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Evaluation of comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry for the diagnosis of inherited metabolic disorders using an automated data processing strategy

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ABSTRACT

Organic acidurias are a large group of inherited metabolic disorders (IMDs), commonly diagnosed by GC–MS analysis of organic acids in urine after acidic extraction and trimethylsilylation. In this study, a GC × GC-ToF-MS method has been optimized for the analysis of pathological metabolites in urine. An automated data processing strategy based on the use of mass spectra and GC retention times for the target search and quantification of pathological metabolites has been developed. Using this procedure, each unknown sample is automatically examined for the presence of markers of several diseases at the same time. The method has been applied for the analysis of 6 challenging proficiency testing samples from patients with IMDs (thymidine phosphorylase deficiency, mevalonic aciduria, hawkinsinuria, aromatic L-amino acid decarboxylase deficiency, propionic acidemia and medium-chain acyl-CoA dehydrogenase deficiency). Using the GC × GC-ToF-MS method, we were able to determine complete sets of markers for all the IMDs. The quality of the mass spectral matches for the pathological markers was higher than 800 (out of 1000).

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1. Introduction

Inherited metabolic disorders (IMDs) are a diverse group of diseases caused by genetic defects affecting enzyme functions. With the exception of ethnic-specific diseases, the general incidence of IMDs ranges from 1 in 5000 (e.g. hyperphenylalaninemia) to individual cases of very rare diseases. The collective incidence of IMDs is estimated to 0.04% [1]. Thanks to recent advances in therapeutic approaches, such as enzyme replacement therapy or allogeneic hematopoietic stem cell transplantation, an increased proportion of IMDs can be treated, when diagnosed in time. This fact emphasizes the need for accurate and effective diagnosis methods.

A large subgroup of IMDs called organic acidurias is characteristic by the presence of organic acids in urine, or their increased levels. The diagnosis of organic acidurias is commonly performed by the analysis of urine after acidic extraction. Besides organic acids, some other types of metabolites (see below) also serving as pathological markers, are extracted. As concerns the analytical techniques used for the determination of these compounds, GC–MS after trimethylsilylation is widely used, being applicable for a wide range of metabolites of interest.

GC–MS analysis of urine represents a challenging task for the following reasons: (i) urine is an extremely complex matrix where hundreds of structurally different compounds, such as organic acids, purines, pyrimidines, acylglycines, exogenous compounds (e.g. drugs, dietary artifacts) or N-acetyl-amino acids, are present [2]; (ii) concentrations of individual compounds vary by orders of magnitude; this makes the method optimization difficult, especially as concerns the amount of injected sample; (iii) highly abundant natural metabolites can mask the target markers and overload GC columns [3]; (iv) some diseases exhibit very mild changes in urine composition, often difficult to detect by GC–MS, which typically happens in patients well compensated by pharmacological treatment.

In the last decade, comprehensive two-dimensional gas chromatography (GC × GC) has been shown as a powerful technique in the analysis of complex matrices. In GC × GC, two columns of different selectivity are coupled via a modulator and the separation of the whole sample by two different separation mechanisms is performed. In GC × GC, the overall separation power is increased [4,5] and analyte detectability can be improved [6,7] compared to one-dimensional GC. The principle advantages and applications of GC × GC have been described in recent reviews [4,8–11]. For the coupling of GC × GC with MS detection, a mass spectrometer fast enough is preferred to enable the description of the very narrow peaks (typically 0.1–0.3 s) produced by this separation technique.

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Hence, high-speed ToF-MS is the technique of choice in most studies [6].

Recently, the importance of GC × GC-ToF-MS has increased in the field of metabolomics. However, few papers on human urine analysis have been published, including studies related to the development of an algorithm for metabolites searching [12], analysis of anabolic drugs for doping control [13], diabetes biomarker discovery [14], and diagnosing of organic acidurias [15]. In the last-mentioned paper the authors studied the abnormal profiles obtained from patients with several IMDs.

In the work presented here, we have developed a GC × GC-ToF-MS method for routine determination of pathological markers of organic acidurias. This method employs an automated data processing strategy for the target detection of all the known pathological metabolites for several IMDs at the same time.

2. Experimental

2.1. Reagents and chemicals

Internal standard (4-phenylbutyric acid, 99%), ethoxyamine hydrochloride, methanol (HPLC grade), pyridine (p.a.) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), containing 1% of trimethylchlorosilane (TMCS) were purchased from Sigma–Aldrich (St. Louis, USA). Other chemicals for sample preparation, i.e. hydrochloric acid, sodium chloride, anhydrous sodium sulphate, ethyl acetate (p.a.) and acetone (p.a.), were supplied by LACH-NER (Neratovice, Czech Republic). All chemicals and reagents were of analytical grade or higher.

2.2. Urine samples

Urine samples used for building the reference (Section 3.2.2) were from infants from routine diagnostic processes performed in the authors' laboratory. Besides the pathological samples, 10 healthy urines were analyzed.

For the purpose of GC × GC-ToF-MS method evaluation, five samples from quality assurance schemes of the European Research Network for the evaluation and improvement of screening Diagnosis and treatment of Inherited disorders of Metabolism – ERNDIM [16] were analyzed. In all the patients the diagnoses had been previously confirmed by enzyme or molecular-genetic analyses. In addition, urine from an asymptomatic patient, who had been diagnosed with medium-chain acyl-CoA dehydrogenase deficiency (MCADD) by neonatal screening sixth days after birth, was analyzed.

Collected urine samples were stored at −20 °C. Prior to extraction, the samples were allowed to thaw at room temperature.

2.3. Extraction and derivatization

For extraction and derivatization a method based on Tanaka et al. [17] was used.

Two milliliters of urine were pipetted into a ground-joint glass tube, and 40 μL of internal standard solution (4-phenylbutyric acid, 2.105 mg/mL in methanol) and 10 mg of ethoxyamine hydrochloride were added. The mixture was shaken and left at room temperature for 30 min. After ethoxymation the mixture was saturated with sodium chloride and acidified by 100 μL of 5 mol/L hydrochloric acid. Organic acids were then extracted three times with 4 mL of ethyl acetate for 10 min using a shaker. The organic layers were combined in another tube, dried with anhydrous sodium sulphate, transferred to a new tube, and evaporated to dryness under N_2 at room temperature. The dry matter was subsequently extracted with two 400 μL portions of methanol and one 300 μL portion of acetone. Liquid phases were combined, transferred into an injection vial, and again evaporated to dryness under N_2 at room temperature. For trimethylsilylation, 400 μL of pyridine and 150 μL of N-methyl-N-trimethylsilyltrifluoroacetamide (activated with 1% of trimethylchlorosilane) were added to the dry extracts and incubated at 60 °C for 60 min.

2.4. Instrumentation

2.4.1. GC × GC-ToF-MS

A Pegasus 4D system consisting of an Agilent 7890A gas chromatograph equipped with a split/splitless injector, a 7683 B Series Autosampler (Agilent Technologies, Palo Alto, CA, USA) and a Pegasus HT time-of-flight mass spectrometer (LECO Corporation, St. Joseph, MI, USA), was used. The GC × GC system employed a dual-stage, quad-jet modulator and a secondary oven, both built-in to the Agilent GC oven. A consumable-free option of the modulator was employed. Compressed air was used for both hot and cold modulation jets. For the hot jets the air was resistively heated, while for the cold jets the air passed through a moisture filter and was cooled by immersion cooling.

The first dimension column was a 30m × 0.25 mm × 0.25 μm Rxi-5ms (Restek, Bellefonte, PA, USA) and the second dimension column was a 2.5m × 0.10 mm × 0.10 μm BPX-50 (SGE, Ringwood, Australia). The columns were connected using a SilTite Mini Union (SGE, Ringwood, Australia). The connection was done between the modulator and secondary oven, so modulation occurred on the primary column. The operating conditions were as follows:

GC × GC: primary oven temperature: 40 °C (2 min), 8 °C/min to 155 °C (0.2 min), 10 °C/min to 255 °C (0.20 min), and balistically heated to 300 °C (11 min); secondary oven temperature: +5 °C above the primary oven temperature; modulator temperature: +50 °C above the primary oven temperature; modulation period: 3 s; carrier gas: helium; corrected constant flow: 1 mL/min; injection mode: splitless 2 min; injection temperature: 250 °C, injection volume: 0.2 μL.

ToF-MS: electron ionization (−70 eV); ion source temperature: 250 °C; acquired mass range: 35–550 m/z; acquisition rate: 125 spectra/s; solvent delay: 650 s; detector voltage: −1600 V; transfer line temperature: 250 °C.

ChromaTOF software v. 4.24 (LECO Corporation, USA) was used for system control, data acquisition, and data processing. The NIST/EPA/NIH Mass Spectral Library (2008) was used for tentative identification of compounds, with confirmations by retention indices comparisons.

2.4.2. GC-qMS

A system consisting of a Trace GC Ultra, a split/splitless injector, a TriPlus autosampler, and DSQ II quadrupole mass spectrometer (Thermo Electron Corporation, USA) was used. The GC column and the oven temperature program were the same as the primary column and its program used in the GC × GC-ToF-MS experiments (Section 2.4.1). Other operating conditions were as follows:

GC: Injection mode – split 1:10; injection volume: 2 μL.

MS: electron ionization (−70 eV); ion source temperature: 200 °C; acquired mass range: 60–489 m/z; acquisition rate: 1.1 spectra/s; solvent delay: 615 s; detector voltage: −1117 V; transfer line temperature: 250 °C.

For the GC–MS data processing, Xcalibur 1.4 software (Thermo Electron Corporation, USA) was used.

2.5. Quantification

The analyte concentration (in mmol/mol creatinine) was calculated from the dTIC peak area (Section 3.2.1) ratio between analyte and internal standard, the known internal standard concentration (250 μmol/L urine), and the measured creatinine concentration of

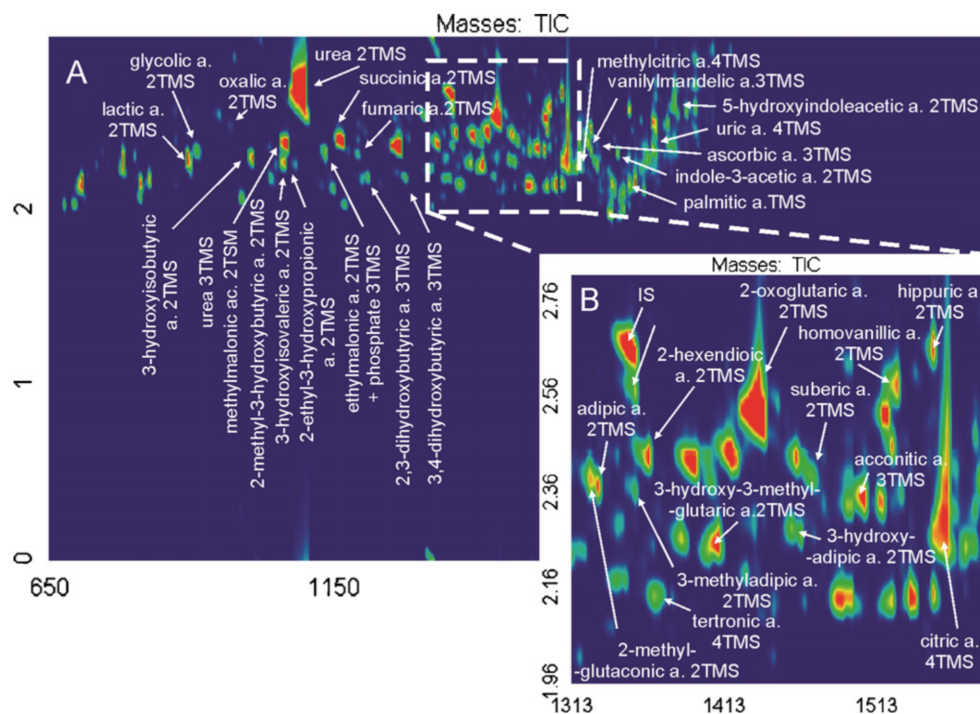

Fig. 1. GC × GC-ToF-MS contour plot from the analysis of healthy urine (A) and enlarged part (B).

the urine sample (the measurement of creatinine was performed by the Jaffe method on a Hitachi 917 automatic analyzer – Roche, Germany).

3. Results and discussion

3.1. Separation and identification capability of GC × GC-ToF-MS

Fig. 1A and B show the contour plot from the GC × GC-ToF-MS analysis of healthy urine, where the main metabolites are identified. It is obvious that urine extract represents a chromatographically challenging material. Using GC × GC, the additional separation in the second dimension enables resolution of peaks originally coeluted when only using the primary column.

Automated data processing with peak finding above S/N 200 was applied to the data, which resulted in the detection of 1353–3420 peaks in the set of healthy urines. After sorting out GC column bleed peaks and the peaks belonging to the derivatization reagent, some 60% of the peaks remained. Tentative identification of those peaks was based on comparing their deconvoluted spectra with reference spectra in the NIST library. Using further confirmation of identity based on retention indices, we were able to identify at least 200 compounds, which will be the subject of a separate paper.

3.2. Automated data processing strategy

3.2.1. Quantification strategy and deconvoluted TIC

Reference materials of pathological metabolites are not always easily available and/or their cost is considerably high. Therefore, it is a common practice to use the total ion chromatogram (TIC) signal for quantification along with the internal standard use. This approach was used also in this work – see Section 2. TIC quantification can, however, produce overestimated results in case of chromatographic coelutions, which cannot be completely avoided even with GC × GC.

This obstacle was overcome in our work by using deconvoluted total ion chromatogram (dTIC) for the quantification of the analytes. Deconvolution is a mathematical algorithm that is based on the absence of spectral skew and faster acquisition rates for peak apex definition in ToF-MS data. This algorithm mathematically separates mass spectra of compounds that chromatographically coelute [18]. In addition to producing deconvoluted spectra, ChromaTOF software also allows the calculation of dTIC peak area. Deconvoluted TIC is the portion of TIC area corresponding to a particular analyte in a coelution. An example is shown in Fig. 2. The peak of suberylglycine 2TMS in the sample from an MCADD patient (see below) is only partially separated from the coeluted compound, as seen from the TIC trace. The real profile of the analyte peak is obvious from the trace of its characteristic mass 360. The dTIC trace calculated by the software and used for quantification corresponds to the part of the TIC belonging to the suberylglycine 2TMS peak.

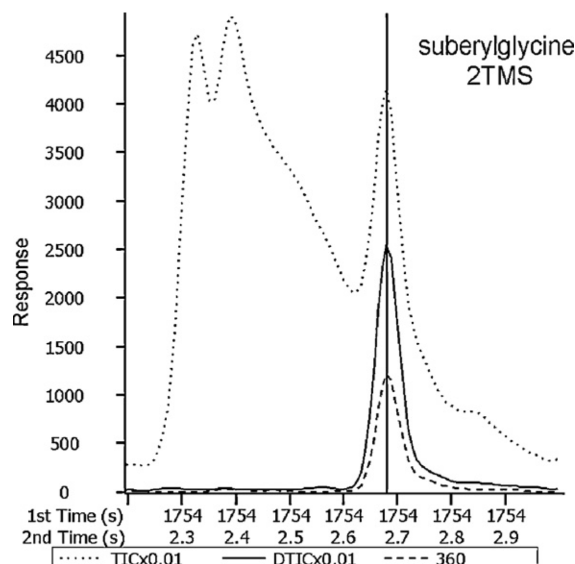

Fig. 2. Example of deconvoluted TIC (dTIC) for suberylglycine 2TMS in urine from MCAD deficient patient.

Table 1
Repeatability of retention times for selected urine metabolites. Determined for the analysis of 10 urine samples within 2 consecutive days of analysis.

Compound	Mean t^R 1st dim. (s)	RSD (%)	Mean t^R 2nd dim. (s)	RSD (%)
Lactic acid 2TMS	893	0.17	2.289	0.47
3-Hydroxypropionic acid 2TMS	986	0.10	2.338	0.56
Uracil 2TMS	1195	0.17	2.468	1.20
Mevaloniclactone TMS	1229	0.08	2.834	0.24
Thymine 2TMS	1253	0.08	2.441	0.60
Adipic acid 2TMS	1332	0.10	2.370	0.22
4-Phenylbutyric acid TMS (IS)	1352	0.13	2.649	1.74
5-Oxoproline 2TMS	1355	0.07	2.573	0.43
N-Acetylaspartic acid 3TMS	1457	0.12	2.286	0.38
Orotic acid 3TMS	1503	0.14	2.392	1.04
Citric acid 4TMS	1559	0.12	2.264	1.24
4-Hydroxyphenyllactic acid 3TMS	1606	0.13	2.256	0.69
N-Acetyltyrosine 2TMS	1709	0.06	2.506	0.47

3.2.2. Using the reference for the automated determination of markers

When a classical GC–MS is used, the chromatogram is typically reviewed manually by plotting characteristic masses in the segment of the expected retention time. To avoid such a time-consuming procedure, we applied a ChromaTOF feature called "Automated sample comparison". This procedure consisted of the following steps:

(i) The sample is processed using a general peak finding method (e.g. S/N 200).
(ii) Peaks of interest (pathological metabolites in this case) are exported to the reference, which is a set of information containing the retention times and mass spectrum of each analyte, among other data. Criteria such as the retention time window-width in both dimensions and minimum spectral match are defined by the user.
(iii) The reference is applied to search for each analyte in the unknown sample and for the quantification of positively identified analytes.

To create a reference applicable for the diagnosing of all 6 IMDs, markers were imported from different samples where the given metabolite was present. Using this approach, no reference materials (and standards) were necessary. In some cases the NIST library mass spectrum was used in the reference.

Minimum spectral match was set to 700 (out of 1000) and the retention time window was ±4.5 s for the first dimension (corresponds to the range of three modulations) and ±0.05 s for the second dimension. These retention time tolerances were determined based on the repeatability of retention times in healthy urines as shown in Table 1.

To avoid missing the target analyte in case of retention time shift, a retention time reference was applied. This option uses a retention reference compound, which is found first by the algorithm based on mass spectral search and retention time (a wider retention time window is set for this compound) and the retention times of all remaining peaks in the sample are corrected by the shift found for the retention reference compound. In our work, the retention time reference was the peak of internal standard, 4-phenylbutyric acid TMS.

3.3. Biochemical characterization of the selected IMDs

Thymidine phosphorylase deficiency (MNGIE). The deficiency of the cytosolic enzyme thymidine phosphorylase (EC 2.4.2.4) causes a multisystem disorder called mitochondrial neurogastrointestinal encephalomyopathy (MNGIE, OMIM 603041) [19]. The disease is biochemically characterized by the accumulation of thymidine, deoxyuridine, thymine, and uracil in body fluids. Within this group of compounds, uracil and thymine can be analyzed by GC after derivatization; the remaining compounds are LC amenable. Some of the MNGIE patients also exhibit elevated lactic acid as typical for mitochondrial dysfunctions. The analyzed sample was from a 36-year-old man with thymidine phosphorylase deficiency.

Mevalonic aciduria (OMIM 610377) caused by a mevalonate kinase (EC 2.7.1.36) deficiency is an inborn defect of cholesterol biosynthesis characterized by dysmorphology, psychomotor retardation, progressive cerebellar ataxia, and recurrent febrile crises, usually manifesting themselves in early infancy. This disease is characterized by slightly, to massively, increased excretion of mevalonic acid and its lactone [20]. The analyzed sample was from a 7-year-old girl suffering from febrile illness with an elevation of serum IgA and IgD.

Hawkinsinuria. 4-Hydroxyphenylpyruvic acid dioxygenase (HPD, EC 1.13.11.27) plays a role in the tyrosine degradation pathway, where it catalyzes the conversion of 4-hydroxyphenylpyruvic acid to homogentisate. Mutation of the HPD gene causes disorders, either of the tyrosinemia type III, or hawkinsinuria (OMIM 140350). In the presence of defective HPD, the final rearrangement step of the reaction is not completed and instead of homogentisate, either hawkinsin ((2-L-cystein-*S*-yl, 4-hydroxycyclohex-5-en-1-yl)acetic acid) or 4-hydroxycyclohexylacetic acids [21] are formed. The latter compounds serve as markers detectable by urinary organic acids analysis. The analyzed proficiency-testing sample was from an adult woman suffering from hawkinsinuria.

Aromatic L-amino acid decarboxylase deficiency (AADC). Aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28) is a central enzyme in the synthesis of biogenic monoamine neurotransmitters (serotonin, dopamine, and norepinephrine). A deficiency of AADC (OMIM 608643) is a recessively inherited severe neurometabolic disorder with developmental delay, abnormal movements, oculogyric crises, and vegetative symptoms. Within the neonatal screening procedures performed by urinary organic acid analysis, the diagnosis of AADC depends on the elevated level of vanillyllactic acid as the main marker [22,23]. The analyzed proficiency-testing sample was from a 4-year-old girl with severe seizures and myoclonus.

Propionic acidemia. This disorder (OMIM 606054) is caused by propionyl-CoA carboxylase (EC 6.4.1.3) deficiency. Propionyl-CoA is an intermediate in the metabolism of some amino acids (e.g. isoleucine, threonine), odd-chain fatty acids, and cholesterol. If further conversion of propionyl-CoA is impossible due to the enzyme dysfunction, it reacts with oxaloacetic acid to form methylcitric acid, a reliable indicator of propionic acidemia. Besides this compound, other markers are also useful. The sample was from an 8-year-old girl with a propionyl-CoA carboxylase deficiency [24].

Medium-chain acyl-CoA dehydrogenase (MCADD). Medium-chain acyl-CoA dehydrogenase (EC 1.3.99.3) deficiency (MCADD, OMIM #201450; *607008) is a potentially fatal disorder of the beta-

Table 2
Quantitative data of pathological metabolites in healthy and pathological urines.

Metabolite name	Concentrations of metabolites [mmol/mol creatinine]							
	Healthy urines[a]	MNGIE	Mevalonic aciduria	Hawkinsinuria	AADC	Propionic acidemia	MCADD	Reference range[b]
Lactic acid	9.53–73.2	67.7	40.2	25.9	59.4	53.5	35.8	<80
3-Hydroxypropionic acid	1.3–6.2	18.6	7.5	5.1	4.2	**54.9**	7.0	3–10
3-Hydroxyvaleric acid	<1.8	n.d.	n.d.	n.d.	n.d.	**16.6***	n.d.	<2
Uracil	1.7–9.5	**30.7**	3.9	5.1	3.8	1.9	2.8	2–22
5-Hydroxyhexanoic acid	<1.5	0.2	0.1	0.1	0.3	1.1	0.4	<7
Mevaloniclactone	<1.1	2.1	**51.8**	0.4	0.2	0.4	0.4	<2
Thymine	<0.9	**27.3***	0.7	0.4	0.5	0.1	0.3	<2
Propionylglycine	n.d.	n.d.	n.d.	n.d.	n.d.	**11.2***	n.d.	<2
Adipic acid	1.7–11.9	8.6	4.4	3.9	11.3	12.0	6.0	<12
Mevalonic acid	n.d.	n.d.	**1.8***	0.2	0.8	n.d.	n.d.	<1
4-Hydroxycyclohexylacetic a.	<2.2	0.2	2.2	**58.8***	2.0	2.1	n.d.	<2
Hexanoylglycine	<0.1	n.d.	0.3	0.1	0.6	1.6	**4.6***	<2
Suberic acid	<4.7	1.9	5.0	n.d.	9.1	9.3	**14.8***	<12
Sebacic acid	<0.5	0.6	0.3	n.d.	n.d.	0.6	0.6	<2
Methylcitric acid	<4.3	11.4	8.3	2.9	11.7	**97.1**	3.8	<12
Decenedioic acid	<1.5	n.d.	1.5	n.d.	0.5	2.0	1.0	<2
4-Hydroxyphenyllactic acid	<6	**11.6***	2.4	5.6	2.1	5.7	2.7	<6
Vanillylactic acid	<6	1.3	0.7	0.1	**5.5***	0.2	0.5	<2
Vanillylpyruvic acid	<0.4	n.d.	n.d.	0.1	**3.8***	0.5	n.d.	<2
N-Acetyltyrosine	<0.3	1.6	0.6	0.4	**2.8***	0.6	0.2	<2
Suberylglycine	<0.1	n.d.	n.d.	0.1	n.d.	n.d.	1.3	<2

In grey fields are the possible diagnostic metabolites known from literature; in bold are metabolites exceeding the reference range; metabolites with an asterisk (*) are the ones not found by the GC-qMS method – see text; n.d. – not detected.

[a] Range of concentration of metabolites in 10 healthy urines analyzed by GC × GC-ToF-MS.

[b] Reference ranges obtained by GC-qMS method.

oxidation of fatty acids. Individuals with undiagnosed MCADD typically present clinically with failure of fatty acid oxidation after fasting and an inability to generate energy during periods of increased energy demands. A number of fatty acids (adipic, suberic, and sebacic acids) and glycine conjugates are present in the urine of patients during a crisis; however, their levels can be in normal in asymptomatic patients. Another compound advocated as a definitive diagnostic marker for MCADD is hexanoylglycine, which is usually only slightly elevated in non-crisis patients. Data from the ERNDIM scheme demonstrates a failure to detect low concentrations of hexanoylglycine in up to 30% of samples [25].

3.4. Determination of pathological markers by GC × GC-ToF-MS

To evaluate the newly developed GC × GC-ToF-MS method as well as the data processing strategy described above, we selected five ERNDIM samples with suboptimal average analytical results reported by the participants of the proficiency test (more than 15% of participants failed in the criteria of analytical performance) and one sample from an asymptomatic patient with MCADD.

Table 2 summarizes the quantitative results from the GC × GC-ToF-MS analysis of the testing samples. Since we used an automated processing with one combined reference (see Section 3.2.2), all

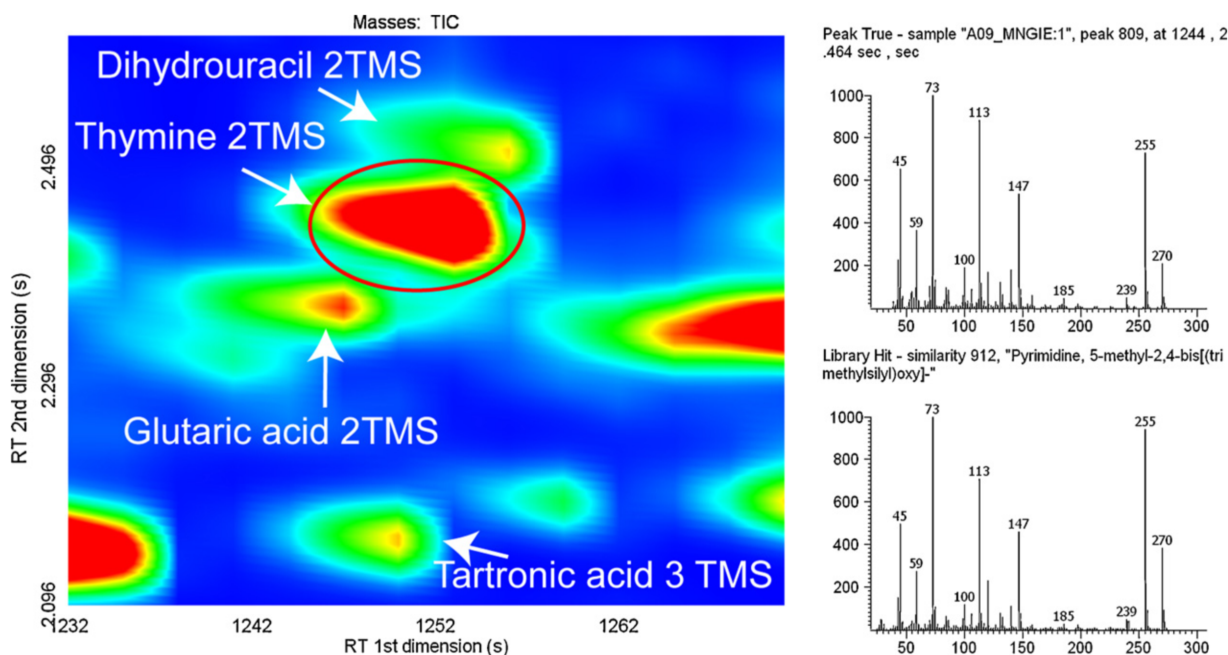

Fig. 3. Enlarged part of GC × GC-ToF-MS contour plot from the analysis of urine from a patient with thymidine phosphorylase deficiency. Thymine 2TMS is resolved from dihydrouracil 2TMS, glutaric acid 2TMS and tartronic acid 3TMS and identified with NIST spectral match 912.

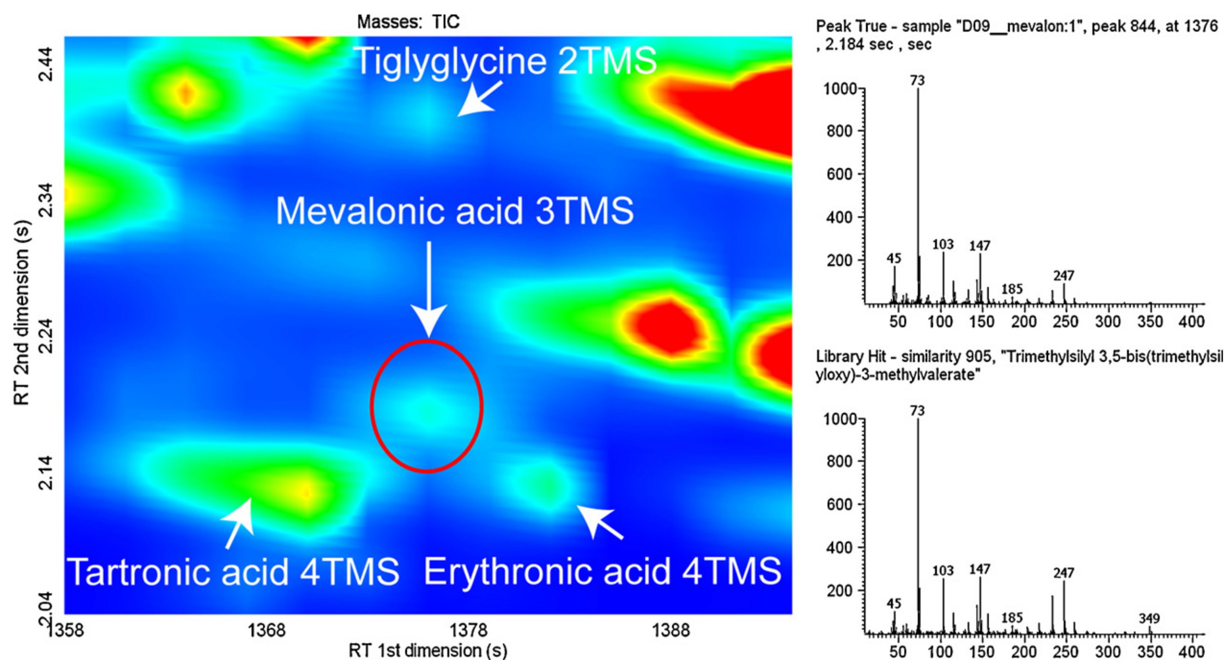

Fig. 4. Enlarged part of GC × GC-ToF-MS contour plot from the analysis of urine from a patient with mevalonate kinase deficiency. The peak of mevalonic acid 3TMS at very low concentration is resolved from erythronic acid 4TMS, tartonic acid 4TMS and tiglylglycine 2TMS and is identified with NIST spectral match 905.

markers were quantified in all samples, regardless of their relevance for the particular disease. The relevant markers associated with a particular IMD are highlighted by the grey fields in the table. In the diagnosing process, the quantity of the relevant marker is compared to the reference range, which is the result of the analysis of 300 disease-free samples (obtained by GC-qMS, GC-ToF-MS data not available yet). The values exceeding the reference range and hence being diagnostic are in bold in Table 2. Besides pathological samples, quantitative data from 10 healthy urines are shown.

In Table 2, the "problematic" markers, i.e. those that could not be found by our traditional GC-qMS method, are marked by an asterisk. Using the GC × GC-ToF-MS method, these markers were reliably detected and identified with library match factors higher than 800. In these cases, the markers were separated from natural metabolites present in high quantities, as documented in more detail in Figs. 3–7. Particularly important were the results obtained for the samples of hawkinsinuria and AADC. In these samples, we were unable to perform the diagnosis from GC-qMS data and they generally yielded poor results by the participants of the proficiency test, too. In hawkinsinuria sample, using the GC × GC-ToF-MS, both *cis*- and *trans*-isomers of the diagnostic compound 4-hydroxycyclohexylacetate 2TMS were resolved from coeluting

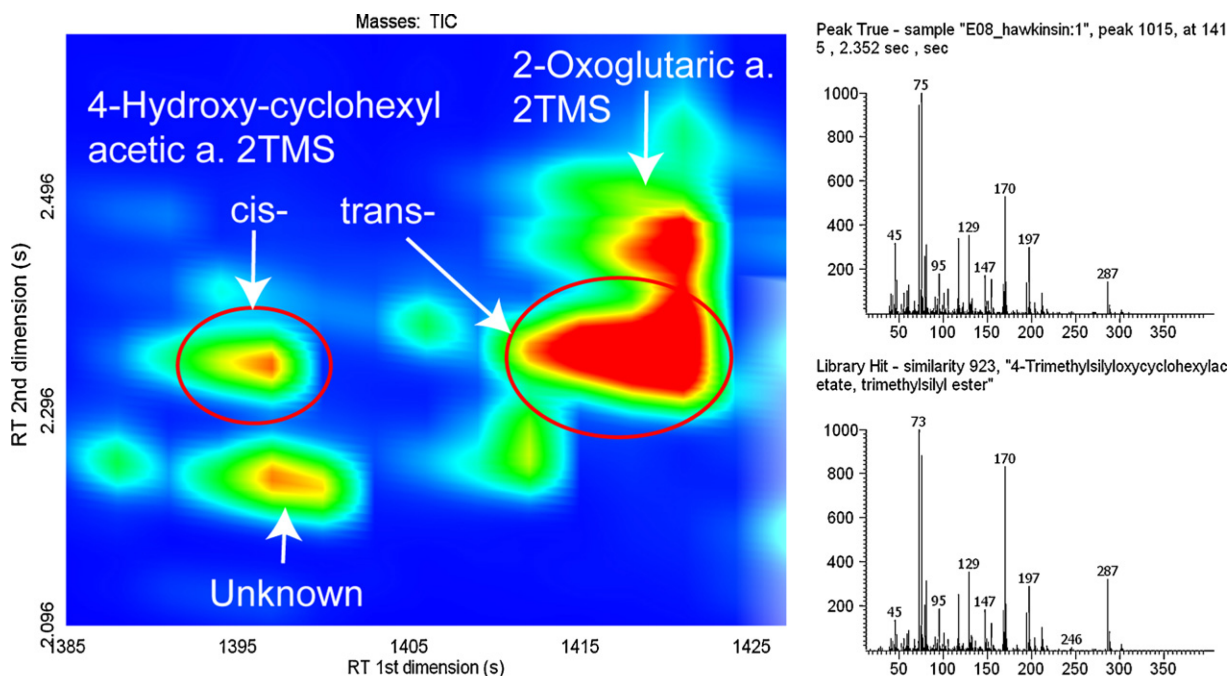

Fig. 5. Enlarged part of GC × GC-ToF-MS contour plot from the analysis of urine from a patient with hawkinsinuria. The peaks of *cis*- and *trans*-4-hydroxycyclohexylacetic acid 2TMS are resolved from coextracts and identified with NIST spectral match values 793 and 923, respectively. The spectrum shown belongs to the *trans*-isomer.

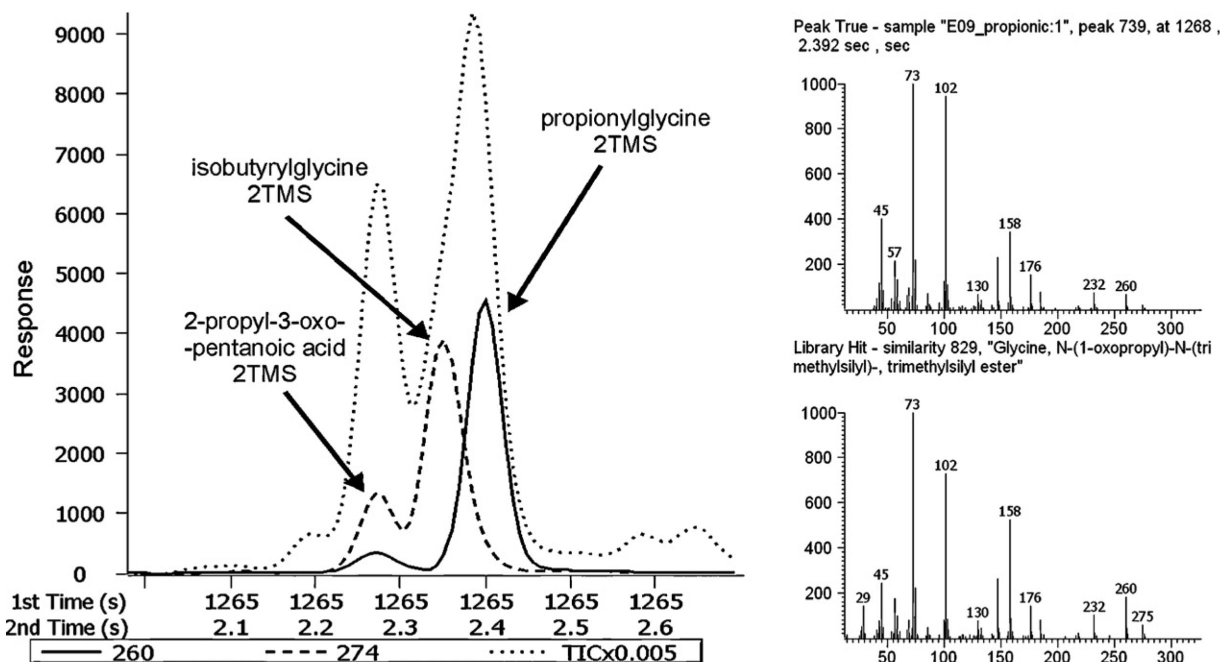

Fig. 6. Part of chromatogram of urine from patient suffering from propionic acidemia. Plotted are specific masses for propionylglycine 2TMS and isobutyrylglycine 2TMS. The peak of 2-propyl-3-oxopentanoic acid 2TMS is most probably from valproic acid therapy. Propionylglycine 2TMS was identified with NIST spectral match 829.

compounds and determined with high quality spectrum (Fig. 5). For AADC sample, many participants of the proficiency test were unable to determine proper diagnosis and erroneously reported another disease, neuroblastoma, on the basis of the increased homovanillic and vanillylmandelic acid excretion. In our laboratory, using a GC-qMS method we also did not detect the slight elevation of vanillactic acid in the sample. On the contrary, using GC × GC-ToF-MS, the compound has been detected and identified with the NIST similarity factor of 827.

Another important result was obtained for MCADD sample where the patient was in a non-crisis state and the diagnosis was missed by GC-qMS. Using GC × GC-ToF-MS we found both hexanoylglycine peaks separated with from a large peak that would coelute in a one-dimensional GC analysis (Fig. 7).

3.5. Time efficiency of the automated data processing

As mentioned above, the classic approach to diagnosing IMDs involves manual searching of chromatograms, peak identifications, and quantification. The typical review time for manually evaluating one urine sample analyzed by GC-qMS is 30–60 min. Using our approach, after setting up a reference, the time of automated data

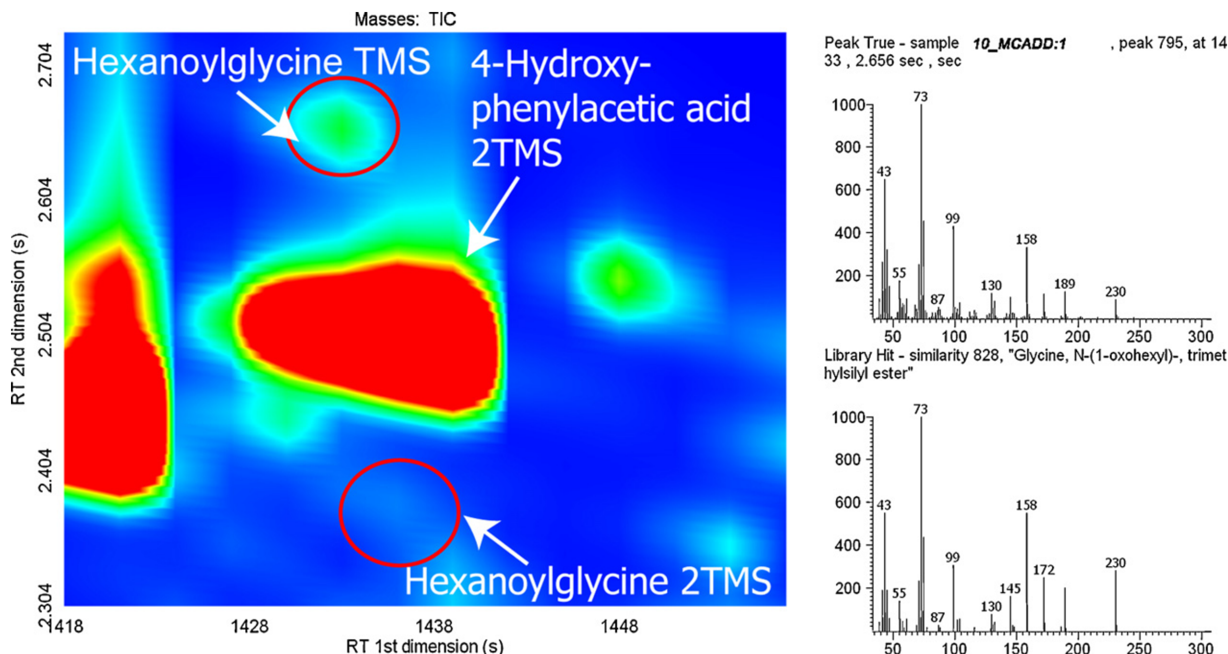

Fig. 7. GC × GC-ToF-MS contour plot from the analysis of urine from a patient with medium-chain acyl-CoA dehydrogenase deficiency. Two peaks of hexanoylglycine derivatives (1 and 2TMS) are resolved from a large peak of 4-hydroxyphenylacetic acid 2TMS and detected with spectral match values 828 and 798, respectively. The spectrum shown belongs to the mono TMS isomer.

processing by the software was approximately 10 min per sample. Additional time to verify the software results (correct assignment of internal standard and checking the GC × GC peak combination) was 5–10 min per sample. Potentially, the reference can be expanded to more IMDs by including more markers.

4. Conclusions

A GC × GC-ToF-MS method has been developed for the purpose of determination of organic acidurias markers for IMDs. In the analysis of challenging proficiency-testing samples (ERNDIM) it has been demonstrated that the difficult markers, for which the classic GC-qMS method failed, were reliably detected and identified with good spectral match quality. This was achieved mainly due to the second dimension separation of GC × GC, allowing resolution of small marker peaks from large naturally occurring metabolites. An automated data processing procedure, based on the use of a reference containing pathological markers for 6 IMDs, was applied for target search and quantitative determination of markers. As a result, all pathological compounds were correctly assigned in all samples and the quantitative data allowed proper diagnosis. The data processing strategy provided significant time-savings compared to the classic manual approach.

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