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Rapid and efficient LC-MS/MS diagnosis of inherited metabolic disorders: a semi-automated workflow for analysis of organic acids, acylglycines, and acylcarnitines in urine

Abstract

Objectives: The analysis of organic acids in urine is an important part of the diagnosis of inherited metabolic disorders (IMDs), for which gas chromatography coupled with mass spectrometry is still predominantly used.
Methods: Ultra-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for urinary organic acids, acylcarnitines and acylglycines was developed and validated. Sample preparation consists only of dilution and the addition of internal standards. Raw data processing is quick and easy using selective scheduled multiple reaction monitoring mode. A robust standardised value calculation as a data transformation together with advanced automatic visualisation tools are applied for easy evaluation of complex data.
Results: The developed method covers 146 biomarkers consisting of organic acids (n=99), acylglycines (n=15) and acylcarnitines (n=32) including all clinically important isomeric compounds present. Linearity with $r^2>0.98$ for 118 analytes, inter-day accuracy between 80 and 120 % and imprecision under 15 % for 120 analytes were achieved. Over 2 years, more than 800 urine samples from children tested for IMDs were analysed. The workflow was evaluated on 93 patient samples and ERNDIM External Quality Assurance samples involving a total of 34 different IMDs.
Conclusions: The established LC-MS/MS workflow offers a comprehensive analysis of a wide range of organic acids, acylcarnitines and acylglycines in urine to perform effective, rapid and sensitive semi-automated diagnosis of more than 80 IMDs.

Keywords: inherited metabolic disorders; liquid chromatography; mass spectrometry; organic acidurias.

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Introduction

Organic acidurias are a heterogeneous group of inherited metabolic disorders (IMDs) resulting in the accumulation of organic acids (OA) in body fluids. The accumulation of OA, caused by a deficiency of an enzyme or transport protein in one of the cellular pathways [1, 2], disrupts the metabolic homeostasis (predominantly acid-base balance) leading to metabolic acidosis, ketosis, and other metabolic consequences [3, 4]. The typical clinical presentation of these disorders appears within the first weeks of life of the newborn and may present metabolic difficulties that develop further into hypotonia, failure to thrive, poor feeding, vomiting,

lethargy, and developmental delay [3, 4]. Early correct diagnosis and supportive treatment can prevent the most severe manifestations of the disease, thereby improving the quality and length of the child's life [5].

OA are characterised as polar substances containing one or more carboxyl or other (hydroxy-, keto-, side chain) functional groups. In some cases of organic acidurias, toxic acyl-CoAs accumulated in the body are eliminated by the kidneys as OA and conjugated with glycine/carnitine in the mitochondria as part of the detoxification mechanism. The resulting conjugates, acylglycines (AG) and acylcarnitines (AC) are eliminated from the body in the urine along with other OA. These are key metabolites in the diagnosis of organic acidurias [6].

The analysis of OA has been a domain of gas chromatography coupled with mass spectrometry (GC-MS) used IMD diagnosis for many years [7, 8]. For clinical use, however, this is a time-consuming and laborious technique and is prone to matrix effects and other issues due to the necessity of analyte extraction and derivatization. In addition, due to complex sample preparation, loss of analytes may occur. Even the data evaluation itself is challenging and protracted, requiring experienced personnel. Therefore, in recent years, this technique has been replaced by liquid chromatography with mass spectrometry (LC-MS), including [9–11] or excluding sample derivatization [12–14]. In addition, the analysis of OA in urine can also be performed using nuclear magnetic resonance spectroscopy [15, 16], also used in the screening of IMDs [17–20] however it is not widely applied due to limited sensitivity.

The objective of this work was to develop and validate semi-automated workflow for rapid and efficient diagnosis of inherited metabolic disorders based on ultra-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of a wide metabolic spectrum of OA, AG and AC in urine as an advanced alternative to GC-MS.

Materials and methods

Overall workflow including analytical strategy of the development and validation of the method is schematically represented in Figure 1.

Chemicals

Water, acetonitrile, formic acid, and methanol were purchased in LC-MS grade from Honeywell Riedel-de Haën (Seelze, Germany). Sigmatrix Urine Diluent was retrieved from Sigma-Aldrich (St. Louis, USA). A list of all chemical standards (99 OA, 15 AG, 30 AC) used, including internal standards (22 IS), is provided in Supplementary Table 1.
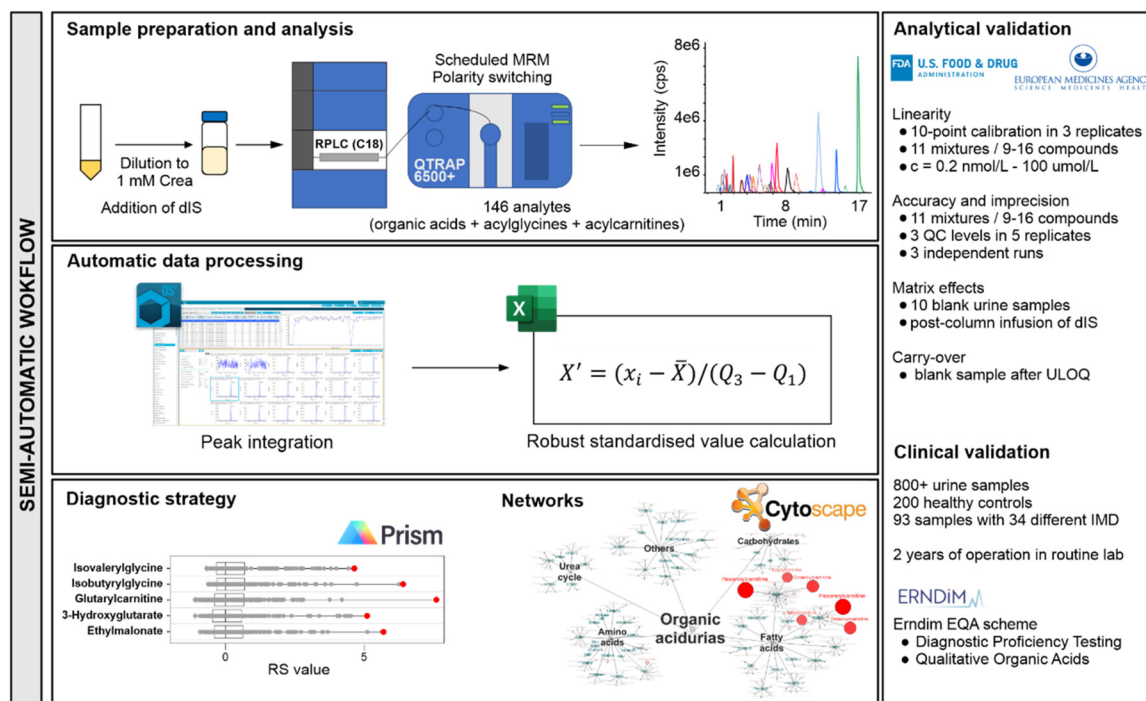

Figure 1: Schematic representation of the workflow and validation.

Preparation of stock solutions and internal standard mixture

All OA, AG, and AC standards were prepared as 10 mmol/L stock solutions in LC-MS water and stored at −20 °C. IS mixture was prepared by mixing IS stock solutions into a 10 mL flask as follows: 22 μL of 224 mmol/L lactic acid-$^{13}C_3$, 50 μL of 41.3 mmol/L methyl-$^2H_3$-malonic acid, 10 μL of 100 μmol/L isovaleryl-$^2H_9$-carnitine, 200 μL of 10 mmol/L homovanillic acid-$^{13}C_6,^{18}O$, 11.5 μL of 10 mmol/L hexanoylglycine-$^{13}C_2,^{15}N$ and 1 mL of 100 μmol/L orotic acid-$^{15}N_2$. The 10 mL volume was topped up with LC-MS grade water. The IS mixture was stored in aliquots at −20 °C.

Biological material

All procedures followed were in accordance with the tenets of the Helsinki Declaration (as revised in 2013) and were approved by the ethical committee of the Faculty of Medicine and Dentistry, Palacky University Olomouc, and University Hospital Olomouc (licence number: 66-19). To date, more than 800 clinical samples have been analysed using this method in the Laboratory of Inherited Metabolic Disorders, Department of Clinical Biochemistry, University Hospital Olomouc, Czechia. Samples from IMD-positive patients (n=93) come from patients (n=46) and residual urine samples of External Quality Assurance (EQA) ERNDIM qualitative schemes – Diagnostic Proficiency Testing (DPT) scheme (n=28) and Qualitative Organic Acid in Urine (QLOU) scheme (n=19). A list of all patient samples is provided in Supplementary Table 5.

For analytical validation procedures, blank surrogate urine matrix samples (n=38) of healthy controls were used. Pooled urine samples were prepared by mixing 38 healthy controls. These samples were diluted to a creatinine concentration of 2 mmol/L and for analysis, equal aliquots of samples were mixed. Pooled urine was stored in aliquots at −20 °C. Basic clinical information about the samples is given in Supplementary Table 7.

Preparation of calibration curve standards and quality control samples

The calibration range for all analytes was determined according to their physiological concentrations in human urine found in the literature [21, 22] or according to the expected concentration when no concentration data were found. The ten-point calibration curve was prepared by binary dilution series in LC-MS water and stored at −20 °C. A calibrator (50 μL), 50 μL of synthetic urine Sigmatrix Urine Diluent (SUD), and 10 μL of IS mixture were mixed into the glass vials with an insert. The highest (Cal10) and the lowest (Cal1) calibration points for each compound are provided in Supplementary Table 2.

Quality control (QC) samples were prepared at three levels, i.e. High (HQC), Medium (MQC), and Low (LQC). HQC was set at 75 % of the calibration range, MQC was set at 25 % of the calibration range and LQC was set at the lower level of physiological concentration of an appropriate analyte. QC samples were prepared in 11 mixtures and stored at −20 °C. QC concentration levels are provided in Supplementary Table 3.

Sample preparation

Urine samples from healthy controls, patients and EQA controls were diluted to the creatinine concentration of 1 mmol/L to a volume of 100 μL into glass vials with an insert. To each sample, 10 μL of the IS mixture was added. The final concentration of IS in samples was as follows: methyl-$^2H_3$-malonic acid (18.8 μmol/L), hexanoylglycine-$^{13}C_2,^{15}N$ (1.0 μmol/L), homovanillic acid-$^{13}C_6,^{18}O$ (18.2 μmol/L), isovaleryl-$^2H_9$-L-carnitine (9.0 nmol/L), orotic acid-$^{15}N_2$ (0.9 μmol/L), lactic acid-$^{13}C_3$ (44.8 μmol/L). For urine samples with creatinine concentrations below 1 mmol/L, the IS volume (in μL) was reduced and the injection (in μL) increased according to Eqs. (1) and (2).

$$V_{IS} = c_{creatinine} \times 10 \qquad (1)$$
$$V_{injection} = 1/c_{creatinine} \qquad (2)$$

LC-MS/MS analysis

The analysis was performed on the HPLC instrument Exion LC (SCIEX, Framingham, MA, USA) using Acquity UPLC HSS T3 C18 column (1.8 μm, 100 × 2.1 mm) (Waters, Milford, MA, USA) and a mass spectrometer QTRAP 6500+ (SCIEX, Framingham, MA, USA). Eluent A contained 0.5 % formic acid in water and eluent B contained 100 % acetonitrile. The flow rate was set at 0.37 μL/min, sample injection at 1 μL and the run time was 26 min. The autosampler was set at 5 °C and the column temperature was 30 °C. The gradient profile for this HPLC solvent run was as follows: t=0–2 min 100 % A; t=2–9 min 80 % A; t=9–17 min 5 % A; t=17–20 min 5 % A; t=21–26 min 100 % A. The analysis was performed in the scheduled multiple reaction monitoring (MRM) mode under polarity switching (simultaneously in positive and negative polarity) with an ion spray voltage=−4,500.0 V, +5,500.0 V, temperature=450.0 °C, curtain gas=35.0 arb, collision gas=medium, ion source gas 1 and 2=50.0 arb using nitrogen as collision gas. The instrument was operated in the MRM mode in which two selective transitions are chosen for each compound during quantification.

Conditions of detection in the mass spectrometer (MS) were optimised for all analytes separately. Standards of OA, AG and AC were diluted in the mobile phases A and B (1:1, v/v) to final concentrations of 10, 3, and 1 μmol/L, respectively. Each standard solution was directly injected into the MS by the syringe pump and specific MRM transitions (mass/charge ratio of first and third quadrupole), collision energy of the second quadrupole and declustering and exit cell potentials were optimised in both positive and negative polarities. For compounds that are not commercially available (2-methylacetoacetate, 3-hydroxybutyrylcarnitine, 3-hydroxyisovalerylcarnitine and hawkinsin), MS parameters were found and optimised from patient urine samples. First, MS spectra were found online [21], and preliminary MRM transitions were generated. Subsequently, the MS2 spectrum of the identified chromatographic peak of the compound was measured, and then MRM transitions were optimised.

For the optimization of separation conditions and parameters of MS detection, standards of all compounds with concentrations of 100 μmol/L OA, 10 μmol/L AC, and 30 μmol/L AG were prepared and analysed separately. Isomeric compounds were analysed both separately and in a mixture of related isomers for unequivocal identification of each isomer. MS detection conditions were selected based on the highest S/N value for a given MRM transition separately in the Analyst 1.7 software (SCIEX, Framingham, MA, USA). Identification of the isomers was done according to their separation or, if possible, by finding a specific MRM transition.

Method validation

The method was validated according to European Medicines Agency (EMA) and Food and Drug Administration (FDA) validation guidelines

[23, 24], and the following parameters were evaluated: linearity, accuracy, imprecision, matrix effect and carry over. The methods and procedures for the analytical validation process are given in the Supplementary Text.

Patient diagnosis workflow

The identification and diagnosis of patients were made by a robust standardised (RS) value calculation [25–27]. For this purpose, a group of initial 200 samples from healthy controls was selected as a control group for computation RS reference values. Subsequently, RS values were calculated for patient samples according to the Eq. (3), where X' is a robust standardised value, x_i is a value of the ith observation, $\overline{X}$ is a median of the healthy control population and $Q_3 - Q_1$ the interquartile range of the healthy control population.

$$X' = (x_i - \overline{X}) / (Q_3 - Q_1) \tag{3}$$

RS values of healthy controls with their age and sex are provided in Supplementary Table 8. Calculated quantiles of RS values for healthy controls are given in Supplementary Table 9. IMD network for over 80 disorders was created in Cytoscape software. RS values of patients for each biomarker were imported to the Cytoscape software and the level change of the respective biomarker was visually monitored.

Data analysis and statistics

For MS data acquisition and processing, Analyst® 1.7 and SCIEX OS 2.0 software (SCIEX, Framingham, MA, USA) were used. The peak area of the analyte relative to the corresponding IS were used to robustly transform the data for all urine samples from healthy controls, EQA, and patients, as well as for long-term routine use in diagnostic practice. IS of each analyte are listed in Supplementary Table 2. Data analysis including RS values calculation and visualization was carried out in MS Excel 365 (Redmond, Washington, USA) and GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). IMD network was made in Cytoscape software 3.8.2 [28] (Bethesda, MD, USA).

Results

LC-MS/MS method development and optimization

Overall, 146 analytes as biomarkers of IMDs consisting of 99 OA, 15 AG, 32 AC, and 22 stable isotope IS were included in the developed method. All isomeric compounds were successfully separated on the HSS T3 column due to optimisation of mobile phase composition. All mass spectrometric method parameters are provided in Supplementary Table 4 and the separation of all compounds divided into 4 classes for better display is shown in Figure 2.

Analytical validation

Analytical validation was performed on standards of 140 analytes and the following parameters were determined: linearity, accuracy, imprecision, matrix effect and carry over. The results of the analytical validation (including graphical representation) and its discussion are presented in Supplementary Text and Supplementary Figures 3 and 4.

Patient diagnosis workflow

For diagnostic purposes, the calculation of RS values is fully automated and requires only copying area ratio values of analytes. Multianalyte scatterplots in GraphPad Prism are also done automatically involving the import of the RS values into the data table. Next, the operator can display an individual or multiple patients at once. Based on outlying values of disease-specific biomarkers it was possible to diagnose samples with 34 IMDs listed in Supplementary Table 5 along with RS values. The workflow also includes plotting IMD network, where IMDs are divided into separate groups according to affected metabolism (amino acids, branched-chain amino acids, urea cycle, carbohydrates, fatty acids, mitochondrial disorders, peroxisomal disorders, and others). This network was created in Cytoscape freeware, which is an interactive tool highly user adjustable. The original Cytoscape file with all data is available in Supplementary File 1. The representative graphical presentation of 5 individual patient samples (primary hyperoxaluria type 2 (OMIM#260000), succinic semialdehyde dehydrogenase deficiency (OMIM#271980), 2-hydroxyglutaric aciduria (OMIM#600721), medium-chain acyl-CoA dehydrogenase deficiency (OMIM#201450) and methylmalonic aciduria (OMIM#251000)) are shown in Supplementary Figure 1B–F. A blank IMD network is shown in Supplementary Figure 1A. IMD abbreviations used in the network are in accordance with the official OMIM nomenclature [29].

As an example, the results of the RS values graphical plotting of a patient suffering from glutaric aciduria type II (GA2; OMIM#231680) (Patient 14; 1.5 years, female) are presented in Figure 3. Due to multiple acyl-CoA dehydrogenase deficiency, patients excrete increased amounts of not only glutarate but also other OA and their conjugates. However, abnormalities in OA levels may be present only when patients are under stress [30], which can make GC-MS-based diagnosis difficult. In this sample, pathology was found not only at the level of OA (ethylmalonate, adipate, suberate, sebacate) but also AG (glutarylglycine, hexanoylglycine, suberylglycine) and AC (butyrylcarnitine, glutarylcarnitine,
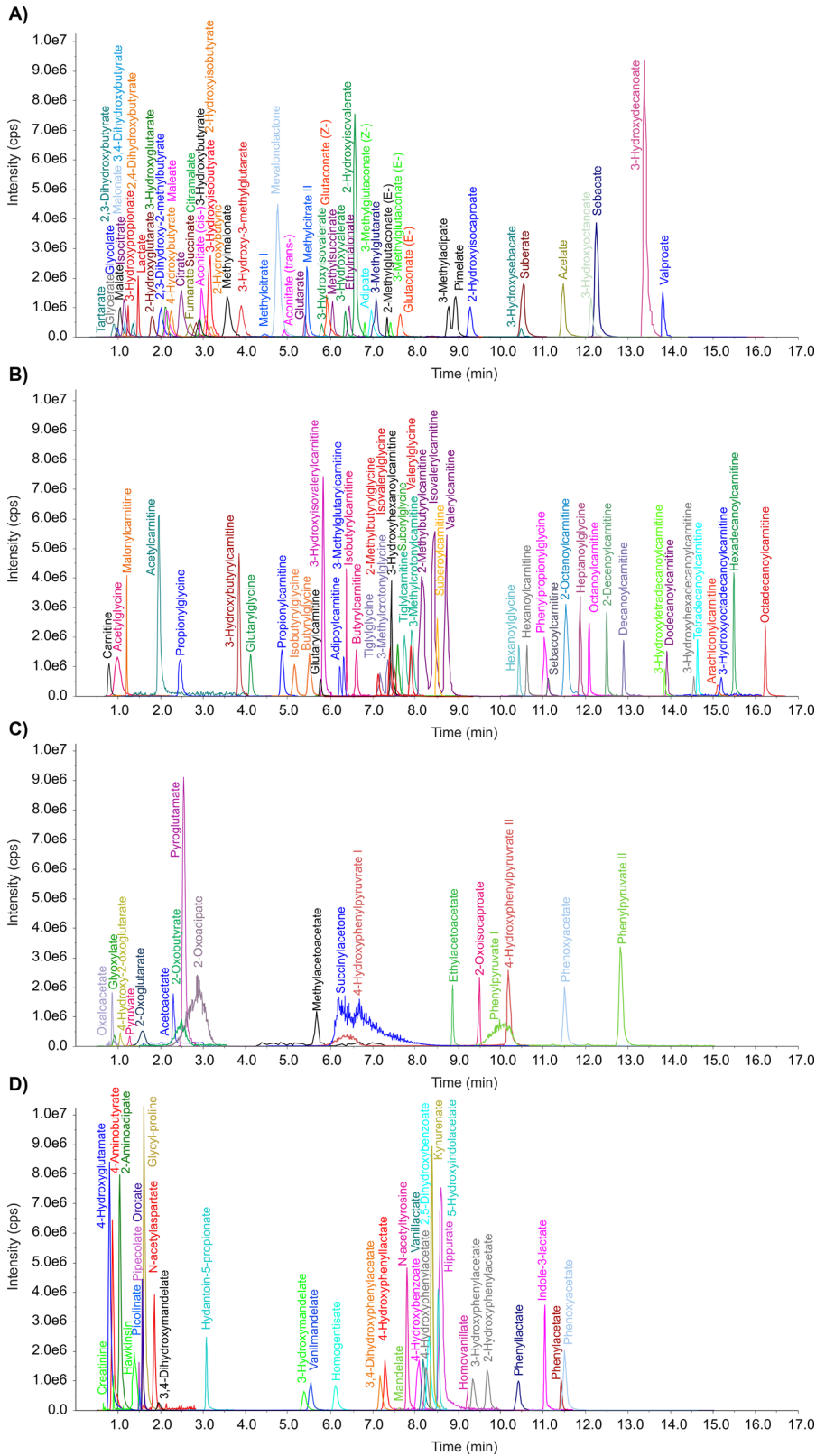
Figure 2: Standards separation of hydroxy- and carboxylic acids (A), acylglycines and acylcarnitines (B), oxoacids (C) and aromatic, heterocyclic and nitrogen-containing compounds (D).
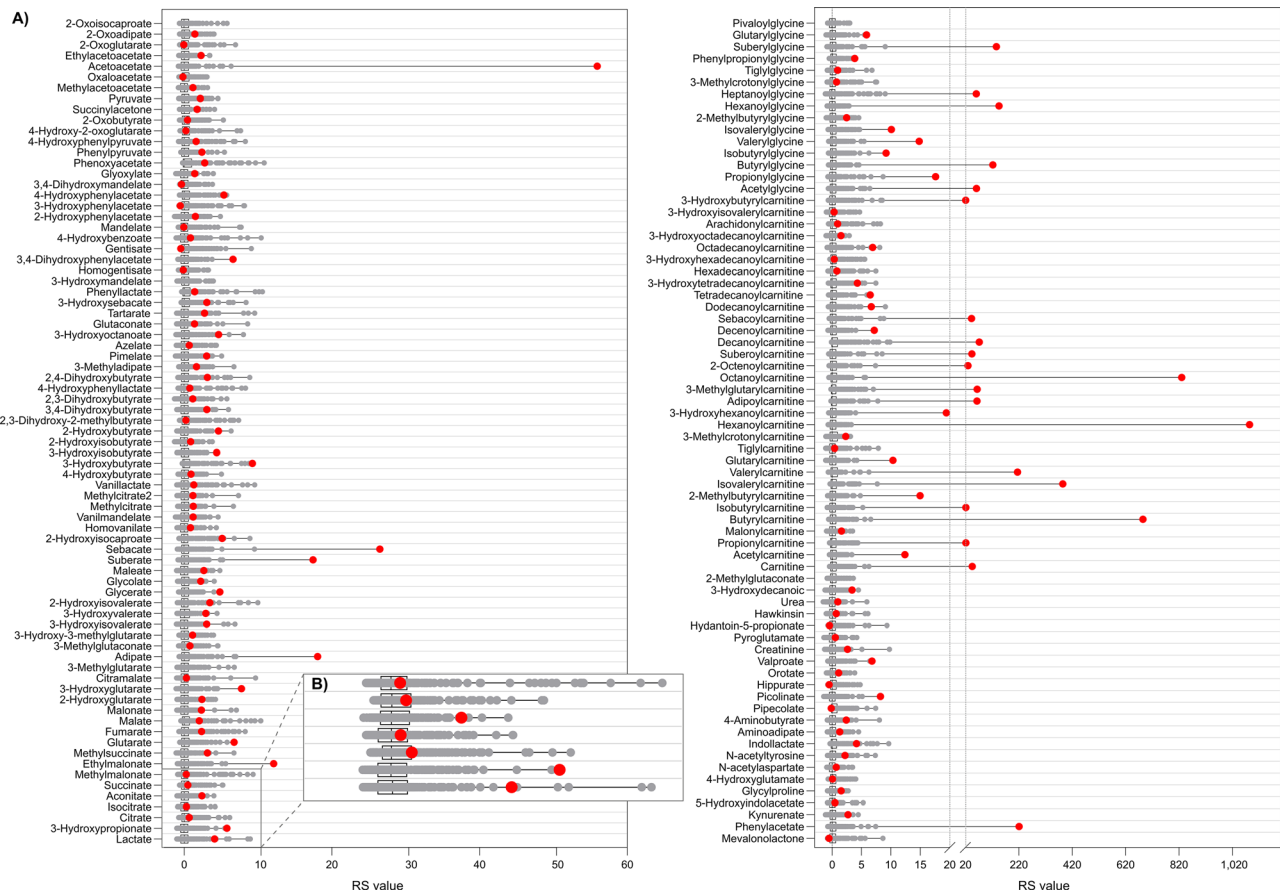
Figure 3: Plotted results of RS values (x-axis) of measured analytes (y-axis) for patient with glutaric aciduria type II (Patient 14) represented by red dots. Grey dots represent values of healthy controls (n=200) (A). The cut-out zoom demonstrates a closer distribution of healthy controls (B).

hexanoylcarnitine, octanoylcarninite) as confirmatory bio-markers. Further, for a better orientation in affected pathways, the metabolic network is depicted in Figure 4.

Long-term usage

This method has been used in our laboratory for more than 2 years in the routine diagnostic process. It has become the method of the first choice and indispensable for acute cases such as metabolic crises. The previously used GC-MS has been side-lined for control purposes only. It suffers from difficult sample preparation, loss of analytes due to variable extraction and derivatization efficiencies, contamination of GC-MS instrument by derivatisation reagents and time-consuming data evaluation requiring highly qualified personnel. Without multi-algorithmic peak detection, run-by-run and peak-by-peak evaluation are common, making this approach completely unsuitable for patients requiring urgent care diagnosis. The advantage of our new robust method is the ease of pre-selected peak integration using

Sciex OS software and the subsequent RS values calculation. Sample analysis can be performed immediately after blank analysis, making this approach suitable for laboratories with urgent emergency tasks. In our experience, one sample result can be obtained within 1 h of sample delivery.

Mass spectrometry proved to be a sufficiently stable platform, as confirmed by the coefficient of variation (CV) of the selected analytes (n=23) included in the ERNDIM Internal Quality Control System at 2 levels. Median and interquartile range (IQR) of CV for Level 1 and Level 2 are 16.2 and 19.8 %, with IQR of 14.9–20.4 % and 18.9–21.3 %, respectively. Consequently, frequent maintenance of the MS is not required, which is a great advantage in its routine use in a clinical laboratory. With respect to the robustness of retention times (RTs), the method provides average stability of 1.6 % (median) for all analytes when used routinely over 1 year (Supplementary Figure 5A). Within batch average reproducibility was 0.4 % (median; Supplementary Figure 5B).

Although this method was developed for urine analysis, in our laboratory it has been also applied to dry blood
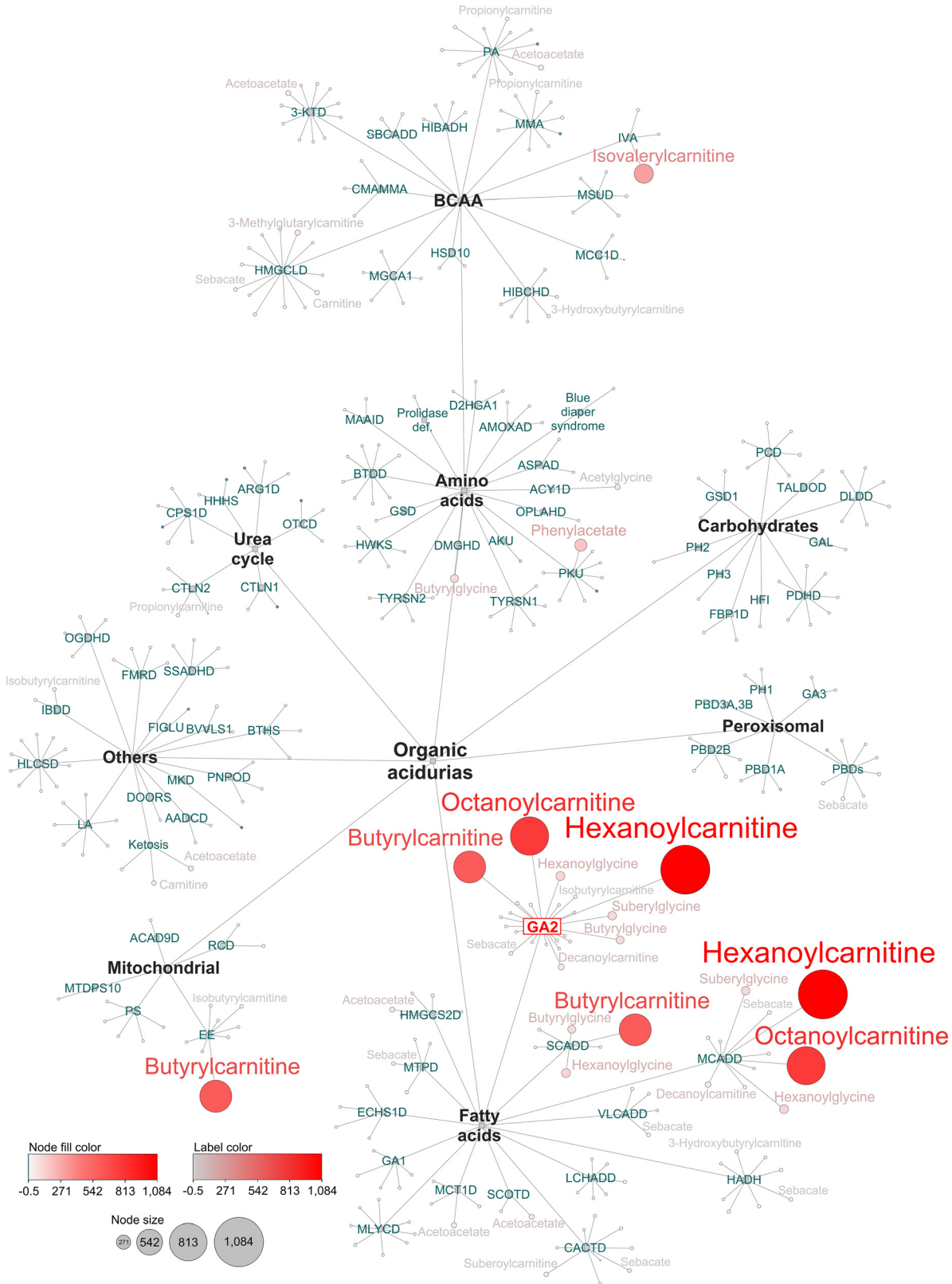
Figure 4: IMD network with visualisation of the patient with glutaric aciduria type II (Patient 14). Organic acidurias are divided into separate groups according to affected metabolism. Biomarkers (end nodes with biomarker names) are connected to a particular disease (rectangle node with green title) and expressed by size and colours according to the calculated RS values. For better display and clarity of the image, the cut-off value of 20 was set for biomarker labelling. IMD abbreviations are in accordance with the official OMIM nomenclature.
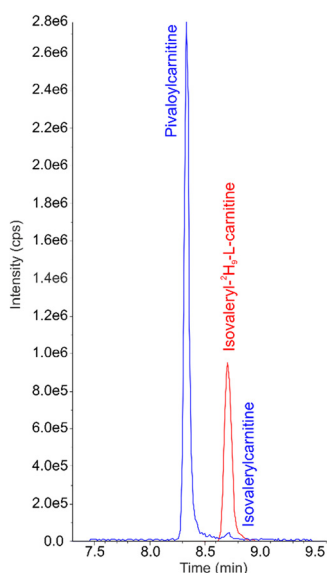
Figure 5: Chromatographic separation of C5 carnitines in DBS sample. Extracted chromatogram of DBS analysis of a patient suspected of IVA with a high amount of interfering pivaloylcarnitine (blue). Internal standard isovaleryl-^{2}H$_9$-L-carnitine (red) coeluates at the same time as isovalerylcarnitine.

spot (DBS) samples in the context of differentiating true and false positivity of isovaleric acidemia (IVA) due to Pivinorm treatment of pregnant women (Figure 5). Pivaloyloxymethyl ester of mecillinam as the main component of the drug is converted into pivaloylcarnitine interfering with isovalerylcarnitine. Both carnitines are baseline separated by the method and semiquantitation is performed directly from the newborn screening sample containing stable labelled internal standard.

External quality assessment

EQA samples – ERNDIM DPT and QLOU (Heidelberg) in the years 2021–2022 have been analysed as a part of the routine operation. The diagnosis was performed by this platform with a 100 % success rate. A list of samples from ERNDIM DPT and QLOU and diseases diagnosed by the developed LC-MS/MS approach are provided in Supplementary Table 6. Furthermore, the RS values plots of each ERNDIM sample with the biomarkers on which the diagnosis was based are shown below in Figure 6.

Discussion

In the routine diagnosis of IMD, due to acute clinical manifestations, organic acidurias are one of the important groups of diseases monitored in all laboratories. The ability to provide a rapid laboratory response with maximum coverage is very important. Compared to GC-MS, which is currently exclusively used, here we present an advanced semi-automated workflow based on LC-MS/MS that allows both rapid analysis with minimal sample preparation and additionally provides complementary information at the level of OA together with the corresponding AG and AC. In our experience, this makes this method a very clinically powerful tool for the diagnosis of a wide range of IMDs.

Recently, there has been widespread use of LC-MS/MS for IMD diagnosis. Körver-Keularts et al. [12] established liquid chromatography-quadrupole-time of flight-mass spectrometry (LC-QTOF/MS) method which allows the diagnosis of 32 IMDs in urine without the lengthy extraction and derivatization step. The above approach was further extended [31] to 78 IMDs, which, however, consisted in expanding the number of biomarkers but not in improving the diagnosis of organic acidurias. Another LC-QTOF/MS method [32] focusing on plasma samples allowed the diagnosis of 42 IMDs using up to 340 known IMD-related metabolites. Considering the laborious sample preparation and data processing, it is questionable whether this approach can be used for rapid clinical diagnostics. The CLAM-2030 automated sample pretreatment system directly coupled to LC-QTOF/MS appears promising and has been demonstrated on 9 acidurias [11]. Nevertheless, this system is time consuming and financially demanding and sample derivatization and incubation are required. A recently published work [14] showed a rapid analysis of 5 common serum and urinary OA. Despite being a fast quantitative method, it is limited to measuring a very small profile, although the authors mention the possibility of extending it to more OA.

Important parameter of the developed workflow is the requirement for a very small sample volume, which can be crucial, especially in neonates and infants. It allows analysis even from a few tens of μL of urine compared to GC-MS where the standard requirement is around 2 mL. Very often we encountered creatinine levels lower than 0.5 mmol/L in young children, which was easily solved in our approach by adjusting the sample preparation and compensating with a higher LC-MS injection.

The big advantage of the developed workflow lies also in straightforward data evaluation. Although the method has been subjected to a complete validation to verify the analytical parameters of all analytes, relative quantification is used in routine operation, where the peak areas of the analytes are related to the corresponding internal standards. Due to the high number of 140 analytes, it is in principle impossible to achieve absolute quantification based on calibration standards in the long term. For internal quality control purposes, commercially available ERNDIM QC
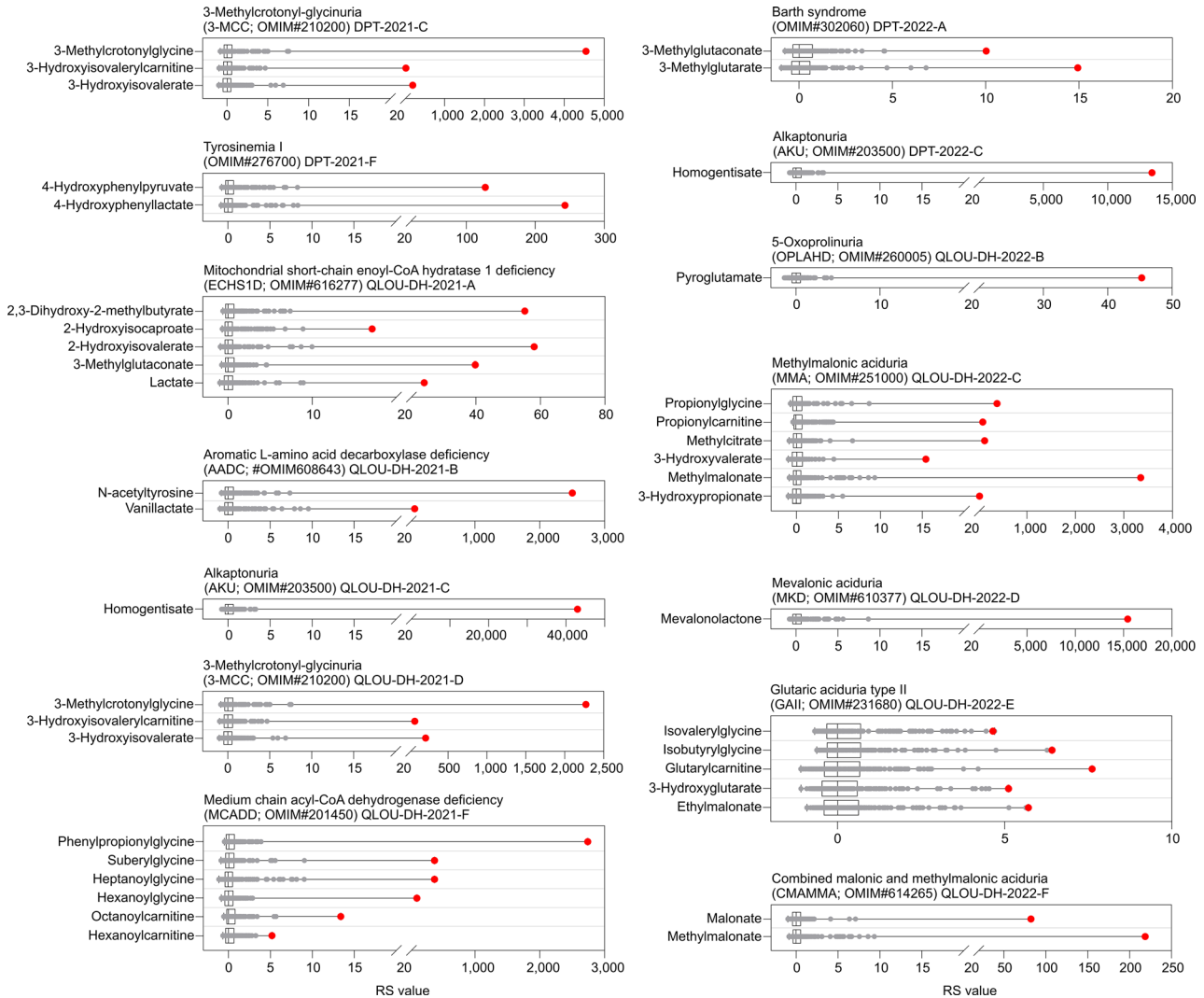
Figure 6: Graphical plotting of RS values (x-axis) of biomarkers (y-axis) used to elucidate the diagnosis. The results are shown for samples of the ERNDIM DPT and QLOU (Heidelberg) schemes from the years 2021–2022 which were analysed during standard routine operation.

materials can then preferably be used. To achieve maximum analytical quality, it is then essential to maintain the long-term consistency of internal standards. The use of the automated robust scaling method has proven to be a fast, convenient, and reliable tool for IMD diagnosis. Data transformation using z-scores is not fully new in data interpretation [12, 31, 33]. However, for urine metabolites, robust data transformation method was used due to the large scale and variability across all measured analytes and the skewed distribution of the data including outliers. To calculate RS values, medians and interquartile range (IQR) as a robust scale estimator was used instead of median absolute deviation (MAD), which is less effective for nonsymmetric distributions with present outliers [25–27]. As an outcome, the data are visualised using a multianalyte scatter plot and IMD network in GraphPad Prism and Cytoscape software,

respectively. The result of the analysis is carried out within 1 h after the sample delivery. Routine raw data processing involving integration of the peaks of 12 samples in a batch (10 urine and 2 IQC samples), transfer to a spreadsheet, conversion to RS values, upload to statistical and visualization software, subsequent printing and initial evaluation of the results takes a total of 90 min. This calculation is valid for experienced personnel familiar with IMD and the software used. In addition, the method can be easily expanded by adding more relevant biomarkers for other IMDs, e.g. MS and LC conditions were optimised for hawkinsin (specific for hawkinsinuria, OMIM#140350) from an EQA sample during its routine laboratory operation. In the future, the method is likely to be extended to include other new or known relevant IMD biomarkers. In addition, the method allows the separation of all 4 isomers of carnitine C5 (i.e. pivaloyl-,

2-methylbutyryl-, isovaleryl- and valeryl-), which is used in the expanded newborn screening as a second-tier method to detect false positive IVA from treatment with the antibiotic Pivinorm. The use of the same sample eliminates subsequent requests for an additional patient sample and thus the burden on the patient and parents themselves. Some of the findings in EQA samples in Figure 6 may not be directly related to the disease, but are associated with metabolic ketoacidosis, or supportive treatment (carnitine, amino acids, vitamins or cofactors supplementation, etc.). That is the case of sample QLOU-DH-2021-A (mitochondrial short-chain enoyl-CoA hydratase 1 deficiency, OMIM#616277). Here, higher concentrations of 2-hydroxyisovalerate and 2-hydroxyisocaproate were also present, referring to lactic acidosis [34]. This is also supported by the increased lactate excretion. Increased concentrations of certain AC were also observed in this sample, probably due to carnitine supplementation. On the other hand, it is common to encounter samples that do not have elevated levels of typical biomarkers. For instance, sample DPT-2021-F (tyrosinemia type I, OMIM#276700) was found to have increased only 4-hydroxyphenylpyruvate and 4-hydroxyphenyllactate. However, aminoacidopathies are investigated by amino acid analysis, for which this method is not suitable. Furthermore, sample QLOU-DH-2021-F with medium-chain acyl-CoA dehydrogenase deficiency (MCADD, OMIM#204450) did not show pathogenicity at the level of OA, but only at the level of AC and AG. This just demonstrates the importance of comprehensive testing in the diagnosis of IMD.

Although the use of the advanced LC-MS/MS method is very convenient, it has some pitfalls as described below. In terms of analyte structure, compounds containing the amino group are not retained on the reversed-phase column. Oxoacids have poor peak shape (as depicted in Figure 2C), low sensitivity and poor validation parameters due to known low stability in aqueous solutions. Thus, many oxoacids can only be detected at elevated pathological conditions as shown in Supplementary Figure 2. These drawbacks of analysing oxoacids can be also seen in their reproducibility of RT. Further, primary hyperoxaluria can be detected based on secondary biomarkers (glycerate and glycolate) as shown in Supplementary Figure 1B. Oxalate as a primary biomarker is not retained on the reversed-phase column due to its low molecular weight and high polarity. One limitation of the study is that the effect of age on the reference limits was not taken into account. This could, for example, affect the assessment of a mild increase of methylmalonic acid in the case of vitamin B12 deficiency. Based on the literature sources, major differences in the definition of age groups can be found and this will be further addressed as part of method improvements.

In conclusion, a robust LC-MS/MS approach enabling rapid diagnosis of a broad spectrum of more than 80 IMDs at the level of OA, AG and AC (146 analytes) in urine has been developed and validated. Compared to routinely used GC-MS, the LC-MS/MS method offers fast and easy sample preparation, low sample consumption, higher coverage of metabolites significantly improving the diagnostic process, comfortable data processing with clear identity of analytes and advanced result visualization and statistical evaluation. This approach has been in use at our institution for 2 years and has been successfully tested on EQA samples in addition to routine samples. As the method is cost-effective, fast, and simple, it has a high potential for implementation as the first-choice method in other laboratories for IMD diagnosis and finally to replace the mostly used GC-MS for urinary OA analysis.

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Informed consent: Not applicable.
Ethical approval: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013) and has been approved by the authors' Institutional Review Board or equivalent committee. (Faculty of Medicine and Dentistry, Palacky University Olomouc, and University Hospital Olomouc, 66-19).
Data availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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