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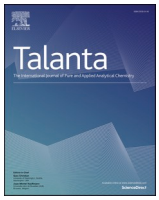

Wide metabolite coverage LC-MS/MS assay for the diagnosis of inherited metabolic disorders in urine

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ABSTRACT

Objective: The laboratory diagnosis of inherited metabolic disorders (IMD) has undergone significant development in recent decades, mainly due to the use of mass spectrometry, which allows rapid multicomponent analysis of a wide range of metabolites. Combined with advanced software tools, the diagnosis becomes more efficient as a benefit for both physicians and patients.

Methods: A hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry assay for determination of urinary purines, pyrimidines, *N*-acylglycines, *N*-acetylated amino acids, sugars, sugar alcohols and other diagnostically important biomarkers was developed and validated. Evaluation of the results consisting of utilisation of robust scaling and advanced visualization tools is simple and even suitable for urgent requirements.

Results: The developed method, covering 65 biomarkers, provides a comprehensive diagnostic platform for 51 IMD. For most analytes, linearity with $R^2 > 0.99$, intra and inter-day accuracy between 80 and 120 % and precision lower than 20 % were achieved. Diagnostic workflow was evaluated on 47 patients and External Quality Assurance samples involving a total of 24 different IMD. Over seven years, more than 2300 urine samples from patients suspected for IMD have been routinely analysed.

Conclusions: This method offers the advantage of a broad coverage of intermediate metabolites of interest and therefore may be a potential alternative and simplification for clinical laboratories that use multiple methods for screening these markers.

1. Introduction

Inherited metabolic disorders (IMD) represent a large group of >1880 rare genetic disorders [1] that are associated with morbidity and mortality, particularly in the neonatal period and childhood [2]. These IMD are caused by mutations in genes encoding enzymes or transport

Abbreviations: AA, amino acids; ACN, acetonitrile; ACY1D, aminoacylase 1 deficiency; AG, acylglycines; BSUM, blank surrogate urine matrix; C3 Gly, propionylglycine; C4 Gly, (iso)butyrylglycine; DMSO, dimethyl sulfoxide; DPT, Diagnostic Proficiency Testing; EMA, European Medicines Agency; EQA, External Quality Assurance; GALAC1, Galactosemia type I (Duarte variant); GC, gas chromatography; GC-MS, gas chromatography coupled with mass spectrometry; HILIC-MS/MS, hydrophilic interaction chromatography coupled with tandem mass spectrometry; HPLC, high performance liquid chromatography; HQC, high quality control sample; IMD, inherited metabolic disorders; IQC sample, internal quality control sample; IS, internal standard; LC, liquid chromatography; LC-MS, liquid chromatography coupled with mass spectrometry; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; LQC, low quality control sample; MQC, medium quality control sample; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; NBS, newborn screening; PP, purine and pyrimidine; PP-Cal, Purine and Pyrimidine calibrator; QC, quality control sample; RS, robustly standardised; RT, retention time; SA-Cal, Special Assays calibrator; S/N, signal-to-noise ratio; SAICAr, succinylaminoimidazole carboxamide riboside; SUD, Sigmatrix urine diluent; TIC, total ion chromatograms.

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proteins, resulting in a clinically significant blockage in a metabolic pathway. This defect leads to accumulation of the substrate or lack of the product of the enzyme reaction. The accumulated metabolites are usually small molecules that are readily excreted by the mother's placenta in the foetal period. Therefore, the first symptoms of IMD almost always begin to manifest after the birth of the child, often after only a few days or weeks of life [3]. However, outbreaks can also occur later in life. This already implies the complexity of IMD, which certainly does not make the work of physicians any easier. Therefore, it is necessary to monitor early symptoms from the foetal period onwards. With early diagnosis, removal of the worst manifestations and appropriate treatment, a good prognosis for patient can be expected [4].

Currently, differential diagnostics based on the measurement of specific metabolites is indispensable. The key analytical technique is tandem mass spectrometry (MS/MS), which is often coupled with gas/liquid chromatography (GC/LC) for analyses of a wide range of biomarkers of the main IMD groups [5]. However, a definitive diagnosis must be confirmed by genetic analysis, which is not easy in terms of cost, time and efficiency [4]. Therefore, routine metabolite biomarker investigations are not abandoned. Instead, there is an effort to develop more multicomponent and faster methods in order to speed up diagnosis and thus make IMD treatment more effective.

A prominent group of IMD is organic acidurias, which have long been diagnosed by gas chromatography coupled with mass spectrometry (GC-MS). In recent years, neverthel ess, the use of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been on the rise [6,7]. Disorders in the purine and pyrimidine (PP) metabolism spectrum are also widely studied. One of the most recent LC-MS/MS methods developed by Cremonesi et al. allows the quantification of 41 PP in urine, thus potentially diagnosing more than 25 disorders in the metabolism of PP [8]. They extended the commonly analysed PP panel with several other new relevant biomarkers such as dihydroorotic acid, uric acid, β-alanine and new biomarkers upstream of SAICAr. Given that SAICAr was not included in LC-MS/MS methods at all until 2019 and was analysed separately using the Bratton-Marshall test [9], the extension of the method to metabolites upstream of SAICAr is a major advance in PP analysis. However, the narrow metabolite profile offered by the PP panel may be insufficient in many cases, and it would be preferable to use an approach with the broadest possible metabolite coverage.

Considering the requirements of our clinical laboratory, the aim of this work was to develop a diagnostic approach based on hydrophilic interaction chromatography with tandem mass spectrometry (HILIC-MS/MS) covering a PP panel extended with other diagnostically important analytes such as amino acids (AA), acylglycines (AG), sugars, sugar alcohols and others, which is fast, simple, efficient and suitable for urgent requirements.

2. Materials and methods

2.1. Chemicals and Reagents

Detailed information about the reference and internal standards is summarised in Supplemental Table 1. All the standards used were of analytical grade or higher. LC-MS grade acetonitrile (ACN) and water were shipped by Honeywell Riedel-de Haën (Seelze, Germany). Sigmatrix Urine Diluent (SUD) as well as acetic acid, ammonium hydroxide solution and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide was obtained from CentralChem (Bratislava, Slovakia).

2.2. Biological material

Purine and Pyrimidine calibrator (PP-Cal) level 1 and 2 and Special Assays calibrator (SA-Cal) level 1 and 2 was obtained from ERNDIM (MCA Laboratory, BN Winterswijk, The Netherlands).

For analytical validation, a blank surrogate urine matrix (BSUM) sample was obtained by pooling samples from healthy controls ($n = 38$), which were selected to match the urine of patients with IMD. IMD preferably occur in neonates or young children (the median age corresponds to 1 year). The diagnostic workflow was developed using urine samples from healthy controls ($n = 140$), patients ($n = 22$) and ERNDIM External Quality Assurance (EQA) samples ($n = 25$). All healthy controls were obtained from the Laboratory for Inherited Metabolic disorders, Department of Clinical Biochemistry, University Hospital Olomouc (Czech Republic). Basic clinical data of patients and controls are collected in the Supplemental Table 2. Detailed characteristics of patient samples including their origin is summarised in Supplemental Table 3. All procedures were approved by the Ethics Committee of the Faculty of Medicine and Dentistry, Palacky University and University Hospital Olomouc (licence number: 66-19).

2.3. Standard preparation

2.3.1. Preparation of standard stock solutions

Due to the diverse solubility of the standards, these substances were dissolved at different concentrations in various solvents. All detailed information about the preparation of standard stock solutions is provided in Supplemental Table 4. All standards were stored at −20 °C.

2.3.2. Preparation of internal standard mixture

Internal standard (IS) mixture was prepared by mixing 10 IS stock solutions according to Supplemental Table 5. The mixture of IS was filled to a final volume of 50 ml with 20 mM ammonium acetate (pH 9.75), aliquoted, and stored at −20 °C.

2.3.3. Preparation of calibration standards

A 10-point calibration standards ($n = 3$) were prepared by binary dilution series in SUD or 20 mM ammonium acetate (pH 9.75) (30 μL) according to Table 1 with a spike of the working solution with a known amount of analytes (30 μL) and mixture of IS (10 μL).

2.3.4. Preparation of quality control (QC) samples

Intra-day ($n = 5$) and inter-day ($n = 15$) accuracy and precision were determined by using quality control (QC) samples (at low, medium, and high concentrations, i.e. LQC, MQC, and HQC, respectively) according Table 1. BSUM sample (30 μL, Chapter 2.4.1) of was mixed with 30 μL of the working solution with the known amount of analytes and 10 μL of IS mixture.

2.3.5. Preparation of internal quality control (IQC) sample

Internal quality control (IQC) sample was prepared by mixing equal volumes of 4 samples from Purines and Pyrimidines control scheme 2021 and 4 samples from Special assays urine 2021.

2.4. Sample preparation

2.4.1. Preparation of blank surrogate urine matrix

BSUM sample was prepared by pooling urines from healthy controls ($n = 38$). The samples of healthy controls were diluted to creatinine concentration of 2 mmol/L (if its concentration was <2 mmol/L, this sample was used without dilution) and then, equal aliquots of samples were mixed. The aliquots of BSUM were stored at −20 °C and further used for validation experiments.

2.4.2. Preparation of urine samples from healthy controls, patients and ERNDIM diagnostic Proficiency testing (DPT) control scheme

Urine samples from healthy controls, patients and ERNDIM DPT control scheme were continuously stored at −80 °C. Before the LC-MS/MS analysis, samples were thawed to room temperature and diluted to a creatinine concentration of 1 mmol/L (25 μL) and mixture of IS (25 μL) was added. Urine with creatinine concentration under 1 mmol/L was analysed without dilution.

Table 1
Working range of standards for determining linearity parameters and nominal concentrations of quality control (QC) samples.

Compound	Working range (μmol/L)	LQC (μmol/L)	MQC (μmol/L)	HQC (μmol/L)
2-Deoxyadenosine	0.04–20	1	5	15
2-Deoxyguanosine	0.04–20	1	5	15
2-Deoxyinosine	0.04–20	1	5	15
2-Deoxyuridine	0.04–20	1	5	15
2,8-Dihydroxyadenine[a]	0.04–20	1	5	15
Adenine	0.04–20	1	5	15
Adenosine[a]	0.04–20	1	5	15
AICAr	0.04–20	1	5	15
Dihydrothymine	0.08–40	2	10	30
Dihydrouracil	0.2–100	5	25	75
Guanosine	0.04–20	1	5	15
Hypoxanthine	0.27–140	7	35	105
Inosine[a]	0.04–20	1	5	15
Orotic acid	0.12–60	3	15	45
Orotidine	0.04–20	1	5	15
SAICAr[a]	0.04–20	1	5	15
Succinyladenosine	0.04–20	1	5	15
Thymidine	0.04–20	1	5	15
Thymine	0.04–20	1	5	15
Uracil[a]	0.12–60	3	15	45
Uric acid[a]	3.91–2000	100	500	1500
Uridine	0.04–20	1	5	15
Xanthine	0.39–200	10	50	150
N-Acetylaspartic acid	0.16–80	4	20	60
N-Acetylglutamine	0.20–100	5	25	75
N-Acetylleucine	0.04–20	1	5	15
N-Acetylserine	0.12–60	3	15	45
N-Acetylvaline	0.04–20	1	5	15
Argininosuccinic acid	0.04–20	1	5	15
Homocitrulline	0.08–40	2	10	30
Succinylacetone[a]	0.04–20	1	5	15
N-3-Methylcrotonylglycine	0.04–20	1	5	15
N-Acetylglycine	0.08–40	2	10	30
N-Butyrylglycine	0.04–20	1	5	15
N-Glutarylglycine	0.04–20	1	5	15
N-Hexanoylglycine	0.04–20	1	5	15
N-3-Phenylpropionylglycine	0.04–20	1	5	15
N-Propionylglycine	0.04–20	1	5	15
N-Suberylglycine	0.04–20	1	5	15
N-Tiglylglycine	0.04–20	1	5	15
N-Valerylglycine	0.04–20	1	5	15
Erythritol	1.17–600	30	150	450
Galactitol	1.17–600	30	150	450
Galactose	1.17–600	30	150	450
Perseitol	0.04–20	1	5	15
Ribitol	0.39–200	10	50	150
Sedoheptulose	0.39–200	10	50	150
Stachyose	0.08–40	2	10	30
Guanidinoacetic acid[a]	0.59–300	15	75	225
Creatine[a]	3.91–2000	100	500	1500
Creatinine[a]	5.86–3000	150	750	2250
Sialic acid[a]	0.59–300	15	75	225

[a] Calibration curve of the analyte prepared within one mixture along with others labeled (*) in 20 mM ammonium acetate (pH 9.75) due to the presence of creatine and creatinine in Sigmatrix Urine Diluent (SUD).

2.5. LC-MS/MS method development

The newly developed HILIC-MS/MS method was adopted from Bajad et al. (2006) [10] and optimised according to the Yuan et al. (2012) [11] and other publications with similar application [12,13]. The method has been continuously expanded with new analytes with a purpose to cover biomarkers from as wide a spectrum of IMD as possible.

Automated MS/MS optimization was performed for each analyte. Standards were diluted by mobile phases A and B (1:1, v/v) to a concentration of 10–100 μmol/L and injected directly into the mass spectrometer. Values of declustering potential, entrance potential, collision energy and collision cell exit potential of the second quadrupole were optimised for each transition (mass/charge ratio of the first and third quadrupole), mostly in negative polar mode. For the final method, only one transition was selected for each analyte, which was characterised by the highest S/N ratio, while no interference was observed.

2.5.1. Column efficiency

As part of the method optimization, an injection experiment (in the range of 0.25–3 μL) was performed to test column efficiency, based on which the optimal injection volume was selected.

2.6. LC-MS/MS analysis

LC-MS/MS analysis was performed on HPLC instrument UltiMate 3000 RS (Dionex, Sunnyvale, CA, USA) using NH_2 Luna column (3 μm, 100 × 2 mm, Phenomenex, Torrance, USA) and triple quadrupole mass spectrometer Triple Quad 6500 (Sciex, Framingham, MA, USA). Mobile phase A contained 20 mM ammonium acetate (pH = 9.75) and mobile phase B 100 % ACN. The gradient elution profile, at a flow rate of 0.4 mL/min was as follows: t = 0 min 95 % B; t = 6 min 30 % B; t = 7 min 30 % B; t = 7.5 min 95 % B; t = 11.5 min 95 % B. The column was thermostated at 30 °C, the injection volume was 0.5 μL and the autosampler

temperature was 10 °C.

The analysis was performed in the scheduled multiple reaction monitoring (MRM) mode in polarity switching system with an ion spray voltage ± 4500 V, temperature 400 °C, curtain gas 35 arb, collision gas 6 arb, 1 and 2 ion source gas 40 arb using nitrogen as collision gas.

The typical batch for routine settings consisted of: first run, blank, PP-Cal level 1 and 2, SA-Cal level 1 and 2, in-house calibrator level 1 and 2, IQC, samples from patients (typically 10–20) and IQC. Instrument stability and sensitivity are monitored based on analysis of IQC sample measured before and after patient samples. The intensity of ISs and retention time (RT) of analytes are monitored throughout the sequence.

2.7. Method validation

The LC-MS/MS method was validated according to guidelines for Bioanalytical Method Validation published by European Medicines Agency (EMA) [14].

2.7.1. Linearity

The calibration range was determined according to the analyte's physiological concentration in urine using database tools [15,16]. Each calibration curve consisted of a blank sample, a zero calibrator (blank sample spiked with IS mixture), and 10 concentration levels of calibration standards. The calibration standards were analysed for three separate days. During the evaluation, the number of calibration points for each analyte was adjusted according to the linearity achieved. However, at least 6 calibration points (according to the requirements of EMA guideline) for each analyte were defined.

The method linearity was determined by regression curves and expressed by the coefficient of determination (R^2), which was required to be > 0.99 for acceptance (for all three runs). By regression analysis were also determined slopes and intercepts. The limit of detection (LOD) and limit of quantification (LOQ) were evaluated according to the following Eqs. (1) and (2):

$$LOD = 3 \times c/(\mathrm{S/N}) \quad (1)$$

where c is the concentration of the analyte in the calibration standard and S/N is signal-to noise ratio,

$$\mathrm{LOQ} = 3.33 \times \mathrm{LOD} \quad (2)$$

The S/N ratio was obtained using Sciex OS software 2.0 (Sciex, Framingham, MA, USA).

2.7.2. Accuracy and precision

The accuracy and precision of the method were determined as within-run ($n = 5$) and between-runs ($n = 15$) using QC samples at three concentration levels: LQC, MQC, and HQC in five replicates for three separate runs analysed together with a set of calibration standards. The concentration levels of the QC samples were set such that LQC corresponded to 5 %, MQC to 25 % and HQC to 75 % of the highest calibration standard (ULOQ). Acceptable limits for accuracy were set to 85–115 % (except for LQC where 80–120 % was allowed). Precisions were satisfactory when the CV values were ≤15 % (LQC ≤20 %).

2.7.3. Matrix effects

Matrix effects were evaluated by post-column infusion of adenosine-$^{13}C_5$ (−/+ MRM). For this experiment, 10 blank urine samples of healthy controls diluted to a creatinine concentration of 1 mmol/L were used in addition to the blank sample (LC-MS water). Subsequently, these were analysed with the continuous addition of adenosine-$^{13}C_5$ (c = 2 μmol/L) using an injection pump at a rate of 7 μL/min. Total ion chromatograms of the blank urine samples and blank sample were compared and regions of observable ion suppression/enhancement were identified.

2.7.4. carry over

Carry over was evaluated by analysing a blank sample (LC-MS water) following the ULOQ. To determine the percentage of carry over, the ratio of the analyte area in the blank sample was related to the analyte area in the ULOQ. Carry over effects can be deemed insignificant if the interference observed in the blank samples is less than 5 % of ULOQ.

2.8. Evaluation workflow

The evaluation involves creating multianalyte scatterplot with the robustly standardised (RS) values of analytes [17,18] and forming the IMD network with the corresponding biomarkers, which are marked with different sized and coloured nodes depending on the RS value of biomarkers.

The calculated concentrations have been taken as the basis for calculating the RS values according to the following Eq. (3):

$$X' = (c_i - \bar{c}) / (Q_3 - Q_1) \quad (3)$$

where X' is a robust standardised value, c_i is a value of the ith observation, $\bar{c}$ is a median of the healthy control population and $Q_3 - Q_1$ the interquartile range of the healthy control population.

First, the population was divided into two groups (<1 year and >1 year). To establish the reference range, RS values of the healthy population <1 year ($n = 39$) and >1 year ($n = 101$) for each metabolite were calculated. The RS values of the patients were then automatically computed with respect to age and summarised including control samples in the Supplemental Tables 6 and 7. Multi-analyte scatterplots were generated in GraphPad Prism software to present the results graphically.

Creating a network consists of importing the RS values of the respective patient into Cytoscape software, which is an interactive freeware highly user-adjustable. The network connects IMD that are divided into separate groups according to the affected metabolism (PP, AA, fatty acids, carbohydrates, urea cycle, etc.). The further branches are the respective IMD, marked by the abbreviation according to OMIM nomenclature or full name [19]. The end nodes represent the corresponding diagnostic biomarkers [taken from 1, 15, 16] of each IMD. Depending on the RS value, the end nodes and their labels change colour and size. The original Cytoscape file with all data is freely available based on request of authors and blank network is shown in Supplemental Fig. 1A. The schematic representation of the workflow is depicted in Fig. 1.

2.9. Data analysis

MS operation was conducted using Analyst® software 1.6.2 while data processing was carried out by SciexOS 2.0 (Sciex, Framingham, MA, USA). The concentrations of the analytes were calculated using the PP-Cal (2 levels) and SA-Cal (2 levels). An in-house calibrator was prepared to monitor analytes not included in the EQC. The reliability of analytes quantification was ensured using 10 ISs, with the most suitable one selected primarily on the basis of similarity of chemical structure and retention time. Obtained concentrations were transformed to the RS values using Microsoft Excel 365 (Redmond, Washington, USA). The transformed data were imported into GraphPad Prism 9.3.1 (San Diego, CA, USA) and Cytoscape 3.8.2 (Bethesda, MD, USA) [20] for better graphical representation of patients diagnosed with a specific IMD.

3. Results

3.1. LC-MS/MS method development

The developed method enables the analysis of 75 analytes consisting of PP ($n = 23$), AA ($n = 8$), AG ($n = 13$), sugars and sugar alcohols ($n = 17$), guanidinoacetic acid, sialic acid, creatine, creatinine and 10 ISs. Most analytes are detected in the negative ionisation mode, precisely

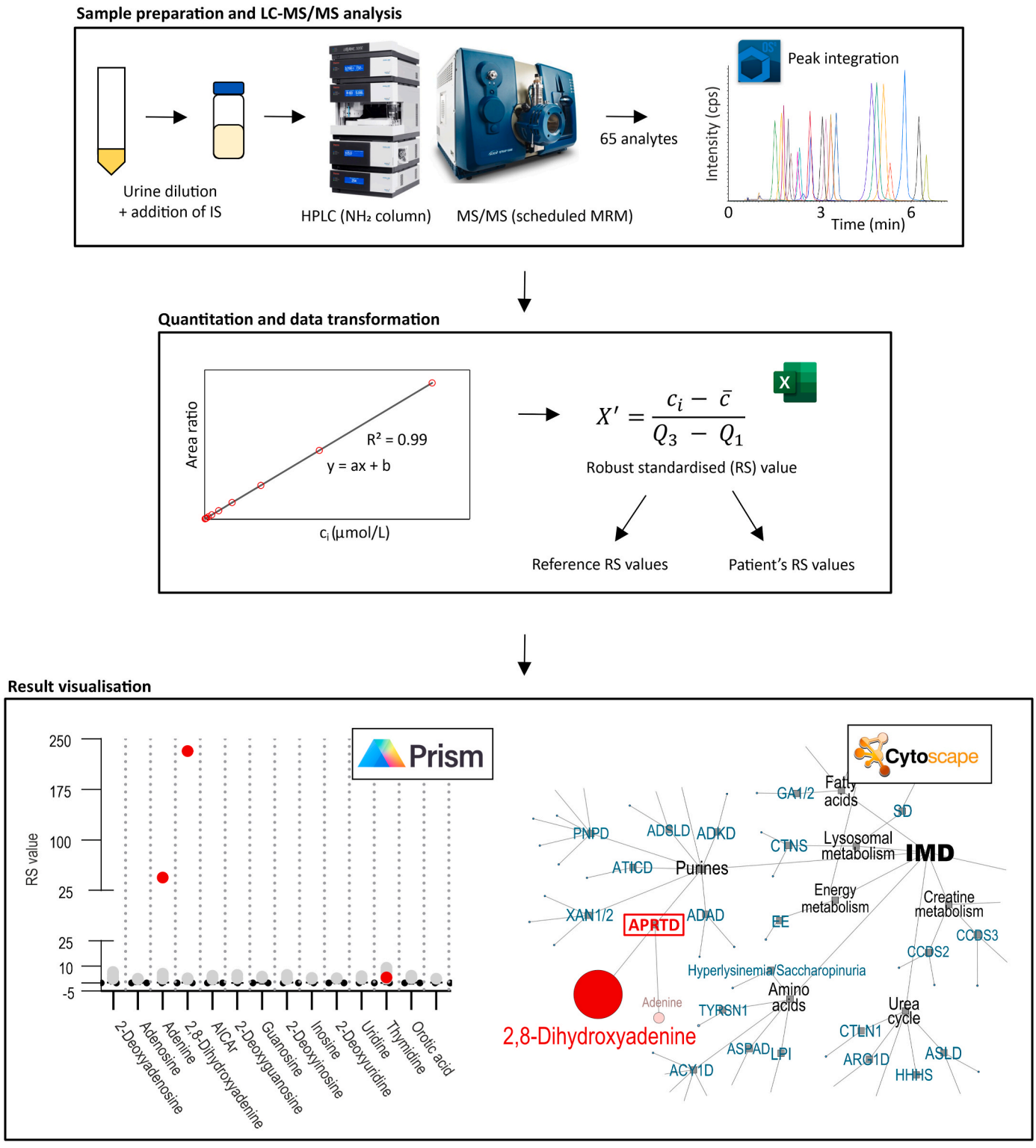

Fig. 1. Schematic representation of the workflow.

because of minimal interference compared to the positive mode. Moreover, although the absolute signal is usually lower in the negative mode, the quantitation parameters are better due to the lower noise. The optimised MS/MS settings and RT are provided in Supplemental Table 8. The extracted ion chromatogram (± MRM mode) of standard mixture in a SUD or 20 mM ammonium acetate (pH 9.75) at a concentration corresponding to the MQC level is shown in Fig. 2. Less retained analytes such as dihydrothymine and dihydrouracil eluted close to void time. Next, the analytes are eluted in the order of sugars, sugar alcohols and AG, followed at the end of the analysis by organic acids such as argininosuccinic and *N*-acetylaspartic acids.

3.1.1. *Column efficiency*

Column efficiency depends significantly on the injection volume. The largest decrease was observed for pyrimidine-based ribosides (Fig. 3). Interestingly, there is an increase for dihyrothymine and dihydrouracil,

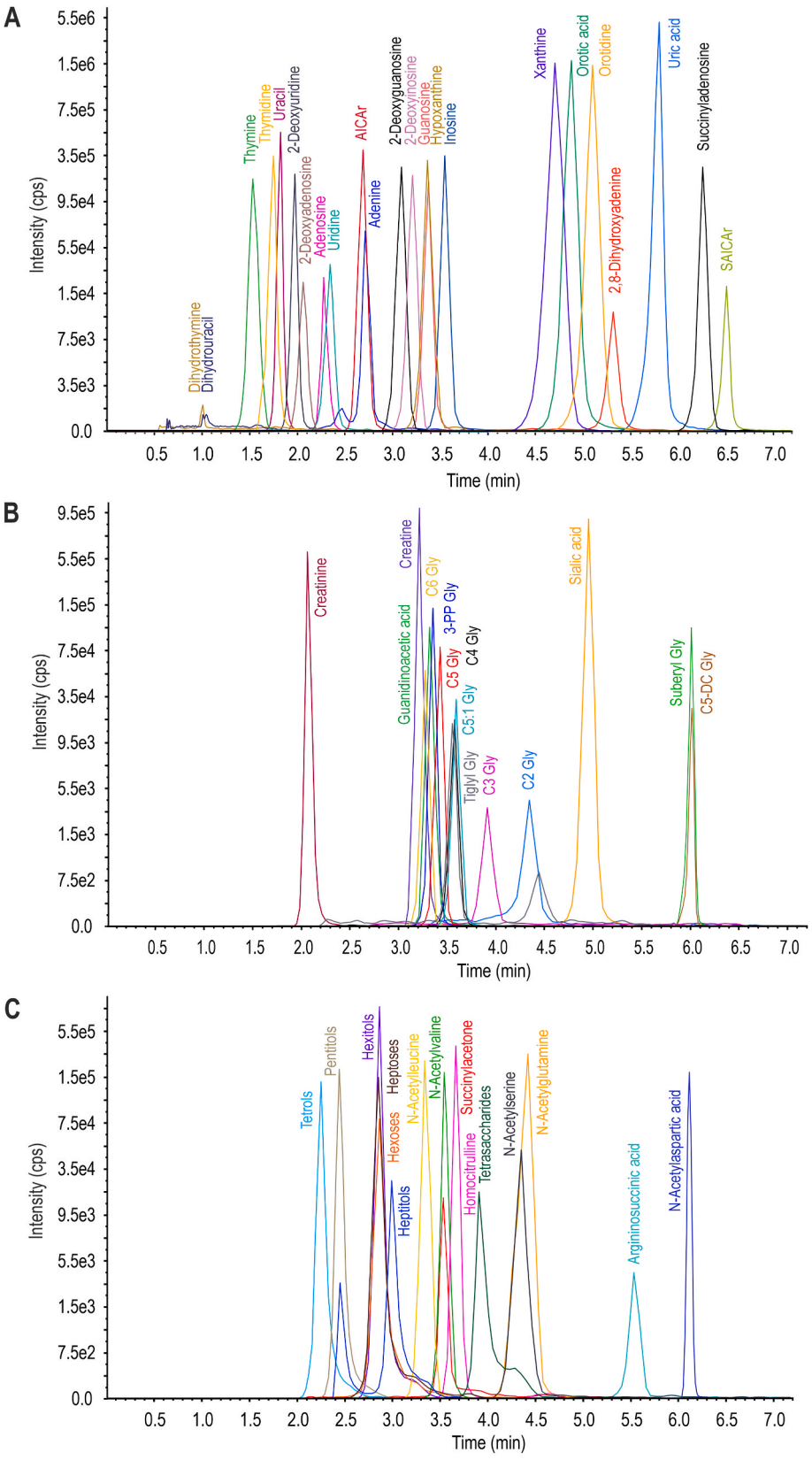

Fig. 2. Extracted ion chromatograms of standards in the Sigmatrix Urine Diluent/20 mM ammonium acetate (pH 9.75) at a concentration corresponding to the MQC level. Profile of purines and pyrimidines (A), *N*-acylglycines, guanidinoacetic acid, creatine, creatinine and sialic acid (B) and amino acids, sugars and sugar alcohols (C).

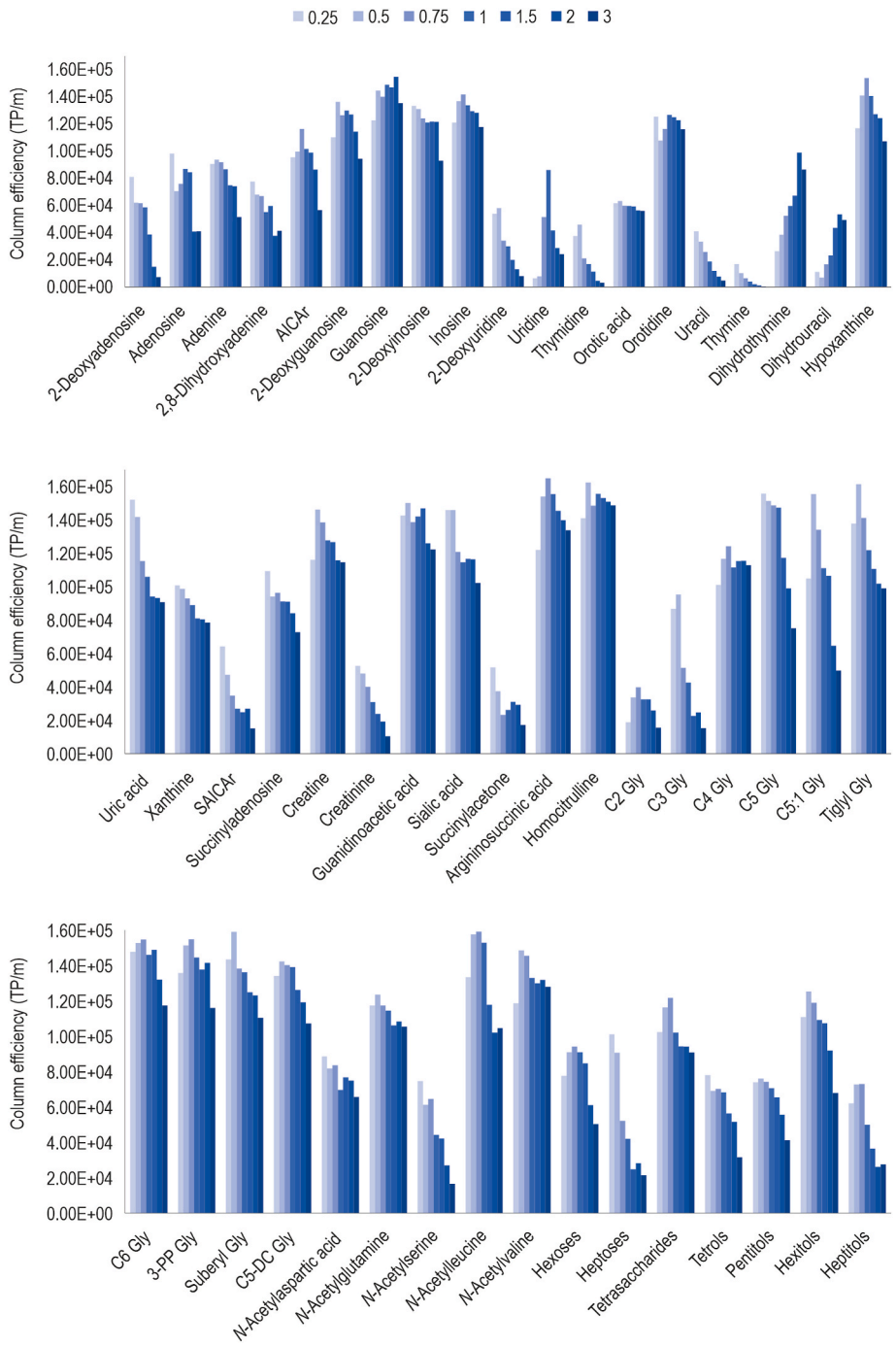

Fig. 3. Column efficiency at different injection dosages (range of 0.25–3 μL).

but these follow closely behind the dead time. An optimal injection volume (V = 0.5 μL) was chosen and the column efficiency at this injection volume is depicted in Fig. 4.

3.2. Method validation

3.2.1. Linearity

The calibration curves of the analytes showed mainly quadratic dependence due to the wide concentration range as in the work on targeted urinary metabolomics by Zheng et al. (2020) [21]. Only adenosine, guanosine, SAICAr, uracil, xanthine, *N*–acetylvaline, succinylacetone, sedoheptulose, guanidinoacetic acid and creatinine achieved a linear regression. The calibration curves contained at least six calibration points for each analyte. Variables *a*, *b* and *c* defining the slope and intercept of the regression equations are defined in Supplemental Table 9. Calibration curves were evaluated using a 1/y weighting. The coefficients of determination (R^2) were >0.9934 except for 2-deoxyadenosine ($R^2 = 0.9871$) and thymidine ($R^2 = 0.9797$) as shown in Fig. 5. All values of the regression parameters of the calibration curves, LOD and LOQ are summarised in Supplemental Table 9.

3.2.2. Accuracy and precision

The intra- and inter-day accuracy (%) and precision (CV, %) values of QC samples are summarised in Supplemental Tables 10–11. The majority of analytes met the acceptance criteria. Only a few analytes showed slight deviations in inter-day values (2,8-dihydroxyadenine,

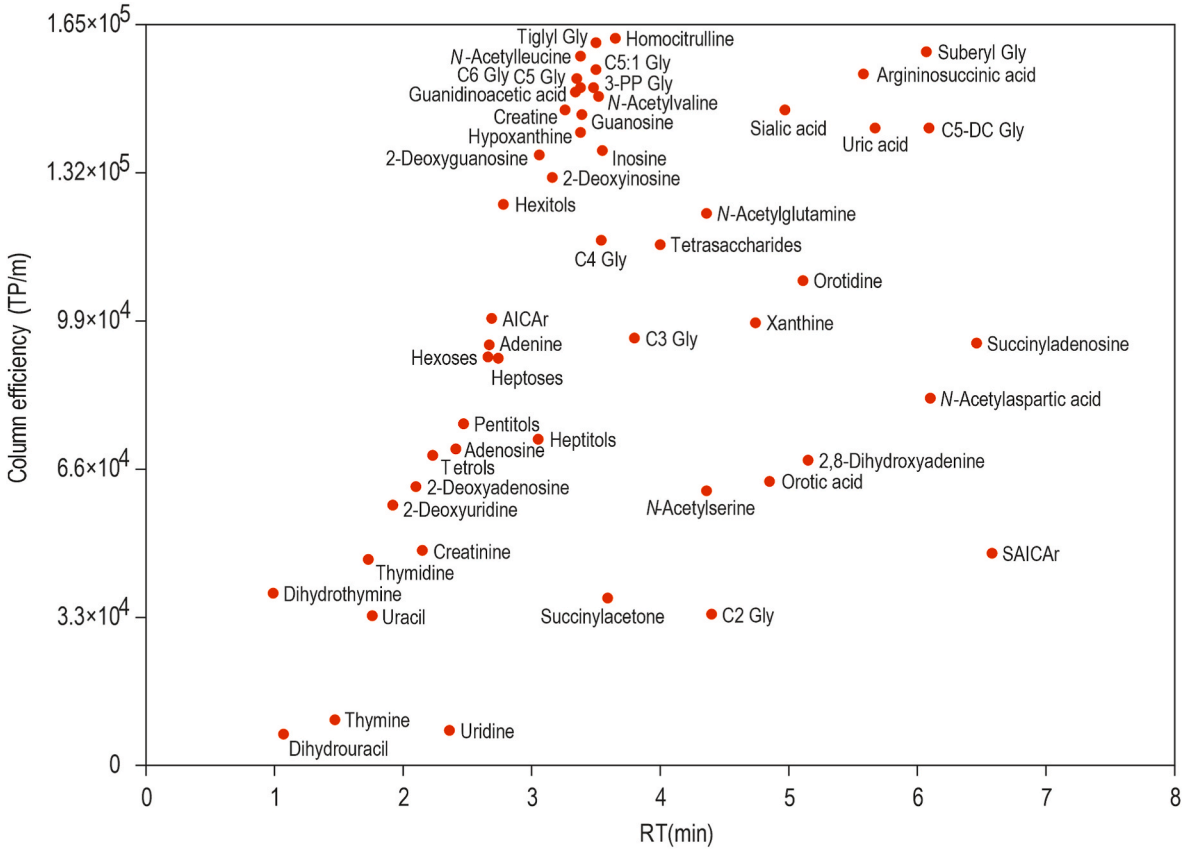

Fig. 4. Column efficiency at optimum injection volume (V = 0.5 μL).

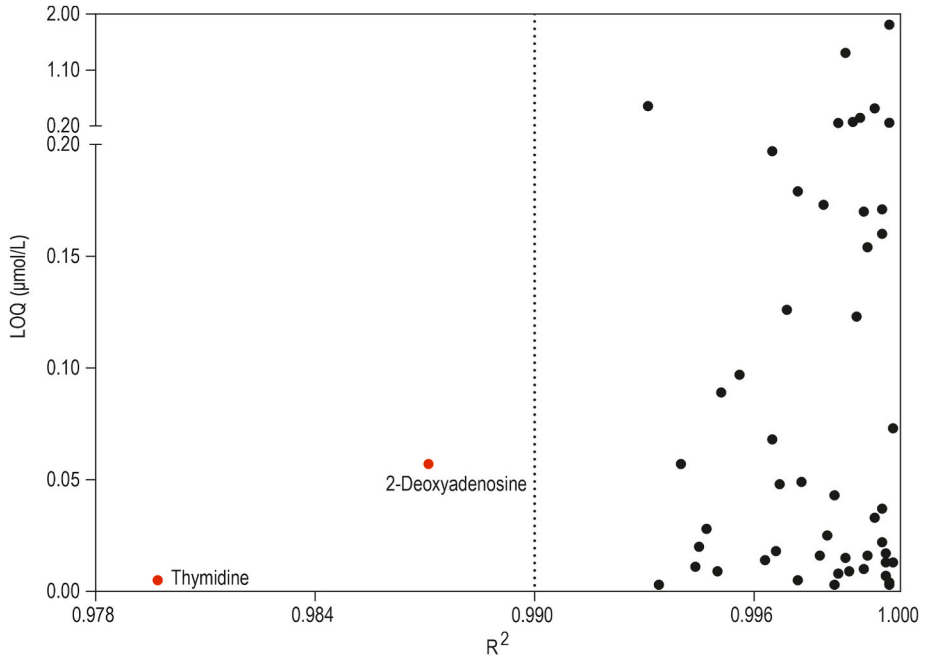

Fig. 5. LOQ and R^2 of measured analytes, where the dotted line shows the acceptance limit ($R^2 > 0.99$). Red coloured analytes not meeting the criteria are indicated by name.

inosine, *N*-acetylglycine, perseitol, argininosuccinic acid, succinyladenosine, *N*-acetylglutamine, *N*-propionylglycine and orotidine) as depicted in Fig. 6 and deviations in intra-day values (2,8-dihydroxyadenine, orotidine, succinyladenosine, N-acetylaspartatic acid, *N*-acetylglutamine, argininosuccinic acid, *N*-propionylglycine and perseitol).

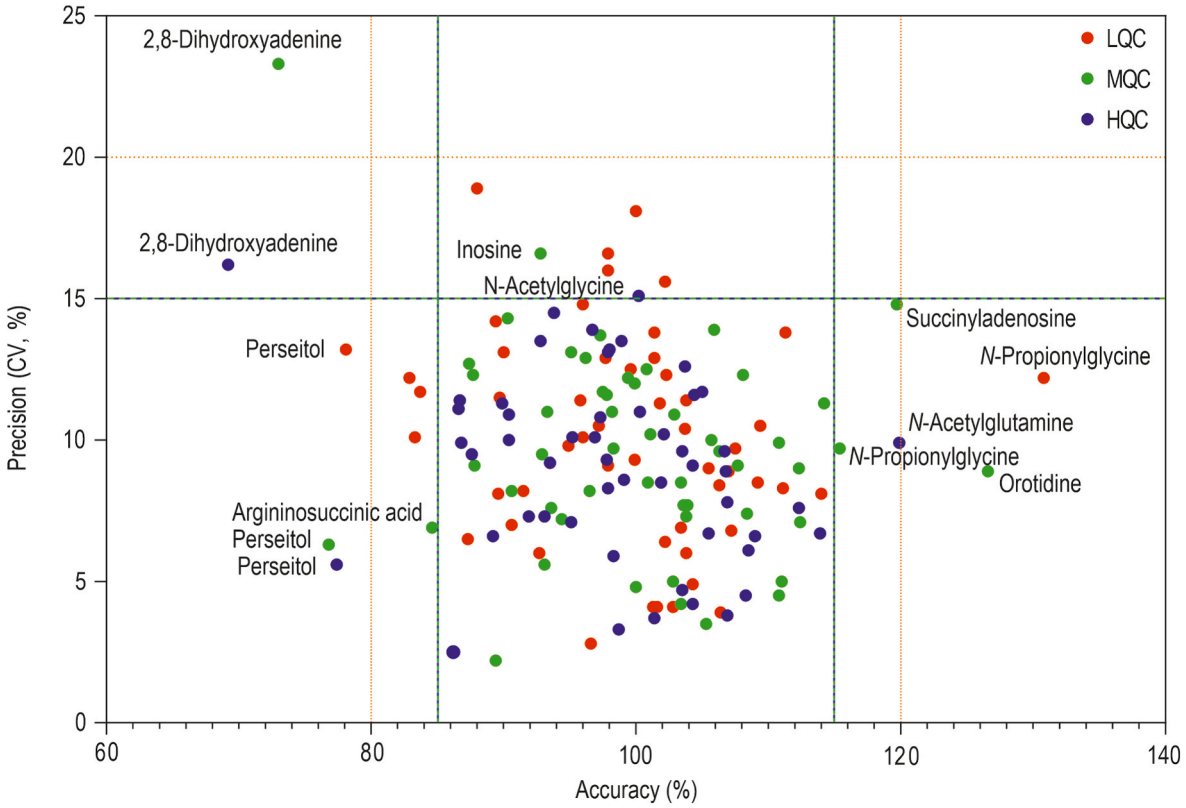

Fig. 6. Inter-day accuracy and precision (n = 15), each analyte is represented by a coloured dot and every colour represents the corresponding QC level of the sample. The red dotted line represents the acceptable criteria for LQC and green-blue line indicates the acceptable criteria for MQC and HQC samples. Analytes not meeting the criteria are indicated by name.

3.2.3. *Matrix effects*

Matrix effects were evaluated by the difference in signal intensity in the elution peak regions of the analytes measured in the method. Ion suppression and enhancement effects are shown in Fig. 7 (-MRM mode) and Fig. 8 (+MRM mode) by comparing signals of urine (colour) to blank (black) sample.

In the negative mode, ion enhancement occurs with a retention time of 0.5–2 min, whereas there were no interference in the positive mode, measuring only three analytes, namely *N*-propionylglycine (RT 3.6 min), *N*-valerylglycine (RT 3.8 min) and *N*-glutarylglycine (RT 6.0 min).

3.2.4. *Carry over*

No signals influencing quantification were found in the carry over experiment (for all analytes the interference was lower than 5 % of ULOQ). The mean responses obtained (%) in the analyte channel are summarised in Supplemental Table 12.

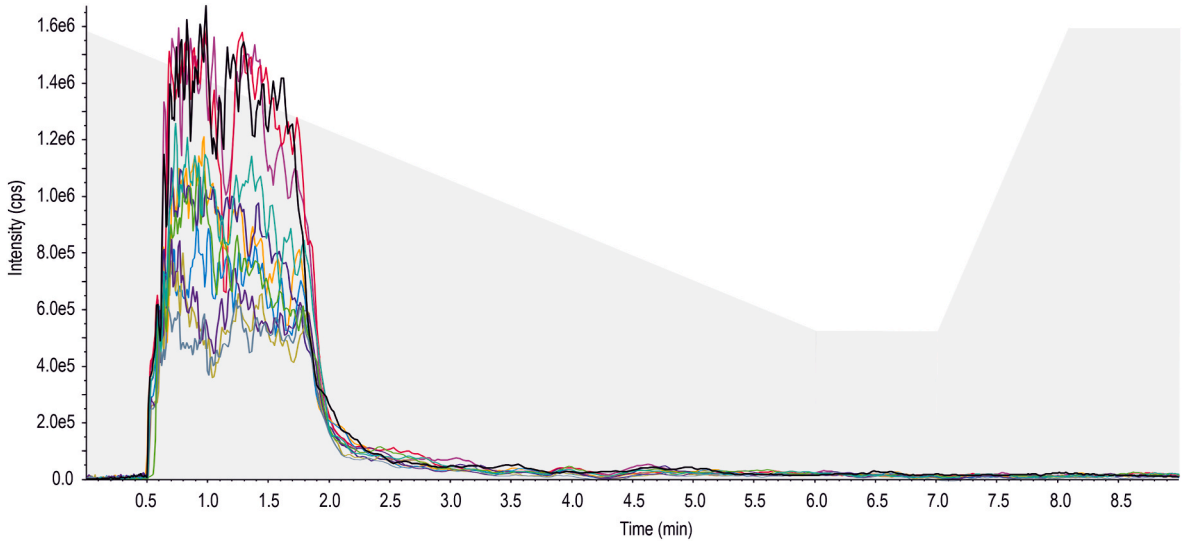

Fig. 7. Total ion chromatograms (TIC) of ion suppression/enhancement of 10 blank urine samples and blank (water) sample in -MRM mode. TIC of urine samples are coloured, while the blank sample is black. The grey area shows the percentage of mobile phase B (100 % ACN).

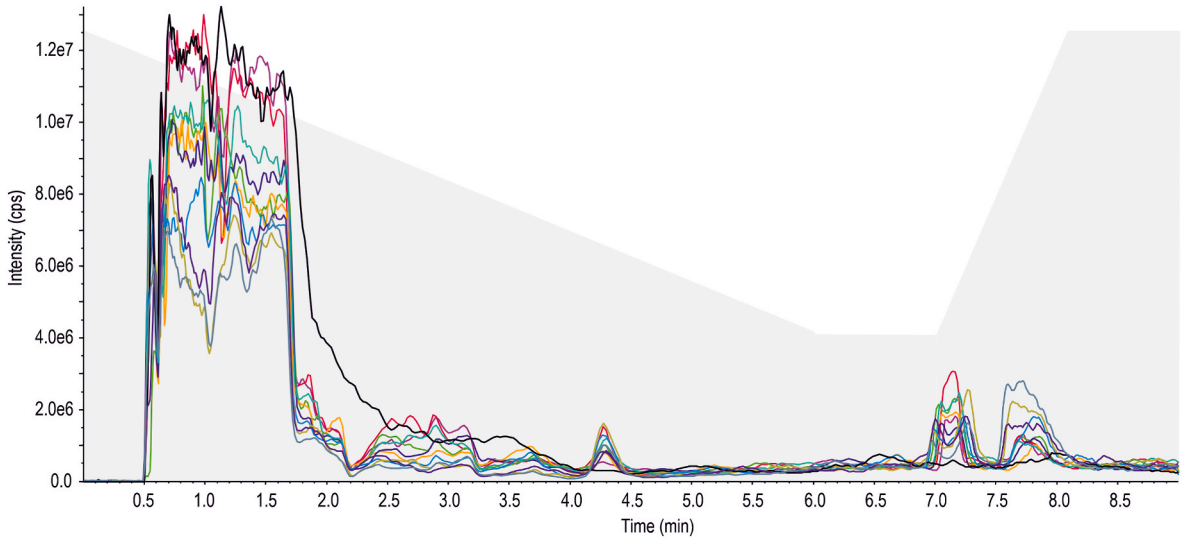

Fig. 8. Total ion chromatograms (TIC) of ion suppression/enhancement of 10 blank urine samples and blank (water) sample in +MRM mode. TIC of urine samples are coloured, while the blank sample is black. The grey area shows the percentage of mobile phase B (100 % ACN).

3.3. Evaluation workflow

During several years of applying this method to samples of patients with suspected IMD, many diagnoses have been revealed. Multi-analyte scatterplots and IMD networks of patients using RS values were constructed. Based on changes in biomarkers of patient levels relative to healthy controls, 47 patients with 24 IMD were diagnosed. A summary of IMD patients diagnosed with the developed method and further information about IMD is presented in Table 2. Representative networks of 9 patients are shown in Supplemental Figs. 1B–J. In the following sections, 2 selected case reports of patients whose diagnosis was captured using this method and the evaluation workflow will be presented.

3.3.1. Patient with aminoacylase 1 deficiency and siblings

The proband (Patient21) was a 15-years-old boy of Czech nationality from the 2nd pregnancy, whose delivery was spontaneous at term, without complications, and the postnatal adaptation was also uneventful. Newborn screening (NBS) was negative. The boy's psychomotor development was normal, although he was attending speech therapy for a pronunciation disorder. He was assessed as socially immature when he started school and was diagnosed with dysgraphia at the age of 10. He had problems with body coordination and balance. At the age of 15, he was examined at a Children clinic for hepatopathy and obesity. The diagnosis appeared to be non-alcoholic steatosis of the liver with obesity and incompletely expressed metabolic syndrome.

The boy was advised to undergo psychological assessment and IMD screening. The psychologists found that his thinking skills were in the upper range of below average. IMD screening using this developed method revealed increased excretion of *N*-acetylated derivatives of serine, leucine, valine and mildly glutamine and glycine (Fig. 9), which are characteristic of aminoacylase 1 deficiency (ACY1D, OMIM #609924). In addition, elevated AG such as propionylglycine (C3 Gly) and (iso)butyrylglycine (C4 Gly) were also observed in this sample, as previously reported [22].

The suspicion of ACY1D was confirmed by molecular genetic analysis, which revealed a pathogenic missense variant in the *ACY1* gene c.1057C > T (p.Arg353Cys; rs21912698, MAF 0.06 %) in the homozygous state. In connection with this finding, subsequent genetic analysis revealed this missense mutation in both parents in the heterozygous form and the familial mutation was also confirmed in two younger brothers in the homozygous form. IMD screening using our developed method also revealed increased levels of characteristic amino acids in the urine of the brothers (Patient 02 - purple and Patient 22 - blue) as shown in Supplemental Fig. 2.

3.3.2. patient with galactosemia type I, Duarte variant and her family

The proband (Patient01) was a 2-month-old girl of Czech nationality. This was the 1st pregnancy of the mother with significant growth restriction, which had to be terminated by caesarean section due to pathological findings on cardiotocography. After unsatisfactory saturation, oxygen therapy was administered, and further adaptation was uneventful. After birth, the newborn was breastfed normally. Because of prolonged jaundice and weight below the 3rd percentile of normal, she was followed up in the neonatal clinic. Artificial nutrition was given for low weight, but was always followed by vomiting. NBS results were negative. The family history did not suggest any genetic disease.

However, a few weeks later, she began to show non-specific symptoms of failure to thrive, bloated abdomen and refusal to feed appeared. In addition, her sclerae were yellowish and she showed signs of subicterus. Abdominal ultrasound showed marked ascites, increased liver echogenicity and 3 mm pericardial effusion. These symptoms did not improve, but they instead developed into acute liver failure. At this time, galactosemia was suspected based on results from urine performed by our developed method (Fig. 10). As the peak of galactitol and galactose is indistinguishable from other hexitols and hexoses, the presence of mannitol/sorbitol was subsequently excluded by GC-MS (additionally, intake of mannitol was excluded by doctors and parents). While galactose-1-phosphate is accumulated in erythrocytes in galactosemia patients, the diagnosis was further confirmed by analysis elevated hexose-monophosphates (RS value = 31.4) in dry blood spot (collected as part of NBS) using the same LC-MS/MS conditions (data not shown).

After revealing the laboratory findings, the mother immediately stopped breastfeeding and the acute symptoms had improved within hours. Finally, DNA analysis of the *GALT* gene revealed mutation in heterozygous form: c.37C > T (p.Gln 13*) and c.200G > A (p.Arg67His). The proband was thus definitively diagnosed as galactosemia type I, Duarte variant (GALAC1, OMIM #230400). The girl was placed under specialist care and prescribed a lifelong galactose-free diet. Today, the girl is 3 years old, and her condition is well established.

3.4. Application of the LC-MS/MS assay on urine samples from diagnostic Proficiency testing (DPT) ERNDIM control scheme

To verify diagnostic possibilities, our developed method and

Table 2
A summary of IMD patients diagnosed with the developed method.

Inherited metabolic disorder	OMIM number	Patients diagnosed (n)	Abnormal metabolites	RS values (median)	Reference RS values for <1y (5; 95 %)	Reference RS values for >1y (5; 95 %)
3-Hydroxy-3-methylglutaryl-CoA lyase deficiency	246450	1	C5:1 Gly	33.5	−0.3; −5.0	−0.5; 3.0
3-Methylcrotonyl-glycinuria	210200	2	C5:1 Gly	751.1, 3104.3	−0.3; −5.0	−0.5; 3.0
Adenine phosphoribosyltransferase deficiency	614723	1	2,8-Dihydroxyadenine	232.1	−0.6; 1.7	−0.7; 2.1
			Adenine	43.8	−0.7; 2.8	−1.0; 2.6
Adenylosuccinate lyase deficiency	103050	1	SAICAr	36.4	−0.5; 2.3	−0.6; 3.1
			Succinyladenosine	11.5	−0.6; 1.6	−0.6; 2.2
Allopurinol treatment	–	1	Orotic acid	40.7	−0.7; 1.4	−0.7; 2.1
			Orotidine	39.4	−1.3; 1.4	−0.7; 1.7
Aminoacylase deficiency	609924	6	*N*-Acetylserine	36.9–74.3 (62.0)	−0.4; 3.7	−0.7; 1.9
			N-Acetylleucine	15.3–49.0 (29.9)	−0.8; 2.3	−0.8; 2.7
			N-Acetylvaline	6.8–17.7 (14.3)	−0.7; 1.3	−0.9; 2.1
			N-Acetylglutamine	5.6–14.8 (8.0)	−0.5; 2.5	−0.5; 1.9
			N-Acetylglycine	22.6–46.8 (41.6)	−0.8; 1.5	−0.7; 2.2
Argininosuccinic aciduria	207900	3	Argininosuccinic acid	1389.7–6252.0 (4441.6)	−0.7; 1.6	−0.9; 1.9
			Orotic acid	1.5–12.7 (7.9)	−0.7; 1.4	−0.7; 2.1
Canavan disease	271900	3	*N*-Acetylaspartic acid	42.3–77.6 (73.8)	−0.8; 1.2	−0.6; 1.7
Citrullinemia type I	215700	1	Orotic acid	95.7	−0.7; 1.4	−0.7; 2.1
Dihydropyrimidine dehydrogenase deficiency	274270	1	Thymine	281.1	−1.0; 1.4	−0.6; 1.9
			Uracil	21.7	−0.8; 1.4	−0.8; 2.4
Galactosemia type I	230400	3	Hexitols	53.0–1969.5 (442.5)	−1.1; 1.9	−0.6; 2.3
			Hexoses	−0.1 - 499.5 (50.8)	−0.4; 3.1	−0.6; 3.6
Glutaric aciduria type I	231670	2	C5-DC Gly	21.4, 51.6	−0.8; 1.0	−0.6; 2.4
Glycogen storage disease type II	232300	1	Tetrasaccharides	13.9	−0.8; 2.7	−0.5; 5.2
Guanidinoacetate methyltransferase deficiency	612736	2	Guanidinoacetic acid	9.8, 18.2	−0.6; 1.0	−0.8; 2.2
			Creatine	−0.2, −0.2	−0.3; 1.6	−0.3; 1.4
HHH syndrome	238970	2	Orotic acid	40.3, 55.3	−0.7; 1.4	−0.7; 2.1
			Homocitrulline	6.5, 10.3	−0.6; 3.2	−0.5; 2.1
Isovaleric aciduria	243500	3	C5 Gly	153.7–1833.3 (471.3)	−0.7; 1.5	−0.8; 2.7
Lysinuric protein intolerance	222700	1	Orotic acid	96.6	−0.7; 1.4	−0.7; 2.1
Medium chain acyl-CoA dehydrogenase deficiency	201450	2	C3 Gly	−0.6, −0.7	−0.5; 1.6	−0.7; 1.9
			C6 Gly	16.4, 440.7	−0.6; 1.8	−0.6; 2.3
			3-PP Gly	360.1, 2679.8	−0.2; 2.8	−0.4; 2.1
			C8-DC Gly	49.1, 1764.6	−0.9; 2.5	−0.8; 1.3
Methylmalonic aciduria	251000	3	C3 Gly	4.0–415.2 (24.8)	−0.5; 1.6	−0.7; 1.9
			Tiglyl Gly	3.0–117.1 (39.1)	−0.3; 1.9	−0.5; 1.7
Mitochondrial neurogastrointestinal encephalomyopathy	603041	2	Thymidine	3159.1, 4757.1	−0.5; 2.2	−0.9; 3.1
			2-Deoxyuridine	516.9, 552.9	−0.7; 3.3	−0.5; 1.7
			Thymine	77.1, 201.8	−1.0; 1.4	−0.6; 1.9
			Uracil	6.5, 17.5	−0.8; 1.4	−0.8; 2.4
Ornithine transcarbamylase deficiency	311250	1	Orotic acid	1942.6	−0.7; 1.4	−0.7; 2.1
			Uridine	272.7	−0.8; 2.3	−0.7; 1.9
			Uracil	42.9	−0.8; 1.4	−0.8; 2.4
			Uric acid	9.9	−0.9; 1.8	−1.0; 2.1
			Homocitrulline	2.0	−0.6; 3.2	−0.5; 2.1
Orotic aciduria	258900	1	Orotic acid	29.6	−0.7; 1.4	−0.7; 2.1
Propionic aciduria	606054	1	C3 Gly	698.6	−0.5; 1.6	−0.7; 1.9
			C4 Gly	22.5	−0.4; 3.1	−0.6; 2.4
			C5 Gly	40.8	−0.7; 1.5	−0.8; 2.7
			C5:1 Gly	67.8	−0.3; −5.0	−0.5; 3.0
β-Ketothiolase deficiency	203750	1	Tiglyl Gly	399.6	−0.3; 1.9	−0.5; 1.7
Xanthinuria type I	278300	2	Xanthine	6.5, 10.0	−1.3; 1.2	−0.7; 1.2
			Uric acid	−1.7, −1.0	−0.9; 1.8	−1.0; 2.1
			Hypoxanthine	−0.8, 0.5	−0.5; 1.4	−0.7; 1.6

diagnostic workflow were also tested on 25 urine samples from EQA - ERNDIM DPT. The samples from years 2010–2022 were used. The list of samples and the corresponding diagnoses are shown in Supplemental Table 13. In all cases, EQA samples were correctly distinguished from control samples based on RS values for pathological metabolite levels. A multi-analyte scatterplots of RS values of the DPT ERNDIM samples is shown in Fig. 11.

3.5. Long-term stability of the method

Long-term stability of the method was monitored by IQC sample measured over the last year and a half ($n = 60$). The average of concentrations of analytes and CV (%) is shown in Table 3. The CV values for majority of analytes ranges in 9–20 %, indicating good stability of the method. The CV values above 20 % were obtained for 2,8-dihydroxyadenine, succinyladenosine, SAICAr and C2 Gly. 2,8-dihydroxyadenine showed a higher CV value probably due to its poor solubility. C2_Gly suffers from poor peak shape with low separation efficiency. In the case

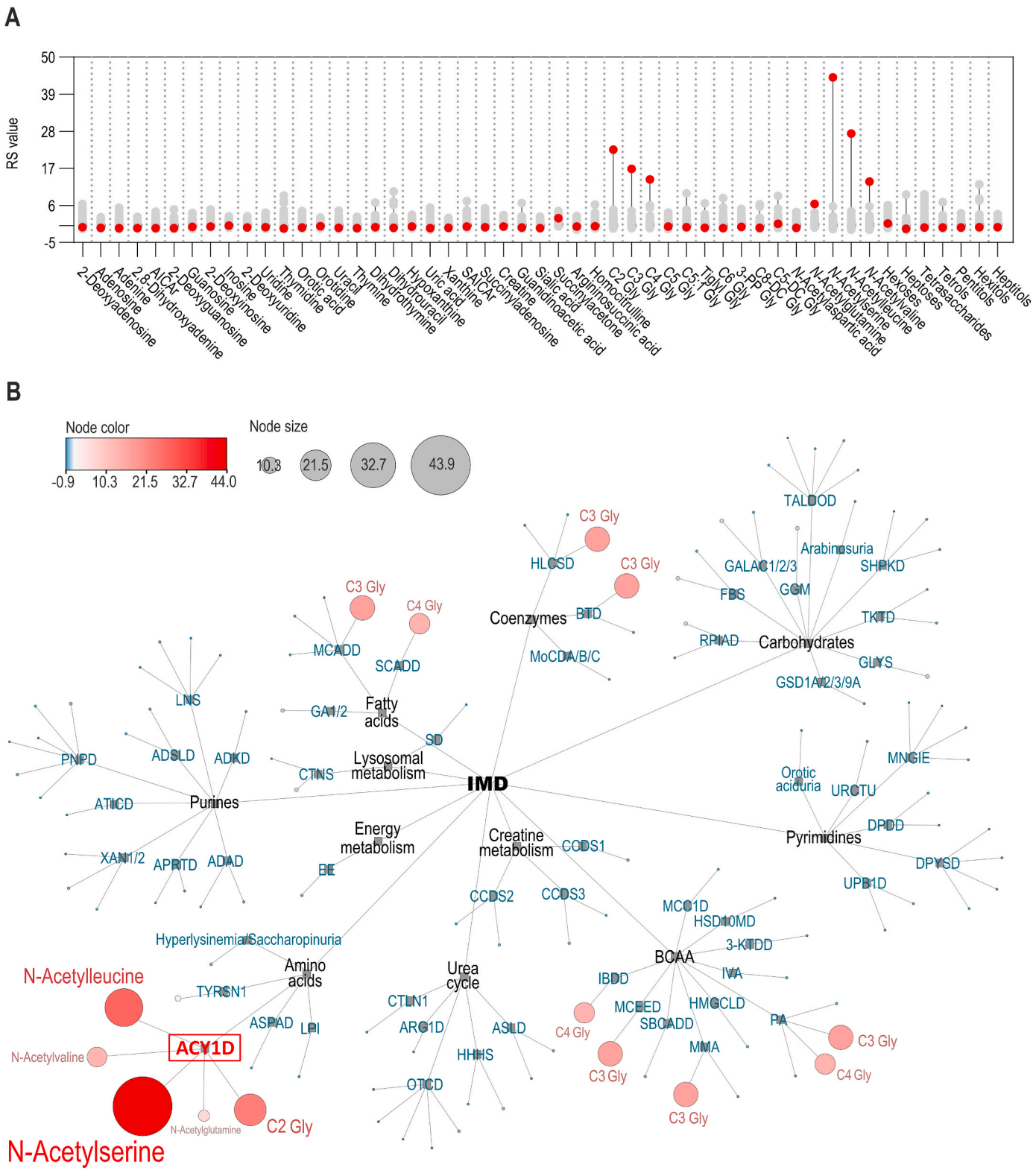

Fig. 9. Aminoacylase 1 deficiency: Multianalyte scatterplot of RS values for Patient21 (red dots) versus controls (grey dots) (A) and corresponding IMD network (B). Based on elevated biomarkers suggestive of ACY1D, the patient was correctly diagnosed.

of succinyladenosine and SAICAr, the reason is not fully clear and can be associated with high retention on the column.

4. Discussion

The diagnosis of IMD is not only complicated by its low incidence and nonspecific symptoms, but also because of the extensive and challenging techniques involved in the analysis of the diagnostic markers. Currently, the trend is to use methods that include a time-efficient analysis of a wide range of metabolites. Therefore, two-dimensional thin-layer chromatography, reversed-phase HPLC or GC-MS technique are being replaced by LC-MS/MS methods using multiple-reaction

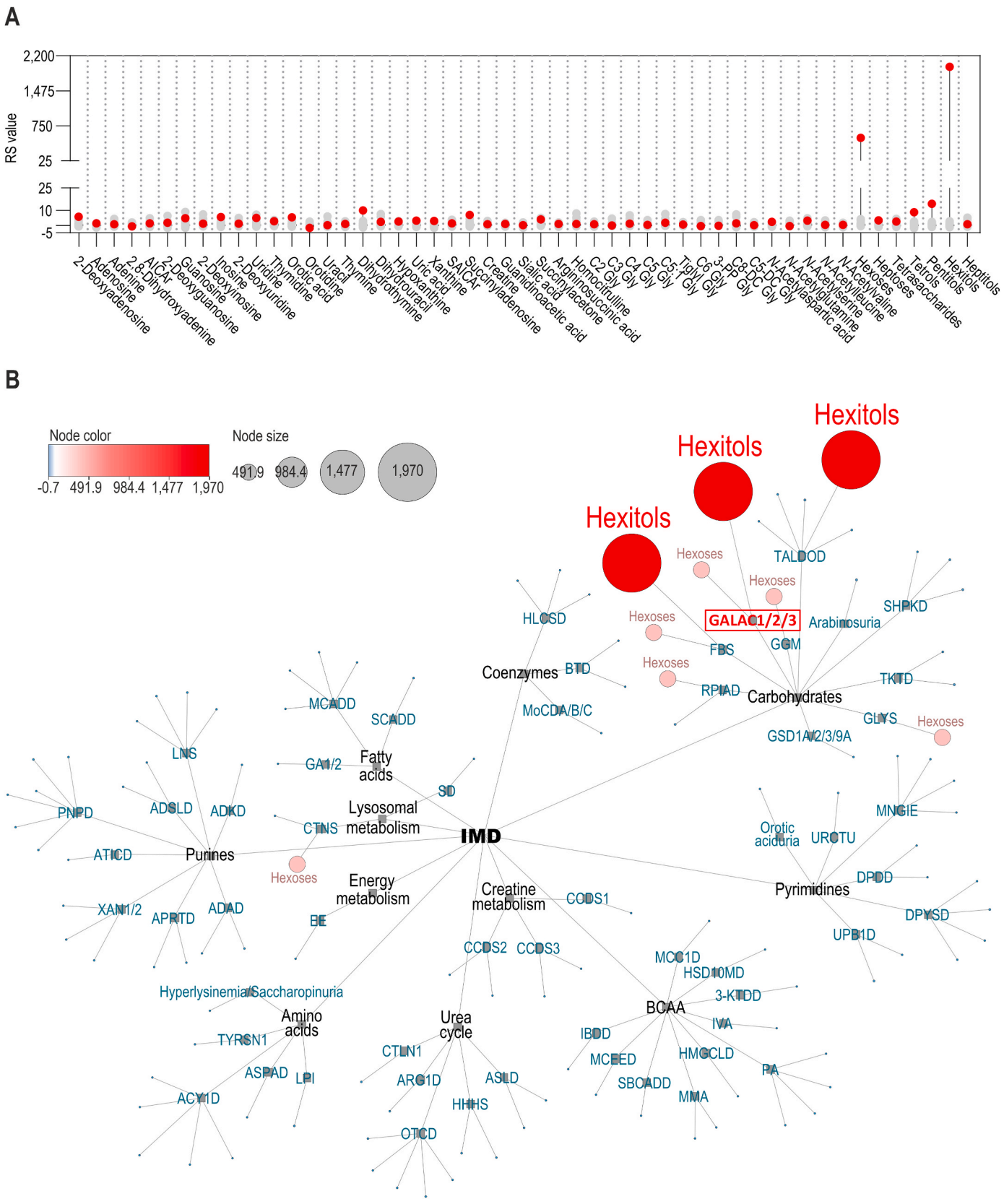

Fig. 10. Galactosemia type I, Duarte variant: Multianalyte scatterplot of RS values for Patient01 (red dots) versus controls (grey dots) (A) and corresponding IMD network (B). Based on elevated biomarkers suggestive of GALAC1/2/3, the patient was correctly diagnosed.

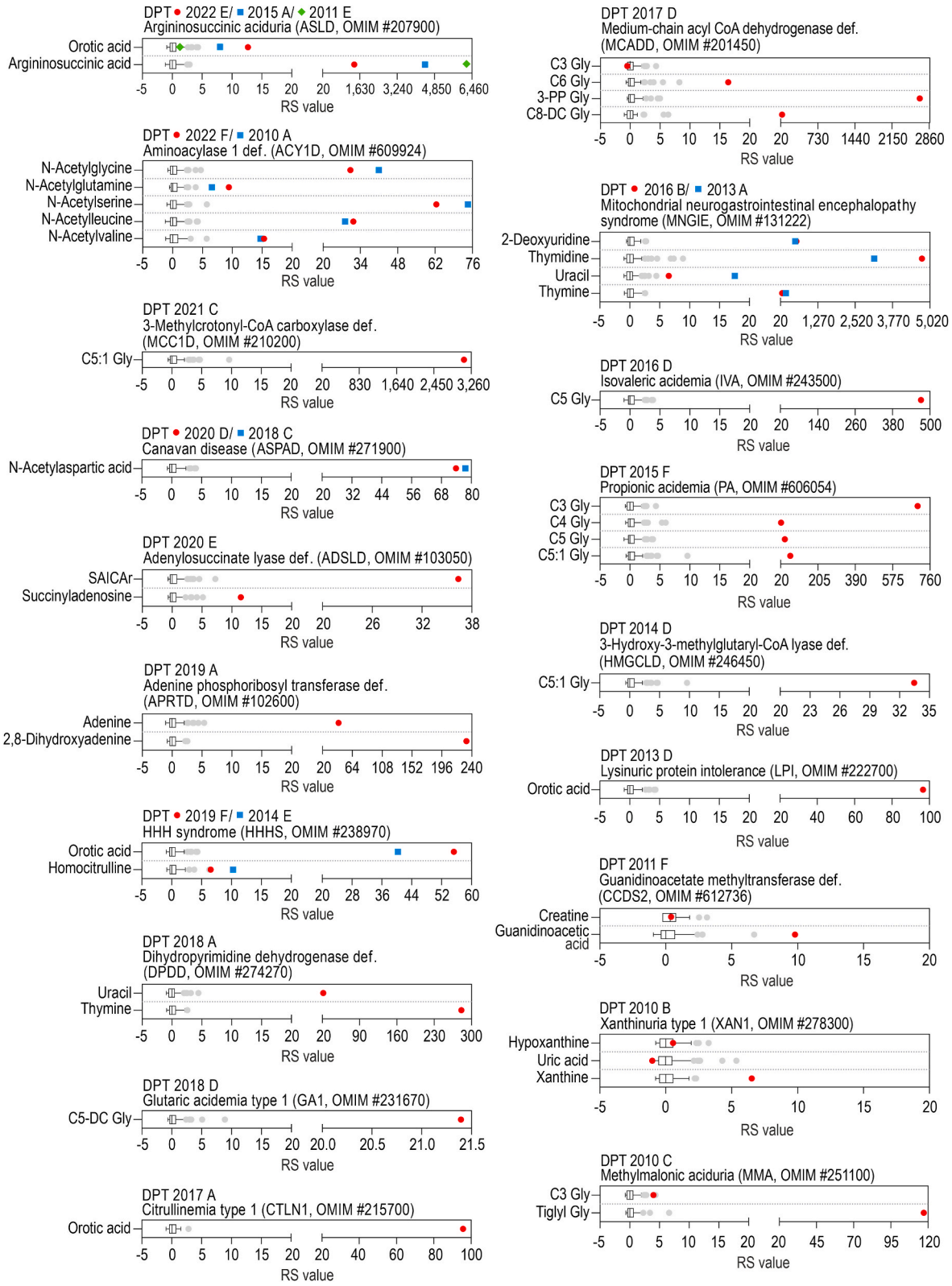

Fig. 11. Multianalyte scatterplots of RS values of 25 samples from DPT ERNDIM control scheme from the years 2010–2022.

monitoring, which is a highly selective and sensitive method for quantification of specific compounds of interest in a shorter time. Our laboratory has extensive experience in the development and optimization of HILIC-MS/MS methods and their application in metabolomic studies [23–26]. However, in this work, the goal was to focus only on IMD-related metabolites (particularly involving metabolism of PP, AA, branched-chain AA, carbohydrates, fatty acids and urea cycle) to make the analysis and evaluation as simple and fast as possible.

Here we present a novel LC-MS/MS approach allowing simultaneous analysis of a wide range of metabolites and concentrations. Unique separation mode based on an aminopropyl stationary phase allows analysis of many polar metabolites with various physico-chemical

Table 3
Stability of the method by IQC sample ($n = 60$).

Compound	Concentration (μmol/L)	CV (%)
2-Deoxyadenosine	17.3	12.6
2-Deoxyguanosine	14.7	12.2
2-Deoxyinosine	15.5	12.9
2-Deoxyuridine	17.0	16.8
2,8-Dihydroxyadenine	1.8	30.2
Adenine	49.4	14.8
Adenosine	40.1	14.0
AICAr	16.1	19.1
Dihydrothymine	42.0	20.1
Dihydrouracil	44.	18.2
Guanosine	14.7	14.0
Hypoxanthine	43.9	18.2
Inosine	120.7	16.9
Orotic acid	59.0	8.6
Orotidine	1.3	15.4
SAICAr	1.7	21.9
Succinyladenosine	1.6	24.2
Thymidine	17.4	14.3
Thymine	86.7	9.7
Uracil	118.7	12.3
Uric acid	158.6	12.9
Uridine	0.2	19.5
Xanthine	72.7	9.8
N-Acetylaspartic acid	5.7	18.7
N-Acetylglutamine	1.8	16.0
N-Acetylleucine	0.4	17.8
N-Acetylserine	0.9	17.4
N-Acetylvaline	0.8	19.3
Argininosuccinic acid	2.2	18.1
Succinylacetone	12.6	18.8
Homocitrulline	2.6	13.1
C2 Gly	1.8	22.5
C3 Gly	0.2	12.8
C4 Gly	0.9	18.8
C5 Gly	1.3	14.9
C5:1 Gly	0.9	18.5
Tiglyl Gly	3.1	20.6
C6 Gly	0.5	16.4
3-PP Gly	0.03	19.5
Suberyl Gly	0.1	15.7
C5-DC Gly	0.3	19.3
Tetrols	51.0	16.5
Pentitols	44.0	10.1
Hexitols	72.2	8.6
Heptitols	0.2	20.2
Hexoses	62.0	17.2
Heptoses	8.0	18.7
Tetrasaccharides	0.2	16.4
Guanidinoacetic acid	139.2	11.8
Creatine	359.9	15.3
Creatinine	4119.5	10.0
Sialic acid	60.4	13.5

properties (e.g. AA, PP, organic acids, nucleotides and others). This new highly sensitive and efficient approach is in line with current trends in disease diagnosis, as it features simple sample preparation (dilution step and addition of IS), short analysis time (11.5 min) and a semi-automated diagnostic procedure. As a part of the method development, it was necessary to consider how to display all metabolites at once to make the evaluation of results fast and convenient. Therefore, a simple data processing workflow was used, involving easy peak integration and quantification of the analytes, conversion of the calculated concentrations by age group, transformation of the data using an RS value and advanced visualization software tools. This workflow can be performed in less than an hour of sample delivery, making it a reliable for IMD diagnosis.

The sensitivity of the newly developed method was compared with a validated method for the diagnosis of purine and pyrimidine disorders from urine [9], in which LOQ for 26 biomarkers were determined (LOQ = 0.0125–2.5 μmol/L; median of LOQ = 0.25 μmol/L). LOQ of analytes in our developed method ranges from 0.003 to 1.829 (median of LOQ = 0.040 μmol/L). Compared to the above mentioned method [9], the newly developed method shows an order of magnitude higher sensitivity.

Analytical validation, based on the EMA guideline [14], was performed for a total of 52 out of 65 analytes, as only one representative per isomer group was selected within AG, sugars and sugar alcohols. Most analytes met the acceptance criteria for inter-day accuracy and precision except for 2,8-dihydroxyadenine or also perseitol, *N*-propionylglycine, orotidine, and inosine. In the case of 2,8-dihydroxyadenine, its low accuracy and higher precision may be related to its very poor solubility. However, 2,8-dihydroxyadenine plays an important role in the diagnosis of adenine phosphoribosyltransferase deficiency (OMIM #614723; APRTD) as it is one of the two diagnostic markers. As it is not physiologically present in urine, its mere presence in urine is indicative of the disease. Therefore, its validation shortcomings do not play a major role and do not cause difficulties in the diagnosis of APRTD in this study (as shown in Supplemental Fig. 1C). Lower accuracy was obtained at all 3 QC levels for perseitol, an additional marker for transladolase deficiency (OMIM #606003), whose diagnosis can be based on more diagnostically specific markers, just as inosine is not the only marker related to purine nucleoside phosphorylase deficiency (OMIM #613179). Higher accuracy values were obtained for orotidine, whose increase in urine may be related to allopurinol or other drug treatment. Patients under treatment have not only elevated urinary orotic acid but also the orotidine. Therefore, it is important that the method ensures detection of potentially false-positive patients for ornithine transcarbamylase deficiency (OMIM #311250), who, in contrast, would not have elevated the orotidine. It can therefore be said that these slight variations did not affect the reliability of the method developed. Higher deviations especially in terms of accuracy were obtained for LQC of N-propionylglycine, although the deviations for MQC and HQC are minimal. Since *N*-propionylglycine tends to be very elevated in patients compared to controls, the MQC and HQC results are more interesting for diagnostic purposes.

Regarding the matrix effect, no interference in the elution time of the analytes was detected in the positive mode. On the contrary, in the negative mode, ion enhancement was observed, mainly influenced by ACN, due to increasing sensitivity of the mass spectrometer. Analytes affected include dihydrothymine, dihydrouracil, thymine, uracil, thymidine and deoxyuridine, which may be the reason why the also have the lowest column efficiency. Moreover, different solvent composition of urine samples (water) and mobile phase (CAN) led to poor peak shape of selected analytes (e.g. C2_Gly) and limited injection volume to achieve good separation selectivity and sensitivity. One practical limitation of the method is the lifetime of the Luna NH_2 column, which allows approximately 500 injections under highly alkaline separation conditions. However, during the 7 years that the method was used for diagnostics, only 5 columns were needed to ensure stable chromatographic selectivity over the entire time. From an analytical/diagnostic perspective, another limitation is the lack of resolution of AG and sugar isomers. Therefore, in case of abnormal findings, confirmation by other methods should be considered.

Current trends focus on the use of low sample volumes, which is consistent with our newly developed method where a very small amount of urine sample (less than 100 μL) is required. This meets the need of physicians to analyse the urine of newborns, which is scarce and is characterised by low creatinine. In the future, the method could also be optimised for plasma and extended to other metabolite groups such as ornithine cycle intermediates (ornithine, citrulline, aspartate, arginine, fumarate) and phosphates such as sedoheptulose-7-phosphate, a marker of transaldolase deficiency (OMIM #606003) [27] and glycerol-3-phosphate, a marker of fructose-1,6,-bisphosphatase deficiency (OMIM #229700) [28].

In conclusion, a quantitative HILIC-MS/MS method was developed and validated enabling the analysis of a wide range of IMD-related biomarkers. This highly sensitive approach introduces a diagnostic platform for >50 IMD enabling the quantitation of >60 urinary biomarkers. Simple sample preparation is complemented by short analysis

time and a straightforward diagnostic workflow, making the developed method suitable for clinical laboratories addressing urgent requirements. The clinical performance of the method has been validated in the laboratory over a period of seven years on patient and EQA DPT samples. The method was applied to other biological matrices with the possibility of optimising metabolite coverage. It is thought the method will be expanded with other diagnostically relevant IMD biomarkers according to medical needs.

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CRediT authorship contribution statement

Eliška Ivanovová: Conceptualization, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Barbora Piskláková:** Data curation, Methodology, Validation, Visualization. **Dana Dobešová:** Data curation, Methodology, Software. **Hana Janečková:** Methodology, Validation, Writing – original draft. **Hana Foltenová:** Resources. **Aleš Kvasnička:** Data curation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Matúš Prídavok:** Methodology, Resources, Validation. **Kateřina Bouchalová:** Data curation, Resources. **Julie de Sousa:** Data curation, Software. **David Friedecký:** Conceptualization, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2024.125699.

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