Chapter 4

Analysis of Organic Acids and Acylglycines for the Diagnosis of Related Inborn Errors of Metabolism by GC- and HPLC-MS

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Abstract

The analysis of organic acids in urine is commonly included in routine procedures for detecting many inborn errors of metabolism. Many analytical methods allow for both qualitative and quantitative determination of organic acids, mainly in urine but also in plasma, serum, whole blood, amniotic fluid, and cerebrospinal fluid. Liquid–liquid extraction and solid-phase extraction using anion exchange or silica columns are commonly employed approaches for sample treatment. Before analysis can be carried out using gas chromatography-mass spectrometry, organic acids must be converted into more thermally stable, volatile, and chemically inert forms, mainly trimethylsilyl ethers, esters, or methyl esters.

Key words: Organic acids, inborn errors of metabolism, gas chromatography-mass spectrometry, organic acidurias.

1. Introduction

Organic acids are small molecules continuously produced, metabolized, and excreted by healthy individuals, as well as by those in abnormal states. Their origins include the metabolic pathways of amino acids, carbohydrates, lipids, and steroids, as well as drug metabolism, bacterial decomposition of food in the intestine, artifacts due to storage, sample preparation, and analysis of the sample (*see* **Note 1**). A list of the most characteristic diagnostic organic acids and their associated disorders is reported in **Table 4.1**. To date, by the analytical methodologies available, more than 250 different compounds (mainly organic acids) and glycine conjugates can be identified in the urine of a normal

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Table 4.1
Characteristic organic acids profile in metabolic disorders

Disorders	Metabolites
Amino acid disorders	
Maple syrup urine disease	2-Keto-isocaproic acid 2-Keto-isovaleric acid 2-Keto-3-methylvaleric acid 2-Hydroxy-isocaproic acid 2-Hydroxy-isovaleric acid 2-Hydroxy-3-methylvaleric acid
Tyrosinemia type I	4-Hydroxy-phenyllactic acid Succinylacetone 4-Hydroxy-phenylpyruvic acid
Tyrosinemia type II	4 Hydroxy-phenyllactic acid 4-Hydroxy-phenylpyruvic acid
Phenylketonuria	Phenyllactic acid Phenylpyruvic acid Mandelic acid di-TMS
Organic acidurias	
Isovaleric acidemia	3-Hydroxy-isovaleric acid Isovalerylglycine Isovalerylglutamic acid
3-Methylcrotonyl-CoA carboxylase deficiency	3-Hydroxy-isovaleric acid 3-Methylcrotonylglycine
Multiple carboxylase deficiency	3-Hydroxy-isovaleric acid Lactic acid Methylcitric acid 3-Hydroxy-propionic acid 3-Methylcrotonylglycine
3-Hydroxy-3-methylglutaric aciduria	3-Hydroxy-3-methylglutaric acid 3-Methylglutaconic acid 3-Methylglutaric acid 3-Hydroxy-isovaleric acid 3-Methylcrotonylglycine
3-Methylglutaconic aciduria	3-Methylglutaconic acid 3-Methylglutaric acid
3-Ketothiolase deficiency	2-Methyl-3-hydroxy-butyric acid 2-Methyl-acetoacetic acid 3-Hydroxy-butyric acid Tiglylglycine
2-Methylbutyryl-CoA dehydrogenase deficiency	2-Methylbutyrylglycine
2-Methyl-3-hydroxy-butyryl-CoA dehydrogenase deficiency	2-Methyl-3-hydroxy-butyric acid Tiglylglycine
Propionic acidemia	3-Hydroxy-propionic acid Methylcitric acid Propionylglycine Tiglylglycine

Table 4.1
(continued)

Disorders	Metabolites
Methylmalonic aciduria	Methylmalonic acid 3-Hydroxy-propionic acid Methylcitric acid
Glutaric aciduria type I	Glutaric acid 3-Hydroxy-glutaric acid Glutaconic acid
Beta-oxidation fatty acid defects	
Glutaric aciduria type II	Glutaric acid Ethylmalonic acid Adipic acid Suberic acid 2-Hydroxy-glutaric acid Isovalerylglycine Isobutyrylglycine 2-Methylbutyrylglycine
Short chain acyl-CoA dehydrogenase deficiency	Ethylmalonic acid Butyrylglycine Methylsuccinic acid Adipic acid Suberic acid Sebacic acid
Medium chain acyl-CoA dehydrogenase deficiency	5-Hydroxy-hexanoic acid 7-Hydroxy-octanoic acid Adipic acid Suberic acid Sebacic acid Octanedioic acid Decanedioic acid Hexanoylglycine Phenylpropionylglicine Suberylglycine
Very long-chain acyl-CoA dehydrogenase deficiency	Suberic acid Sebacic acid
Long-chain 3-hydroxy-acyl-CoA dehydrogenase deficiency	Adipic acid
Mitochondrial trifunctional protein deficiency	Suberic acid Sebacic acid 2-Hydroxy-adipic acid 3-Hydroxy-adipic acid 3-Hydroxy-octenedioic acid 3-Hydroxy-suberic acid 3-Hydroxy-decanedioic acid 3-Hydroxy-sebacic acid 3-Hydroxy-dodecenedioic acid 3-Hydroxy-dodecanedioic acid 3-Hydroxy-tetradecenedioic acid 3-Hydroxy-tetradecanedioic acid
Short-chain 3-hydroxy-acyl-CoA dehydrogenase deficiency	3,4-Dihydroxy-butyric acid 3-Hydroxy-glutaric acid

Table 4.1 (continued)

Disorders	Metabolites
Translocase deficiency	Adipic acid Suberic acid Sebacic acid Dodecanedioic aid Tetradecanedioic acid
Urea cycle defects	
HHH syndrome, OTCD, argininemia	Orotic acid
Citrullinemia, argininosuccinic aciduria	Uracil
Carbamoyl phosphate synthetase deficiency	3-Methylglutaconic acid
Carbohydrate metabolism	
Fructose 1,6-bisphosphatase deficiency	Lactic acid Glycerol Glycerol 3-phosphate
Glycogenosis type I	Lactic acid 3-Methylglutaconic acid 3-Metylglutaric acid
Galactosemia	4-Hydroxy-phenyl-lactic acid
Hereditary fructose intolerance	4-Hydroxy-phenyl-lactic acid
Pyruvate dehydrogenase (subunit E3) deficiency	Lactic acid 2-Ketoglutaric acid Succinic acid Malic acid 2-Keto-isocaproic acid 2-Keto-isovaleric acid 2-Keto-3-methylvaleric acid 2-Hydroxy-isocaproic acid 2-Hydroxy-isovaleric acid 2-Hydroxy-3-methylvaleric acid
Pyruvate carboxylase deficiency	Lactic acid Pyruvic acid Succinic acid Malic acid Fumaric acid
Miscellaneous	
Lactic acidosis	Lactic acid Pyruvic acid 2-Hydroxy-butyric acid 2-Hydroxy-isobutyric acid 4-Hydroxy-phenyl-lactic acid
Hyperoxaluria type I	Glycolic acid Oxalic acid
Hyperoxaluria type II	Oxalic acid Glyceric acid
D-glyceric aciduria	D-glyceric acid

Table 4.1 (continued)

Disorders	Metabolites
Glycerol kinase deficiency	Glycerol
3-Hydroxy-isobutyric aciduria	3-Hydroxy-isobutyric acid
Fumaric aciduria	Fumaric acid
Malonic aciduria	Malonic acid Methylmalonic acid Succinic acid
Mevalonic aciduria	Mevalonic acid
Alkaptonuria	Homogentisic acid
Canavan disease	*N*-acetylaspartic acid
Acylase I deficiency	*N*-acetyl-alanine *N*-acetylaspartic acid *N*-acetylglutamic acid *N*-acetyl-isoleucine *N*-acetyl-methionine *N*-acetyl-serine *N*-acetyl-valine
Pyroglutamic aciduria	Pyroglutamic acid
Xanthurenic aciduria	Xanthurenic acid
Peroxisomal biogenesis disorders	3,6-Epoxyoctanedioc acid 3,6-Epoxydecanedioc acid 3,6-Epoxydodecanedioc acid 3,6-Epoxytetradecanedioc acid 2-Hydroxy-sebacic acid 4-Hydroxy-phenyllactic acid Adipic acid Suberic acid Sebacic acid
Formiminoglutamic aciduria	Hydantoin-3-hydroxy-propionic acid
2-Hydroxy-glutaric aciduria	2-Hydroxy-glutaric acid
Aromatic L-amino acid decarboxylase deficiency	Vanyllactic acid
Dihydropyrimidine dehydrogenase deficiency	Thymine Uracil
Molybdenum cofactor deficiency	Xanthine

individual (1), although a great variability due to age and diet occurs (2). More than 70 inborn errors of metabolism are known to yield a characteristic urinary organic acids pattern, which is essential for diagnosis and follow-up (3). Organic acids can be detected in many biological matrices (e.g., blood, plasma, serum, amniotic fluid, and cerebrospinal fluid), but urine is the best fluid. This is mainly due to the fact that organic acids are concentrated in the urine by the kidneys, and this matrix contains very small quantities of proteins, reducing potential drawbacks during sample analysis.

There are three main steps for the determination of urinary organic acids: (1) oximation; (2) extraction procedures: liquid–liquid extraction or ion-exchange chromatography or solid-phase extraction; (3) derivatization (usually silylation) followed by gas chromatography with mass detection in scan and/or single ion monitoring modes data acquisition. In 2002, Pitt and colleagues (4) reported a new tandem mass spectrometry (MS/MS)-based method to detect some organic acids and acylglycines in urine with a simple dilution of sample by mobile phase followed by direct injection into the mass spectrometer. In 2006, Rebollido and colleagues proposed a modified Pitt method as a neonatal screening tool for the detection of organic acids, acylglycines, acylcarnitines, and amino acids from urine spotted on filter paper (5).

Herein, we describe in detail how to perform the analysis of organic acids and acylglycines in urine for the biochemical diagnosis of organic acidemias.

2. Materials

2.1. Reagent Preparation for Picrate Determination of Creatinine

All reagents (Siemens Healthcare Diagnostics Inc., Newark, NJ) are dissolved in water and ready to use and are of reagent grade unless stated otherwise. Their storage temperature is 2–8°C. These reagents are delivered and mixed automatically by the Dimension Chemistry System during the determination of creatinine.

1. Lithium picrate 25 mM.
2. Sodium hydroxide 100 mM.
3. Potassium ferricyanide 0.13 mM.

2.2. Solvents, Standards, and Reagents (See Note 1)

All solvents are high-purity chromatography grade.

1. Ethyl acetate.
2. Pyridine.
3. Hydroxyl chloride acid, 30%.
4. Methanol.
5. *N*-butanol.
6. Formic acid.
7. Acetic acid.
8. Diethyl ether.
9. $Ba(OH)_2$.
10. H_2SO_4.

11. Hydroxylamine hydroxychloride (25 g/L), dissolved in pyridine.
12. Stock solutions (10 mM) of each organic acid (Sigma-Aldrich, St. Louis, MO) are prepared in water or methanol.
    a. Glycolic acid.
    b. Lactic acid.
    c. 3-Hydroxy-propionic acid.
    d. 3-Hydroxy-butyric acid.
    e. 4-Hydroxy-butyric acid.
    f. 2-Hydroxy-butyric acid.
    g. Glyceric acid.
    h. Fumaric acid.
    i. Maleic acid.
    j. 3-Hydroxy-isovaleric acid.
    k. 2-Hydroxy-isovaleric acid.
    l. Succinic acid.
    m. Methylmalonic acid.
    n. Glutaric acid.
    o. Ethylmalonic acid.
    p. Methylsuccinic acid.
    q. Phosphoethanolamine.
    r. 3-Methylglutaconic acid.
    s. 2-Ketoglutaric acid.
    t. 3-Methylglutaric acid.
    u. Adipic acid.
    v. 2-Hydroxy-glutaric acid.
    w. Mevalonic acid.
    x. 3-Hydroxy-glutaric acid.
    y. 4-Hydroxy-phenylacetic acid.
    z. Succinylacetone.
    aa. 3-Hydroxy-3-methylglutaric acid.
    bb. Homogentisic acid.
    cc. Suberic acid.
    dd. *N*-acetylaspartic acid.
    ee. Homovanillic acid.
    ff. 4-Hydroxy-phenylacetic acid.
    gg. Sulfocysteine.
    hh. Sialic acid.

ii. Uracil.
jj. Thymine.
kk. Xanthine.
ll. Orotic acid.
mm. Uric acid.

13. Acylglycines were synthesized and purchased from Dr. H. Ten Brink (Academic Medical Hospital, the Netherlands). Stock solutions (10 mM) of acylglycines are prepared in water or methanol.
    a. Propionylglycine.
    b. Butyrylglycine.
    c. Isobutyrylglycine.
    d. 3-Methylcrotonylglycine.
    e. Tiglylglycine.
    f. Methylbutyrylglycine.
    g. Isovalerylglycine.
    h. Hexanoylglycine.
    i. 3-Phenylpropionylglycine.
    j. Suberylglycine.
14. Stock solutions (10 mM) of isotopically labeled and unlabeled internal standards are prepared in water or methanol. The storage temperature of the internal standards is –20°C. Under these conditions, the stability of the standard solutions is at least 6 months.
    a. Tricarballylic acid (TCA) (Sigma-Aldrich).
    b. 2-Oxocaproic acid (OA) (Sigma-Aldrich).
    c. 2-Phenylbutyric acid (PBA) (Sigma-Aldrich).
    d. Dimethylmalonic acid (DMMA) (Sigma-Aldrich).
    e. Tropic acid (TPA) (Sigma-Aldrich).
    f. Pentadecanoic acid (PDA) (Sigma-Aldrich).
    g. $^{2}H_3$-methylmalonic acid (Cambridge Isotopes Laboratories, Andover, MA).
    h. $^{2}H_3$-3-hydroxy-isovaleric acid (C.D.N. Isotopes, Pointe-Claire, Quebec, Canada).
    i. $^{13}C_6$-adipic acid (Cambridge Isotopes Laboratories).
    j. $^{2}H_3$-3-hydroxy-3-methylglutaric acid (C.D.N. Isotopes).
    k. $^{2}H_3$-acetylaspartic acid (C.D.N. Isotopes).
    l. 1,3 $^{15}N_2$-uric acid (Cambridge Isotopes Laboratories).
    m. $^{15}N_2$-uracil (C.D.N. Isotopes).

n. $^{2}H_4$-thymine (C.D.N. Isotopes).

o. $^{15}N_2$-orotic acid (C.D.N. Isotopes).

p. $^{2}H_3$-propionylglycine (Dr. H. Ten Brink, Academic Medical Hospital, the Netherlands).

q. $^{2}H_3$-hexanoylglycine (Dr. H. Ten Brink, Academic Medical Hospital, the Netherlands).

15. Silylating reagents.
    a. *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Bellefonte, PA).
    b. *N*-methyl-*N*-trifluoroacetamide (MSTFA) (Supelco).
    c. *N*,*O*-bis-(trimethylsilyl)acetamide (BSA) (Supelco).
    d. *N*-methyl-*N*-(tert-butyldimethylsilyl)trifluoroacetimide (MTBSTFA) (Supelco).

2.3. Glassware

1. Extraction tubes (*see* **Note 1**): 8 mL screw-capped pyrex glass tubes (14 × 100 mm) with Teflon-lined caps (Barcorword Scientific, Ltd., Stone Staff, UK).
2. GC-MS 2 mL glass vials (9 mm) and caps with siliconized rubber septa (Chromacol, Ltd. Welwyn Garden City, UK) (*see* **Note 1**).

2.4. Columns

1. Gas chromatography separation column: Agilent J&W ULTRA2 (methyl siloxane 5% phenyl), 25 m × 0.20 mm × 0.33 μm fused silica capillary column or an HP-5 ms (methyl siloxane 5% phenyl), 25 m × 0.25 mm × 0.25 μm fused silica capillary column.

2.5. Internal Standards for Semiquantitative and Quantitative Determination of Organic Acids and Acylglycines in Urine by GC-MS

A stock calibration solution containing 100 mg/L of each internal standard in ethanol/water (1:1, v/v) is made and stored at 4°C or less (*see* **Note 2**).The final makeup volume of the working calibration solution is 50 μL; therefore, the concentration of the working calibration solution is 1 μg/mol creatinine when the added internal standard volume is 10 μL and 2 μg/mol creatinine when the added internal standard volume is 20 μL.

1. Dimethylmalonic acid (DMMA). Working calibration solution contains 10 μL of DMMA (100 mg/L) in ethanol/water (1:1, v/v). Store at 4°C or less.
2. Tropic acid (TA). Working calibration solution contains 20 μL of TA (100 mg/L) in aqueous solution. Store at 4°C or less.
3. Pentadecanoic acid (PDA). Working calibration solution contains 20 μL of PDA (100 mg/L) in ethanol. Store at 4°C or less.

4. Tricarballylic acid (propane-1,2,3-tricarboxylic acid). Working calibration solution contains 10 μL of tricarballylic acid (100 mg/L) in aqueous solution. Store at 4°C or less.
5. 2-Phenylbutyric acid. Working calibration solution contains 10 μL of a 2-phenylbutyric acid (100 mg/L) in aqueous solution. Store at 4°C or less.
6. 2-Oxocaproic acid (2-oxohexanoic acid). Working calibration solution contains 10 μL of a 2-oxocaproic acid (100 mg/L) in aqueous solution. Store at 4°C or less.
7. $^{2}H_3$-methylmalonic acid. Working calibration solution contains 10 μL of $^{2}H_3$-methylmalonic acid (100 mg/L) in ethanol. Store at 4°C or less.
8. $^{2}H_3$-3-hydroxy-isovaleric acid. Working calibration solution contains 10 μL of $^{2}H_3$-3-hydroxy-isovaleric acid (100 mg/L) in ethanol. Store at 4°C or less.
9. $^{13}C_6$-adipic acid. Working calibration solution contains 10 μL of $^{13}C_6$-adipic acid (100 mg/L) in ethanol. Store at 4°C or less.
10. $^{2}H_3$-3-hydroxy-3-methylglutaric acid. Working calibration solution contains 10 μL of $^{2}H_3$-3-hydroxy-3-methylglutaric acid (100 mg/L) in ethanol. Store at 4°C or less.
11. $^{2}H_3$-acetylaspartic acid. Working calibration solution contains 10 μL of $^{2}H_3$-acetylaspartic acid (100 mg/L) in ethanol. Store at 4°C or less.
12. $^{2}H_3$-propionylglycine. Working calibration solution contains 20 μL of $^{2}H_3$-propionylglycine (100 mg/L) in ethanol. Store at 4°C or less.
13. $^{2}H_3$-hexanoylglycine. Working calibration solution contains 20 μL of $^{2}H_3$-hexanoylglycine (100 mg/L) in ethanol. Store at 4°C or less.

2.6. Internal Standards and Calibrators for Semiquantitative and Quantitative Determination of Organic Acids and Acylglycines in Urine by HPLC-MS/MS

A stock calibration solution containing 100 mg/L of each internal standard in ethanol is made and stored at 4°C or less (*see* **Note 2**). The final makeup volume of the working calibration solution is 200 μL; therefore, the concentration of the working calibration solution is 5 μg/mol creatinine when the added internal standard volume is 10 μL and 10 μg/mol creatinine when the added internal standard volume is 20 μL.

1. $^{2}H_3$-methylmalonic acid. Working calibration solution contains 10 μL of $^{2}H_3$-methylmalonic acid (100 mg/L) in ethanol. Store at 4°C or less.

2. $^{2}H_3$-3-hydroxy-isovaleric acid. Working calibration solution contains 10 μL of $^{2}H_3$-3-hydroxy-isovaleric acid (100 mg/L) in ethanol. Store at 4°C or less.
3. $^{13}C_6$-adipic acid. Working calibration solution contains 10 μL of $^{13}C_6$-adipic acid (100 mg/L) in ethanol. Store at 4°C or less.
4. $^{2}H_3$-3-hydroxy-3-methylglutaric acid. Working calibration solution contains 10 μL of $^{2}H_3$-3-hydroxy-3-methylglutaric acid (100 mg/L) in ethanol. Store at 4°C or less.
5. $^{2}H_3$-acetylaspartic acid. Working calibration solution contains 10 μL of $^{2}H_3$-acetylaspartic acid (100 mg/L) in ethanol. Store at 4°C or less.
6. $^{2}H_3$-propionylglycine. Working calibration solution contains 20 μL of $^{2}H_3$-propionylglycine (100 mg/L) in ethanol. Store at 4°C or less.
7. $^{2}H_3$-hexanoylglycine. Working calibration solution contains 20 μL of $^{2}H_3$-hexanoylglycine (100 mg/L) in ethanol. Store at 4°C or less.
8. 1,3-$^{15}N_2$-uric acid. Working calibration solution contains 10 μL of 1,3 $^{15}N_2$-uric acid (100 mg/L) in ethanol. Store at 4°C or less.
9. $^{15}N_2$-uracil. Working calibration solution contains 10 μL of $^{15}N_2$-uracil (100 mg/L) in water. Store at 4°C or less.
10. $^{2}H_4$ thymine. Working calibration solution contains 10 μL of $^{2}H_4$ thymine (100 mg/L) in water. Store at 4°C or less.
11. $^{15}N_2$-orotic acid. Working calibration solution contains 10 μL of $^{15}N_2$-orotic acid (100 mg/L) in water. Store at 4°C or less.
12. Calibration solutions are prepared in an aqueous matrix to simulate the composition of urine samples. The aqueous matrix is composed of the following:
    a. 30 mM urea (Sigma-Aldrich).
    b. 11.4 mM sodium chloride (Sigma-Aldrich).
    c. 7.8 mM potassium chloride (Sigma-Aldrich).
    d. 1.5 mM potassium dihydrogen orthophosphate (Sigma-Aldrich).
    e. 7.6 mg/L bovine serum albumin (Sigma-Aldrich).
13. For monitoring recoveries and for quality control purposes, a pooled urine sample is prepared and diluted to a creatinine concentration of 1.0 mM. Two enriched urine samples are prepared by diluting the same pooled urine sample to a creatinine concentration of 1.0 mM and adding abnormal amounts of metabolites.

2.7. Instruments

1. Dimension RxL Max Integrated Chemistry System (Siemens Healthcare Diagnostics Inc., Newark, NJ).
2. Gas chromatograph 6890 N with a single-quadrupole mass spectrometer (5975) as detector (Agilent Technologies, Palo Alto, CA).
3. 1100 Series Cap Pump HPLC (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent Micro ALS Autosampler.
4. API 4000 bench-top triple-quadrupole mass spectrometer equipped with the TurboIonSpray source (AB–Sciex, Toronto, Canada).

3. Methods

3.1. Sample Collection

1. Urine samples should be collected over 24 h when possible. However, considering that prolonged urine collections are quite difficult, especially for newborns or very young babies, a random sample, preferably the first morning voiding, is acceptable. Thus, it is possible to obtain results similar to those obtained in a 1-day collection (6), although the amount of many organic acids, excreted per milligram of creatinine, varies considerably according to age. Newborns and small babies have not yet developed complete renal tubular function, so they normally excrete a larger amount of aliphatic acids.
2. Any urine samples must be accompanied by a form containing information about clinical history, therapy, and diet details over the last 48 h. This is a critical point for a correct interpretation of data (*see* **Note 1**).
3. Urine should be collected in clean glass containers to avoid contamination by plastic compounds (e.g., phthalates) (7), and the containers should be preferably sterile.
4. Collect 24-h urine samples by freezing each single sampling. Other protein-free physiological fluids should be collected in the same way (*see* **Note 1**). Considering that most patients are acutely ill, this procedure is almost impractical.

3.2. Storage

1. Analyze urine samples immediately or freeze at –70°C until analysis.
2. If a –70°C freezer is not available, freeze the urine samples at –20°C. Indeed, if containers are not sterile, prolonged exposure at room temperature leads to bacterial growth, production of artifacts, and the formation of new organic acids (8) or loss of volatile acids (*see* **Note 1**). Urine samples should

be transported in a deeply frozen state, preferably packed in solid carbon dioxide at –70°C.

3. Some authors suggested adding 2 mL of chloroform or merthiolate [*o*-(carboxyphenyl)thioethylmercury] sodium salt to urine sample as a preservative (*see* **Note 1**). Merthiolate is toxic by inhalation, ingestion, and contact with skin (EC hazard symbol T+), and it has cumulative effects. It is also very toxic to aquatic organisms and may cause long-term adverse effects in aquatic environments. Therefore, the addition of choloroform or merthiolate as a preservative is very seldom used.

3.3. Creatinine Assay

Creatinine (2-imino-1-methyl-4-imidazolidinone) concentration is the reference to which each organic acid concentration is typically normalized for quantitative purposes. However, this molecule is often declared unsuitable as a reference standard (8), due to its increasing excretion with age (9) and wide variation between individuals, especially small babies and newborns (10). Moreover many differences between males and females have been reported (9). Thus, the accurate determination of creatinine prior to urinary organic acid analysis is extremely important.

The alkaline picrate-based method (a modified Jaffe method) has been reported to be less susceptible than conventional methods to interference from non-creatinine Jaffe-positive compounds (11). Sampling, reagent delivery, mixing, and processing of the analysis are automatically carried out by the Dimension Chemistry System.

1. Add about 100 μL of urine to a cuvette. Creatinine reacts with picrate (lithium picrate 25 mM) in presence of a strong base, like sodium hydroxide (NaOH 100 mM), forming a red chromophore (λ 510 nm). The rate of increasing absorption is directly proportional to the creatinine concentration in the sample.
2. Read the value of creatinine. It is measured by using a bichromatic (absorption wavelengths: λ 510 and λ 600 nm) rate technique (Dimension Chemistry System or similar) (*see* **Note 3**).

3.4. Oximation

1. Dilute urine sample corresponding to 0.5 or 1 mmol of creatinine with distilled water to 1,500 mL.
2. Adjust the pH to 14 with 5 M NaOH.
3. Add 0.5 mL of aqueous hydroxylamine solution.
4. Heat the urine and hydroxylamine mixture at 60°C for 30 min, then cool the solution to room temperature, and acidify to pH 1 with 6 M HCl.
5. After acidification, proceed with extraction as described below.

3.5. Extraction Procedure

In our experience, samples capped well with silicon caps are stable about 1 week at 4°C, prior to the derivatization step (12). The solvent extraction procedure described below is widely used even if polar acids are not efficiently extracted. Moreover, many non-acid compounds are extracted, such as urea and alcohols, and these compounds can interfere with the chromatographic separation. Great attention also must be paid to the contaminants potentially present in the organic extraction solvent (*see* **Note 1**).

a. Place a volume of urine corresponding to 0.5 or 1 mmol of creatinine in a 14 × 100-mm screw-cap culture tube.
b. Dilute the sample with deionized water to 1.5 mL and adjust the pH to 1 by dropwise addition of 6 M HC1 or 2.5 M H_2SO_4.
c. Add internal standards: 10 μL of DMMA (100 mg/L) in ethanol/water (1:1, v/v); 20 μL of TA (100 mg/L) in water; and 20 μL of PDA (100 mg/L) in ethanol.
d. Extract the acidified sample three or four times with 2-mL aliquots of ethyl acetate (or diethyl ether), shaking the tube vigorously. The extraction efficiency has been measured by Tanaka and colleagues (13) who reported that hydroxy acids and low molecular weight dicarboxylic acids were recovered with much higher yields with ethyl acetate than with diethyl ether.
e. After shaking, centrifuge the mixture at 1,620×*g* for 10 min.
f. Combine the organic layers in a second tube (*see* **Note 4**) and then dry the combined extract over about 1–1.5 g of anhydrous Na_2SO_4 for 45–60 min.
g. Wash the Na_2SO_4 twice with 1-mL portions of ethyl acetate. Optionally, the sample can be filtered with filter paper into a third tube prior to washing the Na_2SO_4.
h. Dry the extract under a nitrogen stream at 60°C. When the volume is about 1–1.5 mL, transfer the solution to a 2-mL GC-MS glass vial and dry the remaining solvent.

3.6. Derivatization Procedures

After extraction procedures, in order to make the acids more volatile and stable for gas chromatography, a derivatization step is performed. Most laboratories use trimethylsilylation.

1. Add 50 μL of bis-trimethylsilyl-trifluoroacetamide (BSTFA) to the evaporated sample in a 2-mL GC-MS glass vial, vortex-mix, and incubate at 60°C for 30 min. It is also possible to use *N*-methyl-*N*-trifluoroacetamide (MSTFA), *N*,*O*-bis[trimethylsilyl]acetamide (BSA), or *N*-methyl-*N*-[tert-butyldimethyl-silyl]trifluoroacetimide (MTBSTFA) as alternative derivatization reagents. All of these agents can be used at full strength or diluted with a suitable solvent such as pyridine.

3.7. Gas Chromatography/Mass Spectrometry Analysis

1. The vialed samples should be kept at room temperature until analysis and analyzed no later than 24 h after preparation.
2. Generally, the GC analyses are performed on a gas chromatograph coupled to a single-quadrupole mass spectrometer as analyzer.
3. The capillary column is typically a nonpolar column such as an HP-5 ms or J&W ULTRA2 or similar.
4. The carrier gas is helium at 1 mL/min.
5. The column oven initial temperature is 70°C; the temperature gradient is 10°C/min to 280°C, followed by 280°C for 5 min.
6. The injection volume is 1 μL (*see* **Note 5**) with a split ratio of 10:1, and the front inlet temperature is 280°C.
7. The detector temperature is typically about 280–300°C.
8. The mass spectra are recorded both in selected ion monitoring (SIM) and in scan mode over a mass range of 40–550 *m/z*. A list containing the most characteristic ions and molecular weights of the main organic acids is reported in **Table 4.2**. **Table 4.3** contains the most diagnostic acylglycines molecular ions and characteristic fragments.
9. The retention times are obtained using chemical organic acid standards. Both standards and urine samples are analyzed under the same chromatographic conditions.
10. Mass calibration (*see* **Note 6**) of the instrument by using a mixture containing the target compounds at different concentrations is a critical step. The authors calibrated the mass spectrometer using a mixture containing about 100 different organic acids using a 5-point curve containing 10, 25, 50, 100, and 250 μM of each.
11. The calibration must be performed monthly or more frequently (*see* **Note 6**).
12. For quantitation purposes, a mixture containing 50 μM of standards is analyzed weekly, and if the quantitation report differs from the expected values by more than 25%, a new calibration curve is made.
13. The GC-MS system must have a regular maintenance schedule to avoid artifacts and generation of new compounds in the organic acids profile (*see* **Note 7**).
14. Many labs choose to perform a semiquantitative determination of urinary organic acids using non-labeled internal standards. These standards are non-physiological compounds that elute in proximity to the organic acids to be quantified. In our own experience, dimethylmalonic

Table 4.2
The most characteristic ions and molecular weights of the main organic acids

Characteristic urinary organic acid	MW	M-15	Ion 1	Ion 2
2-Keto-isovaleric acid (oxime) di-TMS	275	260	232	158
2-Keto-isocaproic acid (oxime) di-TMS	289	274	200	172
2-Keto-3-methylvaleric acid (oxime) di-TMS	289	274	200	172
2-Ketoglutaric acid (oxime) di-TMS	377	362	260	
2-Hydroxy-3-methylvaleric acid	276	261	159	
2-Hydroxy-adipic acid	378	363		
2-Hydroxy-butyric acid di-TMS	248	233	131	
2-Hydroxy-glutaric acid	364	349	247	203
2-Hydroxy-isobutyric acid di-TMS	248	233	95	
2-Hydroxy-isocaproic acid di-TMS	276	261	159	103
2-Hydroxy-isovaleric acid di-TMS	262	247	219	145
2-Hydroxy-sebacic acid tri-TMS	419	391	317	
2-Methyl-3-hydroxy-butyric acid di-TMS	262	247	117	
2-Methyl-acetoacetic acid di-TMS	260	245		
3-Hydroxy-3-methylglutaric acid	378	363	273	247
3-Hydroxy-adipic acid	378	363	247	
3-Hydroxy-butyric acid	248	233	191	117
3-Hydroxy-isobutyric acid	248	233	177	103
3-Hydroxy-decanedioic acid	432	417	233	
3-Hydroxy-dodecanedioic acid	462	447	233	
3-Hydroxy-dodecanedioic acid	460	445	233	
3-Hydroxy-glutaric acid	364	349	259	185
3-Hydroxy-isovaleric acid	262	247	205	131
3-Hydroxy-octanedioic acid	404	389	233	217
3-Hydroxy-propionic acid di-TMS	234	219	204	177
3-Hydroxy-sebacic acid tri-TMS	434	419	233	217
3-Hydroxy-suberic acid tri-TMS	406	391	233	217
3-Hydroxy-tetradecanedioic acid tri-TMS	490	475	233	217
3-Hydroxy-tetradecanedioic acid tri-TMS	488	473	233	217
3-Methylglutaconic acid di-TMS	288	273	198	183
3-Methylglutaric acid di-TMS	290	275	204	69
3,4-Dihydroxy-butyric acid tri-TMS	336	233	189	
3,6-Epoxyoctanedioc acid di-TMS	332	317	201	174
3,6-Epoxydecanedioc acid di-TMS	360	345	201	174
3,6-Epoxydodecanedioc acid di-TMS	388	372	201	174
3,6-Epoxytetradecanedioc acid di-TMS	416	401	201	174
4-Hydroxy-butyric acid di-TMS	248	233	204	117

Table 4.2 (continued)

Characteristic urinary organic acid	MW	M-15	Ion 1	Ion 2
4-Hydroxy-phenyllactic acid tri-TMS	398	383	308	179
4-Hydroxy-phenylpyruvic acid tri-TMS	396	381	325	
4-Hydroxy-phenylpyruvic acid (oxime) tri-TMS	412	396	277	190
4,5-Dihydroxy-hexanoic tri-TMS (erythro and threo)	364	349	247	129
4,5-Dihydroxy-hexanoic lactone (erythro and threo)	202	187	158	143
5-Hydroxy-hexanoic acid di-TMS	276	261	204	171
7-Hydroxy-octanoic acid di-TMS	304	289	260	
Adipic acid di-TMS	290	275	172	111
Decanedioic acid di-TMS	344	329	164	119
Dodecanedioic aid	374	359	243	
Ethylmalonic acid di-TMS	276	261	217	
Fumaric acid di-TMS	260	245	147	
Glutaconic acid di-TMS	274	259	217	
Glutaric acid di-TMS	276	261	158	
Glycerol tri-TMS	308	293	218	205
Glycerol 3-phosphate tetra-TMS	460	445	357	299
Glyceric acid tri-TMS	322	307	292	205
Hydantoin-3-hydroxy-propionic acid tri-TMS	388	373	257	230
Homogentisic acid tri-TMS	384	341	252	
Isovaleryl-glutamic acid di-TMS	375	360	258	156
Lactic acid di-TMS	234	219	190	117
Malic acid tri-TMS	350	319	245	233
Malonic acid di-TMS	248	233	133	
Mandelic acid di-TMS	296	281	253	179
Methylcitric acid tetra-TMS	494	479	389	361
Methylmalonic acid di-TMS	262	247	218	157
Methylsuccinic acid di-TMS	276	261	217	
Mevalonic acid tri-TMS	364	349	247	233
Mevalonic lactone mono-TMS	202	187	145	115
Mevalonic lactone di-TMS	274	259	187	175
N-acetyl-alanine di-TMS	275	260	232	158
N-acetylaspartic acid tri-TMS	391	376	274	230
N-acetylaspartic acid di-TMS	319	304	202	158
N-acetylglutamic di-TMS	333	318	216	158
N-acetyl-isoleucine tri-TMS	245	230	128	86
N-acetyl-methionine di-TMS	335	320	274	261
N-acetyl-serine di-TMS	291	276	261	186

Table 4.2 (continued)

Characteristic urinary organic acid	MW	M-15	Ion 1	Ion 2
N-acetyl-valine di-TMS	303	288	260	186
Octanedioic acid di-TMS	316	301	136	137
Orotic acid tri-TMS	372	357	254	
Oxalic acid di-TMS	234	219	190	
Phenyllactic acid di-TMS	310	295	220	193
Phenylpyruvic acid (oxime)	323	308	189	
Pyroglutamic acid di-TMS	273	258	156	
Pyruvic acid (oxime) di-TMS	247	232	204	
Sebacic acid di-TMS	346	331	345	
Suberic acid di-TMS	318	303	169	
Succinic acid di-TMS	262	247	172	
Succinylacetone (oxime)	227	212	182	138
Tetradecanedioic acid di-TMS	402	387	271	
Uracil di-TMS	256	241	99	
Vanillactic acid tri-TMS	428	413	338	209
Xanthurenic acid tri-TMS	420	406	318	

acid, tropic acid, pentadecanoic acid, tricarballylic acid, 2-oxocaproic acid, and 2-phenylbutyrric acid can be used as internal standards. Their use is advantageous only in terms of cost per analysis (*see* **Note 8**).

15. Quantitation is performed according to either Internal Standard Method or the Absolute Calibration Curve Method, generally using the peak height or peak area.
16. Internal Standard Method
    a. Prepare several standard solutions containing a constant amount of the specified labeled or unlabeled internal standard and known graded amounts of the compound to be quantified.
    b. With each of the chromatograms obtained by injecting a constant volume of each standard solution, calculate the ratio of the peak height or peak area of the compound to be quantified to that of the internal standard.
    c. Prepare a calibration curve by plotting these ratios on the ordinate and the ratios of each amount of the compound to be quantified to the amount of the internal standard, or simply the amount of the compound to be quantified, on the abscissa. The calibration curve is usually a straight line through the origin.

Table 4.3
The most diagnostic acylglycines molecular ions and characteristic fragments

Acylglycine mono-TMS derivatives	$[M]^+$	$[M-15]^+$	$[M-117]^+$	$[M-146]^+$	$[M-59]^+$	$[M-45]^+$	$[M-44]^+$
2-Methylbutyrylglycine	231	216	114	85	172	186	187
3-Methylcrotonylglycine	229	214	112	83	170	184	185
Butyrylglycine	217	202	100	71	158	172	173
Hexanoylglycine	245	230	128	99	186	200	201
Isobutyrylglycine	217	202	100	71	158	172	173
Isovalerylglycine	231	216	114	85	172	186	187
Phenylpropionylglycine	270	255	86	57	144	158	159
Propionylglycine	203	188	86	57	144	158	159
Tiglylglycine	229	214	112	83	170	184	185
Suberylglycine	300	285	183	154	241	255	256
Aclglycine di-TMS derivatives	**$[M]^+$**	**$[M-15]^+$**	**$[M-117]^+$**	**$[M-131]^+$**	**$[M-218]^+$**		
2-Methylbutyrylglycine	303	288	186	172	85		
3-Methylcrotonylglycine	301	286	184	170	83		
Butyrylglycine	289	274	172	158	71		
Hexanoylglycine	317	302	200	186	99		
Isobutyrylglycine	289	274	172	158	71		
Isovalerylglycine	303	288	186	172	85		
Phenylpropionylglycine	343	328	226	212	125		
Propionylglycine	275	260	158	144	57		
Tiglylglycine	301	286	184	170	83		
Suberylglycine	375	360	256	242	155		
	$[M-15]^+=[M-CH_3]^+$						
	$[M-117]^+=[M-COOSi(CH_3)_3]^+$						
	$[M-146]^+=[RCO]^+$ R=alkyl radical						
	$[M-59]^+=[M-CH_2COOH]^+$						
	$[M-45]^+=[M-COOH]^+$						
	$[M-44]^+=[M-COO]^+$						
	$[M-131]^+=[M-CH_2COOSi(CH_3)_3]^+$						
	$[M-218]^+=[RCO]^+$ R=alkyl radical						

d. Prepare the urine sample containing the same amount of the internal standard as directed in the individual monograph.

e. Record a chromatogram under the same conditions as for the preparation of the calibration curve.

f. Calculate the ratio of the peak height or peak area of the compound to be quantified to that of the internal standard.

g. Perform the quantitation using the calibration curve.

17. Absolute Calibration Curve Method
    a. Prepare standard solutions containing a graded amount of the compound to be quantified and inject a constant volume of each standard solution.
    b. With the chromatograms obtained, prepare a calibration curve by plotting the peak heights or peak areas of the compound to be quantified on the ordinate and the amounts of the compound to be quantified on the abscissa. The calibration curve is usually a straight line through the origin.
    c. Prepare the urine sample as directed in the individual monograph.
    d. Record a chromatogram under the same conditions used for the preparation of the calibration curve.
    e. Measure the peak height or peak area of the compound to be quantified.
    f. Perform the quantitation using the calibration curve.

3.8. Liquid Chromatography/Mass Spectrometry Analysis

1. Loop injections (20 μL) are made via a HPLC autosampler into a mobile phase of acetonitrile–water (1:1 by volume).
2. The mobile phase and sample are infused at a flow rate of 30 μL/min by an HPLC pumping system into the electrospray ion source of a triple-quadrupole mass spectrometer.
3. The ion source operated with a capillary voltage of 5.0 kV (positive-ion mode) or 4.5 kV (negative-ion mode) and a temperature of 250°C. Nitrogen is used as collision gas at a pressure of 10 mTorr.
4. Fragmentation transitions, declustering potentials, and collision energies should be optimized during manual loop injections of pure compounds.
5. Negative-ion multiple reaction monitoring (MRM) for 49 metabolites and 11 labeled internal standards is performed under the conditions shown in **Table 4.4**.
6. Data are acquired for 1.3 min after injection, and the total cycle time between injections is 2.1 min.
7. Data are processed with MRM signals averaged between 0.6 and 1.3 min and baseline subtraction of signals from 0.15 to 0.45 min.
8. Raw data are exported to an Excel spreadsheet file (Microsoft) for calculations.

Table 4.4
Precursor (Q1), product ion (Q3) masses, and optimized voltages for the 60 compounds studied in negative-ion mode

	Metabolites	Q1/Q3 transition	DP (V)	CE (V)	Internal standard
1	Glycolic acid	75 > 47	–30	–20	14
2	Lactic acid	89 > 43	–40	–20	14
3	3-Hydroxy-propionic acid	89 > 59	–30	–15	14
4	3-Hydroxy-butyric acid	103 > 59	–30	–15	14
5	4-Hydroxy-butyric acid	103 > 57	–15	–20	14
6	2-Hydroxy-butyric acid	103 > 57	–17	–25	14
7	Glyceric acid	105 > 75	–25	–15	14
8	Fumaric acid	115 > 71	–18	–12	14
9	Maleic acid	115 > 71	–19	–13	14
10	3-Hydroxy-isovaleric acid	117 > 59	–21	–14	15
11	2-Hydroxy-isovaleric acid	117 > 71	–31	–16	14
12	Succinic acid	117 > 73	–12	13	14
13	Methylmalonic acid	117 > 73	–10	–13	14
14	$^{2}H_3$-Methylmalonic acid	120 > 76	–10	–13	
15	$^{2}H_3$-3-Hydroxy-isovaleric acid	123 > 59	–21	–15	
16	Glutaric acid	131 > 87	–10	–13	28
17	Ethylmalonic acid	131 > 87	–10	–12	28
18	Methylsuccinic acid	131 > 87	–11	–13	28
19	Phosphoethanolamine	140 > 79	–20	–15	28
20	3-Methylglutaconic acid	143 > 99	–5	–9	28
21	2-Ketoglutaric acid	145 > 101	–20	–13	28
22	3-Methylglutaric acid	145 > 101	–22	–16	28
23	Adipic acid	145 > 83	–10	–18	28
24	2-Hydroxy-glutaric acid	147 > 129	–22	–13	28
25	Mevalonic acid	147 > 59	–25	–21	28
26	3-Hydroxy-glutaric acid	147 > 85	–11	–13	31
27	4-Hydroxy-phenylacetic acid	151 > 107	–8	–20	28
28	$^{13}C_6$-Adipic acid	151 > 88	–10	–18	
29	Succinylacetone	157 > 99	–10	–12	46
30	3-Hydroxy-3-methylglutaric acid	161 > 99	–13	–14	31
31	$^{2}H_3$-3-Hydroxy-3-methylglutaric acid	164 > 102	–13	–14	
32	Homogentisic acid	167 > 123	–34	–15	28
33	Suberic acid	173 > 111	–31	–18	28
34	*N*-acetylaspartic acid	174 > 88	–17	–19	35
35	$^{2}H_3$-acetylaspartic acid	177 > 89	–17	–19	
36	Homovanillic acid	181 > 137	–19	–13	28
37	4-Hydroxy-phenyllactic acid	181 > 163	–30	–16	28

Table 4.4 (continued)

	Metabolites	Q1/Q3 transition	DP (V)	CE (V)	Internal standard
38	Sulfocysteine	200 > 136	–30	–14	28
39	Sialic acid	308 > 87	–33	–23	28
40	Uracil	111 > 42	–20	–20	41
41	$^{15}N_2$-uracil	113 > 43	–17	–20	
42	Thymine	125 > 42	–22	–31	43
43	Thymine D4	129 > 42	–20	–31	
44	Xanthine	151 > 108	–27	–25	48
45	Orotic acid	155 > 111	–13	–13	46
46	$^{15}N_2$-orotic acid	157 > 113	–13	–15	
47	Uric acid	167 > 124	–54	–25	48
48	1,3 $^{15}N_2$-uric acid	169 > 125	–54	–25	
49	Propionylglycine	130 > 74	–30	–13	50
50	$^{2}H_3$-propionylglycine	133 > 74	–30	–13	
51	Butyrylglycine	144 > 74	–45	–15	50
52	Isobutyrylglycine	144 > 74	–45	–15	50
53	3-Methylcrotony lglycine	156 > 74	–45	–15	50
54	Tiglylglycine	156 > 75	–45	–15	50
55	Methylbutyry lglycine	158 > 74	–45	–15	
56	Isovalerylglycine	158 > 74	–50	–15	58
57	Hexanoylglycine	172 > 74	–45	–15	58
58	$^{2}H_3$-hexanoylglycine	175 > 74	–45	–15	
59	3-Phenylpropiony lglycine	206 > 74	–45	–15	58
60	Suberylglycine	230 > 74	–45	–15	58

9. Ratios of the MRM signals of the metabolites relative to the internal standards are used to construct calibration curves and calculate concentrations in the urine samples. The internal standards used for each metabolite are listed in **Table 4.4**.
10. Six calibrators with concentrations 0 (i.e., blank), 10, 25, 50, 100, and 250 μM are run with every batch of samples. The unenriched and enriched urine samples are also included with each batch.
11. Imprecision and analytical recovery from the enriched urine samples are determined from consecutive batches run over a period of 5 weeks. The presence of salts in the urine can lead to significant suppression of negative-ion signals, which in turn affects the linearity, inter-batch variability, and recovery for metabolites quantified without their corresponding labeled internal standards. This ion

Table 4.5
Isobaric interferences in negative ionization mode

Metabolites	Q1/Q3 transition
4-Hydroxy-butyric acid	103 > 57
2-Hydroxy-butyric acid	103 > 57
Succinic acid	117 > 73
Methylmalonic acid	117 > 73
Glutaric acid	131 > 87
Ethylmalonic acid	131 > 87
Methylsuccinic acid	131 > 87
Butyrylglycine	144 > 74
Isobutyrylglycine	144 > 74
2-Ketoglutaric acid	145 > 101
3-Methylglutaric acid	145 > 101
Tiglylglycine	156 > 74
3-Methylcrotonylglycine	156 > 74
Isovalerylglycine	158 > 74
Methylbutyrylglycine	158 > 74

suppression effect is minimized using several labeled internal standards. Apart from routine ion source cleaning performed at approximately weekly intervals, no other precautions are taken to minimize ion source contamination.

12. A careful selection of appropriate MRM transitions makes it possible to measure several isomeric metabolites. In some cases, no suitable transitions to solve isomeric compounds are found (**Table 4.5**) (5). This condition can be solved in the future by coupling the tandem mass spectrometer with HPLC separation.
13. The primary application of the LC tandem mass spectrometer urine screening method is as a first test for suspected inborn errors of metabolism. While the tandem mass spectrometry method can be used to test all urine samples, GC-MS-based organic acid analysis is typically used to confirm abnormal LC tandem mass spectrometer results or to distinguish isomers.

4. Notes

1. The 24-h collected urine should be considered a first choice for the analysis of organic acids. The majority of patients with suspicion of IEM are in neonatal age; thus, it is important to

perform a rapid test, preferably on the first morning voiding. When possible, urine samples must be stored in a freezer at –20°C if a –70°C system is not available. In fact, poor preservation of samples can lead to (1) bacterial conversion of some organic acids to artifacts such as the conversion of all keto acids to their respective hydroxy acids, (2) increased levels of succinic acid from bacterial degradation of glutamine, and (3) an abnormal concentration of pyroglutamic acid due to conversion from glutamine. The concentration of such volatile compounds as small molecule organic acids (e.g. short-chain organic acids) can be shortened if the sample is exposed to high temperature (especially in summer). The administration of drugs should be suspended in patients undergoing organic acids analysis to avoid artifacts due to drugs or drug metabolism. The following are some examples of the interference of drugs on the analysis of organic acids. Valproic acid administration causes an abnormal excretion of 3-hydroxy-isovaleric acid, tiglylglycine, dicarboxylic acids (even saturated, e.g., suberic, adipic, and sebacid acids), 2-methylbutyrylglycine, 7-hydroxy-octanoic acid, 5-hydroxy-hexanoic acid, and hexanoylglycine. An increase of pivalic acid can be due to the administration of the antibiotics pivampicillin and pivmecillinam. Fluvoxamine maleate, a selective serotonin reuptake inhibitor (SSRI) used to treat obsessive–compulsive disorder and depression, can cause an increment of maleic acid. A therapy with the antihyperuricemic drug allopurinol can increase orotate urinary excretion. Artifactual formation of new compounds during sample preparation can also occur. For example, during extraction with ethyl acetate, decarboxylation of keto acids can occur (14). Additionally, it is essential to pay attention to solvent contaminants, plasticizers (phthalate, adipate, and sebacate esters), glassware-cleaning agents, lubricants, resins, and bleeding from the stationary phase of the chromatographic columns.

2. IS work solution stability is only at 4°C or below. For example, the peak intensity of PDA-TMS is considerably larger when analyzed 1 week after preparation and storage at room temperature than when immediately analyzed. This is due to solvent evaporation at room temperature (12).
3. Bilirubine, a potential interferent, is oxidized by using potassium ferricyanide $K_3Fe(CN)_6$ at 0.13 mmol/L.
4. Do not mix organic and aqueous phases during liquid/liquid extraction of urine with solvents.
5. Clean the injector syringe everyday to avoid encrustation, purging, and bleeding.

6. Incorrect mass spectrometer calibration can result in abnormal qualitative and quantitative results.
7. Clean the mass spectrometer source and change the liner every 500 samples. Contamination is usually identified by an abnormal background. In a GC-MS instrument, there are sources of contamination within the gas chromatograph (bleeding septa, dirty injection port liners, air leaks, etc.) and the mass spectrometer (ion source leaks, fluid/oil, manifold dirt). Unfortunately, not all contamination can be removed through running clean carrier gas overnight. When this is the case, the instrument may need extensive cleaning. Cleaning the mass spectrometer ion source to remove contamination is critical to restore the electrostatic properties of the ion source lens. The authors suggest that the ion source be cleaned every 500 runs. Another common source of mass spectrometer contamination is from inadequate venting and maintenance of the diffusion pump. Preventive maintenance ensures that diffusion pump oil/fluids are topped to the correct levels. It is important to maintain proper fluid levels to avoid pump failure and ensure optimum performance of the vacuum system. Air leaks can occur if a seal becomes damaged or is not correctly fastened. This is another common problem for any instruments that use a vacuum and can be identified by a higher than normal vacuum manifold pressure, low relative ion abundance, and poor sensitivity.
8. The use of labeled internal standards and isotope dilution allows one to obtain a correct and absolute quantitation of organic acids, but it can be very expensive.

References

1. Björkman, L., McLean, C., Steen, G. (1976) Organic acids in urine from human newborns. *Clin Chem* 22, 49–52.
2. Chalmers, R. A., Healy, M. J., Lawson, A. M., Watts, R. W. (1976) Urinary organic acids in man. II. Effects of individual variation and diet on the urinary excretion of acidic metabolites. *Clin Chem* 22, 1288–1291.
3. Kumps, A., Duez, P., Mardens, Y. (2002) Metabolic, nutritional, iatrogenic, and artifactual sources of urinary organic acids: a comprehensive table. *Clin Chem* 48, 708–717.
4. Pitt, J. J., Eggington, M., Kahler, S. G. (2002) Comprehensive screening of urine samples for inborn errors of metabolism by electrospray tandem mass spectrometry. *Clin Chem* 48, 1970–1980.
5. Rebollido, M., Cocho, J. A., Castiñeiras, D. E., Bóveda, M. D., Fraga, J. M. (2006) Aplicación de la espectrometría de masas en tándem al análisis de aminoácidos, acilcarnitinas, acilglicinas y ácidos orgánicos en muestras de orina en papel. *Quím Clín* 25, 64–74.
6. Chalmers, R. A., Healy, M. J., Lawson, A. M., Hart, J. T., Watts, R. W. (1976) Urinary organic acids in man. III. Quantitative ranges and patterns of excretion in a normal population. *Clin Chem* 22, 1292–1298.
7. Perry, T. L., Hansen, S. (1974) Artifacts and pitfalls in interpretation of gas chromatograms in Application of Gas Chromatography-Mass Spectrometry to the Investigation of Human Disease. In *Proceedings of a workshop, Montreal, May 1973. McGill University-Montreal Children's*

Hospital Research Institute, Montreal, Quebec, Canada, 89–102.
8. Paterson, N. (1967) Relative constancy of 24-hour urine volume and 24-hour creatinine output. *Clin Chim Acta* 18, 57–58.
9. Zorab, P. A. (1969) Normal creatinine and hydroxyproline excretion in young persons. *Lancet* 29, 1164–1165.
10. Applegarth, D. A., Hardwick, D. F., Ross, P. M. (1968) Creatinine excretion in children and the usefulness of creatinine equivalents in amino acid chromatography. *Clin Chim Acta* 22, 131–134.
11. Larsen, K. (1972) Creatinine assay by a reaction-kinetic principle. *Clin Chim Acta* 41, 209–217.
12. Tanaka, K., West-Dull, A., Hine., D. G., Lynn, T. B., Lowe, T. (1980) Gas-chromatographic method of analysis for urinary organic acids. II. Description of the procedure, and its application to diagnosis of patients with organic acidurias. *Clin Chem* 26, 847–853.
13. Tanaka, K., Hine, D. G., West-Dull, A., Nad Lynn, T. B. (1980) Gas-chromatographic method of analysis for urinary organic acids. I. Retention indices of 155 metabolically important compounds. *Clin Chem* 26, 1839–1846.
14. Thompson, R. M., Belanger, B. G., Wappner, R. S., Brandt, I. K. (1975) An artifact in the gas chromatographic analysis of urinary organic acids from phenylketonuric children: decarboxylation of the phenylpyruvic acid during extraction. *Clin Chim Acta* 61, 367–374.