** H317 May cause an allergic skin reaction.
These components are not classified as dangerous according to European Union legislation: Mobile Phase B (order no. 75002) Rinsing Solution (order no. 75009)	

Appendix II: Manual calculation

The quotients of the signal intensities of the analytes divided by the signal intensities of the internal standard plotted against the analyte concentrations produce a calibration curve by linear regression, 1/x weighted. The resulting equation is applied for each substance.

For manual calculation the following data are required.

- Peak area of the analyte A in the MRM chromatogram =A_{sample}
- Peak area of the internal standard in the MRM chromatogram =IS_{sample}
- Slope of the calibration curve =a
- Y-intercept of the calibration curve =b

Calculate the concentration of the analyte A in the sample C_{sample} as follows:

$$C_{sample} = \frac{(A_{sample} / IS_{sample}) - b}{a}$$

Appendix III: Analytical performance data

The performance features were determined and verified on the following equipment:
– SCIEX 4500™ mass spectrometer with Shimadzu LC-20A Prominence HPLC system
– SCIEX 4500MD™ mass spectrometer with Agilent 1260 UHPLC system
– Waters® Xevo™ TQ-S micro mass spectrometer with ACQUITY™ UPLC® I-Class UHPLC system

Recovery:
Relative recovery was determined with the matrix EDTA plasma, heparin plasma and serum. Each individual matrix was spiked repeatedly with the analytes for this purpose. Three concentration levels inside the working ranges of the analytes were investigated for this purpose. Recovery is calculated using the following formula:

$$\text{Recovery [\%]} = \frac{\text{measured concentration in spiked sample} - \text{measured concentration in plain sample}}{\text{Spike concentration}} \times 100$$

Table 23: Recovery rates, Full Panel, determination with SCIEX 4500™ and Waters® Xevo™ TQ-S micro mass spectrometers

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates, Sciex 4500™		Relative recovery rates, Waters® Xevo™ TQ-S micro	
			average	range	average	range
1-Methylhistidine	serum	2.10	101 %	91.4–114 %	101 %	88.1–113 %
	EDTA plasma		104 %	94.0–112 %	102 %	92.0–113 %
	heparin plasma		100 %	86.9–107 %	104 %	93.5–115 %
	serum	10.5	100 %	98.4–105 %	99 %	86.1–104 %
	EDTA plasma		101 %	95.1–109 %	100 %	90.4–113 %
	heparin plasma		97 %	85.3–101 %	101 %	95.8–111 %
α-Aminobutyric acid	serum	12.9	100 %	94.7–107 %	105 %	92.8–113 %
	EDTA plasma		99 %	88.1–110 %	101 %	90.1–111 %
	heparin plasma		103 %	94.4–111 %	102 %	91.3–111 %
	serum	64.5	103 %	100–107 %	101 %	96.1–107 %
	EDTA plasma		102 %	97.2–108 %	103 %	95.5–112 %
	heparin plasma		98 %	87.0–104 %	102 %	97.9–107 %
β-Aminoisobutyric acid	serum	5.69	104 %	97.9–113 %	101 %	91.5–110 %
	EDTA plasma		99 %	90.7–109 %	99 %	93.2–104 %
	heparin plasma		102 %	95.8–109 %	97 %	85.3–115 %
	serum	28.4	103 %	95.7–111 %	99 %	90.1–105 %
	EDTA plasma		100 %	97.8–105 %	103 %	98.2–108 %

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates, Sciex 4500™		Relative recovery rates, Waters® Xevo™ TQ-S micro	
			average	range	average	range
	heparin plasma		102 %	93.3–107 %	95 %	87.6–105 %
3-Methylhistidine	serum	9.14	98 %	90.5–114 %	98 %	85.8–112 %
	EDTA plasma	11.6	102 %	87.8–114 %	104 %	87.0–114 %
	heparin plasma		100 %	85.1–113 %	100 %	85.7–112 %
	serum	90.1	104 %	101–111 %	97 %	91.6–99.8 %
	EDTA plasma	58.2	100 %	96.0–106 %	99 %	89.8–106 %
	heparin plasma		109 %	101–114 %	109 %	103–115 %
4-Hydroxyproline	serum	24.3	99 %	93.7–106 %	99 %	92.3–102 %
	EDTA plasma		103 %	96.3–113 %	100 %	95.0–105 %
	heparin plasma		99 %	91.4–106 %	98 %	85.5–109 %
	serum	121	101 %	99.1–105 %	99 %	95.1–102 %
	EDTA plasma		102 %	97.4–107 %	101 %	95.3–106 %
	heparin plasma		98 %	89.3–103 %	100 %	94.8–105 %
Acetyltyrosine	serum	5.46	103 %	97.3–106 %	99 %	89.5–112 %
	EDTA plasma		103 %	96.8–107 %	103 %	96.4–109 %
	heparin plasma		101 %	95.9–105 %	100 %	88.4–113 %
	serum	27.3	101 %	98.9–103 %	103 %	89.0–113 %
	EDTA plasma		102 %	97.9–106 %	103 %	97.6–111 %
	heparin plasma		99 %	88.6–102 %	99 %	90.3–112 %
Adenosyl-homocysteine	serum	5.84	103 %	99.6–108 %	102 %	98.3–104 %
	EDTA plasma		101 %	96.9–108 %	100 %	93.0–107 %
	heparin plasma		100 %	91.3–105 %	101 %	93.1–107 %
	serum	29.2	99 %	96.3–102 %	100 %	96.3–103 %
	EDTA plasma		99 %	94.5–106 %	102 %	98.1–109 %
	heparin plasma		97 %	87.5–101 %	99 %	96.1–101 %
Alanine	serum	137	98 %	91.8–106 %	104 %	90.5–114 %
	EDTA plasma		99 %	87.8–111 %	98 %	89.6–106 %
	heparin plasma		103 %	85.8–115 %	102 %	94.1–114 %
	serum	687	99 %	96.0–105 %	100 %	93.8–109 %

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates, Sciex 4500™		Relative recovery rates, Waters® Xevo™ TQ-S micro	
			average	range	average	range
	EDTA plasma		100 %	95.1–107 %	101 %	97.9–107 %
	heparin plasma		99 %	85.9–104 %	102 %	96.2–108 %
Allo-isoleucine	serum	13.7	103 %	101–107 %	97 %	94.3–101 %
	EDTA plasma		104 %	98.1–109 %	100 %	95.1–110 %
	heparin plasma		101 %	94.4–106 %	102 %	95.2–111 %
	serum	68.4	100 %	98.1–105 %	99 %	94.9–103 %
	EDTA plasma		105 %	99.8–110 %	102 %	96.2–108 %
	heparin plasma		98 %	89.6–102 %	101 %	94.9–105 %
α-Aminoadipic acid	serum	2.48	101 %	95.9–105 %	102 %	90.3–114 %
	EDTA plasma		100 %	90.3–112 %	98 %	93.2–107 %
	heparin plasma		97 %	86.7–106 %	100 %	93.8–110 %
	serum	12.4	101 %	96.2–105 %	101 %	97.3–105 %
	EDTA plasma		100 %	96.5–103 %	100 %	90.8–105 %
	heparin plasma		98 %	86.3–106 %	100 %	96.3–103 %
Anserine	serum	6.78	95 %	89.6–104 %	104 %	93.9–111 %
	EDTA plasma	2.35	103 %	94.8–112 %	103 %	87.8–115 %
	heparin plasma	6.78	95 %	88.2–102 %	99 %	89.9–107 %
	serum	15.0	103 %	94.5–111 %	104 %	94.4–110 %
	EDTA plasma	11.8	101 %	89.2–115 %	102 %	85.6–115 %
	heparin plasma	15.0	91 %	85.6–102 %	102 %	100–106 %
Arginine	serum	31.7	98 %	87.0–110 %	98 %	91.1–109 %
	EDTA plasma		103 %	93.1–114 %	104 %	89.4–114 %
	heparin plasma		105 %	92.4–110 %	105 %	90.3–112 %
	serum	159	101 %	95.8–108 %	101 %	91.9–110 %
	EDTA plasma		105 %	95.5–115 %	103 %	95.3–109 %
	heparin plasma		100 %	93.0–111 %	106 %	96.7–111 %
Argininosuccinic acid	serum	6.18	94 %	87.6–97.8 %	95 %	87.4–103 %
	EDTA plasma		97 %	92.7–104 %	98 %	90.6–107 %
	heparin plasma		93 %	85.1–102 %	90 %	85.2–94.0 %

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates, Sciex 4500™		Relative recovery rates, Waters® Xevo™ TQ-S micro	
			average	range	average	range
	serum	30.9	95 %	88.8–104 %	98 %	88.1–104 %
	EDTA plasma		95 %	90.2–100 %	98 %	89.7–109 %
	heparin plasma		93 %	86.1–97.5 %	93 %	85.9–99.7 %
Asparagine	serum	31.6	102 %	97.1–109 %	101 %	94.0–105 %
	EDTA plasma		99 %	89.2–108 %	101 %	93.4–113 %
	heparin plasma		101 %	87.9–110 %	102 %	90.2–111 %
	serum	158	99 %	96.9–102 %	100 %	96.7–104 %
	EDTA plasma		100 %	94.8–107 %	101 %	96.3–106 %
	heparin plasma		98 %	87.7–103 %	100 %	94.5–103 %
Aspartic acid	serum	30.0	95 %	86.2–114 %	97 %	86.1–106 %
	EDTA plasma		92 %	85.2–99,2 %	97 %	91.1–105 %
	heparin plasma		93 %	87.0–108 %	101 %	95.0–108 %
	serum	150	97 %	88.5–102 %	101 %	97.9–104 %
	EDTA plasma		102 %	94.9–109 %	99 %	93.4–106 %
	heparin plasma		95 %	88.0–103 %	97 %	90.5–105 %
β-Alanine	serum	18.9	104 %	94.4–113 %	110 %	97.4–115 %
	EDTA plasma	12.7	108 %	100–113 %	102 %	87.4–113 %
	heparin plasma	18.9	108 %	97.5–115 %	109 %	94.9–115 %
	serum	90.0	108 %	100–114 %	111 %	106–115 %
	EDTA plasma	63.5	107 %	98.0–114 %	104 %	92.7–113 %
	heparin plasma	90.0	110 %	106–114 %	108 %	101–114 %
Carnosine	serum	16.5	98 %	90.3–102 %	94 %	88.4–100 %
	EDTA plasma	7.24	104 %	85.2–114 %	91 %	86.6–96.3 %
	heparin plasma	16.5	104 %	97.0–109 %	94 %	89.8–97.5 %
	serum	37.5	99 %	93.7–102 %	95 %	92.0–1001 %
	EDTA plasma	36.2	101 %	91.0–114 %	99 %	92.3–107 %
	heparin plasma	37.5	99 %	93.2–104 %	92 %	86.7–103 %
Citrulline	serum	12.1	98 %	87.7–113 %	99 %	90.0–108 %
	EDTA plasma		102 %	93.0–114 %	104 %	92.9–115 %

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates, Sciex 4500™		Relative recovery rates, Waters® Xevo™ TQ-S micro	
			average	range	average	range
	heparin plasma		104 %	89.8–114 %	101 %	92.9–107 %
	serum	60.3	99 %	95.4–107 %	102 %	98.2–105 %
	EDTA plasma		103 %	94.6–111 %	100 %	91.2–107 %
	heparin plasma		106 %	96.5–114 %	100 %	95.8–105 %
Cystathionine	serum	5.89	99 %	95.8–102 %	108 %	103–113 %
	EDTA plasma		103 %	99.6–109 %	103 %	98.3–108 %
	heparin plasma		104 %	95.5–112 %	104 %	101–108 %
	serum	29.4	99 %	95.0–103 %	102 %	97.9–105 %
	EDTA plasma		99 %	94.6–105 %	98 %	93.4–103 %
	heparin plasma		100 %	88.8–106 %	100 %	94.7–103 %
Cysteine sulphate	serum	18.4	95 %	85.3–111 %	105 %	98.9–111 %
	EDTA plasma	6.42	105 %	94.8–113 %	92 %	87.3–99.8 %
	heparin plasma	18.4	99 %	93.3–103 %	97 %	93.9–101 %
	serum	45.0	98 %	91.6–105 %	100 %	94.7–107 %
	EDTA plasma	32.1	93 %	85.2–102 %	88 %	85.2–93.5 %
	heparin plasma	45.0	98 %	91.5–103 %	97 %	88.9–104 %
Cystine	serum	9.72	101 %	91.2–113 %	100 %	95.8–102 %
	EDTA plasma	20.2	89 %	85.3–98.0 %	92 %	87.2–99.7 %
	heparin plasma	9.72	108 %	92.5–115 %	102 %	96.5–107 %
	serum	150	101 %	100–104 %	102 %	98.6–104 %
	EDTA plasma	101	90 %	85.1–96.8 %	91 %	85.7–97.8 %
	heparin plasma	150	102 %	97.3–105 %	102 %	98.3–104 %
Ethanolamine	serum	34.9	110 %	104–115 %	99 %	86.8–108 %
	EDTA plasma		98 %	89.1–109 %	98 %	86.0–114 %
	heparin plasma		110 %	102–114 %	104 %	87.9–114 %
	serum	174	107 %	102–111 %	102 %	92.6–109 %
	EDTA plasma		102 %	99.7–106 %	97 %	85.3–115 %
	heparin plasma		103 %	91.3–109 %	104 %	90.6–112 %
	serum	2.10	102 %	90.8–110 %	109 %	106–113 %

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates, Sciex 4500™		Relative recovery rates, Waters® Xevo™ TQ-S micro	
			average	range	average	range
γ-Aminobutyric acid	EDTA plasma	10.5	106 %	94.9–115 %	100 %	85.6–109 %
	heparin plasma		103 %	96.7–107 %	95 %	86.7–102 %
	serum		104 %	100–106 %	107 %	101–114 %
	EDTA plasma		101 %	97.2–106 %	101 %	93.6–111 %
	heparin plasma		102 %	92.0–106 %	99 %	94.9–105 %
Glutamine	serum	200	91 %	87.8–96.8 %	98 %	87.2–108 %
	EDTA plasma		100 %	87.4–112 %	100 %	89.5–112 %
	heparin plasma		102 %	87.3–115 %	101 %	87.6–112 %
	serum	998	93 %	90.5–97.4 %	101 %	97.8–103 %
	EDTA plasma		105 %	96.7–112 %	103 %	95.3–115 %
	heparin plasma		96 %	88.3–105 %	100 %	97.4–104 %
Glutamic acid	serum	90.5	105 %	101–110 %	100 %	87.6–110 %
	EDTA plasma		101 %	92.5–106 %	98 %	93.3–102 %
	heparin plasma		101 %	95.7–105 %	95 %	90.4–100 %
	serum	453	102 %	100–105 %	101 %	95.9–109 %
	EDTA plasma		102 %	98.4–106 %	100 %	96.4–104 %
	heparin plasma		99 %	90.9–103 %	96 %	88.6–100 %
Glycine	serum	160	101 %	97.1–104 %	99 %	88.6–114 %
	EDTA plasma		100 %	92.0–106 %	103 %	96.4–109 %
	heparin plasma		98 %	87.2–109 %	103 %	94.4–111 %
	serum	798	100 %	97.3–102 %	101 %	98.7–107 %
	EDTA plasma		101 %	96.6–109 %	102 %	96.5–107 %
	heparin plasma		98 %	85.3–103 %	101 %	95.2–105 %
Histidine	serum	27.7	108 %	91.2–115 %	101 %	88.8–113 %
	EDTA plasma		98 %	85.6–108 %	98 %	85.7–109 %
	heparin plasma		100 %	86.0–113 %	101 %	91.5–105 %
	serum	139	110 %	104–114 %	108 %	105–112 %
	EDTA plasma		101 %	94.2–108 %	100 %	91.7–107 %

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates, Sciex 4500™		Relative recovery rates, Waters® Xevo™ TQ-S micro	
			average	range	average	range
	heparin plasma		109 %	96.1–114 %	111 %	106–115 %
Homocitrulline	serum	4.36	106 %	96.0–114 %	100 %	91.4–106 %
	EDTA plasma		102 %	96.0–109 %	107 %	101–114 %
	heparin plasma		98 %	90.9–104 %	107 %	103–115 %
	serum	21.8	103 %	101–106 %	99 %	96.2–101 %
	EDTA plasma		101 %	96.4–104 %	102 %	97.6–107 %
	heparin plasma		99 %	90.7–103 %	100 %	95.4–103 %
Homocystine	serum	7.75	99 %	90 .0- 108 %	96 %	93.1–99.9 %
	EDTA plasma		100 %	95.2–108 %	102 %	100–103 %
	heparin plasma		98 %	89.8–104 %	99 %	96.1–102 %
	serum	22.5	101 %	97.0–104 %	101 %	99.5–102 %
	EDTA plasma		103 %	99.3–105 %	101 %	98.6–104 %
	heparin plasma		99 %	94.0–105 %	98 %	95.4–101 %
Hydroxylysine	serum	4.10	102 %	86.8–109 %	102 %	94.8–112 %
	EDTA plasma		101 %	95.1–104 %	107 %	98.2–114 %
	heparin plasma		109 %	98.8–115 %	106 %	100–112 %
	serum	20.5	100 %	91.6–106 %	102 %	97.1–104 %
	EDTA plasma		103 %	97.6–109 %	103 %	96.0–109 %
	heparin plasma		103 %	98.8–111 %	101 %	96.5–106 %
Isoleucine	serum	24.0	99 %	87.2–107 %	98 %	88.4–108 %
	EDTA plasma		98 %	88.2–110 %	100 %	86.8–114 %
	heparin plasma		100 %	85.4–112 %	98 %	86.2–109 %
	serum	120	99 %	94.9–104 %	98 %	92.7–103 %
	EDTA plasma		98 %	91.4–104 %	101 %	88.9–105 %
	heparin plasma		97 %	86.3–107 %	98 %	95.1–102 %
Leucine	serum	54.2	100 %	90.9–108 %	101 %	87.6–108 %
	EDTA plasma		98 %	85.8–107 %	101 %	95.8–106 %
	heparin plasma		98 %	87.8–111 %	99 %	86.2–108 %
	serum	271	100 %	96.3–103 %	101 %	96.4–109 %

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates, Sciex 4500™		Relative recovery rates, Waters® Xevo™ TQ-S micro	
			average	range	average	range
	EDTA plasma		100 %	92.4–105 %	102 %	93.5–107 %
	heparin plasma		98 %	86.5–103 %	98 %	87.7–102 %
Lysine	serum	103	98 %	86.4–113 %	102 %	91.5–113 %
	EDTA plasma		99 %	88.3–115 %	106 %	95.8–114 %
	heparin plasma		97 %	89.1–108 %	101 %	91.2–109 %
	serum	515	99 %	94.3–103 %	102 %	98.2–107 %
	EDTA plasma		107 %	99.8–114 %	105 %	99.3–110 %
	heparin plasma		99 %	92.8–103 %	100 %	94.7–103 %
Methionine	serum	15.1	101 %	89.7–107 %	100 %	96.2–110 %
	EDTA plasma		100 %	91.7–110 %	96 %	90.9–105 %
	heparin plasma		102 %	86.7–111 %	99 %	91.0–109 %
	serum	75.7	100 %	97.3–104 %	100 %	92.7–104 %
	EDTA plasma		101 %	96.4–107 %	98 %	89.6–105 %
	heparin plasma		99 %	86.9–104 %	99 %	90.0–102 %
Ornithine	serum	22.8	100 %	85.1–115 %	107 %	99.7–115 %
	EDTA plasma		100 %	87.3–114 %	101 %	88.2–114 %
	heparin plasma		99 %	85.9–113 %	101 %	85.4–112 %
	serum	114	99 %	89.6–103 %	103 %	98.6–107 %
	EDTA plasma		99 %	91.0–106 %	100 %	94.0–106 %
	heparin plasma		99 %	90.4–102 %	100 %	96.4–104 %
Phenylalanine	serum	32.6	105 %	98.3–113 %	97 %	86.0–108 %
	EDTA plasma		100 %	90.8–114 %	97 %	87.9–110 %
	heparin plasma		101 %	88.6–110 %	97 %	87.6–105 %
	serum	163	102 %	98.9–104 %	101 %	96.7–104 %
	EDTA plasma		101 %	96.9–104 %	101 %	98.9–106 %
	heparin plasma		101 %	88.1–106 %	96 %	88.9–103 %
Phospho-ethanolamin	serum	16.8	91 %	86.5–96.6 %	103 %	92.7–113 %
	EDTA plasma		103 %	97.5–111 %	104 %	98.9–114 %
	heparin plasma		96 %	85.3–104 %	91 %	85.3–97.6 %

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates, Sciex 4500™		Relative recovery rates, Waters® Xevo™ TQ-S micro	
			average	range	average	range
	serum	83.9	97 %	92.0–102 %	100 %	92.4–106 %
	EDTA plasma		101 %	97.8–105 %	102 %	96.4–105 %
	heparin plasma		93 %	85.7–97.1 %	95 %	87.1–99.6 %
Phosphoserine	serum	24.3	91 %	87.8–102 %	92 %	86.3–98.4 %
	EDTA plasma		104 %	102–107 %	106 %	102–110 %
	heparin plasma	9.40	102 %	95.1–113 %	105 %	100–111 %
	serum	122	93 %	87.6–96.4 %	94 %	88.0–97.4 %
	EDTA plasma		103 %	97.3–106 %	103 %	97.0–108 %
	heparin plasma	165	101 %	95.7–105 %	102 %	98.6–104 %
Pipecolic acid	serum	4.01	101 %	96.4–107 %	101 %	97.5–104 %
	EDTA plasma		102 %	95.9–104 %	101 %	98.0–104 %
	heparin plasma		98 %	91.1–106 %	99 %	91.7–104 %
	serum	20.1	100 %	97.4–103 %	100 %	98.3–105 %
	EDTA plasma		101 %	96.2–105 %	101 %	97.5–106 %
	heparin plasma		97 %	86.7–101 %	101 %	96.7–105 %
Proline	serum	76.3	94 %	88.8–103 %	103 %	94.6–112 %
	EDTA plasma		97 %	88.2–104 %	100 %	90.1–111 %
	heparin plasma		98 %	87.2–109 %	99 %	89.2–106 %
	serum	381	95 %	90.5–100 %	99 %	96.0–103 %
	EDTA plasma		98 %	93.5–104 %	101 %	90.9–107 %
	heparin plasma		95 %	85.8–99.5 %	98 %	96.0–105 %
Saccharopine	serum	0.968	97 %	90.0–103 %	100 %	85.6–111 %
	EDTA plasma	6.29	102 %	95.9–112 %	89 %	85.7–99.3 %
	heparin plasma	0.968	103 %	87.3–113 %	88 %	75.0–100 %
	serum	4.84	102 %	93.4–113 %	100 %	93.1–111 %
	EDTA plasma	15.0	100 %	97.2–104 %	100 %	94.6–108 %
	heparin plasma	4.84	98 %	90.0–107 %	99 %	93.6–104 %
Sarcosine	serum	4.33	100 %	86.8–109 %	102 %	85.7–114 %
	EDTA plasma		96 %	86.7–107 %	102 %	90.0–113 %

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates, Sciex 4500™		Relative recovery rates, Waters® Xevo™ TQ-S micro	
			average	range	average	range
	heparin plasma		98 %	85.3–107 %	99 %	86.8–114 %
	serum	21.6	100 %	94.5–105 %	100 %	91.2–109 %
	EDTA plasma		100 %	93.5–108 %	100 %	93.6–109 %
	heparin plasma		98 %	89.5–104 %	100 %	94.7–109 %
Serine	serum	102	105 %	98.3–113 %	103 %	95.0–107 %
	EDTA plasma		102 %	97.4–110 %	100 %	86.3–105 %
	heparin plasma		109 %	96.9–113 %	105 %	93.7–109 %
	serum	509	103 %	102–107 %	103 %	100–106 %
	EDTA plasma		102 %	97.7–107 %	101 %	96.5–108 %
	heparin plasma		102 %	88.0–109 %	102 %	94.3–105 %
Taurine	serum	59.7	101 %	96.3–107 %	96 %	90.9–100 %
	EDTA plasma		101 %	96.8–104 %	101 %	95.3–106 %
	heparin plasma		97 %	86.0–104 %	100 %	90.7–107 %
	serum	299	101 %	99.4–104 %	99 %	94.8–104 %
	EDTA plasma		101 %	97.2–107 %	101 %	96.4–105 %
	heparin plasma		98 %	87.4–103 %	100 %	96.6–103 %
Threonine	serum	71.5	102 %	89.4–109 %	102 %	92.8–113 %
	EDTA plasma		100 %	94.3–107 %	99 %	85.5–106 %
	heparin plasma		105 %	94.6–111 %	101 %	92.7–107 %
	serum	358	101 %	99.7–104 %	100 %	95.1–104 %
	EDTA plasma		102 %	96.7–106 %	102 %	94.9–109 %
	heparin plasma		100 %	86.4–104 %	99 %	96.8–101 %
Tryptophan	serum	29.5	98 %	88.5–106 %	100 %	87.6–111 %
	EDTA plasma		97 %	88.8–106 %	97 %	85.1–112 %
	heparin plasma		98 %	86.5–111 %	97 %	85.0–113 %
	serum	147	100 %	97.0–104 %	101 %	94.8–109 %
	EDTA plasma		101 %	95.2–106 %	100 %	91.4–111 %
	heparin plasma		99 %	87.3–103 %	100 %	95.2–106 %
Tyrosine	serum	31.6	99 %	91.8–103 %	100 %	92.2–106 %

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates, Sciex 4500™		Relative recovery rates, Waters® Xevo™ TQ-S micro	
			average	range	average	range
Valine	EDTA plasma	158	97 %	89.3–103 %	97 %	87.2–110 %
	heparin plasma		104 %	90.2–113 %	101 %	88.9–113 %
	serum		101 %	96.5–108 %	101 %	96.4–106 %
	EDTA plasma		103 %	95.4–112 %	100 %	93.4–104 %
	heparin plasma		98 %	88.9–102 %	99 %	93.9–104 %
	serum	66.5	99 %	89.4–108 %	102 %	87.6–114 %
	EDTA plasma		100 %	89.4–113 %	98 %	89.7–111 %
	heparin plasma		100 %	85.8–115 %	101 %	90.5–113 %
	serum	333	100 %	94.4–106 %	103 %	97.6–109 %
	EDTA plasma		97 %	94.0–102 %	101 %	89.8–105 %
	heparin plasma		100 %	85.5–106 %	102 %	98.0–107 %

Table 24: Recovery rates – PKU/MSUD panel, determination with SCIEX 4500MD™ mass spectrometer

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates	
			average	range
Allo-isoleucine	serum	13.7	99 %	93.7–104 %
	EDTA plasma		101 %	95.1–106 %
	heparin plasma		100 %	93.1–111 %
	serum	68.4	101 %	95.8–106 %
	EDTA plasma		100 %	93.8–106 %
	heparin plasma		99 %	94.6–104 %
Isoleucine	serum	24.0	102 %	88.9–112 %
	EDTA plasma		99 %	85.8–107 %
	heparin plasma		102 %	88.7–113 %
	serum	120	101 %	95.4–105 %
	EDTA plasma		101 %	92.9–107 %
	heparin plasma		101 %	94.7–106 %
Leucine	serum	54.2	100 %	91.0–105 %
	EDTA plasma		100 %	88.9–111 %
	heparin plasma		101 %	91.2–109 %
	serum	271	101 %	97.5–104 %
	EDTA plasma		102 %	98.7–104 %
	heparin plasma		99 %	94.9–101 %
Methionine	serum	15.1	97 %	91.6–105 %
	EDTA plasma		97 %	86.8–110 %

Substance	Matrix	Spike concentration [µmol/l]	Relative recovery rates	
			average	range
	heparin plasma		98 %	86.4–108 %
	serum		96 %	90.6–102 %
	EDTA plasma	75.7	100 %	94.1–111 %
	heparin plasma		96 %	92.4–98.0 %
Phenylalanine	serum		101 %	90.3–109 %
	EDTA plasma	32.6	96 %	87.1–111 %
	heparin plasma		101 %	89.5–115 %
	serum		101 %	96.9–106 %
	EDTA plasma	163	99 %	91.8–105 %
	heparin plasma		100 %	95.5–104 %
Tyrosine	serum		100 %	94.6–107 %
	EDTA plasma	31.6	100 %	90.7–112 %
	heparin plasma		103 %	97.8–107 %
	serum		101 %	97.8–107 %
	EDTA plasma	158	102 %	95.8–110 %
	heparin plasma		101 %	97.7–105 %
Valine	serum		100 %	85.6–111 %
	EDTA plasma	66.5	99 %	93.7–111 %
	heparin plasma		99 %	85.2–111 %
	serum		101 %	92.8–108 %
	EDTA plasma	333	105 %	94.2–113 %
	heparin plasma		107 %	99.5–115 %

Lower limit of quantitation (LLOQ) and linearity (upper limit of quantitation):
Linearity was determined by spiking plasma and serum samples with defined quantities of standard substances. The lower limit of quantitation (LLOQ) was determined using defined dilutions of plasma and serum samples with analyte-free matrix.

The method is linear from the lower limit of quantitation (LLOQ) to the stated upper limit of quantitation (linear range).

Table 25: Limit of quantitation and linearity, Full Panel, determination with SCIEX 4500™ und Waters® Xevo™ TQ-S micro mass spectrometer

Substance	Matrix	Sciex 4500™		Waters® Xevo™ TQ-S micro	
		LLOQ	Linear range up to at least	LLOQ	Linear range up to at least
1-Methylhistidine	plasma	0.534 µmol/l	60.0 µmol/l	0.333 µmol/l	60.0 µmol/l
	serum	0.275 µmol/l	60.0 µmol/l	0.270 µmol/l	60.0 µmol/l
α-Aminobutyric acid	plasma	0.549 µmol/l	360 µmol/l	1.35 µmol/l	360 µmol/l
	serum	0.407 µmol/l	360 µmol/l	2.13 µmol/l	360 µmol/l
β-Aminoisobutyric acid	plasma	0.248 µmol/l	150 µmol/l	1.18 µmol/l	150 µmol/l

Substance	Matrix	Sciex 4500™		Waters® Xevo™ TQ-S micro	
		LLOQ	Linear range up to at least	LLOQ	Linear range up to at least
	serum	0.745 µmol/l	150 µmol/l	0.795 µmol/l	150 µmol/l
3-Methylhistidine	plasma	1.46 µmol/l	360 µmol/l	1.67 µmol/l	360 µmol/l
	serum	1.91 µmol/l	360 µmol/l	1.85 µmol/l	360 µmol/l
4-Hydroxyproline	plasma	2.06 µmol/l	677 µmol/l	4.12 µmol/l	677 µmol/l
	serum	1.45 µmol/l	677 µmol/l	3.61 µmol/l	677 µmol/l
Acetyltyrosine	plasma	0.991 µmol/l	150 µmol/l	1.16 µmol/l	150 µmol/l
	serum	0.331 µmol/l	150 µmol/l	0.845 µmol/l	150 µmol/l
Adenosylhomocysteine	plasma	0.266 µmol/l	150 µmol/l	0.665 µmol/l	150 µmol/l
	serum	0.108 µmol/l	150 µmol/l	0.586 µmol/l	150 µmol/l
Alanine	plasma	17.2 µmol/l	3687 µmol/l	31.7 µmol/l	3687 µmol/l
	serum	11.8 µmol/l	3687 µmol/l	45.9 µmol/l	3687 µmol/l
Allo-isoleucine	plasma	0.275 µmol/l	500 µmol/l	1.33 µmol/l	500 µmol/l
	serum	0.611 µmol/l	500 µmol/l	0.549 µmol/l	500 µmol/l
α-Aminoadipic acid	plasma	1.20 µmol/l	72.1 µmol/l	0.239 µmol/l	72.1 µmol/l
	serum	0.721 µmol/l	72.1 µmol/l	0.719 µmol/l	72.1 µmol/l
Anserine	plasma	0.271 µmol/l	60.0 µmol/l	1.38 µmol/l	60.0 µmol/l
	serum	0.295 µmol/l.	60.0 µmol/l	1.15 µmol/l	60.0 µmol/l
Arginine	plasma	2.62 µmol/l	946 µmol/l	6.35 µmol/l	900 µmol/l
	serum	5.68 µmol/l	946 µmol/l	4.53 µmol/l	946 µmol/l
Argininosuccinic acid	plasma	0.702 µmol/l	1000 µmol/l	0.440 µmol/l	1000 µmol/l
	serum	1.07 µmol/l	900 µmol/l	0.540 µmol/l	1000 µmol/l
Asparagine	plasma	1.40 µmol/l	900 µmol/l	2.76 µmol/l	900 µmol/l
	serum	2.07 µmol/l	900 µmol/l	2.03 µmol/l	900 µmol/l
Aspartic acid	plasma	3.57 µmol/l	840 µmol/l	3.55 µmol/l	840 µmol/l
	serum	4.58 µmol/l	840 µmol/l	4.31 µmol/l	840 µmol/l
β-Alanine	plasma	3.02 µmol/l	363 µmol/l	3.43 µmol/l	363 µmol/l
	serum	4.27 µmol/l	363 µmol/l	4.55 µmol/l	363 µmol/l
Carnosine	plasma	2.65 µmol/l	150 µmol/l	3.51 µmol/l	150 µmol/l
	serum	0.655 µmol/l	150 µmol/l	0.659 µmol/l	150 µmol/l
Citrulline	plasma	3.46 µmol/l	900 µmol/l	1.73 µmol/l	900 µmol/l
	serum	2.59 µmol/l	900 µmol/l	2.50 µmol/l	900 µmol/l
Cystathionine	plasma	0.255 µmol/l	194 µmol/l	0.638 µmol/l	180 µmol/l
	serum	0.204 µmol/l	194 µmol/l	0.972 µmol/l	194 µmol/l
Cysteine sulphate	plasma	2.95 µmol/l	180 µmol/l	1.84 µmol/l	180 µmol/l
	serum	2.80 µmol/l	175 µmol/l	1.60 µmol/l	175 µmol/l

Substance	Matrix	Sciex 4500™		Waters® Xevo™ TQ-S micro	
		LLOQ	Linear range up to at least	LLOQ	Linear range up to at least
Cystine	plasma	1.56 µmol/l	600 µmol/l	0.312 µmol/l	600 µmol/l
	serum	0.667 µmol/l	1000 µmol/l	3.21 µmol/l	1000 µmol/l
Ethanolamine	plasma	5.44 µmol/l	900 µmol/l	20.0 µmol/l	900 µmol/l
	serum	4.94 µmol/l	900 µmol/l	20.0 µmol/l	900 µmol/l
γ-Aminobutyric acid	plasma	1.82 µmol/l	60.0 µmol/l	1.94 µmol/l	60.0 µmol/l
	serum	0.712 µmol/l	60.0 µmol/l	0.700 µmol/l	60.0 µmol/l
Glutamine	plasma	73.3 µmol/l	6600 µmol/l	74.0 µmol/l	6600 µmol/l
	serum	41.3 µmol/l	6600 µmol/l	41.0 µmol/l	6600 µmol/l
Glutamic acid	plasma	8.84 µmol/l	2254 µmol/l	21.2 µmol/l	2254 µmol/l
	serum	4.35 µmol/l	2254 µmol/l	11.1 µmol/l	2254 µmol/l
Glycine	plasma	19.8 µmol/l	4496 µmol/l	47.3 µmol/l	4496 µmol/l
	serum	22.4 µmol/l	4496 µmol/l	79.3 µmol/l	4496 µmol/l
Histidine	plasma	10.5 µmol/l	840 µmol/l	12.3 µmol/l	840 µmol/l
	serum	13.7 µmol/l	840 µmol/l	15.7 µmol/l	840 µmol/l
Homocitrulline	plasma	0.470 µmol/l	160 µmol/l	1.18 µmol/l	160 µmol/l
	serum	0.168 µmol/l	160 µmol/l	1.57 µmol/l	160 µmol/l
Homocystine	plasma	1.24 µmol/l	90.0 µmol/l	0.775 µmol/l	90.0 µmol/l
	serum	1.30 µmol/l	90.0 µmol/l	0.615 µmol/l	90.0 µmol/l
Hydroxylysine	plasma	0.935 µmol/l	120 µmol/l	0.585 µmol/l	120 µmol/l
	serum	1.31 µmol/l	120 µmol/l	0.603 µmol/l	120 µmol/l
Isoleucine	plasma	9.29 µmol/l	820 µmol/l	5.06 µmol/l	1006 µmol/l
	serum	10.9 µmol/l	1006 µmol/l	4.56 µmol/l	1006 µmol/l
Leucine	plasma	2.67 µmol/l	2104 µmol/l	5.22 µmol/l	2007 µmol/l
	serum	5.36 µmol/l	2104 µmol/l	5.22 µmol/l	2104 µmol/l
Lysine	plasma	3.27 µmol/l	2355 µmol/l	5.77 µmol/l	2355 µmol/l
	serum	4.38 µmol/l	2355 µmol/l	3.56 µmol/l	2355 µmol/l
Methionine	plasma	0.972 µmol/l	420 µmol/l	2.00 µmol/l	420 µmol/l
	serum	1.44 µmol/l	420 µmol/l	2.91 µmol/l	420 µmol/l
Ornithine	plasma	1.84 µmol/l	1002 µmol/l	6.16 µmol/l	1002 µmol/l
	serum	2.62 µmol/l	1002 µmol/l	5.80 µmol/l	1002 µmol/l
Phenylalanine	plasma	8.14 µmol/l	1400 µmol/l	8.46 µmol/l	1400 µmol/l
	serum	11.1 µmol/l	1400 µmol/l	5.70 µmol/l	1400 µmol/l
Phosphoethanolamine	plasma	1.25 µmol/l	480 µmol/l	0.783 µmol/l	480 µmol/l
	serum	2.50 µmol/l	480 µmol/l	2.49 µmol/l	480 µmol/l
Phosphoserine	plasma	1.50 µmol/l	660 µmol/l	0.301 µmol/l	660 µmol/l

Substance	Matrix	Sciex 4500™		Waters® Xevo™ TQ-S micro	
		LLOQ	Linear range up to at least	LLOQ	Linear range up to at least
	serum	0.732 µmol/l	660 µmol/l	3.87 µmol/l	660 µmol/l
Pipecolic acid	plasma	0.961 µmol/l	120 µmol/l	0.120 µmol/l	120 µmol/l
	serum	0.680 µmol/l	120 µmol/l	0.619 µmol/l	120 µmol/l
Proline	plasma	3.67 µmol/l	2232 µmol/l	6.89 µmol/l	2232 µmol/l
	serum	5.44 µmol/l	2232 µmol/l	5.39 µmol/l	2232 µmol/l
Saccharopine	plasma	1.89 µmol/l	60.0 µmol/l	2.01 µmol/l	60.0 µmol/l
	serum	0.575 µmol/l	60.0 µmol/l	0.916 µmol/l	60.0 µmol/l
Sarcosine	plasma	1.98 µmol/l	120 µmol/l	1.33 µmol/l	120 µmol/l
	serum	1.27 µmol/l	120 µmol/l	1.33 µmol/l	120 µmol/l
Serine	plasma	14.1 µmol/l	2373 µmol/l	28.5 µmol/l	2373 µmol/l
	serum	4.90 µmol/l	2373 µmol/l	31.8 µmol/l	2373 µmol/l
Taurine	plasma	0.983 µmol/l	1650 µmol/l	4.09 µmol/l	1650 µmol/l
	serum	1.16 µmol/l	1650 µmol/l	4.63 µmol/l	1650 µmol/l
Threonine	plasma	2.05 µmol/l	2018 µmol/l	6.24 µmol/l	2018 µmol/l
	serum	5.08 µmol/l	2018 µmol/l	4.97 µmol/l	2018 µmol/l
Tryptophan	plasma	1.77 µmol/l	840 µmol/l	5.03 µmol/l	840 µmol/l
	serum	0.976 µmol/l	840 µmol/l	3.95 µmol/l	840 µmol/l
Tyrosine	plasma	2.81 µmol/l	1400 µmol/l	3.05 µmol/l	1400 µmol/l
	serum	4.27 µmol/l	1400 µmol/l	4.55 µmol/l	1400 µmol/l
Valine	plasma	20.3 µmol/l	2028 µmol/l	18.7 µmol/l	2028 µmol/l
	serum	14.1 µmol/l	2028 µmol/l	24.8 µmol/l	2028 µmol/l

Table 26: Limit of quantitation and linearity – PKU/MSUD panel, determination with SCIEX 4500MD™ mass spectrometer

Substance	Matrix	LLOQ	Linear range up to at least
Allo-isoleucine	plasma	0.301 µmol/l	500 µmol/l
	serum	0.465 µmol/l	500 µmol/
Isoleucine	plasma	6.06 µmol/l	1000 µmol/l
	serum	10.9 µmol/l	1000 µmol/
Leucine	plasma	5.26 µmol/l	2000 µmol/l
	serum	5.35 µmol/l	2000 µmol/l l
Methionine	plasma	0.958 µmol/l	420 µmol/l
	serum	1.50 µmol/l	420 µmol/l
Phenylalanine	plasma	4.70 µmol/l	1400 µmol/l
	serum	5.83 µmol/l	1400 µmol/

Substance	Matrix	LLOQ	Linear range up to at least
Tyrosine	plasma	5.78 µmol/l	1400 µmol/l
	serum	8.41 µmol/l	1400 µmol/l
Valine	plasma	18.4 µmol/l	2028 µmol/l
	serum	10.9 µmol/l	2028 µmol/l

Table 27: Limit of quantitation and linearity – PKU/MSUD panel, determination with Waters® Xevo™ TQ-S micro mass spectrometer

Substance	Matrix	LLOQ	Linear range up to at least
Allo-isoleucine	plasma	1.02 µmol/l	566 µmol/l
	serum	0.347 µmol/l	566 µmol/
Isoleucine	plasma	5.04 µmol/l	1192 µmol/l
	serum	4.95 µmol/l	1180 µmol/
Leucine	plasma	6.92 µmol/l	2369 µmol/l
	serum	5.65 µmol/l	2352 µmol/l l
Methionine	plasma	1.11 µmol/l	494 µmol/l
	serum	0.903 µmol/l	491 µmol/l
Phenylalanine	plasma	4.01 µmol/l	1641 µmol/l
	serum	5.40 µmol/l	1650 µmol/
Tyrosine	plasma	6.17 µmol/l	1661 µmol/l
	serum	6.09 µmol/l	1656 µmol/l
Valine	plasma	13.7 µmol/l	2493 µmol/l
	serum	20.7 µmol/l	2425 µmol/l

Intra-assay precision:
The coefficients of variation were determined on three different concentrations using the plasma control levels I to III (order no. 0471, 0472, 0473) by repeated preparation (n = 10) of the same plasma sample in one sequence.

Table 28: Intra-assay precision, Full Panel, determination with SCIEX 4500MD™ mass spectrometer

Substance	Coefficient of variation (concentration of substance)		
1-Methylhistidine	8.7 % (2.26 µmol/l)	8.9 % (9.39 µmol/l)	7.5 % (13.4 µmol/l)
α-Aminobutyric acid	2.6 % (7.99 µmol/l)	3.2 % (41.9 µmol/l)	2.0 % (81.4 µmol/l)
β-Aminoisobutyric acid	2.6 % (4.51 µmol/l)	5.5 % (20.5 µmol/l)	4.5 % (36.1 µmol/l)
3-Methylhistidine	8.7 % (6.70 µmol/l)	3.0 % (35.1 µmol/l)	3.7 % (69.7 µmol/l)
4-Hydroxyproline	2.6 % (9.21 µmol/l)	3.1 % (83.5 µmol/l)	2.9 % (160 µmol/l)
Acetyltyrosine	8.8 % (4.96 µmol/l)	4.7 % (20.5 µmol/l)	6.3 % (35.4 µmol/l)
Adenosylhomocysteine	5.2 % (4.81 µmol/l)	4.8 % (18.7 µmol/l)	4.1 % (35.5 µmol/l)

Substance	Coefficient of variation (concentration of substance)		
Alanine	3.1 % (226 µmol/l)	3.0 % (579 µmol/l)	2.6 % (890 µmol/l)
Allo-isoleucine	4.7 % (5.09 µmol/l)	4.6 % (44.6 µmol/l)	4.9 % (91.2 µmol/l)
α-Aminoadipic acid	3.8 % (5.48 µmol/l)	4.5 % (9.93 µmol/l)	4.3 % (15.6 µmol/l)
Anserine	5.3 % (5.08 µmol/l)	9.5 % (10.5 µmol/l)	7.4 % (15.6 µmol/l)
Arginine	5.2 % (11.5 µmol/l)	4.1 % (92.9 µmol/l)	3.4 % (201 µmol/l)
Argininosuccinic acid	3.9 % (3.13 µmol/l)	5.4 % (19.4 µmol/l)	5.2 % (37.5 µmol/l)
Asparagine	2.1 % (40.7 µmol/l)	4.1 % (112 µmol/l)	4.7 % (205 µmol/l)
Aspartic acid	2.5 % (43.2 µmol/l)	1.9 % (89.1 µmol/l)	2.0 % (192 µmol/l)
β-Alanine	5.5 % (9.68 µmol/l)	5.0 % (44.9 µmol/l)	5.9 % (80.2 µmol/l)
Carnosine	5.6 % (12.7 µmol/l)	5.3 % (27.6 µmol/l)	7.4 % (43.3 µmol/l)
Citrulline	2.4 % (13.3 µmol/l)	2.7 % (46.7 µmol/l)	3.0 % (75.5 µmol/l)
Cystathionine	5.4 % (4.84 µmol/l)	4.9 % (20.0 µmol/l)	5.6 % (35.6 µmol/l)
Cysteine sulphate	2.4 % (13.7 µmol/l)	2.7 % (21.6 µmol/l)	2.1 % (41.5 µmol/l)
Cystine	3.0 % (7.01 µmol/l)	4.8 % (65.2 µmol/l)	4.6 % (130 µmol/l)
Ethanolamine	12.6 % (11.4 µmol/l)	3.0 % (114 µmol/l)	3.6 % (215 µmol/l)
γ-Aminobutyric acid	6.0 % (4.93 µmol/l)	4.1 % (9.06 µmol/l)	4.7 % (14.2 µmol/l)
Glutamine	4.6 % (303 µmol/l)	8.8 % (555 µmol/l)	3.0 % (1231 µmol/l)
Glutamic acid	3.6 % (119 µmol/l)	3.3 % (370 µmol/l)	2.8 % (564 µmol/l)
Glycine	2.3 % (251 µmol/l)	2.9 % (612 µmol/l)	2.6 % (1009 µmol/l)
Histidine	3.0 % (47.1 µmol/l)	5.6 % (85.6 µmol/l)	2.6 % (172 µmol/l)
Homocitrulline	6.3 % (8.59 µmol/l)	3.4 % (18.4 µmol/l)	4.2 % (27.6 µmol/l)
Homocystine	7.0 % (5.87 µmol/l)	5.4 % (16.1 µmol/l)	4.4 % (33.1 µmol/l)
Hydroxylysine	4.6 % (4.51 µmol/l)	3.9 % (15.6 µmol/l)	2.9 % (26.5 µmol/l)
Isoleucine	2.8 % (42.9 µmol/l)	3.9 % (53.3 µmol/l)	3.0 % (154 µmol/l)
Leucine	2.8 % (86.2 µmol/l)	3.2 % (209 µmol/l)	2.8 % (341 µmol/l)
Lysine	4.9 % (54.8 µmol/l)	2.9 % (272 µmol/l)	3.7 % (670 µmol/l)
Methionine	5.2 % (12.3 µmol/l)	3.6 % (49.3 µmol/l)	4.1 % (93.6 µmol/l)
Ornithine	3.5 % (20.7 µmol/l)	5.8 % (75.5 µmol/l)	3.4 % (136 µmol/l)
Phenylalanine	4.5 % (51.3 µmol/l)	2.5 % (93.8 µmol/l)	3.2 % (214 µmol/l)
Phosphoethanolamine	3.5 % (5.54 µmol/l)	4.4 % (59.8 µmol/l)	4.8 % (109 µmol/l)
Phosphoserine	6.3 % (6.25 µmol/l)	3.6 % (77.9 µmol/l)	3.1 % (154 µmol/l)
Pipecolic acid	3.6 % (4.58 µmol/l)	3.3 % (14.4 µmol/l)	2.5 % (26.2 µmol/l)
Proline	3.5 % (93.3 µmol/l)	2.5 % (268 µmol/l)	2.5 % (492 µmol/l)
Saccharopine	7.3 % (5.21 µmol/l)	5.5 % (7.71 µmol/l)	6.3 % (5.96 µmol/l)
Sarcosine	2.6 % (4.79 µmol/l)	3.1 % (16.7 µmol/l)	2.8 % (27.6 µmol/l)
Serine	4.4 % (118 µmol/l)	2.4 % (296 µmol/l)	2.1 % (700 µmol/l)
Taurine	4.9 % (21.8 µmol/l)	4.4 % (200 µmol/l)	3.3 % (380 µmol/l)
Threonine	2.5 % (66.6 µmol/l)	2.9 % (226 µmol/l)	2.3 % (448 µmol/l)
Tryptophan	5.8 % (20.0 µmol/l)	4.7 % (101 µmol/l)	3.9 % (186 µmol/l)
Tyrosine	2.6 % (40.4 µmol/l)	2.2 % (119 µmol/l)	2.1 % (211 µmol/l)

Substance	Coefficient of variation (concentration of substance)		
Valine	3.5 % (120 µmol/l)	3.2 % (126 µmol/l)	2.6 % (391 µmol/l)

Table 29: Intra-assay precision, Full Panel, determination with Waters® Xevo™ TQ-S micro mass spectrometer

Substance	Coefficient of variation (concentration of substance)		
1-Methylhistidine	4.7 % (2.67 µmol/l)	2.3 % (10.1 µmol/l)	5.3 % (14.4 µmol/l)
α-Aminobutyric acid	2.6 % (8.71 µmol/l)	1.9 % (45.3 µmol/l)	1.3 % (84.2 µmol/l)
β-Aminoisobutyric acid	3.3 % (4.81 µmol/l)	3.0 % (22.0 µmol/l)	1.0 % (38.0 µmol/l)
3-Methylhistidine	6.7 % (7.39 µmol/l)	3.5 % (39.9 µmol/l)	2.0 % (77.1 µmol/l)
4-Hydroxyproline	1.8 % (10.3 µmol/l)	2.0 % (86.2 µmol/l)	1.8 % (163 µmol/l)
Acetyltyrosine	8.0 % (4.73 µmol/l)	6.6 % (19.8 µmol/l)	7.0 % (35.8 µmol/l)
Adenosylhomocysteine	3.4 % (5.60 µmol/l)	2.8 % (16.9 µmol/l)	2.8 % (42.2 µmol/l)
Alanine	2.9 % (234 µmol/l)	3.3 % (621 µmol/l)	1.9 % (946 µmol/l)
Allo-isoleucine	7.0 % (5.13 µmol/l)	2.4 % (50.0 µmol/l)	2.5 % (95.6 µmol/l)
α-Aminoadipic acid	3.9 % (5.87 µmol/l)	2.5 % (10.6 µmol/l)	1.8 % (16.7 µmol/l)
Anserine	12.0 % (5.37 µmol/l)	10.8 % (10.5 µmol/l)	7.7 % (16.4 µmol/l)
Arginine	4.6 % (12.4 µmol/l)	4.3 % (99.7 µmol/l)	2.9 % (213 µmol/l)
Argininosuccinic acid	11.0 % (3.43 µmol/l)	5.8 % (22.1 µmol/l)	7.3 % (42.0 µmol/l)
Asparagine	2.2 % (41.9 µmol/l)	2.9 % (117 µmol/l)	2.0 % (213 µmol/l)
Aspartic acid	2.8 % (44.6 µmol/l)	3.4 % (94.2 µmol/l)	3.1 % (198 µmol/l)
β-Alanine	6.4 % (10.4 µmol/l)	4.3 % (50.8 µmol/l)	3.2 % (84.0 µmol/l)
Carnosine	8.3 % (13.9 µmol/l)	8.3 % (31.6 µmol/l)	7.4 % (46.8 µmol/l)
Citrulline	4.4 % (14.2 µmol/l)	4.0 % (51.3 µmol/l)	4.5 % (83.2 µmol/l)
Cystathionine	6.1 % (5.24 µmol/l)	4.4 % (22.4 µmol/l)	5.3 % (40.9 µmol/l)
Cysteine sulphate	4.6 % (14.6 µmol/l)	3.5 % (22.6 µmol/l)	3.5 % (43.3 µmol/l)
Cystine	6.1 % (7.83 µmol/l)	3.6 % (59.8 µmol/l)	3.2 % (133 µmol/l)
Ethanolamine	not determinable (<20 µmol/l)	13.6 % (122 µmol/l)	3.5 % (231 µmol/l)
γ-Aminobutyric acid	6.7 % (4.98 µmol/l)	3.6 % (9.63 µmol/l)	4.2 % (14.5 µmol/l)
Glutamine	2.3 % (303 µmol/l)	6.3 % (545 µmol/l)	2.8 % (1225 µmol/l)
Glutamic acid	3.8 % (121 µmol/l)	2.7 % (407 µmol/l)	2.6 % (618 µmol/l)
Glycine	3.3 % (266 µmol/l)	2.3 % (659 µmol/l)	2.3 % (1087 µmol/l)
Histidine	2.8 % (50.3 µmol/l)	3.6 % (92.9 µmol/l)	2.3 % (189 µmol/l)
Homocitrulline	2.9 % (9.67 µmol/l)	3.8 % (19.4 µmol/l)	3.6 % (28.9 µmol/l)
Homocystine	4.5 % (6.42 µmol/l)	5.4 % (8.48 µmol/l)	4.6 % (33.4 µmol/l)
Hydroxylysine	3.4 % (4.65 µmol/l)	4.2 % (16.0 µmol/l)	2.9 % (26.8 µmol/l)
Isoleucine	2.8 % (45.7 µmol/l)	2.3 % (58.3 µmol/l)	1.5 % (165 µmol/l)
Leucine	2.2 % (90.8 µmol/l)	3.2 % (222 µmol/l)	3.0 % (364 µmol/l)
Lysine	4.7 % (54.6 µmol/l)	2.0 % (285 µmol/l)	2.4 % (701 µmol/l)
Methionine	2.7 % (12.7 µmol/l)	1.5 % (52.9 µmol/l)	1.9 % (98.7 µmol/l)

Substance	Coefficient of variation (concentration of substance)		
Ornithine	4.9 % (20.2 µmol/l)	4.0 % (84.4 µmol/l)	1.6 % (151 µmol/l)
Phenylalanine	4.8 % (52.2 µmol/l)	3.1 % (95.1 µmol/l)	4.6 % (215 µmol/l)
Phosphoethanolamine	3.7 % (5.93 µmol/l)	3.7 % (63.4 µmol/l)	2.1 % (114 µmol/l)
Phosphoserine	9.0 % (6.61 µmol/l)	2.5 % (86.5 µmol/l)	2.2 % (168 µmol/l)
Pipecolic acid	2.5 % (4.90 µmol/l)	2.1 % (15.8 µmol/l)	1.5 % (27.6 µmol/l)
Proline	1.8 % (98.7 µmol/l)	2.2 % (281 µmol/l)	1.2 % (505 µmol/l)
Saccharopine	8.1 % (6.04 µmol/l)	8.8 % (8.46 µmol/l)	14.2 % (6.67 µmol/l)
Sarcosine	3.4 % (5.14 µmol/l)	2.5 % (17.7 µmol/l)	2.2 % (29.4 µmol/l)
Serine	3.9 % (126 µmol/l)	2.8 % (320 µmol/l)	0.8 % (746 µmol/l)
Taurine	3.1 % (22.9 µmol/l)	2.2 % (208 µmol/l)	1.8 % (398 µmol/l)
Threonine	1.4 % (71.1 µmol/l)	1.9 % (246 µmol/l)	1.2 % (483 µmol/l)
Tryptophan	3.6 % (21.0 µmol/l)	5.4 % (104 µmol/l)	7.3 % (195 µmol/l)
Tyrosine	2.7 % (41.7 µmol/l)	2.9 % (128 µmol/l)	4.7 % (225 µmol/l)
Valine	2.0 % (121 µmol/l)	2.3 % (151 µmol/l)	1.0 % (449 µmol/l)

Table 30: Intra-assay precision, PKU/MSUD Panel, determination with SCIEX 4500MD™mass spectrometer

Substance	Coefficient of variation (concentration of substance)		
Allo-isoleucine	6.0 % (4.70 µmol/l)	4.9 % (43.7 µmol/l)	1.9 % (89.6 µmol/l)
Isoleucine	2.5 % (43.7 µmol/l)	2.3 % (55.0 µmol/l)	3.4 % (162 µmol/l)
Leucine	1.7 % (86.7 µmol/l)	2.4 % (210 µmol/l)	1.5 % (349 µmol/l)
Methionine	2.9 % (11.9 µmol/l)	5.5 % (50.5 µmol/l)	1.2 % (97.0 µmol/l)
Phenylalanine	4.6 % (50.4 µmol/l)	3.6 % (90.8 µmol/l)	2.7 % (208 µmol/l)
Tyrosine	5.4 % (39.5 µmol/l)	3.5 % (118 µmol/l)	3.1 % (207 µmol/l)
Valine	3.8 % (111 µmol/l)	5.2 % (138 µmol/l)	1.6 % (425 µmol/l)

Inter-assay precision:
Determination of the inter-assay precision was done on three different concentrations using the plasma control levels I to III (order no. 0471, 0472, 0473) by repeated preparation (n = 5) of the same plasma sample on 20 different days.

Table 31: Inter-assay precision, Full Panel, determination with SCIEX 4500MD™mass spectrometer

Substance	Coefficient of variation (concentration of substance)		
1-Methylhistidine	7.2 % (2.65 µmol/l)	5.3 % (10.4 µmol/l)	5.4 % (14.4 µmol/l)
α-Aminobutyric acid	4.2 % (8.64 µmol/l)	4.7 % (47.4 µmol/l)	4.9 % (87.6 µmol/l)
β-Aminoisobutyric acid	5.7 % (5.06 µmol/l)	5.0 % (22.2 µmol/l)	5.5 % (37.8 µmol/l)
3-Methylhistidine	9.7 % (7.51 µmol/l)	7.1 % (40.5 µmol/l)	7.8 % (79.5 µmol/l)
4-Hydroxyproline	5.6 % (10.1 µmol/l)	5.1 % (88.4 µmol/l)	5.3 % (165 µmol/l)
Acetyltyrosine	5.6 % (4.95 µmol/l)	5.2 % (21.3 µmol/l)	5.5 % (36.8 µmol/l)
Adenosylhomocysteine	10.2 % (5.35 µmol/l)	7.4 % (20.7 µmol/l)	7.8 % (38.8 µmol/l)

Substance	Coefficient of variation (concentration of substance)		
Alanine	6.1 % (233 µmol/l)	4.7 % (613 µmol/l)	5.6 % (934 µmol/l)
Allo-isoleucine	5.2 % (5.26 µmol/l)	5.7 % (48.4 µmol/l)	5.4 % (96.2 µmol/l)
α-Aminoadipic acid	4.8 % (5.97 µmol/l)	5.1 % (10.7 µmol/l)	5.0 % (16.6 µmol/l)
Anserine	5.7 % (5.55 µmol/l)	6.3 % (10.6 µmol/l)	6.8 % (15.8 µmol/l)
Arginine	5.6 % (12.2 µmol/l)	6.7 % (101 µmol/l)	6.2 % (212 µmol/l)
Argininosuccinic acid	8.6 % (3.38 µmol/l)	7.7 % (22.5 µmol/l)	9.4 % (42.6 µmol/l)
Asparagine	4.5 % (42.6 µmol/l)	4.6 % (119 µmol/l)	5.1 % (211 µmol/l)
Aspartic acid	5.2 % (44.5 µmol/l)	5.4 % (96.2 µmol/l)	5.3 % (202 µmol/l)
β-Alanine	7.9 % (9.77 µmol/l)	6.0 % (50.9 µmol/l)	6.3 % (88.7 µmol/l)
Carnosine	5.8 % (13.5 µmol/l)	5.3 % (31.9 µmol/l)	5.6 % (51.1 µmol/l)
Citrulline	5.2 % (14.4 µmol/l)	4.8 % (51.7 µmol/l)	5.4 % (82.6 µmol/l)
Cystathionine	6.0 % (5.25 µmol/l)	6.7 % (21.8 µmol/l)	6.5 % (38.5 µmol/l)
Cysteine sulphate	5.4 % (14.8 µmol/l)	5.7 % (23.4 µmol/l)	5.6 % (44.0 µmol/l)
Cystine	5.4 % (7.64 µmol/l)	6.0 % (70.8 µmol/l)	5.8 % (141 µmol/l)
Ethanolamine	9.6 % (13.6 µmol/l)	5.9 % (131 µmol/l)	6.0 % (238 µmol/l)
γ-Aminobutyric acid	5.7 % (4.87 µmol/l)	6.0 % (9.43 µmol/l)	6.1 % (14.1 µmol/l)
Glutamine	4.6 % (312 µmol/l)	7.6 % (572 µmol/l)	6.1 % (1260 µmol/l)
Glutamic acid	5.6 % (121 µmol/l)	4.2 % (400 µmol/l)	5.0 % (603 µmol/l)
Glycine	4.6 % (266 µmol/l)	4.5 % (663 µmol/l)	5.3 % (1075 µmol/l)
Histidine	6.4 % (50.3 µmol/l)	7.7 % (93.1 µmol/l)	7.6 % (191 µmol/l)
Homocitrulline	5.4 % (9.47 µmol/l)	5.2 % (19.6 µmol/l)	5.1 % (29.1 µmol/l)
Homocystine	7.6 % (6.18 µmol/l)	9.9 % (17.6 µmol/l)	6.9 % (34.7 µmol/l)
Hydroxylysine	6.6 % (4.63 µmol/l)	6.6 % (16.6 µmol/l)	5.8 % (27.7 µmol/l)
Isoleucine	5.4 % (46.2 µmol/l)	5.7 % (58.8 µmol/l)	5.2 % (169 µmol/l)
Leucine	4.4 % (92.8 µmol/l)	4.8 % (227 µmol/l)	5.4 % (370 µmol/l)
Lysine	4.5 % (57.0 µmol/l)	5.4 % (298 µmol/l)	5.2 % (712 µmol/l)
Methionine	4.0 % (12.6 µmol/l)	4.5 % (55.0 µmol/l)	5.3 % (103 µmol/l)
Ornithine	7.7 % (20.8 µmol/l)	4.7 % (85.5 µmol/l)	5.1 % (154 µmol/l)
Phenylalanine	4.9 % (54.1 µmol/l)	4.9 % (99.3 µmol/l)	4.9 % (225 µmol/l)
Phosphoethanolamine	8.2 % (5.82 µmol/l)	6.3 % (64.8 µmol/l)	6.9 % (115 µmol/l)
Phosphoserine	6.1 % (6.36 µmol/l)	5.4 % (85.9 µmol/l)	5.5 % (166 µmol/l)
Pipecolic acid	5.7 % (4.84 µmol/l)	5.7 % (15.8 µmol/l)	6.3 % (27.5 µmol/l)
Proline	5.5 % (101 µmol/l)	6.2 % (281 µmol/l)	5.6 % (500 µmol/l)
Saccharopine	6.6 % (5.41 µmol/l)	6.5 % (8.35 µmol/l)	8.1 % (6.29 µmol/l)
Sarcosine	7.3 % (4.75 µmol/l)	5.2 % (17.7 µmol/l)	6.0 % (29.3 µmol/l)
Serine	5.5 % (128 µmol/l)	4.6 % (322 µmol/l)	5.1 % (736 µmol/l)
Taurine	5.1 % (22.6 µmol/l)	4.6 % (210 µmol/l)	4.8 % (400 µmol/l)
Threonine	3.8 % (71.2 µmol/l)	4.7 % (249 µmol/l)	5.0 % (489 µmol/l)
Tryptophan	4.3 % (20.7 µmol/l)	5.0 % (106 µmol/l)	5.0 % (198 µmol/l)
Tyrosine	5.1 % (41.9 µmol/l)	6.1 % (127 µmol/l)	5.9 % (220 µmol/l)

Substance	Coefficient of variation (concentration of substance)		
Valine	6.1 % (122 µmol/l)	6.4 % (150 µmol/l)	5.5 % (449 µmol/l)

Table 32: Inter-assay precision, Full Panel, determination with Waters® Xevo™ TQ-S micro mass spectrometer

Substance	Coefficient of variation (concentration of substance)		
1-Methylhistidine	7.1 % (2.67 µmol/l)	6.9 % (10.3 µmol/l)	7.5 % (14.2 µmol/l)
α-Aminobutyric acid	4.7 % (8.75 µmol/l)	4.4 % (46.6 µmol/l)	4.5 % (87.6 µmol/l)
β-Aminoisobutyric acid	5.4 % (4.89 µmol/l)	3.9 % (22.4 µmol/l)	4.6 % (38.2 µmol/l)
3-Methylhistidine	8.3 % (7.51 µmol/l)	5.2 % (40.6 µmol/l)	5.6 % (78.9 µmol/l)
4-Hydroxyproline	5.3 % (10.1 µmol/l)	4.1 % (88.0 µmol/l)	4.4 % (165 µmol/l)
Acetyltyrosine	10.5 % (4.95 µmol/l)	10.0 % (21.6 µmol/l)	10.3 % (37.2 µmol/l)
Adenosylhomocysteine	10.7 % (5.44 µmol/l)	9.5 % (20.7 µmol/l)	9.3 % (39.5 µmol/l)
Alanine	4.6 % (236 µmol/l)	4.0 % (620 µmol/l)	4.4 % (939 µmol/l)
Allo-isoleucine	5.5 % (5.11 µmol/l)	4.9 % (49.2 µmol/l)	5.0 % (94.8 µmol/l)
α-Aminoadipic acid	4.2 % (6.03 µmol/l)	3.7 % (10.7 µmol/l)	4.3 % (16.7 µmol/l)
Anserine	9.6 % (5.54 µmol/l)	11.9 % (10.8 µmol/l)	11.3 % (15.9 µmol/l)
Arginine	7.6 % (12.3 µmol/l)	5.6 % (104 µmol/l)	5.4 % (216 µmol/l)
Argininosuccinic acid	7.8 % (3.45 µmol/l)	10.6 % (22.4 µmol/l)	10.6 % (42.7 µmol/l)
Asparagine	4.3 % (42.9 µmol/l)	4.0 % (119 µmol/l)	4.4 % (215 µmol/l)
Aspartic acid	6.9 % (44.2 µmol/l)	4.6 % (96.8 µmol/l)	4.7 % (204 µmol/l)
β-Alanine	9.4 % (9.89 µmol/l)	5.6 % (50.1 µmol/l)	6.1 % (87.7 µmol/l)
Carnosine	7.9 % (13.6 µmol/l)	8.3 % (31.5 µmol/l)	9.4 % (50.9 µmol/l)
Citrulline	6.1 % (14.6 µmol/l)	4.8 % (51.2 µmol/l)	5.1 % (82.3 µmol/l)
Cystathionine	6.2 % (5.33 µmol/l)	6.0 % (22.1 µmol/l)	6.8 % (39.0 µmol/l)
Cysteine sulphate	5.5 % (14.9 µmol/l)	5.3 % (23.2 µmol/l)	5.1 % (44.3 µmol/l)
Cystine	5.3 % (7.80 µmol/l)	5.3 % (69.7 µmol/l)	5.2 % (139 µmol/l)
Ethanolamine	not determinable (<20 µmol/l)	6.8 % (132 µmol/l)	6.7 % (239 µmol/l)
γ-Aminobutyric acid	9.2 % (4.90 µmol/l)	8.0 % (9.71 µmol/l)	8.3 % (14.4 µmol/l)
Glutamine	4.9 % (316 µmol/l)	8.0 % (579 µmol/l)	5.5 % (1282 µmol/l)
Glutamic acid	4.8 % (123 µmol/l)	3.8 % (405 µmol/l)	4.0 % (607 µmol/l)
Glycine	4.2 % (269 µmol/l)	3.9 % (669 µmol/l)	4.4 % (1080 µmol/l)
Histidine	5.4 % (51.3 µmol/l)	5.8 % (92.9 µmol/l)	5.8 % (186 µmol/l)
Homocitrulline	6.2 % (9.68 µmol/l)	4.1 % (19.5 µmol/l)	4.3 % (28.8 µmol/l)
Homocystine	6.5 % (6.36 µmol/l)	15.0 % (17.2 µmol/l)	7.3 % (35.2 µmol/l)
Hydroxylysine	6.3 % (4.72 µmol/l)	5.9 % (16.6 µmol/l)	6.3 % (27.8 µmol/l)
Isoleucine	5.9 % (46.8 µmol/l)	4.6 % (59.2 µmol/l)	4.5 % (168 µmol/l)
Leucine	4.3 % (93.2 µmol/l)	3.9 % (226 µmol/l)	4.4 % (371 µmol/l)
Lysine	7.2 % (57.7 µmol/l)	7.0 % (297 µmol/l)	7.1 % (721 µmol/l)
Methionine	4.0 % (13.0 µmol/l)	3.9 % (54.2 µmol/l)	4.0 % (102 µmol/l)

Substance	Coefficient of variation (concentration of substance)		
Ornithine	7.3 % (20.5 µmol/l)	4.9 % (84.9 µmol/l)	4.6 % (153 µmol/l)
Phenylalanine	5.8 % (54.3 µmol/l)	5.4 % (99.6 µmol/l)	5.0 % (224 µmol/l)
Phosphoethanolamine	5.1 % (5.97 µmol/l)	4.5 % (64.6 µmol/l)	5.1 % (113 µmol/l)
Phosphoserine	6.0 % (6.48 µmol/l)	5.5 % (86.5 µmol/l)	5.7 % (166 µmol/l)
Pipecolic acid	4.0 % (4.95 µmol/l)	3.8 % (15.7 µmol/l)	4.3 % (27.6 µmol/l)
Proline	4.5 % (100 µmol/l)	4.0 % (288 µmol/l)	4.5 % (518 µmol/l)
Saccharopine	10.2 % (5.47 µmol/l)	12.5 % (8.53 µmol/l)	12.4 % (6.40 µmol/l)
Sarcosine	5.4 % (5.09 µmol/l)	4.3 % (18.0 µmol/l)	4.3 % (29.4 µmol/l)
Serine	5.0 % (128 µmol/l)	4.2 % (320 µmol/l)	4.6 % (742 µmol/l)
Taurine	4.7 % (23.0 µmol/l)	4.6 % (211 µmol/l)	4.6 % (400 µmol/l)
Threonine	3.1 % (71.7 µmol/l)	3.9 % (246 µmol/l)	4.6 % (486 µmol/l)
Tryptophan	7.4 % (21.1 µmol/l)	5.8 % (106 µmol/l)	6.2 % (197 µmol/l)
Tyrosine	5.5 % (43.1 µmol/l)	4.7 % (127 µmol/l)	4.6 % (217 µmol/l)
Valine	4.8 % (121 µmol/l)	3.9 % (151 µmol/l)	4.5 % (456 µmol/l)

Table 33: Inter-assay precision, PKU/MSUD Panel, determination with SCIEX 4500MD™ mass spectrometer

Substance	Coefficient of variation (concentration of substance)		
Allo-isoleucine	6.2 % (5.11 µmol/l)	6.0 % (49.3 µmol/l)	5.7 % (97.1 µmol/l)
Isoleucine	4.4 % (45.5 µmol/l)	5.6 % (55.8 µmol/l)	6.5 % (167 µmol/l)
Leucine	4.1 % (92.1 µmol/l)	4.4 % (225 µmol/l)	4.7 % (367 µmol/l)
Methionine	4.8 % (12.9 µmol/l)	5.1 % (54.4 µmol/l)	6.1 % (102 µmol/l)
Phenylalanine	5.1 % (53.9 µmol/l)	5.2 % (100 µmol/l)	6.2 % (226 µmol/l)
Tyrosine	5.9 % (42.1 µmol/l)	5.8 % (124 µmol/l)	6.5 % (215 µmol/l)
Valine	4.9 % (123 µmol/l)	5.0 % (152 µmol/l)	5.0 % (455 µmol/l)

These data have been established in our laboratory solely in order to verify the performance of the reagent kit and to fulfil regulatory requirements. We particularly emphasize that these data are not suitable to compare the measurement systems used, nor to make any statement concerning their general performance.

Drift

To identify any drift of analyte concentration over time the concentration of all analytes in the three controls was compared over a 20-day period. No drift was observed for any analytes.

Carry-over

A plasma sample with analyte concentrations in the range of the upper limit of quantification was measured followed immediately by measurement of a blank sample. The resulting concentrations were compared with each other on a percentage basis. Review of the data obtained for all analytes, except the analytes specified below, showed no carry-over effects with the measured concentration of the blank sample below the limit of quantitation.

Table 34: Carry-over rates

Substance	Carry-Over rate		
	Shimadzu LC-20A Prominence HPLC system in combination with Sciex 4500™	Agilent 1260 HPLC system in combination with Sciex 4500MD™	ACQUITY™ UPLC® H-Class UHPLC system in combination with with Waters® Xevo™ TQ-S micro
Argininosuccinic acid	< LLOQ	< LLOQ	0.29%
Cystine	< LLOQ	< LLOQ	0.11%
Phospho-ethanolamine	< LLOQ	< LLOQ	0.27%
Phosphoserine	1.22%	0.52%	1.90%

Stability of patient samples:
The stability of endogenous (not spiked) analytes was investigated by storing plasma / serum samples from 10 donors and testing them at defined intervals. The following stabilities were determined:

Table 35: Stabilities of endogenous analytes in EDTA plasma, heparin plasma and serum

Substance	Stability at +2 to +8 °C			Stability below –18 °C		
	EDTA	Heparin	Serum	EDTA	Heparin	Serum
1-Methylhistidine	unstable	unstable	14 days	unstable	1 day	1 day
α-Aminobutyric acid	14 days	14 days	28 days	28 days	28 days	28 days
3-Methylhistidine	28 days	unstable	unstable	28 days	unstable	unstable
4-Hydroxyproline	14 days	14 days	28 days	28 days	28 days	28 days
Alanine	14 days	7 days	7 days	28 days	unstable	1 day
Arginine	14 days	7 days	5 days	28 days	1 day	28 days
Asparagine	5 days	14 days	28 days	28 days	28 days	28 days
Aspartic acid	< LLOQ	< LLOQ	unstable	< LLOQ	< LLOQ	unstable
β-Alanine	5 days	unstable	unstable	28 days	unstable	unstable
Citrulline	7 days	14 days	1 day	28 days	28 days	1 day
Cystine	unstable	unstable	unstable	unstable	unstable	unstable
Glutamine	28 days	14 days	14 days	28 days	28 days	28 days
Glutamic acid	unstable	unstable	unstable	unstable	unstable	28 days
Glycine	7 days	5 days	1 day	28 days	28 days	28 days
Histidine	28 days	1 day	1 day	28 days	28 days	28 days
Isoleucine	14 days	7 days	7 days	28 days	28 days	28 days
Leucine	28 days	14 days	5 days	28 days	28 days	28 days
Lysine	5 days	5 days	5 days	28 days	28 days	28 days
Methionine	14 days	14 days	14 days	28 days	28 days	28 days
Ornithine	1 days	7 days	1 day	28 days	28 days	28 days

Substance	Stability at +2 to +8 °C			Stability below –18 °C		
	EDTA	Heparin	Serum	EDTA	Heparin	Serum
Phenylalanine	28 days	14 days	1 day	28 days	28 days	28 days
Proline	28 days	14 days	7 days	28 days	28 days	28 days
Serine	14 days	17 days	unstable	28 days	1 day	28 days
Taurine	28 days	28 days	28 days	28 days	28 days	28 days
Threonine	28 days	14 days	28 days	28 days	28 days	28 days
Tryptophan	28 days	14 days	14 days	28 days	28 days	28 days
Tyrosine	28 days	14 days	7 days	28 days	28 days	28 days
Valine	28 days	14 days	28 days	28 days	28 days	28 days

Amino acids present only in individuals with a metabolic disorder were investigated by spiking these analytes in EDTA plasma and serum and measuring the samples at defined intervals. The following stabilities were determined:

Table 36: Stabilities of spiked analytes in EDTA plasma and serum

Substance	Stability at +2 to +8 °C		Stability below –18 °C	
	EDTA Plasma	Serum	EDTA Plasma	Serum
β-Aminoisobutyric acid	3 months	3 months	3 months	3 months
Acetyltyrosine	3 months	3 months	3 months	3 months
Adenosylhomocysteine	3 months	3 months	3 months	3 months
Allo-isoleucine	3 months	3 months	3 months	3 months
α-Aminoadipic acid	3 months	3 months	3 months	3 months
Anserine	3 months	indeterminable*	3 months	indeterminable*
Argininosuccinic acid	2 days	2 days	3 months	3 months
Aspartic acid	3 months	3 months	3 months	3 months
Carnosine	3 months	indeterminable*	3 months	indeterminable*
Cystathionine	3 months	3 months	3 months	3 months
Cysteine sulphate	20 hours	20 hours	20 hours	unstable
Ethanolamine	3 months	20 hours	3 months	3 months
γ-Aminobutyric acid	3 months	3 months	3 months	3 months
Homocitrulline	3 months	3 months	3 months	3 months
Homocystine	unstable	unstable	20 hours	unstable
Hydroxylysine	3 months	3 months	3 months	3 months
Phosphoethanolamine	3 months	20 hours	3 months	3 months
Phosphoserine	3 months	unstable	3 months	3 months
Pipecolic acid	3 months	3 months	3 months	3 months
Saccharopine	3 months	3 months	3 months	3 months
Sarcosine	3 months	3 months	3 months	3 months

* Levels not determinable because these substances are broken down immediately by enzymatic activities. Stability may differ significantly in pathological samples because enzyme activity is altered or absent.

Robustness:
The effect of certain modifications in sample preparation and HPLC system setup were reviewed during verification. The method is robust within the following tolerances provided the particular setup remains constant throughout a measurement series:

Table 37: Tolerance ranges for HPLC system

HPLC system	Tolerance range
Injection volume Full Panel	not more than 6 µl
Injection volume PKU/MSUD Panel	not more than 6 µl
Column temperature Full Panel	22–30 °C
Column temperature PKU/MSUD Panel	22–30 °C

Table 38: Tolerance ranges for sample preparation with Deep Well Plate

Sample preparation	Tolerance range
Centrifugation speed	1800–2600 x g
Centrifugation duration	4–6 min
Shaking speed	1100–1200 rpm

Appendix IV: Clinical performance data

Diagnostic sensitivity and specificity:
Plasma samples from 102 healthy individuals and 27 patients suffering from maple sirup urine disease and phenylketonuria were analysed using the reagent kit ***MassChrom***® Amino Acid Analysis in plasma/serum using both the Full Panel and the PKU/MSUD Panel. The results are summarised in the following tables:

Table 39: Results for the Full Panel

	Healthy individuals	Diseased patients	Total
Test negative	85	0	85
Test positive	17	27	44
Total	102	27	129

Table 40: Results for the PKU/MSUD Panel

	Healthy individuals	Diseased patients	Total
Test negative	86	0	86
Test positive	16	27	43
Total	102	27	129

Table 41: Overall diagnostic sensitivity and specificity results

	Full Panel	PKU/MSUD Panel
Diagnostic sensitivity	100%	100%
Diagnostic specificity	83.3%	84.3%

Appendix V: Medical conditions

This table features amino acid metabolism disorders and associated changes in the amino acid profile, and measures recommended in the literature for reliable diagnosis. This table does not claim to be complete.

Table 42: Amino acid metabolism disorders

Disorder	Markers used in plasma amino acid analysis [15]	Measures to increase diagnostic reliability
Maple syrup urine disease (MSUD)	↑: Val ↑↑: Leu, Ile, Allo-Ile (detection is diagnostic)	– Calculate ratios[18]: (Leu+Ile)/Phe, (Leu+Ile)/Ala, (Leu+Ile)/Tyr, Val/Phe) – Conduct additional tests [19]: Urine organic acid test, fibroblast enzyme activity test, gene mutation analysis of the various enzyme subunits
Antiquitin deficiency and pyridoxine-responsive epilepsy	↑: Pipecolic acid	– Conduct additional tests [15]: ↑ Pipecolic acid in urine and cerebrospinal fluid
Argininaemia	↑↑: Arg	– Calculate ratios[18]: Arg/Orn – Conduct additional tests ↑↑ urine orotic acid [15], red cell enzyme activity [15] ↑ urine Arg [1]
Argininosuccinic aciduria (argininosuccinate lyase deficiency)	↑↑: Asa [1] ↑: Gln, Pro, Gly, Ala, Cit [1] ↓: Arg, Orn [1]	– Calculate ratios[18]: Cit/Arg, Cit/Phe, Asa/Arg – Conduct additional tests [15]: ↑ Urine orotic acid and ↑↑ Asa, red cell and fibroblast enzyme activity

Disorder	Markers used in plasma amino acid analysis [15]	Measures to increase diagnostic reliability
Carbamoyl phosphate synthetase deficiency (CPS)	↑: Gln , Ala, Glu, Gly, Lys [1] n-↓: Cit, Arg	– Conduct additional tests [15]: n-↓ Urine orotic acid
Carnosinaemia	↑: Carnosine	– Conduct additional tests [15]: ↑ urine carnosine, carnosinase enzyme activity
Citrullinaemia type I (argininosuccinate synthetase deficiency)	↑↑: Cit ↓: Arg	– Calculate ratios[18]: Cit/Arg, Cit/Phe, Met/Cit – Conduct additional tests: ↑ Urine orotic acid [15], fibroblast enzyme activity [15], ↑ urine Gln, Gly, Pro, Ala, Lys [1]
Citrullinaemia type II (aspartate-glutamate carrier deficiency)	↑: Cit, Thr, Met, Tyr	– Calculate ratios[18]: (Cit/Arg, Cit/Phe, Met/Cit, Orn/Cit) – Conduct additional tests [15]: SLC25A13 gene mutation analysis
Cystinuria	n: Cys, Arg, Lys, Orn	– Conduct additional tests [15]: ↑↑ Urine Cys, ↑ Arg, Lys and Orn, cyanide-nitroprusside test positive in urine
Glutamine synthetase deficiency	↓: Gln	– Conduct additional tests [15]: ↓ Urine and CSF Gln, GS gene mutation analysis
Glycogenosis type III (Cori/Forbes)	↓: Ala, Leu, Ile, Val	– Conduct additional tests [15]: ↓ Glucose , ↑ transaminases, cholesterol
Gyrate atrophy of the choroid and retina	↑: Orn	– Conduct additional tests [15]: Ornithine aminotransferase (OAT) enzyme activity, OAT gene mutation analysis, ↓ creatinine
Hartnup disease	n-↓: neutral AS (Ala, Ser, Thr, Val, Leu, Ile, Phe, Tyr, Trp, His, Gln, Asn)	– Conduct additional tests [15]: ↑ Urine neutral amino acids, SLC6A19 gene mutation analysis
HHH (hyperammonaemia-hyperornithinaemia-homocitrullinuria) syndrome	↑↑: Orn	– Conduct additional tests [15]: ↑ Urine ornithine and homocitrulline, fibroblast enzyme activity
Histidinaemia	↑: His	– Conduct additional tests [15]: ↑ Urine histidine and imidazole pyruvate
Homocystinuria (cystathionine beta-synthase deficiency, CBS) for further details, see methylenetetrahydrofolate reductase deficiency (MTHFR) and	↑: Met ↓: Cys	– Calculate ratios [18]: Met/Leu+Ile, Met/Phe, Met/Tyr, Met/Cit – Conduct additional tests [15]: Positive urine cyanide-nitroprusside test

Disorder	Markers used in plasma amino acid analysis [15]	Measures to increase diagnostic reliability
methyltransferase deficiency		
Hydroxyprolinaemia	↑: OH-Pro	– Conduct additional tests [1]: ↑ Urine OH-Pro
Hyperlysinaemia type I (2-Aminoadpic semialdehyde synthase deficiency)	↑: Lys	– Conduct additional tests [1]: ↑ Urine and cerebrospinal fluid Lys
Hyperlysinaemia type II (saccharopinuria)	↑: Lys, ↑: Sacch	– Conduct additional tests [2]: ↑ Urine and cerebrospinal fluid Lys and Sacch
Hypermethioninaemia	↑↑: Met	– Calculate ratios[18]: Met/Phe, Met/Cit, Met/(Leu+Ile), Met/Tyr – Conduct additional tests [15]: MAT1A gene mutation analysis
Hyperprolinaemia	↑↑: Pro	– Conduct additional tests: ↑ Urine Pro, OH-Pro, Gly [15], fibroblast proline oxidase deficiency [1]
Hypophosphatasia (HPP)	↑: Phosphoethanolamine	– Conduct additional tests [15]: ↑ Serum and urine PLP
Hypoprolinaemia	↓: Pro, Orn, Arg, Cit	– Conduct additional tests: P5CS gene mutation analysis [15]
Iminoglycinuria	n: Pro, OH-Pro, Gly	– Conduct additional tests 15] ↑ urine Pro, OH-Pro and Gly
Lysinuric protein intolerance	↑: Gln [1] n-↑: Cit n-↓: Arg, Lys, Orn	– Conduct additional tests [15]: ↑ Urine Arg, Lys and Orn
Methionine synthase deficiency (cblG disease)	n-↓: Met	– Conduct additional tests [15]: Positive urine organic acids, cyanide-nitroprusside test, MTR gene mutation analysis
Methylenetrahydrofolate reductase deficiency (MTHFR)	↑: Homocystine[1] n-↓: Met	– Conduct additional tests: Positive urine cyanide-nitroprusside test [15], MTHFR gene mutation analysis [15], ↑ urine homocystine [1]
Methylmalonic aciduria (MMA)	↑: Gly, Ala	– Conduct additional tests [15]: ↑ urine methylmalonic acid, methylcitrate and (3-OH-)propionic acid
Methyltransferase deficiency	↑: Homocystine, cystathionine [1] n-↓: Met [1]	– Conduct additional tests [1]: ↑ Urine homocystine, cystathionine, n-↓: urine methylmalonic acid
Multiple carboxylase deficiency	↑: Ala	– Conduct additional tests [15]: Biotinidase (BTD) and holocarboxylase synthetase (HLCS) enzyme activity, BTD / HLCS gene mutation analysis, ↑

Disorder	Markers used in plasma amino acid analysis [15]	Measures to increase diagnostic reliability
		lactate, ↑ NH3
Non-ketotic hyperglycinaemia (NKH)	↑: Gly	– Conduct additional tests [15]: CSF Gly, GLDC, AMT and GCSH gene mutation analysis
Ornithine transcarbamoylase deficiency (OTC)	↑: Gln, Gly, Ala [1], Lys [15] ↓: Cit, Arg	– Conduct additional tests: OTC gene mutation analysis [15], ↑↑ urine orotic acid [15], ↑: Urine Gln, Lys [1]
PKU - classical phenylketonuria and hyperphenylalaninaemia (phenylalanine hydroxylase deficiency) and atypical phenylketonuria (tetrahydrobiopterin, BH4 biosynthesis / regeneration deficiency)	↑: Phe n-↓: Tyr	– Calculate ratio [18]: Phe/Tyr, Phe/(Leu+Ile) – For exclusion of atypical phenylketonuria [19]: Urine pterin analysis, diihydropterin reductase activity assay, phenylalanine hydroxylase gene mutation analysis
Propionic acidemia (PA)	↑: Gly, Ala	– Conduct additional tests [15]: ↑ urine (3-Oh-)propionic acid, methylcitrate and propionylcarnitine
Pyruvate carboxylase deficiency	↑: Cit, Ala, Lys, Pro	– Conduct additional tests [15]: Urine 2-oxoglutaric acid, PC gene mutation analysis
Pyruvate dehydrogenase deficiency	↑: Ala	– Conduct additional tests [15]: ↑ Lactate, pyruvate and Ala in body fluids
S-adenosylhomocysteine hydrolase deficiency	↑: Met, adenosylhomocysteine	– Conduct additional tests [15]: AHCY gene mutation analysis
Sarcosinaemia	↑: Sarcosine	– Conduct additional tests [15]: ↑ Urine sarcosine
Serine deficiency disorder	↓: Ser, n-↓: Gly	– Conduct additional tests [15]: ↓ CSF Ser , n-↓ Gly, fibroblast enzyme activity
Sulfite oxidase and molybdenum cofactor deficiency	↑: Tau, cysteine sulphate	– Conduct additional tests [15]: Positive urine sulfite test, ↑ urine tau, cysteine sulphate
Tryptophanaemia	↑: Trp	– Conduct additional tests [15]: Tryptophan 2,3-dioxygenase enzyme activity
Tyrosinaemia type I (fumarylacetoacetase deficiency)	n-↑: Tyr, Met	– Conduct additional tests [15]: ↑ Detecting succinylacetone in the urine is diagnostic, ↑ urine 4-OH phenyl derivatives
Tyrosinaemia type II (tyrosine transferase deficiency)	↑↑: Tyr ↑: Phe	– Calculate ratios[18]: Phe/Tyr, Met/Tyr – Conduct additional tests [15]: ↑ 4-OH-phenylpyruvate, lactate, acetate)

↑↑: heavily increased; ↑: increased; ↓: low; n: normal

Ala (alanine), allo-Ile (allo-isoleucine), Asa (argininosuccinic acid), Asn (asparagine), Arg (arginine), Cit (citrulline), Cys (cystine), Gln (glutamine), Glu (glutamic acid), Gly (glycine), His (histidine), Ile (isoleucine), Leu (leucine), Lys (lysine), Met (methionine), OH-Pro (4-hydroxyproline), Orn (ornithine), Phe (phenylalanine), PLP (pyridoxal phosphate), Pro (proline), Ser (serine), Tau (taurine), Thr (threonine), Trp (tryptophan), Tyr (tyrosine), Val (valine)

Appendix VI: Optimisation of the gradient

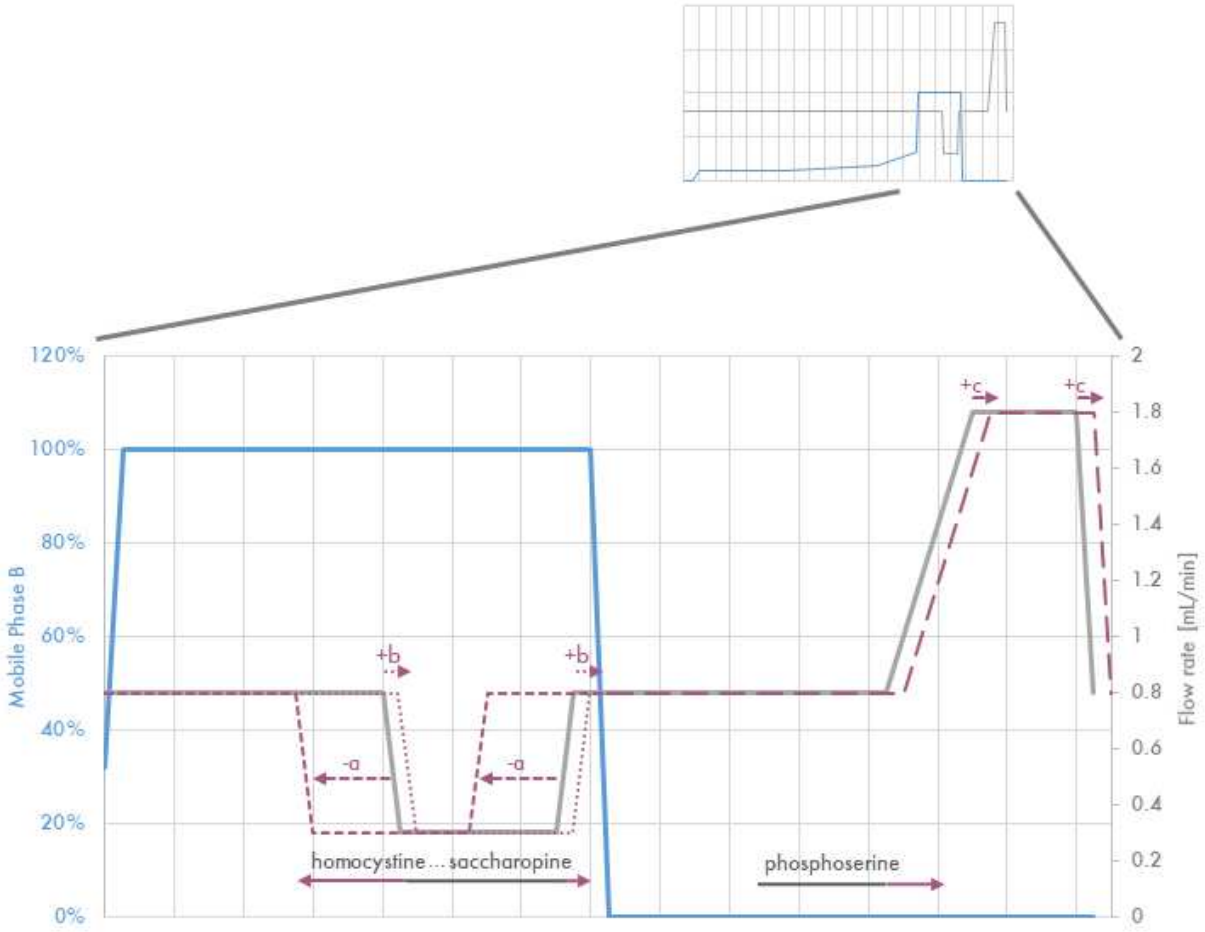

Figure 5: Optimisation of HPLC Gradient

I) Optimisation of the 0.3mL/min flow rate window

In order to ensure that the analytes homocystine, phosphoethanolamine, argininosuccinic acid, cystathionine, cystine and saccharopine elute at a reduced flow rate of 0.3 mL/min, check, upon each column lot change, the retention times of these analytes. If all of them are within the 0.3 mL/min window of your gradient, no adjustment is necessary.

If any of the retention times or parts of the peaks for the listed analytes are outside the 0.3 mL/min window, the following procedure is suggested to optimise the gradient.

Please note that the 0.3mL/min window must entirely be within the 100% Mobile Phase B window.

A) In case the retention time of homocystine has moved to an earlier retention time, move the start and the end of the 0.3 mL/min window forward ("-a" in figure above; recommended shift of flow rate window=retention time shift) as presented in the following example:

If the retention time of homocystine in comparison to previous Analytical Column lot moved from 16.6 min to 15.9 min (=retention time shift of -0.7 min),
move the start of 0.3 mL/min from 16.5 minutes to 15.8 min (-a=retention time shift= -0.7 min) and move the end of the 0.3 mL/min window to the same extent in the same direction (-a = retention time shift = -0.7 min). You do not need to adjust the subsequent steps.

After this adjustment, check that all aforementioned analytes elute in the 0.3 mL/min window.

B) In case the retention time of saccharopine has moved to a later retention time, move the end and the start of the 0.3 mL/min window backwards ("+b" in figure above; recommended shift of flow rate window = 1/2 retention time shift) as presented in the following example:

If the retention time of saccharopine in comparison to previous Analytical Column lot moved from 17.0 min to 17.4 min (retention time shift = +0.4min),
move the end of 0.3 mL/min from 17.4 minutes to 17.6min (+b = 1/2 retention time shift = +0.2 min) and move the start of the 0.3 mL/min window to the same extent in the same direction (+b = 1/2 retention time shift = +0.2 min). You do not need to adjust the subsequent steps.

After this adjustment, check that all aforementioned analytes elute in the 0.3 mL/min window.

II) Optimisation of the 1.8mL/min flow rate window

In order to ensure that all analytes elute by the time the flow rate is increased to 1.8 mL/min, check if phosphoserine elutes within the 0.8 mL/min step prior to the start of flow rate increase to 1.8 mL/min. If this is the case, no adjustment is necessary at this step.
In case the retention time or part of the peak of phosphoserine has moved further back, please follow the described procedure to optimise the gradient.

C) Move the end of the 0.8 mL/min 100% Mobile Phase A window and the subsequent steps backwards ("+c" in figure above; recommended shift of flow rate window=retention time shift) as presented in the following example:

Retention time of phosphoserine in comparison to previous Analytical Column lot moved from 19.1 min to 19.6 min (retention time shift = +0.5min),
then move the end of 0.8 mL/min 100% Mobile Phase A window from 19.3 minutes to 19.8 min (+c = retention time shift = +0.5 min). Move all subsequent steps and the waste switching to the same extent in the same direction (+c = retention time shift = +0.5 min).

Appendix VII: Symbols

We use EN ISO 15223-1 symbols on our labels, specifications and packaging. The meanings of each symbol are given in the table below:

Table 43: Symbols

Symbol	Meaning
	Manufacturer
	Date of manufacture
	Use by
	Order number
	Batch/lot code
	See instructions for use
	Upper temperature limit: Store below a certain temperature
	Temperature limit: Store within a certain temperature range
	*In-vitro* diagnostic medical device
	Sufficient for <n> appliances
	Serial number