CHROMSYSTEMS

NEUGEBORENEN-SCREENING
NEWBORN SCREENING
DEPISTAGE DES NOUVEAUX-NES
SCREENING NEONATALE
ANÁLISIS DE CONTROL PARA NEONATOS

Instruction Manual for LC-MS/MS Analysis

MassChrom® Amino Acids and Acylcarnitines from Dried Blood (non derivatised)

with 96 Well Plates

Order No. 57000

CE 0123 IVD

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	
57000	**LC-MS/MS Reagent Kit**	
	***MassChrom*® Amino Acids and Acylcarnitines from Dried Blood (non derivatised)**	
	Contents for 960 analyses with 96 Well Plates:	
	Mobile Phase	2 x 1000 mL
	Internal Standard	4 x 25.0 mL (lyoph.)
	Rinsing Solution	1 x 1000 mL
	Extraction Buffer	1 x 100 mL
	96 Well Plates	6 x 5 pcs.
	Protective Sheets for 96 Well Plates	2 x 10 pcs.
	Pierceable Heat Seals for 96 Well Plates	2 x 6 pcs.
	***MassCheck*® Amino Acids, Acylcarnitines Dried Blood Spot Control**	
	Level I	1 x 3 spots
	Level II	1 x 3 spots
57111	**Succinylacetone (non derivatised) Upgrade Set**	
	Contents for 960 analyses with 96 Well Plates, consisting of:	
	Internal Standard, Succinylacetone (non derivatised)	4 x 18 mL
	Extraction Buffer, Succinylacetone (non derivatised)	4 x 18 mL
	Protective Sheets for 96 Well Plates	2 x 10 pcs.
	Pierceable Heat Seals for 96 Well Plates	1 x 6 pcs.
	Components available separately	
57001	Mobile Phase	1000 mL
57002	Mobile Phase	10 x 1000 mL
57004	Internal Standard	4 x 25.0 mL (lyoph.)
57044	Internal Standard, Succinylacetone (non derivatised)	4 x 18 mL
57007	Rinsing Solution	1000 mL
57008	Extraction Buffer	100 mL
57012	Extraction Buffer, Succinylacetone (non derivatised)	4 x 18 mL
57010	96 Well Plates	5 pcs.
55011	Protective Sheets for 96 Well Plates	10 pcs.
57014	Pierceable Heat Seals for 96 Well Plates	6 pcs.
	Accessories	
57015	Cross-cut Adhesive Seals for 96 Well Plates	10 pcs.
55015	Restrictor Capillary	1 pc.
57098	Tuning Mix, Succinylacetone (non derivatised), Analyte and Internal Standard	1 mL
57099	Tuning Mix, Analytes and Internal Standards	2 mL
15010	PEEK Prefilter Housing	1 pc.
55033	PEEK Prefilter (2 µm)	5 pcs.
42740	Heat Sealer for 96 Well Plates (suitable insert included)	1 pc.
	Chromsystems *MassCheck*® controls	
0191	***MassCheck*® Amino Acids, Acylcarnitines incl. Succinylacetone Dried Blood Spot Control Bi-Level (I + II)**	2 x 3 spots
0192	***MassCheck*® Amino Acids, Acylcarnitines incl. Succinylacetone Dried Blood Spot Control Level I**	1 x 3 spots
0193	***MassCheck*® Amino Acids, Acylcarnitines incl. Succinylacetone Dried Blood Spot Control Level II**	1 x 3 spots

2 Introduction

2.1 Background information

The aim of newborn screening is the timely diagnosis of hereditary metabolic disorders which cannot be recognized by external signs. The screening makes early treatment of the diseases possible and prevents consequential damages as far as possible.

The diseases are caused by hereditary enzyme defects, leading to decreased or suppressed enzyme activity. Subsequently, non-transformed educts accumulate in the body. According to the degree of the disease the product is lacking completely or is present only at limited levels. This may lead to an accumulation of potential toxic metabolites, causing organ defects and multi-systemic diseases (amino- and organo-acidemias). Not diagnosed, these metabolic disorders cause severe and irreversible injuries of the infant even within days. In the case of PKU this leads to physical and mental retardation of the affected children. Type 1 tyrosinemia leads to the accumulation of toxic metabolites (especially succinylaccetone) resulting in severe liver diseases during early childhood.

However, since the defects are hereditary, these diseases are incurable and therapies are limited to a compensation of the symptoms. Nevertheless, if the disease has been diagnosed in time, i.e. within the first few days of life, children suffering from PKU are able to live a normal life with a phenylalanine-free diet. The incidence of these metabolic disorders lies between 1:10,000 (PKU) and 1:200,000 (maple syrup urine disease, MSUD). On average one of 1,000 newborns is affected by such a disease [1]. The laboratory diagnosis of these diseases is made via measurement of elevated or degraded marker substances respectively in the blood of the newborn. For this purpose blood is taken from the heel onto a filter paper, dried and sent to a laboratory.

In Germany, the examination for 13 target diseases is prescribed by law according to the Kinder-Richtlinie (version dated 18 June 2015, latest revision entered into force on 25 March 2020). Nine of these should be determined by tandem mass spectrometry including disorders of amino acid metabolism such as phenylketonuria (PKU) and maple syrup urine disease (MSUD), disorders of fatty acid metabolism (MCAD deficiency, LCHAD deficiency, VLCAD deficiency) as well as organo acidemia such as isovalerian acidemia. The screening for tyrosinemia type I has been included with the last revision of the directive.

With the technology of tandem mass spectrometry it is possible to screen a broad spectrum of metabolic disorders in a single analytical run. This method allows the reliable and simultaneous detection of many target molecules and the determination of their concentrations. Because of its high selectivity the application does not require an HPLC column, enabling very short run times (< 2 min). To compensate interfering ion suppression effects and to enable precise quantification of the analytes, the use of isotopically labelled standards is essential.

Depending on the type of filter paper used and the hematocrit of the sample, the blood volume on the punched out dried blood disk varies [2]. This allows only semi-quantitative determination of the analytes, and this screening method should therefore not be used as the sole laboratory-diagnostic determination method. Newborn screening with tandem mass spectrometry must always be backed up by confirmation diagnostics based on molecular-genetic and enzymatic methods or based on chromatography.

2.2 Intended use

The Chromsystems reagent kit ***MassChrom***® Amino Acids and Acylcarnitines from Dried Blood (non derivatised) is an *in vitro* diagnostic device to be used in clinical laboratories for the semi-quantitative detection of the following metabolites:

Amino acids and tyrosine metabolites:
Alanine (Ala), arginine (Arg), aspartic acid (Asp), citrulline (Cit), glutamic acid (Glu), glycine (Gly), leucine (Leu), methionine (Met), ornithine (Orn), phenylalanine (Phe), proline (Pro), tyrosine (Tyr), valine (Val), succinylacetone (SUAC)

Free carnitine and acylcarnitines
Free carnitine (C0), acetylcarnitine (C2), propionylcarnitine (C3), butyrylcarnitine (C4), isovalerylcarnitine (C5), glutarylcarnitine (C5DC), hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), dodecanoylcarnitine (C12), tetradecanoylcarnitine (C14), hexadecanoylcarnitine (C16), octadecanoylcarnitine (C18).

Newborn heel prick blood samples dried on filter paper are analysed via liquid chromatography-mass spectrometry (LC-MS/MS). The test is indicated as a screening method for the early detection of disorders of amino acid and fatty acid metabolism.

A diagnostic method should be used for confirmation of presumptive abnormal amino acids, succinylacetone, free carnitine and acylcarnitine profiles.

2.3 Principle of the reagent kit

This Chromsystems reagent kit is designed for the simple and fast, semi-quantitative determination of amino acids, acylcarnitines and succinylacetone in dried blood spots for the newborn screening of amino and fatty acid metabolic disorders via tandem mass spectrometry.

The simple sample preparation for the measurement of amino acids and acylcarnitines is based on an efficient extraction of the analytes out of filter paper without additional butylation step. To ensure reproducible quantification of the analytes, this method uses stable, isotopically labelled internal standards for calibration and measurement.

For the measurement of succinylacetone, the blood spot is extracted a second time after extraction of the amino acids and acylcarnitines using a special reagent. This contains an agent that forms the hydrazone of succinylacetone thus making the extraction of succinylacetone from dried blood possible. An isotopically labelled internal standard is also used for the quantification of succinylacetone. After extraction of succinylacetone, both supernatants are combined and all analytes are measured in one analytical run.

This kit is an *in vitro* diagnostic medical device.
Please note that it is a **screening method**; the results may vary depending on the MS/MS system used and must be seen in relation to other collected laboratory data. The medical interpretation of the results gained with this kit must be carried out only by relevantly medically trained personnel or a metabolic expert. There are no systematic clinical studies about the frequency of false-positive or false-negative results.

3 LC-MS/MS system

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

3.1 Equipment and instrument parameters

The analysis of amino acids (including SUAC), acylcarnitines as well as free carnitine requires a system with an HPLC pump, injector and a tandem mass spectrometer with adequate sensitivity and special evaluation software. To prevent changes to the composition of the mobile phase it should be kept capped even during operation. No HPLC column or column oven is required. For the connection of the HPLC system to the MS/MS system a restrictor capillary (order no. 55015) with PEEK prefilter (order no. 55033 and no. 15010) must be used.

Instrument settings:

Injection volume:	10 µL
Run time:	1.7 min
Flow gradient:	20 to 600 µL/min
Needle rinsing solution for the injector:	Rinsing Solution

Gradient profile:
The robustness of gradient profile depends strongly on the optimisation of gradient flow related to the instrumental setup used.

Because of different void volumes of individual HPLC systems and the length of restrictor capillary the following gradient profile must be optimised to obtain a chromatogram as shown in figure 1. The gradient profile shown is intended as a basis for optimisation. A not optimised gradient profile may give a chromatogramm as shown in figure 2. In that case it is strongly recommended to repeat the optimisation procedure and adjust the flow gradient until a chromatogram as shown in figure 1 is obtained.

The scan time window of the tandem MS system has to be set in the period of time where the signal intensities reach their maximum plateau, e.g. measured on SCIEX API 4000™ coupled to Shimadzu HPLC system approx. 0.32 to 1.51 min.

Table 1: Gradient profile

Time	0 min	0.32 min	0.33 min	1.50 min	1.51 min	1.70 min	1.71 min
Flow rate	200 µL/min	200 µL/min	20 µL/min	20 µL/min	600 µL/min	600 µL/min	200 µL/min

If the HPLC system is unable to maintain a constant flow of 20 µL/min, the method can be run alternatively with a constant flow rate of 100 µL/min. Note, however, that the constant flow method causes reduced sensitivity.

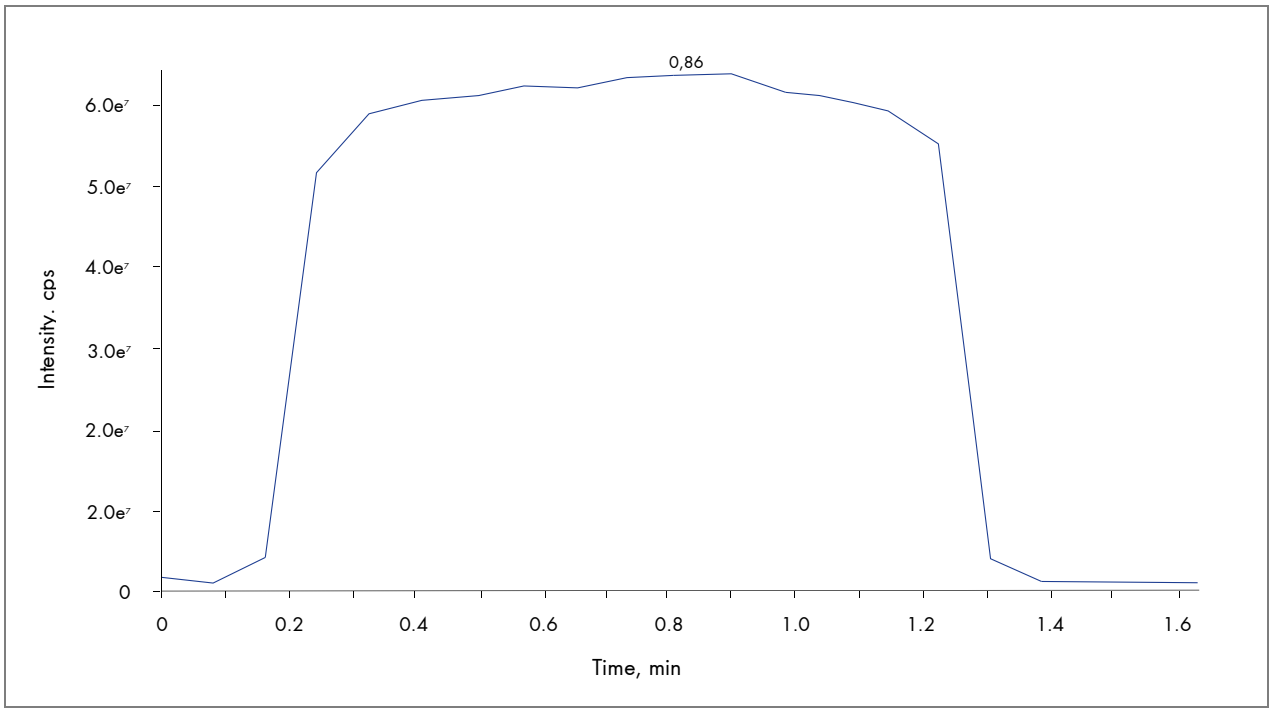

Figure 1: Chromatogram using an optimised flow gradient

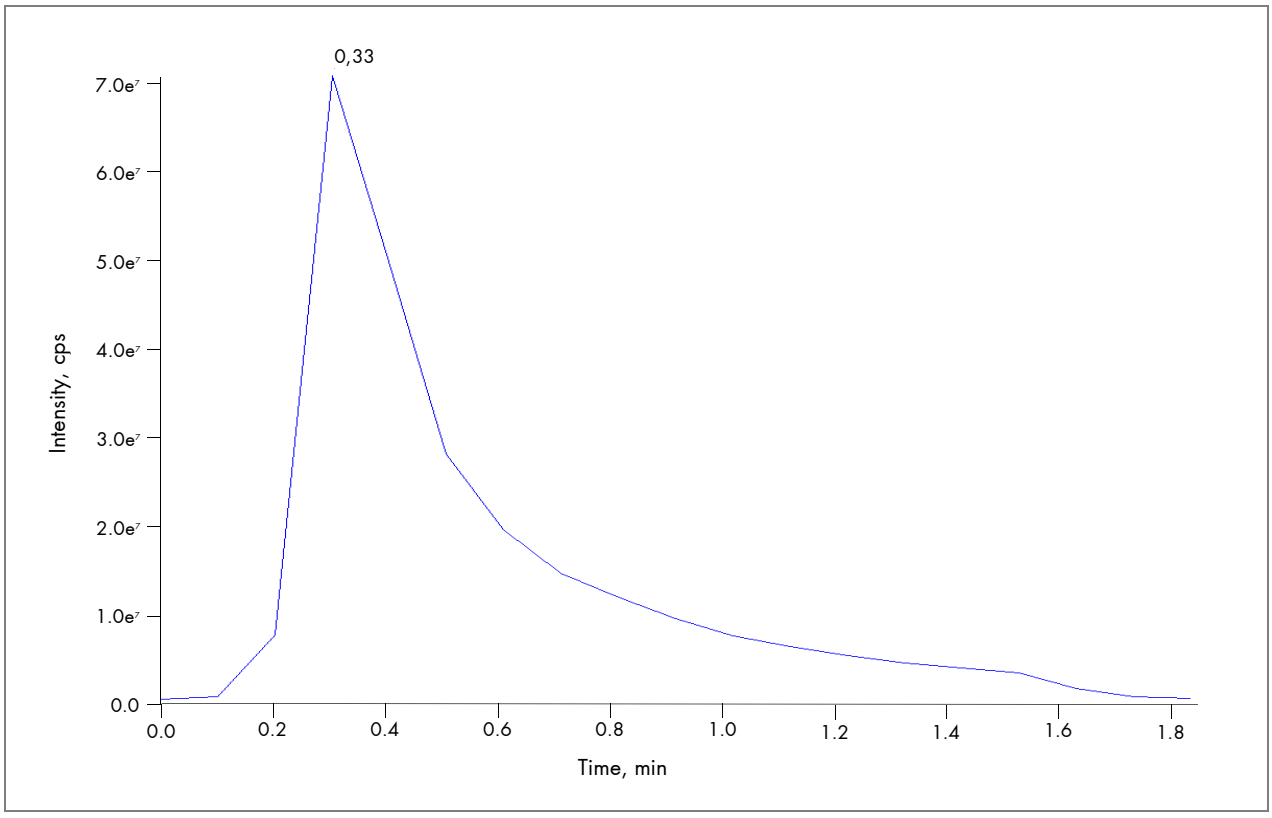

Figure 2: Chromatogram given by a NOT optimised flow gradient

Note:
Use an optimised flow gradient only. Using a not optimised flow gradient will deteriorate the performance of the reagent kit.

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. 57099, 57098) separately with Mobile Phase (order no. 57001) as appropriate for the specific device
2. Inject diluted Tuning Mix or infuse directly by syringe pump (flow rate of 0.02 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. Equilibrate the system for about 15 minutes using the initial conditions of the method.
2. We recommend to equilibrate the system by three matrix-free injections (e.g. Mobile Phase, order no. 57001) followed by at least three injections of the eluate of the Dried Blood Spot Control Level I (order no. 0192) until the signal intensities of analytes and internal standards match in successive injections.
3. The analysis series of all prepared samples can now be started.

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 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.

4 Mass transitions of analytes and internal standards

The following table shows the recommended mass transitions and measuring methods of the analytes and isotopically labelled internal standards. Measure the analytes and the internal standards in positive ionisation mode (ESI).

Table 2: Recommended MRM transitions, amino acids and succinylacetone

Substance	Measuring method	Mass transition
Alanine	MRM	90 > 44
Alanine-D4	MRM	94 > 48
Arginine	MRM	175 > 70
Arginine-D7	MRM	182 > 77
Aspartic acid	MRM	134 > 116
Aspartic acid -D3	MRM	137 > 119
Citrulline	MRM	176 > 113
Citrulline-D2	MRM	178 > 115
Glutamic acid	MRM	148 > 130
Glutamic acid -D5	MRM	153 > 135
Glycine	MRM	76 > 30
Glycine-$^{13}C_2$-^{15}N	MRM	79 > 32
Leucine	MRM	132 > 86
Leucine-D3	MRM	135 > 89
Methionine	MRM	150 > 133
Methionine-D3	MRM	153 > 136
Ornithine	MRM	133 > 70
Ornithine-D6	MRM	139 > 76
Phenylalanine	MRM	166 > 120
Phenylalanine-D5	MRM	171 > 125
Proline	MRM	116 > 70
Proline-D7	MRM	123 > 77
Succinylacetone	MRM	155 > 137
Succinylacetone-13C5	MRM	160 > 142

Substance	Measuring method	Mass transition
Tyrosine	MRM	182 > 136
Tyrosine-D4	MRM	186 > 140
Valine	MRM	118 > 72
Valine-D8	MRM	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 the Tuning Mix for this purpose (order no. 57098 and 57099, see section 3.3 on optimising MRM transitions).

Table 3: Recommended MRM transitions, acylcarnitines and free carnitine

Substance	Measuring method	Mass transition
Carnitine	MRM	162 > 85
Carnitine-D9	MRM	171 > 85
C2-Carnitine	MRM	204 > 85
C2-Carnitine-D3	MRM	207 > 85
C3-Carnitine	MRM	218 > 85
C3-Carnitine-D3	MRM	221 > 85
C4-Carnitine	MRM	232 > 85
C4-Carnitine-D3	MRM	235 > 85
C5-Carnitine	MRM	246 > 85
C5-Carnitine-D9	MRM	255 > 85
C5DC-Carnitine	MRM	276 > 85
C5DC-Carnitine-D6	MRM	282 > 85
C6-Carnitine	MRM	260 > 85
C6-Carnitine-D3	MRM	263 > 85
C8-Carnitine	MRM	288 > 85
C8-Carnitine-D3	MRM	291 > 85
C10-Carnitine	MRM	316 > 85
C10-Carnitine-D3	MRM	319 > 85
C12-Carnitine	MRM	344 > 85
C12-Carnitine-D3	MRM	347 > 85
C14-Carnitine	MRM	372 > 85
C14-Carnitine-D3	MRM	375 > 85
C16-Carnitine	MRM	400 > 85
C16-Carnitine-D3	MRM	403 > 85
C18-Carnitine	MRM	428 > 85
C18-Carnitine-D3	MRM	431 > 85

Quantification of the analytes is done by signal intensity comparison with the corresponding isotopically labelled internal standard. Several diagnostically interesting acylcarnitines, for which isotopically labelled internal standards are not yet available, can also be measured with this reagent kit. Acylcarnitines with equal chain lengths show approximately the same response factor. It is recommended to quantify these disease markers with deuterated standards containing the same chain length. However this reagent kit is validated only for those analytes with available deuterated internal standards shown in the table above.

The following table shows additional acylcarnitines, their mass transitions and the recommended isotopically labelled internal standards. This information is a recommendation only, representing the current practice of many screening laboratories. The reagent kit is suitable for determination of these analytes but it is not validated for this purpose.

Table 4: Recommended MRM transitions, acylcarnitines without explicit standards

Substance	Mass transition	Recommended internal standard
C3DC-Carnitine	248 > 85	C3-Carnitine-D3
C4OH-Carnitine	248 > 85	C4-Carnitine-D3
C4DC-Carnitine	262 > 85	C4-Carnitine-D3
C5:1-Carnitine	244 > 85	C5-Carnitine-D9
C5OH-Carnitine	262 > 85	C5-Carnitine-D9
C8:1-Carnitine	286 > 85	C8-Carnitine-D3
C10:2-Carnitine	312 > 85	C10-Carnitine-D3
C10:1-Carnitine	314 > 85	C10-Carnitine-D3
C12:1-Carnitine	342 > 85	C12-Carnitine-D3
C14:2-Carnitine	368 > 85	C14-Carnitine-D3
C14:1-Carnitine	370 > 85	C14-Carnitine-D3
C14OH-Carnitine	388 > 85	C14-Carnitine-D3
C16:2-Carnitine	396 > 85	C16-Carnitine-D3
C16:1-Carnitine	398 > 85	C16-Carnitine-D3
C16:1OH-Carnitine	414 > 85	C16-Carnitine-D3
C16OH-Carnitine	416 > 85	C16-Carnitine-D3
C18:2-Carnitine	424 > 85	C18-Carnitine-D3
C18:1-Carnitine	426 > 85	C18-Carnitine-D3
C18:2OH-Carnitine	440 > 85	C18-Carnitine-D3
C18:1OH-Carnitine	442 > 85	C18-Carnitine-D3
C18OH-Carnitine	444 > 85	C18-Carnitine-D3

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

For collection and storage of patient specimens different recommendations are available for different countries. Adherence to national regulations is strongly recommended. For example, in Germany blood collection for newborn screening should be done between 48 and 72 hours after birth (see the German "Kinder Richtlinie") whereas according to CLSI Standard NBS01-Ed7, 2021 [3] it is preferable for a screening specimen to be collected after 24 to 48 hours following the birth.

Blood is dripped onto a filter paper and dried. It is recommended to use FDA approved or equivalent filter papers (e.g. Whatman 903). The blood sample should be taken from the heel of the newborn. EDTA or heparin blood must not be used because false negative or false positive results may result!

Brief description of the blood sample collection:

1. Clean the designated spot for the prick at the heel of the newborn with an antiseptic. Dry the heel with a sterile swab.
2. Puncture the infant's heel with a sterile lancet. The tip of the lancet must be smaller than 2 mm, deeper punctures may injure small infants.
3. Wipe off the first drop of blood with a sterile swab.
4. Touch the next big drop of blood with the filter paper. Wait until the blood has soaked through the paper and completely filled the designated circle. Do not apply any drops of blood on top of each other or on both sides of the filter paper. This would change the applied volume of the blood per blood spot and pathological results may be falsely indicated.
5. Fill each of the remaining circles on the filter paper with a single drop of blood.
6. Care of the wound should be according to the common practice of the hospital.
7. Dry the blood spots for 4 hours on a dry, horizontal and non-absorbent surface at +20 to +25 °C.
8. Send the dried filter papers to the laboratory within 24 hours.

Detailed instructions for specimen collection can be found in the manual of the manufacturer of the filter paper and in the German "Kinder-Richtlinie" or other nationally applicable regulations.

Storage in a low humidity environment (less than 30 %) at ambient temperature (+20 to +25°C) is adequate, if analysis is anticipated within 24 to 48 hours. Low humidity and low temperatures (refrigerated or below –18°C) are suggested for storage beyond 48 hours [3]. **Avoid temperatures above 37 °C.** This may lead to a decrease of some amino acids.

Our own tests showed a stability of all analytes of at least 3 months when stored below –18 °C, 6 weeks when stored at +2 to +8 °C and 10 days when stored at +20 to +25 °C packed in an airtight aluminium bag and using desiccants.

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

The Internal Standard (order no. 57004) is used as calibration standard for each sample and is traceable to isotopically labelled reference substances purchased from a certified supplier. After reconstitution, a defined amount of the internal standard is added to the specimen and subjected to the entire sample preparation.

Prior to sample preparation, reconstitute the Internal Standard (order no. 57004) with 25 mL Extraction Buffer. Proceed as folllows:

1. Pipette 5 mL Extraction Buffer (order no. 57008) into the original vial of the Internal Standard
2. Reconstitute for 5 min at +20 to +25 °C, swirl repeatedly
3. Transfer the vial's content into a 25 mL volumetric flask
4. Rinse the Internal Standard vial twice with 5 mL Extraction Buffer and transfer the liquid into the volumetric flask
5. Fill the volumetric flask up to 25 mL with Extraction Buffer and mix thoroughly

Avoid exposure to direct sunlight. The concentrations of the internal standards are batch-dependent. Individual levels are given in the leaflet of the Internal Standard.

Storage life of the Internal Standard after reconstitution:
The Internal Standard dissolved in the Extraction Buffer has the following storage life:

Table 5: Storage life of the Internal Standard after reconstitution

Storage temperature	Storage life	Other storage conditions
+2 to +8 °C	3 weeks	Light protection, tightly closed

5.3 Handling of the reagents of the Succinylacetone Upgrade Set

The Exctraction Buffer, Succinylacetone (order no. 57012) is stored below –18 °C. This results in segregation.

Therefore it is necessary that this reagent has warmed up completely to room temperature and is then mixed thoroughly (vortex) prior to use.

Storage life of the Extraction Buffer after first thawing:
The reagent has the following storage life after first thawing:

Table 6: Storage life of the Extraction Buffer, Succinylacetone after first thawing

Storage temperature	Storage life	Other storage conditions
+2 to +8 °C	2 weeks	tightly closed

5.4 Handling of the controls

The ***MassCheck***® Dried Blood Spot Controls (order no. 0192, 0193) are intended to monitor the trueness and precision of each analytical sequence. They are available in two different concentration levels. The dried blood spots are based on human whole blood. They are handled in the same manner as a patient sample and are analysed under routine conditions analogous to the respective test procedure

The filter cards are sensitive to humidity and temperature. Therefore the packaging includes a temperature indicator, desiccant packets and a humidity indicator card. Check the Dried Blood Spot Controls before each use as follows:

1. Check the condition of the glued-on temperature indicator: The controls may only be used if all fields are white. If a field has turned black, the filter cards must be disposed of.
2. Allow the Dried Blood Spot Control to reach room temperature (+20 to +25 °C), avoiding direct sunlight.
3. Open the packaging.
4. Immediately check the condition of the enclosed humidity indicator: If the humidity indicator has changed colour from blue to pink at the 30 % level, dispose of the filter card.

Achtung:
- When punching out the dried blood spots, use only the area within the dotted line. The concentrations of the analytes may deviate from the specified values in the marginal area.
- As increased humidity has a negative effect on product stability, the time outside the airtight packaging should be minimised.

Immediately after use, return the remaining control material together with the humidity indicator and the desiccant packets to the zip-lock storage bag, seal tightly and freeze below –18 °C.

The analyte concentrations in the controls are batch-dependent. Individual levels are given in the leaflet accompanying each control.

Caution:
This product is manufactured from pooled human whole blood 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.

5.5 Sample preparation procedure

Notices
- Use only the supplied reagents, 96 well plates and protective sheets for sample preparation. Deviations in the sample preparation will alter the performance of the reagent kit.
- Precision and accuracy of the analyses should be monitored by the inclusion of additional controls in each analytical run.

5.5.1 Sample preparation without succinylacetone

1. **Punching out the sample:**
   Punch a 3.2 mm dried blood spot disk out of the filter card into a well of a 96 well plate (order no. 57010).

2. **Extraction:**
   Add 100 µL of the reconstituted internal standard (see chapter 5.2). Seal the 96 well plate with a protective sheet (order no. 55011) and agitate at 600 rpm for 20 min at +20 to +25 °C.

3. **Transfer:**
   Remove the protective sheet from the 96 well plate. Transfer the supernatant into a new 96 well plate (order no. 57010). Seal the 96 well plate with a sealing film (Pierceable Heat Seal, order no. 57014; alternatively: Cross-cut Adhesive Seal, order no. 57015).

4. **Injection:**
   Inject 10 µL of the eluate into the LC-MS/MS system.

5.5.2 Sample preparation with succinylacetone

1. **Punching out the sample:**
   Punch a 3.2 mm dried blood spot disk out of the filter card into a well of a 96 well plate (order no. 57010).

2. **Extraction:**
   Add 100 µL of the reconstituted internal standard (see chapter 5.2). Seal the 96 well plate with a protective sheet (order no. 55011) and agitate at 600 rpm for 20 min at +20 to +25 °C.

3. **Transfer:**
   Remove the protective sheet from the 96 well plate. Transfer the supernatant into a new 96 well plate (order no. 57010) and seal with a new protective sheet (order no. 55011). Take care to transfer supernatant as quantitatively as possible and no liquid remains on the dried blood spot disk.

4. **Extraction of succinylacetone:**
   First add 75 µL Internal Standard, Succinylacetone (order no. 57044), then 75 µL Extraction Buffer, Succinylacetone (order no. 57012) to the remaining dried blood spot disk. Seal the 96 well plate with a protective sheet (order no. 55011) and agitate at 600 rpm for 30 min at 45 °C.

5. **Pooling the extracts:**
   Remove protective sheets from both 96 well plates and pipette the extract from step 4 into the extract from step 3. Seal the 96 well plate with a sealing film (Pierceable Heat Seal, order no. 57014). Then agitate at 500 rpm for 1 min at +20 to +25 °C.

6. **Incubation:**
   Incubate the 96 well plate for 20 min at +20 to +25 °C prior to injection.

7. **Injection:**
   Inject 10 µL of the eluate into the LC-MS/MS system.

Important
- The Extraction Buffer, Succinylacetone segregates during storage below –18 °C. After thawing, this reagent must be mixed thorouhly (vortex) in order to avoid inhomogeneities in the solution.
- The supernatant in step 3 has to be transferred as quantitatively as possible.
- Keep to the described pipetting order in step 4.
- In step 5, the protective sheet must be removed quickly to avoid condensation of solvent on the film.
- Do not use adhesive seals as final sealing films in step 5, but only Piercable Heat Seals (order no. 57014).

5.6 Storage life of prepared samples

5.6.1 Prepared samples without succinylacetone

Samples prepared for analysis according to chapter 5.5.1
- in 96 Well Plates (order no. 57010), sealed with Pierceable Heat Seals (order no. 57014)
- in tightly closed glass vials

have the following storage life:

Table 7: Storage life of prepared samples in 96 Well Plates (order no. 57010), sealed with Pierceable Heat Seals (order no. 57014) or in tightly closed autosampler vials

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	10 days	Light protection, tightly closed
+2 to +8 °C	10 days	Light protection, tightly closed

Note
Once the Pierceable Heat Seals have been pierced, the eluates start to evaporate and the eluate volume decreases. This is dependant on autosampler settings and conditions, e.g. temperature and ventilation. As long as injection of sufficient volume is possible, samples can be analysed.

5.6.2 Prepared samples with succinylacetone

Samples prepared for analysis according to chapter 5.5.2
- in 96 Well Plates (order no. 57010), sealed with Pierceable Heat Seals (order no. 57014)
- in tightly closed glass vials

have the following storage life:

Table 8: Storage life of prepared samples in 96 Well Plates (order no. 57010), sealed with Pierceable Heat Seals (order no. 57014) or in tightly closed autosampler vials

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	10 days	Light protection, tightly closed
+2 to +8 °C	21 days	Light protection, tightly closed
below –18 °C	21 days	Light protection, tightly closed

Note
Once the Pierceable Heat Seals have been pierced, the eluates start to evaporate and the eluate volume decreases. This is dependant on autosampler settings and conditions, e.g. temperature and ventilation. As long as injection of sufficient volume is possible, samples can be analysed.

6 Additionally required equipment

The LC-MS/MS determination of amino acids and acylcarnitines in dried blood (non derivatised) requires the following additional materials not supplied in the reagent kit:
- Tandem mass spectrometer with evaluation software
- HPLC system with pump, injector and autosampler
- Manual or automatic puncher for punching out the sample, 3 mm in diameter
- Thermostatically-controlled shaker for 96 well plates for extraction of the samples. For sample preparation with succinylacetone, 2 shakers are recommended, one for extraction at room temperature, the second for extraction at 45 °C.
- Rubber roller for sealing the 96 well plates with protective sheets
- Pipette or multi-channel pipette
- Pipette tips
- 25 mL volumetric flask
- Optional: Heat Sealer for 96 well plates (Heat Sealer, order no. 42740, suitable insert included)

7 Data acquisition and evaluation

The internal standard is used as an individual calibrator for each sample, so matrix effects are reduced to a minimum. For this purpose the sample (control, specimen) is mixed with a defined amount of the internal standard. The concentrations of the isotopically labelled compounds in the internal standard depend on the batch and will be found on the information leaflet accompanying the standards.

The volume of blood used is necessary for successful quantification of the samples. The blood volume in a punched out disk of dried blood depends on the disk's diameter, the hematocrit of the sample and the filter paper material used.

The CLSI standard NBS01-Ed7, 2021 [3] recommends to use only filter paper intended for the analysis of dried blood spots in newborn screening. Some commercial filter papers are available, e.g. Whatman 903 (GE Healthcare), which meet the requirements of the CLSI standard. The punch should be approximately 1/8 inch (3.2 mm) in diameter, which corresponds to an assumed volume of 3.1 µL for all samples, regardless of haematocrit [4].

This screening method is a quantitative determination method, which is, however, significantly influenced by different blood volumes used, depending on the sample. Therefore, further laboratory diagnostic tests must always be carried out to confirm positive screening results.

Depending on the software used, the values of the compounds of the internal standard and the blood volume or the sample related concentration of the internal standard is necessary. Enter the concentrations (see information sheet) of the internal standard in the analysis table.

If any other sizes of punched out dried blood spots are used (sample volume), the sample related concentrations of the internal standard have to be corrected.

To ensure that the LC-MS/MS conditions haven't changed in the course of an analytical run, the prepared controls should be injected during the course of the run and again at the end.

For the correct handling of the analytical software, contact the manufacturer of your MS system if necessary. Notes on manual calculation can be found in Appendix II.

8 Quality control

Monitor precision and accuracy of the analyses by including additional controls (***MassCheck***® Dried Blood Spot Controls, order no. 0192, 0193) in each analytical run at least at the beginning and end of the measuring sequence. If the values are outside the ranges indicated on the information leaflet of the controls, you must check the system, the sample preparation procedure, as well as the calculation of the analysis values.

As described in CLSI Standard NBS04-Ed2, 2017 [5], each laboratory should establish its own acceptance criteria for results of samples from each analytical run. These criteria are mainly based on analysis of quality controls.

9 Cut-off values

In a pilot study in the screening centre of the University Hospital of Dresden the following cut-off values given as 99.9 % percentile have been determined using the reagent kit 57000 (without succinylacetone). The datas are differentiated between the week of gestation (WG) and the blood collection after birth [a].

Table 9: Cut-off values, amino acids, [a]

Substance	Cut-off value 32–42 WG, > 36 h	Cut-off value 38–42 WG, > 36 h	Cut-off value all WG, > 0 h
Alanine	583 µmol/L	564 µmol/L	736 µmol/L
Arginine	52 µmol/L	49 µmol/L	55 µmol/L
Aspartic acid	420 µmol/L	402 µmol/L	420 µmol/L
Citrulline	50 µmol/L	51 µmol/L	50 µmol/L
Glutamic acid	1074 µmol/L	1087 µmol/L	1073 µmol/L
Glycine	1003 µmol/L	962 µmol/L	1001 µmol/L
Leucine	277 µmol/L	299 µmol/L	276 µmol/L
Methionine	35 µmol/L	35 µmol/L	37 µmol/L
Ornithine	454 µmol/L	455 µmol/L	453 µmol/L
Phenylalanine	127 µmol/L	124 µmol/L	141 µmol/L
Proline	not determined	not determined	not determined

Substance	Cut-off value 32–42 WG, > 36 h	Cut-off value 38–42 WG, > 36 h	Cut-off value all WG, > 0 h
Tyrosine	248 µmol/L	217 µmol/L	248 µmol/L
Valine	192 µmol/L	199 µmol/L	212 µmol/L

Table 10: Cut-off values, acylcarnitines and free carnitine, [a]

Substance	Cut-off value 32–42 WG, > 36 h	Cut-off value 38–42 WG, > 36 h	Cut-off value alle WG, > 0 h
C0-Carnitine	55.87 µmol/L	54.96 µmol/L	55.83 µmol/L
C2-Carnitine	71.17 µmol/L	73.40 µmol/L	71.08 µmol/L
C3-Carnitine	6.35 µmol/L	6.41 µmol/L	6.34 µmol/L
C4-Carnitine	1.07 µmol/L	1.18 µmol/L	1.07 µmol/L
C5-Carnitine	0.43 µmol/L	0.37 µmol/L	0.48 µmol/L
C5DC-Carnitine	0.56 µmol/L	0.58 µmol/L	0.56 µmol/L
C6-Carnitine	0.17 µmol/L	0.17 µmol/L	0.17 µmol/L
C8-Carnitine	0.24 µmol/L	0.24 µmol/L	0.24 µmol/L
C10-Carnitine	0.35 µmol/L	0.29 µmol/L	0.35 µmol/L
C12-Carnitine	0.35 µmol/L	0.31 µmol/L	0.35 µmol/L
C14-Carnitine	0.50 µmol/L	0.49 µmol/L	0.50 µmol/L
C16-Carnitin	9.90 µmol/L	10.03 µmol/L	9.89 µmol/L
C18-Carnitine	2.06 µmol/L	2.07 µmol/L	2.06 µmol/L

In a pilot study with the reagent kit 57000 in combination with 57111 (with succinylacetone) on 1642 newborns carried out in the Universitary Hospital Institute for Clinical Chemistry and Pathobiochemistry Newborn Screening and Metabolism Laboratory of the University of Magdeburg, the following cut-off values were determined as 99.9 % percentile [b].

Table 11: Cut-off values, amino acids, [b]

Substance	Cut-off value
Alanine	544 µmol/L
Arginine	70.9 µmol/L
Aspartic acid	not determined
Citrulline	93.9 µmol/L
Glutamic acid	not determined
Glycine	742 µmol/L
Leucine	349 µmol/L
Methionine	51.1 µmol/L
Ornithine	not determined
Phenylalanine	136 µmol/L
Proline	not determined
Tyrosine	335 µmol/L
Valine	276 µmol/L

Substance	Cut-off value
Succinylacetone	1.23 µmol/L

Table 12: Cut-off values, acylcarnitines and free carnitine, [b]

Substance	Cut-off value
C0-Carnitine	55.4 µmol/L
C0, lower value (0.1% percentile)	4.76 µmol/L
C2-Carnitine	66.7 µmol/L
C3-Carnitine	7.19 µmol/L
C4-Carnitine	1.08 µmol/L
C5-Carnitine	0.58 µmol/L
C5DC-Carnitine	0.79 µmol/L
C6-Carnitine	0.24 µmol/L
C8-Carnitine	0.27 µmol/L
C10-Carnitine	0.35 µmol/L
C12-Carnitine	0.37 µmol/L
C14-Carnitine	0.56 µmol/L
C16-Carnitine	7.66 µmol/L
C18-Carnitine	2.32 µmol/L

These cut-off values are for guidance purpose only and may vary depending on the patients group and the MS/MS system used. Every screening laboratory should determine its own cut-off values in a pilot study in consultantion with a metabolism expert.

10 Conversion factors

The following tables list conversion factors between mass and molar concentrations and conversely.

Table 13: Conversion factors, amino acids and tyrosine metabolite (SUAC)

Substance	µmol/L to mg/L	mg/L to µmol/L
Alanine	x 0.0891	x 11.223
Arginine	x 0.1742	x 5.7405
Aspartic acid	x 0.1331	x 7.5126
Citrulline	x 0.1752	x 5.7081
Glutamic acid	x 0.1471	x 6.7967
Glycine	x 0.0751	x 13.321
Leucine	x 0.1312	x 7.6237
Methionine	x 0.1492	x 6.7020

Substance	µmol/L to mg/L	mg/L to µmol/L
Ornithine	x 0.1322	x 7.5666
Phenylalanine	x 0.1652	x 6.0536
Proline	x 0.1151	x 8.6858
Succinylacetone	x 0.1581	x 6.3231
Tyrosine	x 0.1812	x 5.5191
Valine	x 0.1172	x 8.5361

Table 14: Conversion factors, acylcarnitines and free carnitine

Substance	µmol/L to mg/L	mg/L to µmol/L
C0-Carnitine	x 0.1612	x 6.2019
C2-Carnitine	x 0.2032	x 4.9203
C3-Carnitine	x 0.2172	x 4.6032
C4-Carnitine	x 0.2312	x 4.3245
C5-Carnitine	x 0.2452	x 4.0776
C5DC-Carnitine	x 0.2753	x 3.6319
C6-Carnitine	x 0.2593	x 3.8559
C8-Carnitine	x 0.2874	x 3.4789
C10-Carnitine	x 0.3154	x 3.1701
C12-Carnitine	x 0.3435	x 2.9109
C14-Carnitine	x 0.3715	x 2.6915
C16-Carnitine	x 0.3996	x 2.5023
C18-Carnitine	x 0.4276	x 2.3384

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 15: Transport conditions for the reagent kit

Product	Transport temperature
Reagent kit (order no. 57000)	+18 to +30 °C
Succinylacetone Upgrade Set (order no. 57111)	+18 to +30 °C

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

Table 16: Storage conditions for the reagents

Product	Storage temperature
Mobile Phase (order no. 57001, 57002)	+18 to +30 °C
Rinsing Solution (order no. 57007)	+18 to +30 °C
Extraction Buffer (order no. 57008)	+18 to +30 °C
Tuning Mix (order no. 57099)	+2 to +8 °C
Internal Standard (order no. 57004)	below –18 °C
Dried Blood Spot Controls (order no. 0191, 0192, 0193)	below –18 °C
Internal Standard, Succinylacetone (order no. 57044)	+2 to +8 °C
Extraction Buffer, Succinylacetone (order no. 57012)	below –18 °C
Tuning Mix, Succinylacetone (order no. 57098)	+2 to +8 °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 on the stability of the internal standards after reconstitution, the Extraction Buffer Succinlyacetone after first thawing and the controls are given in sections 5.2 to 5.4.

12 Waste disposal

Mobile Phase (order no. 57001, 57002), Rinsing Solution (order no. 57007), Extraction Buffer (order no. 57008 and 57012), Tuning Mix (order no. 57098 and 57099)), the reconstituted Internal Standard (order no. 57004, solved in order no. 57008) and Internal Standard for succinylacetone (order no. 57044) contain organic solvents. Dispose product residues into a collection container for organic halogen-free solvents.

Residues of patient samples and prepared samples as well as controls must be collected and disposed of as potentially infectious waste.

The mentioned solutions must not be disposed together 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 only access permitted to authorized parties.

13 Interference testing

Please note:

- Isoleucine interferes with leucine. The measured concentration in the sample is a sum of both amino acids.

- Hydroxyproline interferes with leucine. The standard value of hydroxyproline is negligible compared to leucine. Hydroxyproline shouldn't cause any false increased leucine concentrations during routine measurement.

- Methioninesulfone interferes with tyrosine. Methioninesulfone is a degradation product of methionine. The normal range of methionine in newborns is approx. 20 µmol/L. The maximum methioninesulfone concentrations are expected to be in this range. The pathological range of tyrosine is at approx. 300 µmol/L. Therefore methioninesulfone shouldn't cause any essential increased tyrosine concentrations during routine measurement.

- Methioninesulfoxide interferes with tyrosine and phenylalanine. Methioninesulfoxide is an oxidation product of methionine. The normal range of methionine in newborns is approx. 20 µmol/L. The maximum methioninesulfoxide concentrations are expected to be in this range. The pathological range of tyrosine and phenylalanine is at approx. 100–300 µmol/L. Therefore methioninesulfoxide shouldn't cause any essential increased concentrations during routine measurement.

- Asparagine interferes with ornithine. The maximum concentration of asparagine in infants is up to 140 µmol/L. Only asparagine concentrations over 300 µmol/L lead to an increased ornithine concentration of 20 %. Asparagine shouldn't cause any false increased ornithine concentrations during routine measurement.

- Sarosine interferes with alanine. The concentration of sarcosine in relation to alanine, however, is neglectable. Therefore, sarcosine should not cause falsely increased alanine results.

- Creatine, an endogenous substance which is also used as dietary supplement to increase muscle building, interferes with alanine and leucine.

- 4-aminoantipyrine, a metabolite of the analgesic metamizol (e.g. Novalgin®), interferes with C2-carnitine.

- The drug substances allopurinol (a drug to decrease uric acid levels), triamterene (a diuretic), gabapentine (an antiepileptic) and acetylcysteine (an expectorant) interfere with the mass transition (MRM) of succinylacetone and lead to falsely positive succinylacetone values.

- The drug substances azathioprine (an immunosuppressant) and alloxanthine/oxypurinol (metabolite of allopurinol) interfere with the mass transition (MRM) of the internal standard of succinylacetone and lead to falsely positive succinylacetone values.

- Lidocaine interferes with the mass transition (MRM) of the internal standard of C4-carnitine and thus can lead to falsely negative values of C4-carntine.
- Gabapentin (an antiepileptic) interferes with the mass transition (MRM) of the internal standard of C5DC-carnitine and can lead to falsely positive C5DC-carnitine values.

- Levetiracetam, an antiepileptic, interferes with the mass transition (MRM) of C12-carnitine and thus can lead to falsely negative values of C12-carnitine.

- Pregabalin, an antiepileptic, interferes with the mass transition (MRM) of the internal standard of succinylacetone and can thus lead to falsely negative values of succinylacetone.

- Prilocaine, a local anesthetic, interferes with the mass transition (MRM) of the internal standard of C3-carnitine and thus can lead to falsely negative values of C3-carnitine.

- Isobaric acylcarnitines with identical fragmentations are measures as a sum. This applies to following acylcarnitine pairs: C3DC/C4OH; C4DC/C5OH; C5DC/C6OH etc.

- Additives in plastic materials (96 well plates, protective sheets) used in the sample preparation, may interfere considerable with some acylcarnitines causing false positive results. Hence this reagent kit contains all required reagents and sample preparation receptacles, which have all been tested to be suitable for interference-free analysis.

- In patients with parenterale diet, replacing tyrosine by acetyl tyrosine, tyrosine concentrations may be degraded. The elevated Phe/Tyr quotient may falsely indicate phenylketonuria, though the phenylalanine concentration is in the normal range.

- In patients treated with certain antibiotics (e.g. pivmecillinam, pivampicillin), pivalic acid may be formed by metabolisation and, by further processes, pivaloylcarnitine. Pivaloylcarnitine is isobaric to isovalerylcarnitine (C5-carnitine), which can lead to a peak overlap that suggests isovalerianacidemia, although no metabolic disorder is present.

The following isobaric compounds, drug substances, drugs of abuse and metabolites were tested. No interferences of the analysis were observed.

Isobaric compounds:
Acetylserine, malic acid, aminocaproic acid, amitriptyline, aripiprazole, atomoxetine, atropine, betamethasone, budesonide, bupivacaine, carbimazole, cefalexin, cetirizine, chlorprothixene, clemastine (meclastin), clenbuterol, clomipramine, clonidine, clotrimazole, colchicine, cyclophosphamide, cyproterone, diphenhydramine, domperidone, ethambutol, etilefrine, formiminoglutamic acid, fluticasone, fluvoxamine, fosfomycin, hydroxychloroquine, imipramine, irbesartan, isoniazid, lactulose, maprotiline, melperone, mepivacaine, mercaptopurine, mesalazine, metronidazole, moxifloxacin, naproxen, nitrofurantoin, norfloxacin, nortriptyline, ofloxacin, ondansetron, opipramol, oxcarbazepine, oxymetazoline, paliperidone, penicillamine, phenylbutazone, pipamperone, proguanil, propofol, propranolol, propylthiouracil, propyphenazone, pseudoephedrine, pyrazinamide, pyridostigmine, pyridoxine, pyrimethamine, ramipril, ranitidine, reproterol, ropivacaine, sotalol, spironolactone, sulbactam, sulfasalazine, terbinafine, theophylline, thiamazole, thioridazine, timolol, tolperisone, tranylcypromine, trimethoprim, venlafaxine, xylometazoline, zolmitriptan, zuclopenthixol

Drugs/metabolites:
Acetazolamide, acetylsalicylic acid, aciclovir, alprazolam, α-hydroxyalprazolam, amikacin, amlodipine, amoxicillin, ampicillin, azithromycin, benzocaine, betaine, bisoprolol, bromazepam, 3-OH-bromazepam, brotizolam, buprenorphine, captopril, carbamazepine, carbamazepine-10,11-epoxide, carbamylglutamate, cephradine, chloramphenicol, chlordiazepoxide, chlorhexidine, cimetidine, ciprofloxacin, clarithromycin, clobazam, clonazepam, 7-aminoclonazepam, codeine, demoxepam, dexamethasone, dextromethorphane, diazepam, diclofenac, digitoxin, digoxin, dihydrocodeine, 2,3-dihydroxybenzoic acid, disopyramide, EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine), enalaprilat, erythromycin, estazolam, fentanyl, norfentanyl, flunitrazepam, desmethylflunitrazepam, 7-aminoflunitrazepam, flurazepam, desalkylflurazepam, furosemide, ganciclovir, gentamicin, hydrochlorothiazide, ibuprofen, isosorbiddinitrate, itraconazole, ketamine, norketamine, ketoconazole, levofloxacin, levothyroxine, lorazepam, lormetazepam, medazepam, meperidine, normeperidine, metformin HCl (1,1-dimethylbiguanide), methadone, methicillin, methylphenidate, methylprednisolone, , metoclopramide, metoprolol, midazolam, α-hydroxymidazolam, morphine, mycophenolic acid, mycophenolic acid glucuronide, *N*-acetyl-procainamide, nadolol, naloxone, naltrexone, sodium benzoate, sodium fluoride, sodium phenylbutyrate, *N*-desmethyldiazepam, neomycin, nitrazepam, 7-aminonitrazepam, nifedipine, nitisinone, norbuprenorphine, norclobazam, norverapamil, omeprazole, oxazepam, oxycodone, paracetamol (4-acetamidophenol or acetaminophen), penicillin G, penicillin V, phenobarbital, phenytoin, prazepam, prazosin, prednisolone, prednisone, procainamide, promethazine, (±)-propranolol, propoxyphene, quetiapine, norpropoxyphene, ranitidine, rifampicin, risperidone, ritalinic

acid, salbutamol (albuterol), salicylic acid (2-hydroxybenzoic acid), sapropterin, streptomycin, sufentanil, sulfamethoxazole, tapentadol, nortapentadol, temazepam, thiopental, tilidine, nortilidine, tramadol, O-DM-tramadol, triazolam, α-hydroxytriazolam, trimethoprim, valproic acid, vancomycin, verapamil, zaleplon, zolpidem, zopiclone.

Drugs of abuse/metabolites:
2C-B (4-bromo-2,5-dimethoxyphenylethylamine), 2C-I (2,5-dimethoxy-4-iodophenethylamine), 2-Oxo-3-hydroxy-LSD, 6-monoacetylmorphine, acetylcodeine, allobarbital, amobarbital, amphetamine, barbital, BDB (1-(1,3-benzodioxole-5-yl)-2-butylamine), benzoylecgonine, butalbital, butylone, cathinone, cocaethylene, cocaine, d, l-11-nor-Δ9-THC-carboxylic acid, hexobarbital, hydrocodone, hydromorphone, LSD (lysergic acid diethylamide), MBDB (2-methylamino-1-(3,4-methylenedioxyphenyl)butane), MDA (3,4-methylenedioxyamphetamine), MDEA (3,4-methylenedioxy-*N*-ethylamphetamine), MDMA (3,4-methylenedioxy-*N*-methylamphetamine), MDPV (methylenedioxypyrovalerone), meconin, mephedrone, mescaline, methamphetamine, methaqualone, methylone, norcocaine, norcodeine, oxymorphone, papaverine, PCP (phenylcyclohexylpiperidine), pentobarbital, PMA (4-methoxyamphetamine), secbutabarbital, secobarbital, thebaine.

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.

14 Troubleshooting

Table 17: Troubleshooting

Problem	Possible cause	Remedy
Gradient profile can not be generated	HPLC pump	Check pump (air, leaks)
	Air in the system	Degas HPLC system (purge)
	Flow rate not constant	Check pump
Interferering signals	Injection system contaminated	Clean with methanol or inject mobile phase 10 x
	Autosampler vials contaminated	Use new vials, or clean vials with methanol
	Vial seals	Use other seals
No signal	Defective injector	Check injector
	Defective pump	Check pump
	MS/MS-System not ready	Check MS/MS system
Weakened sensitivity	Ion source contaminated	Clean ion source
	Mass spectrometer contaminated	Clean mass spectrometer
	Injection valve leaking	Check injector
	Multiplier aged	Replace multiplier or increase voltage
Small signal intensities	Inaccurate masses	Perform mass calibration
	Autosampler not accurate	Check accuracy

Problem	Possible cause	Remedy
No vacuum	Defective vacuum pump	Check all vacuum pumps
	Vacuum system leaks	Check vacuum tubes and connections
No gas supply	Defective nitrogen generator	Check nitrogen generator
	Defective compressor	Check compressor
	Empty gas cylinder	Replace gas cylinder
	Gas pressure out off rated value	Adjust gas pressure

If you have any questions concerning trouble shooting, 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 Literature

1 Gemeinsamer Bundesausschuss: Richtlinie des Gemeinsamen Bundesausschusses über die Früherkennung von Krankheiten bei Kindern. Kinder-RL, 2021.

2 Hall EM, Flores SR, Jesús VR de: Influence of Hematocrit and Total-Spot Volume on Performance Characteristics of Dried Blood Spots for Newborn Screening. *Int J Neonatal Screen* 2015; 1:69–78.

3 Clinical Laboratory Standards Institute (CLSI): NBS01-Ed7: Dried Blood Spot Specimen Collection for Newborn Screening. Approved Standard 2021.

4 Miller IV JH: An On-card Approach for Assessment of Hematocrit on Dried Blood Spots which Allows for Correction of Sample Volume. *J Anal Bioanal Techniques* 2013; 04.

5 Clinical Laboratory Standards Institute (CLSI): NBS04: Newborn Screening by Tandem Mass Spectrometry 2017.

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 18: Hazard and precautionary statements

Pictograms	Hazard and precautionary statements
Mobile Phase (order no. 57001, 57002)	
	**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 precautionary measures against static discharge. P280 Wear protective gloves/protective clothing/eye protection/face protection.
Rinsing Solution (order no. 57007)	
	**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 precautionary measures against static discharge. P280 Wear protective gloves/protective clothing/eye protection/face protection.
Extraction Buffer (order no. 57008)	
	**Danger** H225 Highly flammable liquid and vapour. H301+H311+H331 Toxic if swallowed, in contact with skin or if inhaled. H370 Causes damage to the central nervous system and the visual organs. P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P280 Wear protective gloves/protective clothing/eye protection/face protection. P301+P310 IF SWALLOWED: Immediately call a POISON CENTER/ doctor. P302+P352 IF ON SKIN: Wash with plenty of water. P403+P233 Store in a well-ventilated place. Keep container tightly closed.

Pictograms	Hazard and precautionary statements
Extraction Buffer (for the Succinylacetone (non derivatised) Upgrade Set, order no. 57012)	
	**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 precautionary measures against static discharge. P280 Wear protective gloves/protective clothing/eye protection/face protection.
Internal Standard (for the Succinylacetone (non derivatised) Upgrade Set, order no. 57044)	
	**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 precautionary measures against static discharge. P280 Wear protective gloves/protective clothing/eye protection/face protection.
Tuning Mix (order no. 57099 and 57098 for the Succinylacetone (non derivatised) Upgrade Set)	
	**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 precautionary measures against static discharge. P280 Wear protective gloves/protective clothing/eye protection/face protection.
These components are not classified as dangerous according to European Union legislation: Internal Standard (order no. 57004) ***MassCheck***® Dried Blood Spot Controls (order no. 0191, 0192, 0193)	

Appendix II: Notes on calculation

The quantification of the analytes is done by comparing the signal intensity with the corresponding isotope-labelled internal standards. Several diagnostically interesting acylcarnitines for which isotopically labelled internal standards are not yet available can also be measured with this reagent kit. Acylcarnitines with the same chain length show approximately the same response factor. It is therefore recommended to quantify these disease markers with deuterated standards of the same chain length.

The concentration of the analyte of interest in the sample is calculated according to the following principle: The ratio between the peak intensity of the analyte and that of the internal standard is determined and multiplied by the concentration of the internal standard. In addition, there are a number of other parameters that are included in the calculation (see below).

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

$$C_{sample}\ [\mu mol/l] = \frac{A_{sample} \times Volume_{ISTD}}{IS_{Sample} \times Volume_{blood\ in\ disk} \times RRF} \times C_{ISTD}$$

–	Concentration of analyte A in the sample	= C_{sample}
–	Signal intensity of analyte A in the sample	= A_{sample}
–	Signal intensity of the internal standard in the sample	= IS_{sample}
–	Sample-related concentration C of the internal standard	= C_{ISTD}
–	Volume of blood in the dried blood spot disk	= $Volume_{blood\ in\ disk}$
–	Volume of the internal standard	= $Volume_{ISTD}$
–	Relative Response Factor	= RRF

The volume of the blood in the dried blood spot disk is usually about 3.11–3.2 µl.

Relative response factors (RRF) can be used according to CLSI standard NBS04-Ed2, 2017 [5] when using different MS instruments and applying the same cut-off values. In this way, for example, different ionisation efficiencies of the different mass spectrometers are taken into account and mathematically compensated. Based on the measurement results of reference samples with known analyte concentrations (e.g. ***MassCheck***® Dried Blood Spot Controls, order no. 0192 and 0193) over several days of analysis, the RRF are calculated for each mass spectrometer according to the following formula:

$$RRF = \frac{\text{Measured analyte concentration}}{\text{Target analyte concentration}}$$

Note on the use of special software for quantitative evaluation:
The volumes of the Internal Standards *57004* and *57044* used for sample preparation differ from each other. When using evaluation programs that allow only one ISTD volume to be entered globally for all analytes, the concentration of the ISTD entered for SUAC-^{13}C5 must be multiplied by the quotient of the volume entered in the software and the volume actually added.

Example:
Volume entered in the software = 100 µL
Volume of *57044* actually added = *75* µL
Concentration of ISTD (batch-dependent, see leaflet) = 0.141 µmol/L

Concentration to be entered in µmol/L:

$$0.141\ \mu mol/L \times \frac{75\ \mu L}{100\ \mu L} = 0.106\ \mu mol/L$$

Appendix III: Performance data

The performance features were determined and verified on the following equipment:

- SCIEX API 3200™ mass spectrometer with Shimadzu LC-20A Prominence HPLC system
- Waters® Quattro Micro™ API mass spectrometer with Waters® Alliance™ HT 2795 HPLC system
- SCIEX API 4000™ mass spectrometer with Shimadzu LC-20A Prominence HPLC system
- Waters® ACQUITY™ TQD mass spectrometer with ACQUITY™ UHPLC® system

Ionisation was done by Electrospray Ionisation (ESI+) for all measurements.

IIIa: Preparation without succinylacetone

Recovery:
The analytical recovery was determined from the slope of the calibration curve of spiked blood samples and diluted standard solutions.

Table 19: Recovery rates, determination with Waters® Quattro Micro™ API mass spectrometer

Substance	Recovery rate	Substance	Recovery rate
Alanine	79 %	C0-Carnitine	85 %
Arginine	80 %	C2-Carnitine	86 %
Aspartic acid	88 %	C3-Carnitine	89 %
Citrulline	94 %	C4-Carnitine	88 %
Glutamic acid	81 %	C5-Carnitine	92 %
Glycine	81 %	C5DC-Carnitine	91 %
Leucine	86 %	C6-Carnitine	90 %
Methionine	90 %	C8-Carnitine	91 %
Ornithine	86 %	C10-Carnitine	92 %
Phenylalanine	94 %	C12-Carnitine	94 %
Proline*	89 %	C14-Carnitine	94 %
Tyrosine	93 %	C16-Carnitine	94 %
Valine	90 %	C18-Carnitine	87 %

* The determination of this data was done on an SCIEX API 4000™.

Table 20: Recovery rates, determination with SCIEX API 3200™ mass spectrometer

Substance	Recovery rate	Substance	Recovery rate
Alanine	73 %	C0-Carnitine	85 %
Arginine	64 %	C2-Carnitine	80 %
Aspartic acid	74 %	C3-Carnitine	72 %
Citrulline	80 %	C4-Carnitine	76 %
Glutamic acid	68 %	C5-Carnitine	88 %
Glycine	78 %	C5DC-Carnitine	102 %
Leucine	76 %	C6-Carnitine	73 %
Methionine	73 %	C8-Carnitine	72 %
Ornithine	85 %	C10-Carnitine	70 %
Phenylalanine	77 %	C12-Carnitine	64 %
Proline	74 %	C14-Carnitine	63 %
Tyrosine	78 %	C16-Carnitine	66 %
Valine	74 %	C18-Carnitine	65 %

Lower limit of quantitation (LLOQ) and linearity (upper limit of quantitation):
The limit of determination and linearity were determined by diluting an eluate of a prepared blood sample with internal standard.

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

Table 21: Limit of quantitation and linearity, determination with Waters® Quattro Micro™ API mass spectrometer, amino acids

Substance	Limit of quantitation (LLOQ), ca.	Linear range up to at least
Alanine	15.6 µmol/L	2000 µmol/L
Arginine	7.8 µmol/L	2000 µmol/L
Aspartic acid	15.6 µmol/L	2000 µmol/L
Citrulline	7.8 µmol/L	2000 µmol/L
Glutamic acid	15.6 µmol/L	2000 µmol/L
Glycine	15.6 µmol/L	2000 µmol/L
Leucine	15.6 µmol/L	2000 µmol/L
Methionine	7.8 µmol/L	2000 µmol/L
Ornithine	7.8 µmol/L	2000 µmol/L
Phenylalanine	7.8 µmol/L	2000 µmol/L
Proline*	4.8 µmol/L	2400 µmol/L
Tyrosine	15.6 µmol/L	2000 µmol/L
Valine	15.6 µmol/L	2000 µmol/L

* The determination of this data was done on an SCIEX API 4000™.

Table 22: Limit of quantitation and linearity, determination with SCIEX API 3200™ mass spectrometer, amino acids

Substance	Limit of quantitation (LLOQ), ca.	Linear range up to at least
Alanine	13.5 µmol/L	2000 µmol/L
Arginine	2.5 µmol/L	2000 µmol/L
Aspartic acid	30.0 µmol/L	2000 µmol/L
Citrulline	2.5 µmol/L	2000 µmol/L
Glutamic acid	25.0 µmol/L	2000 µmol/L
Glycine	10.5 µmol/L	2000 µmol/L
Leucine	13.0 µmol/L	2000 µmol/L
Methionine	2.5 µmol/L	2000 µmol/L
Ornithine	8.0 µmol/L	2000 µmol/L
Phenylalanine	6.0 µmol/L	2000 µmol/L
Proline	5.0 µmol/L	2400 µmol/L
Tyrosine	12.0 µmol/L	2000 µmol/L
Valine	16.5 µmol/L	2000 µmol/L

Table 23: Limit of quantitation and linearity, determination with Waters® Quattro Micro™ API mass spectrometer, acylcarnitines and free carnitine

Substance	Limit of quantitation (LLOQ), ca.	Linear range up to at least
C0-Carnitine	1.6 µmol/L	200 µmol/L
C2-Carnitine	1.6 µmol/L	200 µmol/L
C3-Carnitine	0.2 µmol/L	50 µmol/L
C4-Carnitine	0.2 µmol/L	25 µmol/L
C5-Carnitine	0.2 µmol/L	25 µmol/L
C5DC-Carnitine	0.2 µmol/L	25 µmol/L
C6-Carnitine	0.2 µmol/L	25 µmol/L
C8-Carnitine	0.2 µmol/L	25 µmol/L
C10-Carnitine	0.2 µmol/L	25 µmol/L
C12-Carnitine	0.2 µmol/L	25 µmol/L
C14-Carnitine	0.1 µmol/L	25 µmol/L
C16-Carnitine	0.1 µmol/L	33 µmol/L
C18-Carnitine	0.1 µmol/L	33 µmol/L

Table 24: Limit of quantitation and linearity, determination with SCIEX API 3200™mass spectrometer, acylcarnitines and free carnitine

Substance	Limit of quantitation (LLOQ), ca.	Linear range up to at least
C0-Carnitine	1.5 µmol/L	200 µmol/L
C2-Carnitine	1.0 µmol/L	200 µmol/L
C3-Carnitine	0.1 µmol/L	50 µmol/L
C4-Carnitine	0.2 µmol/L	25 µmol/L
C5-Carnitine	0.2 µmol/L	25 µmol/L
C5DC-Carnitine	0.1 µmol/L	25 µmol/L
C6-Carnitine	0.1 µmol/L	25 µmol/L
C8-Carnitine	0.2 µmol/L	25 µmol/L
C10-Carnitine	0.1 µmol/L	25 µmol/L
C12-Carnitine	0.1 µmol/L	25 µmol/L
C14-Carnitine	0.1 µmol/L	25 µmol/L
C16-Carnitine	0.1 µmol/L	33 µmol/L
C18-Carnitine	0.1 µmol/L	33 µmol/L

Intra-assay precision:
The intra-assay precision was determined at three concentrations by means of multiple clean up (n = 10) of the same specimen.

Table 25: Intra-assay precision, determination with SCIEX 4500MD™ mass spectrometer, amino acids, n = 10

Substance	Coefficient of variation (concentration of analyte)		
Alanine	5.2 % (281 µmol/L)	4.7 % (390 µmol/L)	4.6 % (745 µmol/L)
Arginine	4.2 % (7 µmol/L)	10.4 % (12 µmol/L)	3.4 % (125 µmol/L)
Aspartic acid	4.7 % (96 µmol/L)	5.1 % (147 µmol/L)	5.8 % (345 µmol/L)
Citrulline	6.8 % (20 µmol/L)	7.3 % (61 µmol/L)	5.0 % (256 µmol/L)
Glutamic acid	4.9 % (218 µmol/L)	3.0 % (388 µmol/L)	3.8 % (749 µmol/L)
Glycine	10.9 % (241 µmol/L)	6.0 % (336 µmol/L)	5.0 % (1085 µmol/L)
Leucine	6.4 % (181 µmol/L)	2.9 % (281 µmol/L)	2.8 % (592 µmol/L)
Methionine	6.8 % (19 µmol/L)	4.0 % (62 µmol/L)	2.9 % (268 µmol/L)
Ornithine	5.6 % (95 µmol/L)	7.1 % (217 µmol/L)	5.4 % (626 µmol/L)
Phenylalanine	4.7 % (69 µmol/L)	3.4 % (129 µmol/L)	3.0 % (566 µmol/L)
Proline*	8.8 % (231 µmol/L)	6.4 % (525 µmol/L)	5.4 % (834 µmol/L)
Tyrosine	4.7 % (69 µmol/L)	2.9 % (158 µmol/L)	3.5 % (488 µmol/L)
Valine	4.8 % (111 µmol/L)	2.3 % (171 µmol/L)	4.3 % (365 µmol/L)

* The determination of this data was done on an SCIEX API 4000™.

Table 26: Intra-assay precision, determination with SCIEX API 3200™ mass spectrometer, amino acids, n = 10

Substance	Coefficient of variation (concentration of analyte)		
Alanine	3.5 % (378 µmol/L)	2.6 % (411 µmol/L)	4.0 % (752 µmol/L)
Arginine	2.5 % (19 µmol/L)	1.7 % (80 µmol/L)	3.3 % (167 µmol/L)
Aspartic acid	3.9 % (180 µmol/L)	2.5 % (231 µmol/L)	4.0 % (461 µmol/L)
Citrulline	3.8 % (26 µmol/L)	4.6 % (73 µmol/L)	3.8 % (275 µmol/L)
Glutamic acid	2.0 % (482 µmol/L)	3.8 % (433 µmol/L)	4.3 % (876 µmol/L)
Glycine	8.3 % (285 µmol/L)	5.9 % (389 µmol/L)	7.2 % (1032 µmol/L)
Leucine	3.4 % (348 µmol/L)	2.0 % (351 µmol/L)	4.3 % (637 µmol/L)
Methionine	4.7 % (36 µmol/L)	3.3 % (73 µmol/L)	4.3 % (238 µmol/L)
Ornithine	2.3 % (251 µmol/L)	1.9 % (220 µmol/L)	4.0 % (677 µmol/L)
Phenylalanine	1.8 % (117 µmol/L)	2.1 % (196 µmol/L)	4.2 % (572 µmol/L)
Proline	3.5 % (332 µmol/L)	2.6 % (481 µmol/L)	4.2 % (716 µmol/L)
Tyrosine	2.2 % (96 µmol/L)	1.9 % (192 µmol/L)	4.3 % (585 µmol/L)
Valine	3.1 % (221 µmol/L)	1.9 % (267 µmol/L)	4.0 % (520 µmol/L)

Table 27: Intra-assay precision, determination with Waters® Quattro Micro™ API mass spectrometer, acylcarnitines und free carnitine, n = 10

Substance	Coefficient of variation (concentration of analyte)		
C0-Carnitine	4.7 % (26.4 µmol/L)	3.0 % (48.0 µmol/L)	4.1 % (105 µmol/L)
C2-Carnitine	5.5 % (15.2 µmol/L)	2.7 % (39.5 µmol/L)	2.6 % (81.6 µmol/L)
C3-Carnitine	6.1 % (1.78 µmol/L)	2.7 % (6.54 µmol/L)	2.7 % (15.1 µmol/L)
C4-Carnitine	6.2 % (0.34 µmol/L)	4.9 % (1.15 µmol/L)	4.0 % (4.42 µmol/L)
C5-Carnitine	8.1 % (0.14 µmol/L)	4.0 % (0.60 µmol/L)	3.9 % (2.35 µmol/L)
C5DC-Carnitine	12.2 % (0.19 µmol/L)	12.2 % (0.66 µmol/L)	9.0 % (2.38 µmol/L)
C6-Carnitine	9.6 % (0.06 µmol/L)	3.2 % (0.48 µmol/L)	4.1 % (2.11 µmol/L)
C8-Carnitine	8.5 % (0.10 µmol/L)	3.9 % (0.57 µmol/L)	4.2 % (2.31 µmol/L)
C10-Carnitine	8.2 % (0.16 µmol/L)	3.7 % (0.63 µmol/L)	3.1 % (2.44 µmol/L)
C12-Carnitine	8.1 % (0.07 µmol/L)	3.8 % (0.52 µmol/L)	3.5 % (2.25 µmol/L)
C14-Carnitine	10.3 % (0.10 µmol/L)	4.6 % (0.50 µmol/L)	3.8 % (2.00 µmol/L)
C16-Carnitine	5.7 % (0.72 µmol/L)	4.9 % (4.56 µmol/L)	3.3 % (12.2 µmol/L)
C18-Carnitine	6.6 % (0.54 µmol/L)	4.9 % (2.26 µmol/L)	5.2 % (7.69 µmol/L)

Table 28: Intra-assay precision, determination with SCIEX API 3200™ mass spectrometer, acylcarnitines and free carnitine, n = 10

Substance	Coefficient of variation (concentration of analyte)		
C0-Carnitin	4.0 % (26.5 µmol/L)	3.1 % (40.7 µmol/L)	4.5 % (108 µmol/L)
C2-Carnitin	2.8 % (10.3 µmol/L)	3.2 % (28.5 µmol/L)	4.4 % (75.4 µmol/L)
C3-Carnitin	5.5 % (1.30 µmol/L)	3.1 % (5.84 µmol/L)	4.9 % (16.7 µmol/L)
C4-Carnitin	3.5 % (0.19 µmol/L)	3.3 % (1.04 µmol/L)	5.5 % (4.84 µmol/L)
C5-Carnitin	6.6 % (0.09 µmol/L)	2.9 % (0.60 µmol/L)	4.9 % (2.70 µmol/L)
C5DC-Carnitin	9.0 % (0.16 µmol/L)	8.7 % (0.58 µmol/L)	5.1 % (2.16 µmol/L)
C6-Carnitin	9.4 % (0.04 µmol/L)	2.5 % (0.49 µmol/L)	4.7 % (2.43 µmol/L)
C8-Carnitin	3.0 % (0.04 µmol/L)	4.6 % (0.51 µmol/L)	4.1 % (2.53 µmol/L)
C10-Carnitin	3.1 % (0.08 µmol/L)	4.9 % (0.50 µmol/L)	6.0 % (2.47 µmol/L)
C12-Carnitin	6.8 % (0.04 µmol/L)	4.1 % (0.47 µmol/L)	5.8 % (2.36 µmol/L)
C14-Carnitin	7.8 % (0.08 µmol/L)	4.6 % (0.52 µmol/L)	3.9 % (2.45 µmol/L)
C16-Carnitin	2.9 % (0.79 µmol/L)	4.2 % (4.91 µmol/L)	5.5 % (14.3 µmol/L)
C18-Carnitin	5.1 % (0.56 µmol/L)	2.8 % (2.54 µmol/L)	5.5 % (9.38 µmol/L)

Inter-assay precision:
The inter-assay precision was determined at three concentrations by means of multiple clean up (n = 10) of the same specimen in 10 different test series:

Table 29: Inter-assay precision, determination with Waters® Quattro Micro™ API mass spectrometer, amino acids, n = 100

Substance	Coefficient of variation (concentration of analyte)		
Alanine	6.6 % (281 µmol/L)	7.9 % (390 µmol/L)	5.6 % (745 µmol/L)
Arginine	9.3 % (7 µmol/L)	17.6 % (12 µmol/L)	10.3 % (125 µmol/L)
Aspartic acid	6.4 % (96 µmol/L)	6.7 % (147 µmol/L)	6.1 % (345 µmol/L)
Citrulline	11.4 % (20 µmol/L)	7.6 % (61 µmol/L)	5.7 % (256 µmol/L)
Glutamic acid	6.1 % (218 µmol/L)	7.0 % (388 µmol/L)	5.4 % (749 µmol/L)
Glycine	8.4 % (241 µmol/L)	8.3 % (336 µmol/L)	6.0 % (1085 µmol/L)
Leucine	5.8 % (181 µmol/L)	6.3 % (281 µmol/L)	5.5 % (592 µmol/L)
Methionine	6.7 % (19 µmol/L)	6.3 % (62 µmol/L)	5.4 % (268 µmol/L)
Ornithine	9.4 % (95 µmol/L)	8.8 % (217 µmol/L)	8.6 % (626 µmol/L)
Phenylalanine	5.9 % (69 µmol/L)	6.6 % (129 µmol/L)	5.0 % (566 µmol/L)
Proline*	7.7 % (241 µmol/L)	8.2 % (562 µmol/L)	7.1 % (869 µmol/L)
Tyrosine	5.3 % (69 µmol/L)	5.8 % (158 µmol/L)	4.9 % (488 µmol/L)
Valine	7.3 % (111 µmol/L)	8.2 % (171 µmol/L)	5.7 % (365 µmol/L)

* The determination of this data was done on an SCIEX API 4000™.

Table 30: Inter-assay precision, determination with mass spectrometer SCIEX API 3200™, amino acids, n = 100

Substance	Coefficient of variation (concentration of analyte)		
Alanine	10.1 % (392 µmol/L)	10.2 % (411 µmol/L)	7.7 % (752 µmol/L)
Arginine	4.2 % (18 µmol/L)	3.6 % (80 µmol/L)	3.5 % (167 µmol/L)
Aspartic acid	6.2 % (177 µmol/L)	6.3 % (231 µmol/L)	4.8 % (461 µmol/L)
Citrulline	6.1 % (25 µmol/L)	4.6 % (73 µmol/L)	5.2 % (275 µmol/L)
Glutamic acid	5.7 % (469 µmol/L)	5.1 % (433 µmol/L)	5.9 % (876 µmol/L)
Glycine	12.0 % (295 µmol/L)	11.9 % (389 µmol/L)	10.3 % (1032 µmol/L)
Leucine	5.1 % (345 µmol/L)	5.5 % (351 µmol/L)	5.2 % (637 µmol/L)
Methionine	5.7 % (35 µmol/L)	5.3 % (73 µmol/L)	5.0 % (238 µmol/L)
Ornithine	4.8 % (236 µmol/L)	3.8 % (220 µmol/L)	5.0 % (677 µmol/L)
Phenylalanine	5.2 % (118 µmol/L)	7.0 % (196 µmol/L)	5.4 % (572 µmol/L)
Proline	6.3 % (330 µmol/L)	5.3 % (481 µmol/L)	5.2 % (716 µmol/L)
Tyrosine	6.1 % (96 µmol/L)	7.3 % (192 µmol/L)	5.5 % (585 µmol/L)
Valine	6.2 % (216 µmol/L)	5.0 % (267 µmol/L)	5.4 % (520 µmol/L)

Table 31: Inter-assay precision, determination with Waters® Quattro Micro™ API mass spectrometer, Acylcarnitine und freies Carnitin, n = 100

Substance	Coefficient of variation (concentration of analyte)		
C0-Carnitine	6.7 % (26.4 µmol/L)	7.1 % (48.0 µmol/L)	6.1 % (105 µmol/L)
C2-Carnitine	6.1 % (15.2 µmol/L)	6.5 % (39.5 µmol/L)	5.1 % (81.6 µmol/L)
C3-Carnitine	5.8 % (1.78 µmol/L)	6.8 % (6.54 µmol/L)	5.3 % (15.1 µmol/L)
C4-Carnitine	7.0 % (0.34 µmol/L)	6.8 % (1.15 µmol/L)	5.2 % (4.42 µmol/L)
C5-Carnitine	8.5 % (0.14 µmol/L)	7.0 % (0.60 µmol/L)	5.8 % (2.35 µmol/L)
C5DC-Carnitine	14.3 % (0.19 µmol/L)	11.7 % (0.66 µmol/L)	9.0 % (2.38 µmol/L)
C6-Carnitine	13.3 % (0.06 µmol/L)	7.4 % (0.48 µmol/L)	5.6 % (2.11 µmol/L)
C8-Carnitine	9.3 % (0.10 µmol/L)	7.6 % (0.57 µmol/L)	5.6 % (2.31 µmol/L)
C10-Carnitine	7.8 % (0.16 µmol/L)	7.0 % (0.63 µmol/L)	5.5 % (2.44 µmol/L)
C12-Carnitine	9.1 % (0.07 µmol/L)	6.9 % (0.52 µmol/L)	5.7 % (2.25 µmol/L)
C14-Carnitine	8.8 % (0.10 µmol/L)	7.1 % (0.50 µmol/L)	6.1 % (2.00 µmol/L)
C16-Carnitine	6.4 % (0.72 µmol/L)	6.6 % (4.56 µmol/L)	5.5 % (12.2 µmol/L)
C18-Carnitine	6.3 % (0.54 µmol/L)	6.8 % (2.26 µmol/L)	5.5 % (7.69 µmol/L)

Table 32: Inter-assay precision, determination with SCIEX API 3200™ mass spectrometer, acylcarnitines and free carnitine, n = 100

Substance	Coefficient of variation (concentration of analyte)		
C0-Carnitine	6.4 % (26.5 µmol/L)	5.0 % (40.7 µmol/L)	5.3 % (108 µmol/L)
C2-Carnitine	5.4 % (10.2 µmol/L)	5.1 % (28.5 µmol/L)	5.4 % (75.4 µmol/L)
C3-Carnitine	5.9 % (1.27 µmol/L)	5.3 % (5.84 µmol/L)	5.8 % (16.7 µmol/L)
C4-Carnitine	6.6 % (0.18 µmol/L)	5.2 % (1.04 µmol/L)	5.8 % (4.84 µmol/L)
C5-Carnitine	7.6 % (0.08 µmol/L)	5.3 % (0.60 µmol/L)	6.0 % (2.70 µmol/L)
C5DC-Carnitine	13.7 % (0.18 µmol/L)	8.9 % (0.58 µmol/L)	6.6 % (2.16 µmol/L)
C6-Carnitine	9.8 % (0.04 µmol/L)	5.6 % (0.49 µmol/L)	5.5 % (2.43 µmol/L)
C8-Carnitine	6.8 % (0.04 µmol/L)	5.8 % (0.51 µmol/L)	5.4 % (2.53 µmol/L)
C10-Carnitine	6.1 % (0.08 µmol/L)	6.1 % (0.50 µmol/L)	5.7 % (2.47 µmol/L)
C12-Carnitine	7.2 % (0.04 µmol/L)	6.0 % (0.47 µmol/L)	5.7 % (2.36 µmol/L)
C14-Carnitine	7.0 % (0.08 µmol/L)	5.9 % (0.52 µmol/L)	5.0 % (2.45 µmol/L)
C16-Carnitine	5.7 % (0.77 µmol/L)	5.1 % (4.91 µmol/L)	4.8 % (14.3 µmol/L)
C18-Carnitine	5.1 % (0.54 µmol/L)	4.8 % (2.54 µmol/L)	4.9 % (9.38 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.

IIIb: Preparation with succinylacetone

Losses from preparation:
Losses from preparation were determined from the slope ratios of dried whole blood samples which were spiked before and after sample preparation. The losses from preparation are specified as absolute recovery rate.

Table 33: Losses from preparation, determination with Waters® ACQUITY™ TQD mass spectrometer

Substance	Recovery rate	Substance	Recovery rate
Alanine	119 %	C0-Carnitine	92 %
Arginine	74 %	C2-Carnitine	89 %
Aspartic acid	108 %	C3-Carnitine	92 %
Citrulline	130 %	C4-Carnitine	92 %
Glutamic acid	113 %	C5-Carnitine	96 %
Glycine	119 %	C5DC-Carnitine	103 %
Leucine	111 %	C6-Carnitine	99 %
Methionine	102 %	C8-Carnitine	100 %
Ornithine	85 %	C10-Carnitine	98 %
Phenylalanine	114 %	C12-Carnitine	91 %
Proline	107 %	C14-Carnitine	92 %
Tyrosine	119 %	C16-Carnitine	96 %
Valine	111 %	C18-Carnitine	96 %
Succinylacetone	41 %	———	———

Recovery:
Relative recovery was determined with the matrix of whole blood. For this purpose individual samples were spiked repeatedly with the analytes and dried blood spots were produced. Three concentration levels inside the working ranges of the analytes were investigated for this purpose. Recovery is calculated using the following formula:

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

Table 34: Recovery rates, determination with Waters® ACQUITY™ TQD mass spectrometer, amino acids

Substance	Recovery rate in dried blood (spiked concentration)		
Alanine	94 % (124 µmol/L)	96 % (243 µmol/L)	113 % (344 µmol/L)
Arginine	103 % (49.6 µmol/L)	94 % (108 µmol/L)	108 % (147 µmol/L)
Aspartic acid	108 % (45.4 µmol/L)	94 % (106 µmol/L)	114 % (144 µmol/L)
Citrulline	107 % (44.5 µmol/L)	95 % (98.9 µmol/L)	109 % (136 µmol/L)
Glutamic acid	90 % (112 µmol/L)	91 % (231 µmol/L)	105 % (329 µmol/L)
Glycine	98 % (95.7 µmol/L)	99 % (195 µmol/L)	115 % (274 µmol/L)
Leucine	98 % (41.7 µmol/L)	99 % (83.4 µmol/L)	114 % (121 µmol/L)
Methionine	98 % (35.8 µmol/L)	98 % (72.2 µmol/L)	110 % (102 µmol/L)
Ornithine	110 % (37.8 µmol/L)	94 % (87.4 µmol/L)	113 % (112 µmol/L)
Phenylalanine	100 % (53.0 µmol/L)	98 % (107 µmol/L)	110 % (153 µmol/L)
Proline	101 % (39.0 µmol/L)	96 % (81.8 µmol/L)	114 % (114 µmol/L)
Tyrosine	97 % (55.3 µmol/L)	96 % (112 µmol/L)	110 % (158 µmol/L)
Valine	97 % (36.7 µmol/L)	99 % (73.3 µmol/L)	114 % (106 µmol/L)
Succinylacetone	95 % (2.31 µmol/L)	96 % (4.57 µmol/L)	105 % (6.60 µmol/L)

Table 35: Recovery rates, determination with Waters® ACQUITY™ TQD mass spectrometer, acylcarnitines

Substanz	Recovery rate in dried blood (spiked concentration)		
C0-Carnitine	100 % (24.1 µmol/L)	98 % (48.6 µmol/L)	110 % (65.6 µmol/L)
C2-Carnitine	101 % (29.0 µmol/L)	97 % (58.1 µmol/L)	107 % (77.8 µmol/L)
C3-Carnitine	101 % (3.79 µmol/L)	97 % (7.71 µmol/L)	107 % (10.4 µmol/L)
C4-Carnitine	101 % (4.79 µmol/L)	97 % (9.74 µmol/L)	108 % (13.1 µmol/L)
C5-Carnitine	100 % (2.62 µmol/L)	96 % (5.34 µmol/L)	107 % (7.17 µmol/L)
C5DC-Carnitine	100 % (1.85 µmol/L)	96 % (3.77 µmol/L)	106 % (4.98 µmol/L)
C6-Carnitine	102 % (2.42 µmol/L)	96 % (4.98 µmol/L)	106 % (6.70 µmol/L)
C8-Carnitine	101 % (2.33 µmol/L)	97 % (4.76 µmol/L)	107 % (6.38 µmol/L)
C10-Carnitine	102 % (1.99 µmol/L)	98 % (4.06 µmol/L)	106 % (5.52 µmol/L)
C12-Carnitine	102 % (2.40 µmol/L)	99 % (4.88 µmol/L)	106 % (6.69 µmol/L)
C14-Carnitine	104 % (2.23 µmol/L)	99 % (4.57 µmol/L)	107 % (6.30 µmol/L)
C16-Carnitine	105 % (8.81 µmol/L)	101 % (17.8 µmol/L)	106 % (24.9 µmol/L)
C18-Carnitine	109 % (4.37 µmol/L)	103 % (8.94 µmol/L)	106 % (12.9 µmol/L)

Lower limit of quantitation (LLOQ) and linearity (upper limit of quantitation):
The linearity for dried blood spots was determined by spiking whole blood samples with defined amounts of standard substances and dilutions with analyte-free matrix. Dried blood spots were produced from the respective levels. The lower limit of quantification (LLOQ) for dried blood spots was determined by defined dilution of 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 36: Limit of quantitation and linearity, determination with Waters® ACQUITY™ TQD mass spectrometer, amino acids

Substance	LLOQ	Linear range up to at least
Alanine	12 µmol/L	7000 µmol/L
Arginine	7 µmol/L	2500 µmol/L
Aspartic acid	9 µmol/L	2500 µmol/L
Citrulline	1 µmol/L	2500 µmol/L
Glutamic acid	7 µmol/L	7000 µmol/L
Glycine	10 µmol/L	7000 µmol/L
Leucine	4 µmol/L	2500 µmol/L
Methionine	10 µmol/L	2500 µmol/L
Ornithine	3 µmol/L	2500 µmol/L
Phenylalanine	2 µmol/L	2500 µmol/L
Proline	6 µmol/L	2500 µmol/L
Tyrosine	4 µmol/L	2500 µmol/L
Valine	3 µmol/L	2500 µmol/L
Succinylacetone	0.6 µmol/L	120 µmol/L

Table 37: Limit of quantitation and linearity, determination with Waters® ACQUITY™ TQD mass spectrometer, acylcarnitines and free carnitine

Substance	LLOQ	Linear range up to at least
C0-Carnitine	2.0 µmol/L	120 µmol/L
C2-Carnitine	0.15 µmol/L	400 µmol/L
C3-Carnitine	0.04 µmol/L	50 µmol/L
C4-Carnitine	0.03 µmol/L	50 µmol/L
C5-Carnitine	0.02 µmol/L	25 µmol/L
C5DC-Carnitine	0.03 µmol/L	15 µmol/L
C6-Carnitine	0.01 µmol/L	25 µmol/L
C8-Carnitine	0.01 µmol/L	25 µmol/L
C10-Carnitine	0.01 µmol/L	25 µmol/L
C12-Carnitine	0.01 µmol/L	20 µmol/L
C14-Carnitine	0.02 µmol/L	20 µmol/L
C16-Carnitine	0.02 µmol/L	80 µmol/L
C18-Carnitine	0.1 µmol/L	40 µmol/L

Intra-assay precision:
The coefficients of variation were determined on three different concentrations by repeated preparation (n = 21) of the same sample.

Table 38: Intra-assay precision, determination with Waters® ACQUITY™ TQD mass spectrometer, amino acids, n= 21

Substance	Coefficient of variation (concentration of substance)		
Alanine	3.3 % (614 µmol/L)	4.3 % (434 µmol/L)	5.0 % (864 µmol/L)
Arginine	3.4 % (63.7 µmol/L)	2.8 % (105 µmol/L)	5.1 % (282 µmol/L)
Aspartic acid	6.3 % (241 µmol/L)	7.8 % (150 µmol/L)	4.3 % (393 µmol/L)
Citrulline	5.4 % (42.9 µmol/L)	8.6 % (65.1 µmol/L)	6.1 % (265 µmol/L)
Glutamic acid	3.4 % (644 µmol/L)	4.9 % (442 µmol/L)	6.1 % (739 µmol/L)
Glycine	3.7 % (516 µmol/L)	4.7 % (305 µmol/L)	4.2 % (842 µmol/L)
Leucine	2.5 % (281 µmol/L)	4.3 % (328 µmol/L)	5.1 % (636 µmol/L)
Methionine	4.9 % (35.6 µmol/L)	4.8 % (53.5 µmol/L)	5.3 % (201 µmol/L)
Ornithine	3.5 % (275 µmol/L)	5.1 % (178 µmol/L)	5.0 % (435 µmol/L)
Phenylalanine	2.8 % (153 µmol/L)	4.2 % (130 µmol/L)	4.8 % (537 µmol/L)
Proline	2.4 % (283 µmol/L)	3.8 % (218 µmol/L)	4.5 % (600 µmol/L)
Tyrosine	2.9 % (211 µmol/L)	4.0 % (193 µmol/L)	4.4 % (558 µmol/L)
Valine	2.7 % (212 µmol/L)	4.1 % (240 µmol/L)	4.8 % (469 µmol/L)
Succinylacetone	9.2 % (0.61 µmol/L)	6.9 % (1.59 µmol/L)	5.7 % (5.36 µmol/L)

Table 39: Intra-assay precision, determination with Waters® ACQUITY™ TQD mass spectrometer, acylcarnitines and free carnitine, n = 21

Substance	Coefficient of variation (concentration of substance)		
C0-Carnitine	2.7 % (63.9 µmol/L)	3.8 % (53.1 µmol/L)	5.4 % (112 µmol/L)
C2-Carnitine	2.8 % (29.8 µmol/L)	4.2 % (19.6 µmol/L)	5.0 % (54.9 µmol/L)
C3-Carnitine	3.0 % (2.48 µmol/L)	4.0 % (4.41 µmol/L)	4.7 % (12.4 µmol/L)
C4-Carnitine	3.6 % (0.55 µmol/L)	6.1 % (0.93 µmol/L)	5.9 % (4.08 µmol/L)
C5-Carnitine	3.5 % (0.50 µmol/L)	4.3 % (0.54 µmol/L)	3.9 % (2.23 µmol/L)
C5DC-Carnitine	6.7 % (0.17 µmol/L)	6.4 % (0.45 µmol/L)	7.3 % (1.85 µmol/L)
C6-Carnitine	4.6 % (0.16 µmol/L)	4.9 % (0.43 µmol/L)	4.9 % (2.01 µmol/L)
C8-Carnitine	3.5 % (0.21 µmol/L)	4.3 % (0.46 µmol/L)	5.4 % (2.06 µmol/L)
C10-Carnitine	4.0 % (0.16 µmol/L)	5.7 % (0.41 µmol/L)	5.0 % (1.75 µmol/L)
C12-Carnitine	5.3 % (0.14 µmol/L)	5.6 % (0.45 µmol/L)	5.4 % (2.12 µmol/L)
C14-Carnitine	3.6 % (0.30 µmol/L)	5.2 % (0.47 µmol/L)	5.1 % (2.11 µmol/L)
C16-Carnitine	3.4 % (3.23 µmol/L)	5.7 % (4.75 µmol/L)	5.0 % (13.0 µmol/L)
C18-Carnitine	4.3 % (1.49 µmol/L)	5.8 % (2.58 µmol/L)	5.4 % (9.04 µmol/L)

Inter-assay precision:
Determination of the inter-assay precision was done on three different concentrations by repeated preparation (n =8) of the same sample with double injection on 20 different days.

Table 40: Inter-assay precision, determination with Waters® ACQUITY™ TQD mass spectrometer, amino acids, n = 320

Substance	Coefficient of variation (concentration of substance)		
Alanine	5.5 % (603 µmol/L)	5.6 % (459 µmol/L)	5.5 % (919 µmol/L)
Arginine	6.4 % (60.4 µmol/L)	6.2 % (107 µmol/L)	7.0 % (284 µmol/L)
Aspartic acid	7.0 % (243 µmol/L)	8.1 % (146 µmol/L)	8.0 % (390 µmol/L)
Citrulline	7.8 % (42.5 µmol/L)	7.2 % (65.4 µmol/L)	6.4 % (268 µmol/L)
Glutamic acid	4.9 % (623 µmol/L)	4.6 % (451 µmol/L)	5.8 % (752 µmol/L)
Glycine	5.0 % (513 µmol/L)	4.6 % (310 µmol/L)	5.2 % (854 µmol/L)
Leucine	4.6 % (277 µmol/L)	4.4 % (336 µmol/L)	5.2 % (660 µmol/L)
Methionine	6.7 % (35.4 µmol/L)	5.3 % (53.7 µmol/L)	5.4 % (205 µmol/L)
Ornithine	6.7 % (258 µmol/L)	7.1 % (190 µmol/L)	7.7 % (468 µmol/L)
Phenylalanine	4.9 % (149 µmol/L)	5.1 % (136 µmol/L)	5.5 % (564 µmol/L)
Proline	4.3 % (280 µmol/L)	4.3 % (226 µmol/L)	5.7 % (628 µmol/L)
Tyrosine	4.9 % (209 µmol/L)	4.7 % (194 µmol/L)	5.1 % (571 µmol/L)
Valine	5.7 % (210 µmol/L)	6.0 % (257 µmol/L)	6.2 % (507 µmol/L)
Succinylaceton	10.0 % (0.58 µmol/L)	6.5 % (1.55 µmol/L)	8.6 % (5.75 µmol/L)

Table 41: Inter-assay precision, determination with Waters® ACQUITY™ TQD mass spectrometer, acylcarnitines and free carnitine, n = 320

Substance	Coefficient of variation (concentration of substance)		
C0-Carnitine	4.7 % (63.0 µmol/L)	5.1 % (55.1 µmol/L)	5.9 % (118 µmol/L)
C2-Carnitine	4.3 % (29.5 µmol/L)	4.3 % (19.6 µmol/L)	5.4 % (55.9 µmol/L)
C3-Carnitine	4.6 % (2.46 µmol/L)	4.3 % (4.44 µmol/L)	5.4 % (12.7 µmol/L)
C4-Carnitine	5.0 % (0.55 µmol/L)	5.0 % (0.92 µmol/L)	5.8 % (4.11 µmol/L)
C5-Carnitine	5.5 % (0.50 µmol/L)	4.9 % (0.53 µmol/L)	5.2 % (2.22 µmol/L)
C5DC-Carnitine	10.8 % (0.17 µmol/L)	7.5 % (0.44 µmol/L)	6.7 % (1.80 µmol/L)
C6-Carnitine	6.1 % (0.16 µmol/L)	5.2 % (0.43 µmol/L)	5.5 % (2.03 µmol/L)
C8-Carnitine	6.0 % (0.21 µmol/L)	5.0 % (0.45 µmol/L)	5.4 % (2.08 µmol/L)
C10-Carnitine	5.7 % (0.16 µmol/L)	4.8 % (0.41 µmol/L)	5.8 % (1.76 µmol/L)
C12-Carnitine	6.0 % (0.14 µmol/L)	4.7 % (0.45 µmol/L)	6.1 % (2.14 µmol/L)
C14-Carnitine	5.0 % (0.31 µmol/L)	4.7 % (0.47 µmol/L)	6.1 % (2.13 µmol/L)
C16-Carnitine	4.7 % (3.24 µmol/L)	4.8 % (4.71 µmol/L)	6.1 % (13.0 µmol/L)
C18-Carnitine	5.1 % (1.48 µmol/L)	5.2 % (2.53 µmol/L)	6.6 % (8.91 µmol/L)

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 42: Tolerance ranges HPLC system

HPLC system	Tolerance range
Injection volume	≤ 10 µL

Table 43: Tolerance ranges sample preparation

Sample praparation	Tolerance range
Extraction of succinylacetone Temperature and shaking speed (chap. 5.5.2, step 4)	+45 to +50 °C and 500 to 700 rpm

Appendix IV: 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 44: 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