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# Metabolic profiling of intracellular metabolites in fermentation broths from β-lactam antibiotics production by liquid chromatography–tandem mass spectrometry methods

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ABSTRACT

An analytical platform comprising three LC–ESI-MS/MS methods is presented for qualitative and quantitative profiling of more than 200 intracellular metabolites. Employing a silica based zwitterionic stationary phase in the HILIC mode, in total 223 hydrophilic metabolites can be determined. In particular, amino acids, organic acids as well as nucleotide sugars were found to be well separable and detectable under acidic mobile phase conditions, while in comparison especially phosphates such as nucleotides, coenzymes or sugar phosphates as well as sugars and sugar acids performed better at higher pH. Additionally, 21 less polar analytes turned out to be amenable for separation and analysis on a pentafluorophenyl modified silica stationary phase in RP mode. Solutes were detected by tandem mass spectrometry on a triple quadrupole instrument in the selected reaction monitoring (SRM) mode and specific SRM transitions for 258 metabolites are provided. All three methods were validated with respect to the limit of quantification, linear dynamic range, precision and accuracy. Applicability of the analytical platform was evaluated by analysis of the targeted metabolites in extracts of β-lactam antibiotics fermentation broths. Thereby, 87 metabolites were determined qualitatively in penicillin fermentation broths, and 94 compounds were found in cephalosporin extracts. In addition, a number of selected metabolites that can be determined by at least two of the presented LC–MS/MS methods was analyzed quantitatively by both, external calibration using pure standards as well as by matrix-matched calibration performing standard addition. Quantitative results obtained with the different methods agreed well, however, for some analytes external calibration was found to be ill-suited due to matrix effects.

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## 1. Introduction

Metabolomics is an emerging field of systems biology [1] and aims at the comprehensive analysis of as many metabolites as possible present in a cell at a certain time and under given environmental conditions [2]. Recently, it has also become a popular tool in biotechnology [3,4].

Two complementary methodologies are commonly used, including targeted approaches (metabolic profiling and target analysis) as well as non-targeted, holistic approaches (metabolic fingerprinting and footprinting) [5]. Metabolic profiling deals with the quantitative analysis of a set of known metabolites related to a specific biochemical pathway or a class of compounds, while in target analysis a limited number of selected metabolites is quantitatively analyzed, such as biomarkers of a certain disease or status. Accurate, reliable and robust analytical methods are needed for these purposes in which the chemical identity of the metabolites is known and the obtained quantitative results are ideally independent of the technology used. In comparison, metabolic fingerprinting and footprinting attempt to measure the entirety of intracellular and extracellular metabolites, respectively, in a non-quantitative manner [6]. The goal is to compare patterns, i.e. "fingerprints" in the metabolic state of different samples and, aided by bioinformatics tools, to find out features in which they differ [7].

Both approaches put high demands on the analytical technologies employed with regards to specificity, sensitivity, accuracy and robustness. The current progress in the field of metabolomics is only possible due to the tremendous technical improvements in analytical and bioinformatics techniques in recent years. Various analytical platforms have been used for metabolomic studies, including Fourier transform-infrared (FT-IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy as well as mass spectrometry (MS) coupled to separation techniques such as capillary electrophoresis (CE), gas chromatography, liquid chromatogra-

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phy and ultra-high performance liquid chromatography (GC, LC, UHPLC) [8–14]. A great number of review articles has already been published dealing with the applicability of these techniques in metabolomics studies, covering NMR and MS [15,16] with particular focus on the specific characteristics of NMR [17–21], MS in general [22], high resolution MS techniques, such as Fourier transform-ion cyclotron resonance-MS (FT-ICR-MS) [23,24] as well as hyphenated MS based techniques, such as CE–MS [25], GC–MS [26] and LC–MS [27–32]. Also the importance and application of bioinformatics and chemometrics in the field of metabolomics have been reviewed in several articles [33–35].

In the present work, an analytical platform based on three HPLC–ESI-MS/MS methods is presented for the targeted quantitative analysis of more than 200 intracellular metabolites comprising free, acetylated and phosphorylated amino acids (AAs), vitamins, biogenic amines, free and phosphorylated organic acids, nucleobases (purines and pyrimidines), (deoxy-)nucleosides, (deoxy-)nucleotides (mono-, di-, tri- and cyclic phosphates), sugar nucleotides, coenzymes (e.g. CoA esters, NAD/H, NADP/H, *etc.*), sugars, sugar acids and sugar phosphates as well as pyrophosphates. With the technical progress in the field of MS detection, the reliable simultaneous quantitation of dozens of metabolites (>100 compounds) in one single run has become possible. Besides high mass resolution techniques such as FT-ICR-MS [36], also single quadrupole [37], quadrupole-linear iontrap [38] as well as triple quadrupole (QqQ) instruments [39–41] have been employed. For quantitative analyses of known compounds, especially ESI-QqQ-MS performed in the selected reaction monitoring (SRM) mode (MS/MS approach) offers high specificity and sensitivity since selected fragments (product ions) can be generated by fragmenting the molecule of interest (precursor ion) under controlled conditions which offers an additional dimension of selectivity for the discrimination of analytes. However, knowledge of suitable SRM transitions for all metabolites of interest is a prerequisite which may impede the application of LC–QqQ-MS/MS for comprehensive metabolic profiling studies. Recently, specific SRM transitions have been published for 90 nitrogen containing intracellular metabolites [39], 163 phosphorus metabolites [40] as well as 164 hydrophilic cellular compounds [41]. In the present study, specific SRM transitions for 258 compounds are provided.

Lack of tandem MS for isomers and isobaric compounds, respectively, as well as in-source decay that frequently generates fragments of the same mass-to-charge ($m/z$) ratio requires a suitable chromatography. Herein, various HILIC as well as RP stationary phases were tested upon their usefulness for qualitative and quantitative analysis of the targeted metabolites. Two columns – a zwitterionic HILIC phase and a pentafluorophenyl (PFP) modified RP column – were selected for the development of the three LC–MS/MS methods which were then validated with regards to linear range, LOQ, precision and accuracy. Finally, the applicability of the methods was evaluated by qualitative and quantitative analysis of cold methanolic extracts of fermentation broths from β-lactam antibiotics production. Occurrence of matrix effects was examined for several selected metabolites by comparison of quantitative results obtained by external calibration with pure standards and by matrix-matched calibration using a standard addition procedure.

## 2. Materials and methods

### 2.1. Chemicals

Solvents (acetonitrile, methanol) were of HPLC gradient grade from VWR International (Leuven, Belgium). Ultra-pure water (Chromasolv plus) and formic acid (FA, 98–100%) were from Sigma–Aldrich (Steinheim, Germany), acetic acid (AcOH, >99.8%), trifluoroacetic acid (TFA, 99%) and ammonium hydroxide solution (25%) were obtained from Fluka (Buchs, Switzerland). Potassium hydroxide p.a. was from Riedel-de Haen (Seelze, Germany). Analytical standards of the target compounds were purchased from Sigma–Aldrich. Single stock solutions of all analytes were prepared at concentrations of 0.5–2 mg/mL in ACN/$H_2O$ 50:50 (v/v) or 20:80 (v/v). If necessary, acid (0.1–1% FA or TFA) or base (0.5% 3 M KOH) was added to enable complete dissolution. All standard solutions were stored at −18 °C.

### 2.2. Instrumentation and MS/MS parameter optimization

Throughout the studies an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an Applied Biosystems 4000 QTrap (triple quadrupole-linear iontrap hybrid) mass spectrometer (operated in QqQ mode) equipped with a TurboV electrospray ion source (Applied Biosystems, Foster City, CA, USA) was used. Data were processed with the Analyst 1.5 software (Applied Biosystems). Analyte detection was performed in SRM mode applying the following general settings: ESI voltage was 4300 V in both positive and negative ionization mode, temperature of the ion source was set to 600 °C, entrance potential (EP) was 10 V. Nitrogen was used as nebulizer, heater as well as curtain gas, the pressure of which was set to 60, 50 and 10 psi, respectively. Scan time for each SRM transition (dwell time) was 10 ms, pause between two consecutive SRM transitions was 5 ms and settling time when changing between positive and negative mode was set to 700 ms. Compound specific MS parameters, i.e. declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were optimized for every analyte individually using the instrument's automated fragmentation optimization tool in order to find optimum SRM transitions (specific fragments) and corresponding instrument settings yielding optimum signal-to-noise ratios. For that purpose standard solutions of the target compounds were infused using a 500 μL Hamilton syringe and a syringe pump at a flow rate of 30 μL/min.

For the comparison of the separation performance of citric acid and isocitric acid (*cf.* Fig. 2) an Agilent 1100 HPLC system coupled to an MSD ion trap from Agilent equipped with an ESI interface was employed in addition to the above specified QTrap system.

### 2.3. Optimized HPLC conditions

During chromatographic runs, autosampler temperature was set to 5 °C, temperature of the column compartment was 25 °C, injection volume was 10 μL. Polar compounds were separated in HILIC mode using a ZIC-HILIC stationary phase (150 mm × 4.6 mm, 5 μm) purchased from Merck SeQuant (Marl, Germany) at a flow rate of 700 μL/min. Mobile phases were 20 mM ammonium formiate (adjusted to pH 3.5 for the acidic HILIC method) and 20 mM ammonium acetate (adjusted to pH 7.5 for the neutral HILIC method), respectively, in (A) $H_2O$ and (B) ACN. Mobile phases were prepared from a 200 mM solution of the acid adjusted to the respective pH with $NH_4OH$ which was diluted 1:10 with water and ACN, respectively. Gradient elution was performed starting with 0% A increasing to 80% A in 30 min, then back to starting conditions (80–0% A) in 1 min followed by a re-equilibration period (0% A) of 14 min (total run time 45 min). Less polar analytes were separated in RP mode on a Phenomenex Luna PFP(2) column (150 mm × 4.6 mm, 3 μm) (Phenomenex, Aschaffenburg, Germany) at a flow rate of 400 μL/min, using 20 mM ammonium formiate adjusted to pH 3.5 in (A) $H_2O$ and (B) MeOH, respectively, prepared as described above for the HILIC eluents. Gradient conditions were 5% B to 100% B in 30 min, 100% B to 5% B in 1 min, re-equilibration at 5% B for 14 min (total run time 45 min).

## 3. Results and discussion

### 3.1. Optimization of MS/MS parameters

A prerequisite for the development of a LC–MS/MS method employing the SRM mode is the knowledge of suitable product ions, i.e. SRM transitions as well as of instrumental parameters such as the declustering potential (DP) which determines the ionization efficiency of the analyte in the ESI source or the collision energy (CE) to specifically fragment the molecule of interest. The employed QTrap (that was operated in QqQ mode) offers a software tool that allows an automated fragmentation optimization when infusing standards of high purity. In the present study, fragmentation patterns were examined for about 280 metabolites using commercially available pure standards. For several compounds no reliable SRM transitions could be determined since they turned out to be insufficiently stable in solution, such as ascorbic acid, prephenic acid, orotic acid, oxalic acid, oxaloacetic acid or dihydrofolic acid (DHF) or for any other reason fragmentation optimization failed. These analytes were omitted from further method development. Fragmentation optimization was successfully accomplished for 258 compounds and the optimum product ion was chosen for each metabolite to give best signal-to-noise ratio and as far as possible, selectivity from potential interferences, respectively. In Table 1, the optimized MS/MS parameters, i.e. parent ion and product ion as well as DP and CE, are given.

In many cases the optimum product ion was not necessarily the fragment with the highest signal intensity since interferences from other compounds of the same parent mass could compromise selective detection of the targeted analyte. Especially in the case of compounds with similar physico-chemical characteristics and yet similar chromatographic retention behavior such as isomers, possible occurrence of interferences has to be considered. For instance, the list of metabolites addressed in the present study comprises eight isobaric analytes with a molecular mass of 131, *viz.* L-leucine (L-Leu), L-isoleucine (L-Ile), L-norleucine (L-Nle), L-hydroxyproline (L-OH-Pro), creatine, 3-guanidinopropionic acid, 5-aminolevulinic acid and 6-aminocaproic acid which cannot be distinguished according to their $m/z$ ratio by the herein utilized low resolution ESI-MS instrument (Table 1). In Fig. 1a, total ion chromatogram (TIC) of the separation of these eight compounds is depicted. Under the chosen LC conditions (*vide infra*), only 5-aminolevulinic acid was well separated from the other isobaric compounds, while especially the isomeric amino acids L-Leu, L-Ile and L-Nle showed very similar chromatographic behavior with coelution of L-Leu and L-Nle and partial resolution of L-Ile. For all three AAs a fragment with $m/z$ of 86 (generated by loss of the carboxyl group) exhibited the highest signal intensity (Fig. 1b). However, due to the coelution of L-Leu and L-Nle specific detection of the analytes was not possible using this SRM transition. Nevertheless, it turned out that baseline separation was not necessary since specific transitions were found for L-Leu and L-Nle allowing largely unimpaired detection of all three isomers (Fig. 1c and d). Also L-OH-Pro, creatine, 3-guanidinopropionic acid and 6-aminocaproic acid which eluted close to each other were found to be detectable without interferences due to the existence of selective SRM transitions (Fig. 1b–e).

For several metabolites for which intensity of the product ions was too low or generated fragments were not specific enough for reliable qualitative and quantitative determination so-called pseudo-SRM transitions were used for which the $m/z$ of the intact unfragmented parent ion was measured also in the third quadrupole. This was especially the case for small compounds ($M < 150$) that were almost exclusively ionizable in negative mode such as organic acids (Table 1).

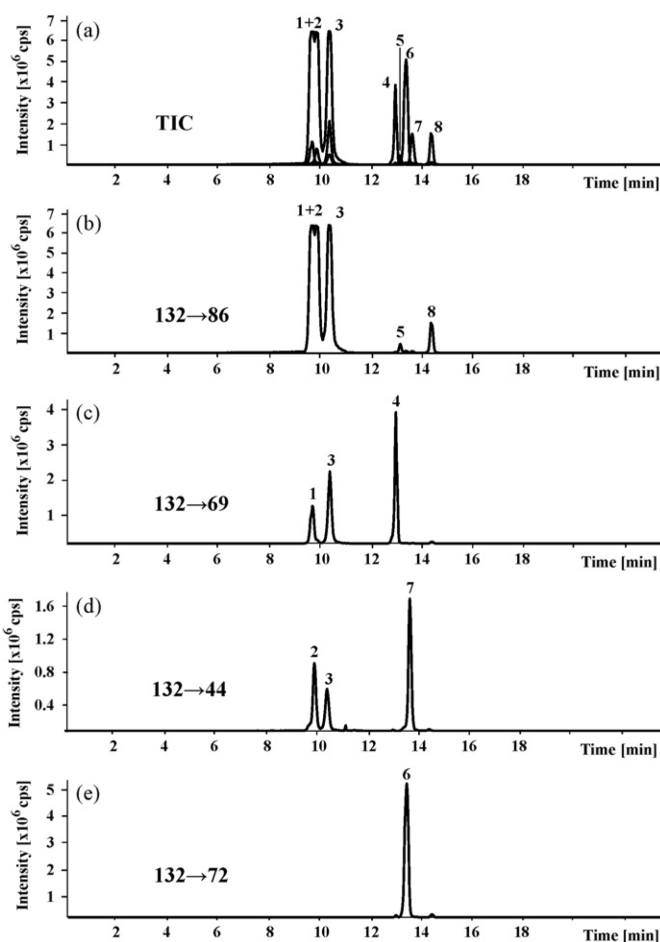


**Fig. 1.** Chromatographic separation of eight isobaric metabolites ($M$ = 131) with MS/MS detection in the positive ionization mode. (a) Total ion chromatogram (TIC) and extracted ion chromatograms (XICs) of SRM transition, (b) 132 → 86, (c) 132 → 69, (d) 132 → 44 and (e) 132 → 72. Peak assignment: 1, L-norleucine; 2, L-leucine; 3, L-isoleucine; 4, 6-aminocaproic acid; 5, L-hydroxyproline; 6, 3-guanidinopropionic acid; 7, creatine; 8, 5-aminolevulinic acid. Experimental conditions: acidic HILIC method (pH 3.5) (see Section 2 for details).

Regarding identity of the product ions and the fragments lost in the collision cell, respectively, structures were not identified for each analyte, however, in several cases common features have been recognized. For instance, phosphates such as nucleotides or sugar phosphates were best determinable in the negative ionization mode by detection of the group specific fragment ion with $m/z$ of 79 (corresponding to $PO_3^-$) or 97 ($H_2PO_4^-$) and in the case of di- and triphosphates the fragment with $m/z$ = 159 ($HP_2O_6^-$) the latter, however, typically showing much lower intensity. Nucleotide sugars were determined by detection of the cleaved sugar moiety or sugar phosphate in the negative ionization mode. Nucleosides and deoxynucleosides were well detectable in positive mode by determination of the corresponding nucleobase as product ion. Also for NAD, NADP and NAADP, cleaved adenine ($m/z$ = 136 in positive mode) was found to be a specific SRM transition. In the case of coenzyme A esters the lost fragment (−507 mass units) was identified as being the adenosine 3′-phosphate 5′-diphosphate residue. Less specific fragments and their unspecific cleavage, respectively, such as carboxyl ($m/z$ = 44), acetyl ($m/z$ = 59) or amino groups ($m/z$ = 17) had to be used as SRM transitions in particular for low molecular weight compounds such as small organic acids or amines and AAs.

**Table 1**
SRM transitions for the metabolites addressed in the present study and chromatographic conditions under which the analytes are determinable. Compounds are listed according to their molecular weight ($M$). + and − indicate whether the analytes can be determined with the respective method.

| Metabolite | $M$[a] | MS/MS parameters | | | | HPLC method | | |
|---|---|---|---|---|---|---|---|---|
| | | Parent ion | Product ion | DP[b] [V] | CE[c] [V] | HILIC pH 3.5 | HILIC pH 7.5 | RP pH 3.5 |
| Ethanolamine | 61.0 | 62 | 45 | 31 | 21 | + | – | – |
| Glyoxylic acid | **74.0** | 73 | 73 | −30 | −6 | + | – | – |
| Propionic acid | **74.1** | 73 | 73 | −15 | −6 | – | – | + |
| Glycine | 75.0 | 76 | 76 | 16 | 5 | + | – | – |
| Glycolic acid | 76.0 | 75 | 75 | −30 | −6 | + | – | – |
| Cysteamine | 77.2 | 78 | 61 | 31 | 17 | – | + | – |
| Butyric acid | **88.1** | 87 | 87 | −40 | −8 | – | – | + |
| Putrescine | **88.0** | 89 | 72 | 31 | 13 | + | – | – |
| Pyruvic acid | **88.0** | 87 | 87 | −30 | −6 | + | – | – |
| L-Alanine | **89.0** | 90 | 44 | 31 | 21 | +[d] | – | – |
| β-Alanine | **89.1** | 90 | 73 | 39 | 25 | +[d] | – | – |
| Sarcosine | **89.1** | 90 | 44 | 31 | 21 | +[d] | – | – |
| Lactic acid | 90.0 | 89 | 89 | −45 | −5 | + | + | – |
| Betaine aldehyde | **102.2** | 103 | 88 | 21 | 11 | + | – | – |
| Cadaverine | **102.2** | 103 | 86 | 26 | 15 | + | – | – |
| Isovaleric acid | **102.1** | 101 | 101 | −35 | −6 | – | – | + |
| 2-Ketobutyric acid | **102.1** | 101 | 101 | −35 | −6 | – | – | + |
| 2-Methylbutyric acid | **102.1** | 101 | 101 | −35 | −6 | – | – | + |
| Valeric acid | **102.1** | 101 | 101 | −45 | −6 | – | – | + |
| γ-Aminobutyric acid | 103.1 | 102 | 102 | −10 | −6 | + | – | – |
| Choline | **104.2** | 105 | 105 | 15 | 20 | – | + | – |
| DL-β-Hydroxybutyric acid | **104.1** | 103 | 59 | −35 | −14 | + | – | – |
| β-Hydroxypyruvic acid | **104.1** | 103 | 103 | −35 | −6 | – | – | + |
| Malonic acid | **104.0** | 103 | 41 | −25 | −36 | + | + | – |
| L-Serine | 105.0 | 106 | 60 | 16 | 15 | + | – | – |
| Glyceric acid | 106.1 | 105 | 75 | −40 | −16 | + | – | – |
| Hypotaurine | 109.2 | 110 | 92 | 31 | 13 | + | + | – |
| Cytosine | **111.1** | 112 | 95 | 45 | 25 | + | – | – |
| Histamine | **111.2** | 112 | 95 | 45 | 25 | + | – | – |
| Uracil | 112.1 | 113 | 70 | 56 | 23 | – | + | – |
| Creatinine | 113.1 | 114 | 114 | 26 | 7 | + | – | – |
| Dihydrouracil | 114.1 | 115 | 115 | 16 | 5 | – | + | – |
| L-Proline | 115.0 | 116 | 70 | 26 | 25 | + | – | – |
| 2,2-Dimethylbutyric acid | **116.2** | 115 | 115 | −60 | −6 | – | – | + |
| Fumaric acid | **116.0** | 115 | 71 | −35 | −12 | + | – | + |
| Hexanoic acid | **116.2** | 115 | 115 | −60 | −6 | – | – | + |
| Maleic acid | **116.1** | 115 | 71 | −30 | −12 | – | – | + |
| 2-Methylvaleric acid | **116.2** | 115 | 115 | −60 | −6 | – | – | + |
| Betaine | **117.2** | 118 | 118 | 31 | 5 | + | + | – |
| Guanidinoacetic acid | **117.2** | 118 | 72 | 31 | 17 | + | + | – |
| DL-Norvaline | **117.1** | 118 | 72 | 31 | 17 | + | + | – |
| L-Valine | **117.0** | 118 | 72 | 31 | 17 | + | + | – |
| Methylmalonic acid | **118.1** | 117 | 73 | −35 | −16 | +[d] | – | +[d] |
| Succinic acid | **118.0** | 117 | 73 | −35 | −16 | +[d] | – | +[d] |
| L-Homoserine | **119.1** | 120 | 74 | 49 | 17 | + | – | – |
| L-Threonine | **119.0** | 120 | 74 | 49 | 17 | + | – | – |
| Purine | 120.1 | 121 | 67 | 46 | 43 | + | + | – |
| Nicotinamide | 122.1 | 123 | 79 | 11 | 15 | + | + | – |
| Isonicotinic acid | **123.1** | 124 | 80 | 46 | 31 | + | – | + |
| Nicotinic acid | **123.0** | 124 | 80 | 46 | 31 | + | – | + |
| 5-Methylcytosine | **125.1** | 126 | 126 | 56 | 5 | + | + | – |
| Taurine | **125.2** | 124 | 80 | −25 | −12 | + | + | – |
| Thymine | 126.1 | 127 | 110 | 21 | 20 | – | + | – |
| *N*-Acetylputrescine | **130.2** | 131 | 114 | 16 | 15 | – | – | + |
| Citraconic acid | **130.1** | 129 | 85 | −15 | −14 | – | – | + |
| Itaconic acid | **130.1** | 129 | 85 | −15 | −14 | – | – | + |
| 6-Aminocaproic acid | **131.2** | 132 | 69 | 46 | 25 | + | + | – |
| 5-Aminolevulinic acid | **131.1** | 132 | 86 | 41 | 17 | + | + | – |
| Creatine | **131.1** | 132 | 44 | 51 | 35 | + | + | – |
| 3-Guanidinopropionic acid | **131.1** | 132 | 72 | 51 | 23 | + | + | – |
| L-Hydroxyproline | **131.1** | 132 | 86 | 41 | 17 | + | + | – |
| L-Isoleucine | **131.0** | 132 | 69 | 46 | 25 | + | + | – |
| L-Leucine | **131.0** | 132 | 44 | 51 | 35 | + | + | – |
| L-Norleucine | **131.2** | 132 | 69 | 46 | 25 | + | + | – |
| L-Asparagine | **132.0** | 133 | 74 | 41 | 21 | + | + | – |
| Glutaric acid | **132.0** | 131 | 87 | −25 | −18 | – | + | – |
| L-Ornithine | **132.2** | 133 | 70 | 36 | 31 | + | + | – |
| L-Aspartic acid | 133.0 | 134 | 74 | 51 | 23 | + | – | – |
| Adenine | 135.1 | 136 | 119 | 16 | 33 | + | – | – |
| Hypoxanthine | **136.1** | 137 | 110 | 61 | 29 | – | + | + |
| p-Toluic acid | **136.2** | 135 | 91 | −55 | −16 | – | – | + |
| m-Toluic acid | **136.2** | 135 | 91 | −55 | −16 | – | – | + |

Table 1 (*Continued* )

| Metabolite | $M^a$ | MS/MS parameters | | | | HPLC method | | |
|---|---|---|---|---|---|---|---|---|
| | | Parent ion | Product ion | DP[b] [V] | CE[c] [V] | HILIC pH 3.5 | HILIC pH 7.5 | RP pH 3.5 |
| Anthranilic acid | 137.1 | 138 | 120 | 31 | 17 | – | – | + |
| Salicylic acid | **138.1** | 137 | 93 | –35 | –24 | – | – | + |
| m-Hydroxybenzoic acid | **138.1** | 137 | 93 | –35 | –24 | – | – | + |
| p-Hydroxybenzoic acid | **138.1** | 137 | 93 | –35 | –24 | – | – | + |
| Acetylphosphate | 140.0 | 139 | 79 | –20 | –12 | – | + | – |
| Carbamoylphosphate | 141.0 | 140 | 79 | –20 | –15 | – | + | – |
| *O*-Phosphorylethanolamine | 141.1 | 140 | 79 | –20 | –15 | – | + | – |
| Caprylic acid | 144.2 | 143 | 143 | –65 | –6 | – | – | + |
| Acetylcholine | **146.2** | 147 | 88 | 26 | 20 | + | – | – |
| L-Glutamine | **146.0** | 147 | 84 | 36 | 29 | + | – | – |
| α-Ketoglutaric acid | **146.0** | 145 | 101 | –20 | –12 | + | – | – |
| L-Lysine | **146.0** | 147 | 84 | 36 | 29 | + | + | – |
| *O*-Acetyl-L-serine | **147.1** | 148 | 88 | 26 | 15 | + | – | – |
| L-Glutamic acid | **147.0** | 148 | 130 | 31 | 12 | + | + | – |
| *trans*-Cinnamic acid | 148.2 | 147 | 103 | –60 | –16 | – | – | + |
| L-Methionine | 149.0 | 150 | 133 | 56 | 15 | + | – | – |
| L-Tartric acid | 150.0 | 149 | 87 | –25 | –18 | – | + | – |
| Guanine | 151.1 | 152 | 135 | 31 | 29 | + | + | + |
| Xanthine | 152.1 | 153 | 110 | 46 | 27 | + | + | + |
| L-Histidine | 155.0 | 156 | 110 | 46 | 21 | + | – | – |
| Allantoin | **158.1** | 159 | 116 | 31 | 11 | + | – | – |
| Dihydroorotic acid | **158.1** | 157 | 113 | –30 | –15 | – | + | – |
| Tryptamine | 160.2 | 161 | 144 | 26 | 15 | + | + | – |
| L-Carnitine | 161.2 | 162 | 103 | 46 | 25 | + | + | – |
| o-Coumaric acid | **164.2** | 163 | 119 | –60 | –19 | – | – | + |
| p-Coumaric acid | **164.2** | 163 | 119 | –60 | –19 | – | – | + |
| Phenylpyruvic acid | **164.2** | 163 | 91 | –25 | –15 | – | + | + |
| L-Phenylalanine | 165.0 | 166 | 120 | 51 | 21 | + | – | – |
| Pyridoxal | 167.1 | 168 | 150 | 31 | 17 | + | + | – |
| PEP | **168.1** | 167 | 79 | –25 | –16 | + | + | – |
| Uric acid | **168.1** | 167 | 124 | –50 | –22 | + | + | – |
| L-Cysteic acid | **169.2** | 170 | 170 | 46 | 7 | + | +[d] | – |
| Pyridoxine | **169.0** | 170 | 152 | 41 | 19 | + | +[d] | – |
| Dihydroxyacetone phosphate | 170.1 | 169 | 79 | –40 | –36 | + | + | – |
| Glycerol 3-phosphate | 172.1 | 171 | 79 | –40 | –26 | – | + | – |
| *N*-Acetyl-L-ornithine | **174.2** | 175 | 70 | 31 | 39 | + | – | – |
| *cis*-Aconitic acid | **174.0** | 173 | 85 | –25 | –18 | + | – | – |
| *trans*-Aconitic acid | **174.0** | 173 | 85 | –25 | –18 | + | – | – |
| L-Arginine | **174.0** | 175 | 70 | 31 | 39 | + | – | – |
| Dehydroascorbic acid | **174.1** | 173 | 173 | –75 | –6 | + | – | – |
| Shikimic acid | **174.2** | 173 | 93 | –75 | –15 | – | + | – |
| L-Citrulline | **175.0** | 176 | 159 | 36 | 15 | + | – | – |
| Indole 3-acetic acid | **175.2** | 176 | 130 | 46 | 25 | – | + | – |
| Allantoic acid | 176.1 | 175 | 132 | –25 | –12 | + | + | – |
| Glucosamine | 179.2 | 180 | 162 | 31 | 13 | – | + | – |
| L-Tyrosine | 181.0 | 182 | 136 | 46 | 21 | + | – | – |
| DL-Homocysteic acid | 183.2 | 182 | 80 | –25 | –12 | + | – | – |
| *O*-Phospho-L-serine | 185.1 | 186 | 88 | 36 | 35 | + | + | – |
| 2-Phosphoglyceric acid | **186.1** | 185 | 79 | –45 | –32 | – | + | – |
| 3-Phosphoglyceric acid | **186.1** | 185 | 79 | –45 | –32 | – | + | – |
| *N*-Acetyl-L-glutamine | **188.2** | 189 | 130 | 16 | 21 | + | – | – |
| Azelaic acid | **188.2** | 187 | 125 | –50 | –22 | – | – | + |
| *N*-Acetyl-L-glutamic acid | 189.2 | 190 | 84 | 26 | 33 | + | + | + |
| *N*-Acetyl-L-methionine | 191.3 | 192 | 144 | 21 | 15 | + | + | – |
| Citric acid | **192.1** | 191 | 87 | –25 | –24 | +[e] | – | – |
| *threo*-D-Isocitric acid | **192.1** | 191 | 73 | –35 | –30 | +[e] | – | – |
| Glucuronic acid | 194.1 | 193 | 113 | –45 | –18 | – | + | – |
| Gluconic acid | 196.2 | 195 | 75 | –50 | –26 | – | + | – |
| Erythrose 4-phosphate | 200.1 | 199 | 97 | –55 | –14 | – | + | – |
| Sebacic acid | 202.3 | 201 | 183 | –40 | –20 | – | – | + |
| L-Tryptophan | 204.0 | 205 | 188 | 51 | 15 | + | – | – |
| α-Lipoamide | **205.3** | 206 | 189 | 26 | 13 | – | – | + |
| Xanthurenic acid | **205.2** | 204 | 160 | –45 | –20 | – | + | – |
| Lipoic acid | 206.3 | 205 | 171 | –45 | –12 | – | – | + |
| L-Kynurenine | 208.2 | 209 | 192 | 31 | 13 | + | – | – |
| Glucaric acid | 210.2 | 209 | 191 | –35 | –14 | – | + | – |
| 2-Deoxyribose 5-phosphate | 214.1 | 213 | 97 | –55 | –22 | – | + | – |
| 5-Hydroxy-DL-tryptophan | 220.2 | 221 | 204 | 36 | 15 | + | + | – |
| GlcNAc | **221.2** | 222 | 204 | 26 | 11 | +[d] | +[d] | – |
| ManNAc | **221.2** | 222 | 204 | 26 | 11 | +[d] | +[d] | – |
| L-Cystathionine | 222.3 | 223 | 88 | 36 | 41 | + | – | – |
| 3-Hydroxy-DL-kynurenine | 224.2 | 225 | 208 | 36 | 13 | + | + | – |
| L-Carnosine | 226.2 | 227 | 110 | 31 | 33 | + | + | – |
| 2′-Deoxycytidine | 227.2 | 228 | 112 | 31 | 15 | + | + | + |

Table 1 (*Continued* )

| Metabolite | M[a] | MS/MS parameters | | | | HPLC method | | |
|---|---|---|---|---|---|---|---|---|
| | | Parent ion | Product ion | DP[b] [V] | CE[c] [V] | HILIC pH 3.5 | HILIC pH 7.5 | RP pH 3.5 |
| 2′-Deoxyuridine | 228.2 | 229 | 113 | 26 | 21 | – | – | + |
| Ribose 5-phosphate | **230.1** | 229 | 97 | −35 | −18 | – | +[d] | – |
| Ribulose 5-phosphate | **230.1** | 229 | 97 | −35 | −18 | – | +[d] | – |
| Xylulose 5-phosphate | **230.1** | 229 | 97 | −35 | −18 | – | + | – |
| L-Cystine | 240.0 | 241 | 74 | 46 | 37 | + | – | – |
| Thymidine | 242.2 | 243 | 127 | 26 | 15 | – | + | – |
| Cytidine | 243.2 | 244 | 112 | 26 | 19 | + | + | + |
| Biotin | **244.0** | 245 | 97 | 46 | 43 | – | + | + |
| Uridine | **244.2** | 245 | 113 | 31 | 15 | + | + | + |
| Isopentenyl PPi | 246.1 | 245 | 79 | −30 | −34 | – | + | – |
| Pyridoxal 5-phosphate | 247.1 | 248 | 150 | 36 | 23 | + | + | – |
| Pyridoxamine 5-phosphate | 248.2 | 249 | 134 | 36 | 33 | – | + | – |
| 2′-Deoxyadenosine | 251.2 | 252 | 136 | 36 | 21 | – | + | – |
| 2′-Deoxyinosine | 252.2 | 253 | 137 | 26 | 13 | + | + | – |
| Glucosamine 1-phosphate | **259.2** | 260 | 162 | 26 | 15 | – | + | – |
| Glucosamine 6-phosphate | **259.2** | 260 | 126 | 31 | 19 | – | + | – |
| Fructose 6-phosphate | **260.1** | 259 | 79 | −45 | −46 | – | +[d] | – |
| Galactose 1-phosphate | **260.2** | 259 | 79 | −50 | −46 | – | +[d] | – |
| Glucose 1-phosphate | **260.1** | 259 | 79 | −45 | −46 | – | +[d] | – |
| Glucose 6-phosphate | **260.1** | 259 | 79 | −45 | −46 | – | +[d] | – |
| Mannose 1-phosphate | **260.1** | 259 | 79 | −45 | −46 | – | +[d] | – |
| Mannose 6-phosphate | **260.1** | 259 | 79 | −45 | −46 | – | +[d] | – |
| Adenosine | **267.2** | 268 | 136 | 51 | 25 | + | + | – |
| 2′-Deoxyguanosine | **267.2** | 268 | 152 | 41 | 15 | + | + | – |
| Homocystine | **268.2** | 269 | 136 | 26 | 15 | + | + | – |
| Inosine | **268.2** | 269 | 137 | 36 | 15 | + | + | – |
| Gluconic acid 6-phosphate | 276.1 | 275 | 97 | −35 | −20 | – | + | – |
| Guanosine | 283.2 | 284 | 152 | 36 | 19 | + | + | – |
| Xanthosine | 284.2 | 285 | 153 | 46 | 17 | + | – | – |
| Argininosuccinic acid | 290.3 | 291 | 70 | 56 | 63 | + | + | – |
| D-Sphingosine | 299.5 | 300 | 282 | 31 | 17 | + | + | – |
| GlcNAc 1-phosphate | 301.2 | 302 | 204 | 26 | 11 | – | + | – |
| cTMP | 304.2 | 303 | 125 | −65 | −28 | + | + | – |
| cCMP | 305.2 | 304 | 110 | −70 | −32 | + | + | – |
| dCMP | 307.2 | 306 | 79 | −70 | −56 | – | + | – |
| dUMP | 308.2 | 307 | 195 | −60 | −22 | – | + | – |
| NANA | 309.3 | 310 | 274 | 31 | 15 | – | + | – |
| Ribulose 1,5-bisphosphate | 310.1 | 309 | 97 | −45 | −34 | – | + | – |
| Geranyl PPi | 314.2 | 313 | 79 | −25 | −45 | + | + | – |
| TMP | 322.2 | 321 | 195 | −65 | −24 | – | + | – |
| CMP | 323.2 | 322 | 79 | −75 | −62 | – | + | – |
| UMP | 324.2 | 323 | 79 | −60 | −60 | – | + | – |
| cAMP | 329.2 | 328 | 134 | −85 | −36 | + | + | – |
| PQQ | 330.2 | 331 | 285 | 51 | 27 | – | + | – |
| dAMP | 331.2 | 330 | 79 | −60 | −62 | – | + | – |
| dIMP | 332.2 | 331 | 135 | −55 | −32 | – | + | – |
| Lactose | **342.3** | 341 | 161 | −65 | −12 | – | +[d] | – |
| Maltose | **342.3** | 341 | 161 | −65 | −12 | – | +[d] | – |
| Sucrose | **342.3** | 341 | 59 | −75 | −55 | – | + | – |
| Trehalose | **342.3** | 341 | 59 | −75 | −55 | – | +[d] | – |
| cGMP | **345.2** | 344 | 150 | −75 | −34 | + | + | – |
| Thiamine monophosphate | **345.3** | 346 | 123 | 51 | 29 | – | + | – |
| AMP | **347.2** | 346 | 79 | −73 | −65 | – | + | – |
| dGMP | **347.2** | 346 | 79 | −73 | −65 | – | + | – |
| IMP | 348.2 | 347 | 79 | −60 | −58 | – | + | – |
| GMP | 363.2 | 362 | 79 | −65 | −62 | – | + | – |
| XMP | 364.2 | 363 | 79 | −55 | −66 | – | + | – |
| Riboflavin | 376.0 | 377 | 243 | 71 | 35 | – | – | + |
| Farnesyl PPi | 382.4 | 381 | 79 | −25 | −45 | + | + | – |
| S-(5′-adenosyl)-L-homocysteine | 384.4 | 385 | 134 | 51 | 27 | + | – | – |
| dCDP | 387.2 | 386 | 79 | −65 | −72 | – | + | – |
| S-(5′-adenosyl)-L-methionine | 398.5 | 399 | 250 | 51 | 23 | + | – | – |
| TDP | 402.2 | 401 | 79 | −65 | −78 | – | + | – |
| CDP | 403.2 | 402 | 79 | −65 | −78 | – | + | – |
| UDP | 404.2 | 403 | 79 | −60 | −76 | – | + | – |
| dADP | 411.2 | 410 | 79 | −60 | −74 | – | + | – |
| ADP | **427.2** | 426 | 79 | −75 | −88 | – | + | – |
| dGDP | **427.2** | 426 | 79 | −70 | −88 | – | + | – |
| IDP | 428.2 | 427 | 79 | −60 | −86 | – | + | – |
| Folic acid | 441.0 | 442 | 295 | 41 | 23 | + | – | – |
| GDP | 443.2 | 442 | 79 | −70 | −82 | – | + | – |
| THF | 445.4 | 446 | 299 | 46 | 31 | – | + | – |
| CDP-ethanolamine | 446.3 | 445 | 79 | −45 | −82 | + | – | – |
| Riboflavin 5′-monophosphate | 456.3 | 457 | 439 | 56 | 25 | – | + | – |

Table 1 (*Continued* )

| Metabolite | *M*[a] | MS/MS parameters | | | | HPLC method | | |
|---|---|---|---|---|---|---|---|---|
| | | Parent ion | Product ion | DP[b] [V] | CE[c] [V] | HILIC pH 3.5 | HILIC pH 7.5 | RP pH 3.5 |
| 5-Methyl-THF | 459.6 | 460 | 313 | 51 | 29 | + | – | – |
| Adenylosuccinic acid | 463.3 | 464 | 252 | 61 | 29 | – | + | – |
| dCTP | 467.2 | 466 | 79 | –55 | –82 | – | + | – |
| dUTP | 468.1 | 467 | 79 | –60 | –90 | – | + | – |
| TTP | 482.2 | 481 | 79 | –60 | –96 | – | + | – |
| CTP | 483.2 | 482 | 79 | –60 | –92 | – | + | – |
| UTP | 484.2 | 483 | 79 | –65 | –90 | – | + | – |
| CDP-choline | 488.4 | 487 | 428 | –80 | –18 | + | + | – |
| dATP | 491.2 | 490 | 79 | –60 | –90 | – | + | – |
| dITP | 492.2 | 491 | 79 | –55 | –90 | – | + | – |
| ATP | **507.2** | 506 | 79 | –70 | –90 | – | + | – |
| dGTP | **507.2** | 506 | 79 | –70 | –90 | – | + | – |
| APPS | **507.3** | 508 | 136 | 41 | 47 | – | + | – |
| ITP | 508.2 | 507 | 79 | –60 | –90 | – | + | – |
| GTP | 523.2 | 522 | 79 | –60 | –90 | – | + | – |
| UDP-Gal | **566.3** | 565 | 323 | –70 | –34 | +[d] | – | – |
| UDP-Glc | **566.3** | 565 | 323 | –70 | –34 | +[d] | – | – |
| UDP-glucuronic acid | 580.3 | 579 | 403 | –75 | –32 | + | + | – |
| ADP-Glc | **589.4** | 588 | 346 | –40 | –34 | + | – | – |
| GDP-Fuc | **589.3** | 588 | 442 | –75 | –34 | + | – | – |
| GDP-Glc | **605.3** | 604 | 362 | –60 | –36 | +[d] | – | – |
| GDP-Man | **605.3** | 604 | 79 | –60 | –90 | +[d] | – | – |
| UDP-GalNAc | **607.4** | 606 | 385 | –85 | –38 | +[d] | – | – |
| UDP-GlcNAc | **607.4** | 606 | 385 | –85 | –38 | +[d] | – | – |
| L-Glutathione oxidized | 612.6 | 613 | 355 | 71 | 33 | + | + | – |
| CMP-NANA | 614.5 | 613 | 322 | –60 | –28 | + | + | – |
| NAD | 663.4 | 664 | 136 | 71 | 69 | + | + | – |
| NADH | 665.4 | 666 | 649 | 61 | 25 | + | + | – |
| NADP | 743.4 | 744 | 136 | 51 | 79 | – | + | – |
| NAADP | 744.4 | 745 | 136 | 86 | 97 | – | + | – |
| NADPH | 745.4 | 746 | 625 | 36 | 23 | – | + | – |
| Coenzyme A | 767.5 | 768 | 261 | 91 | 41 | – | + | – |
| FAD | 785.6 | 786 | 348 | 76 | 31 | + | – | – |
| Acetyl coenzyme A | 809.6 | 810 | 303 | 36 | 45 | – | + | – |
| *n*-Propionyl coenzyme A | 823.6 | 824 | 317 | 81 | 47 | – | + | – |
| Malonyl coenzyme A | 853.6 | 854 | 347 | 96 | 45 | – | + | – |
| Methylmalonyl coenzyme A | **867.6** | 868 | 361 | 71 | 41 | – | +[d] | – |
| Succinyl coenzyme A | **867.6** | 868 | 361 | 71 | 41 | – | +[d] | – |

*Abbreviations*: PEP: phospho(enol)pyruvic acid; GlcNAc: *N*-acetyl-D-glucosamine; ManNAc: *N*-acetyl-D-mannosamine; PPi: pyrophosphate; NANA: *N*-acetylneuraminic acid; PQQ: pyrroloquinoline quinone; THF: tetrahydrofolic acid; APPS: adenosine 3′-phosphate 5′-phosphosulfate.

[a] *M* in bold indicates isobaric compounds.

[b] Declustering potential.

[c] Collision energy. The sign on DP and CE indicates the ionization mode used.

[d] Coelution of isobaric compounds under the respective LC conditions.

[e] Separation not reproducible.

## 3.2. Optimization of chromatographic conditions

Development and optimization of the chromatographic methods were based on a comprehensive screening of all 258 metabolites included in the final set of analytes on various stationary phases under acidic as well as basic gradient elution conditions using ACN as organic modifier. Standard mixtures comprising 40–60 compounds were prepared in such a way that no isobaric compounds or such that could interfere detection of other analytes were mixed together. Both, HILIC as well as RP columns were tested, the former including a zwitterionic phase (Merck SeQuant ZIC-HILIC), two different amino phases (Phenomenex Luna Amino, Unison UK-Amino) and an amide phase (Tosoh Bioscience TSKgel Amide), the latter comprising two conventional C18 phases from different manufacturers (Waters X-Bridge, Phenomenex Gemini C18), a polar embedded RP column (Phenomenex Synergi Fusion-RP) as well as a PFP modified phase (Phenomenex PFP(2)). In addition, a zwitterionic mixed-mode phase was tested in both, HILIC as well as RP mode (Sielc Obelisc R). Chromatographic runs were evaluated with respect to retention time, peak width and peak asymmetry in order to obtain a comprehensive data matrix about retention behavior and peak performance of the metabolites. Furthermore, information about MS detectability as well as stability of the compounds under the respective conditions was gained. Results of this comprehensive column testing with regards to separation characteristics of the different compound classes in HILIC and RP mode are described elsewhere.

During screening several metabolites turned out to be problematic with respect to detectability and/or chromatographic retentivity or eluability. Detection sensitivity for some of the small organic acids was poor even when pseudo-SRM transitions were measured. This was the case for propionic, butyric, 2-methylbutyric, 2,2-dimethylbutyric, γ-aminobutyric, valeric, isovaleric, 2-methylvaleric, hexanoic and β-hydroxypyruvic acid. Due to their physico-chemical characteristics, the analytical method of choice for these compounds should be GC–MS. Regarding chromatographic performance, two analytes which exhibited unexpected behavior shall be mentioned here, *viz.* citric acid and its isomer isocitric acid. Under the chromatographic conditions used during the screening, these metabolites exhibited strong peak tailing which was attributed to interactions with stainless steel parts of the system. Since these compounds are known to be strong chelators of metal ions they can be adsorbed by $Fe^{III}$ ions present at stainless steel surfaces which affects their zonal band shapes. This

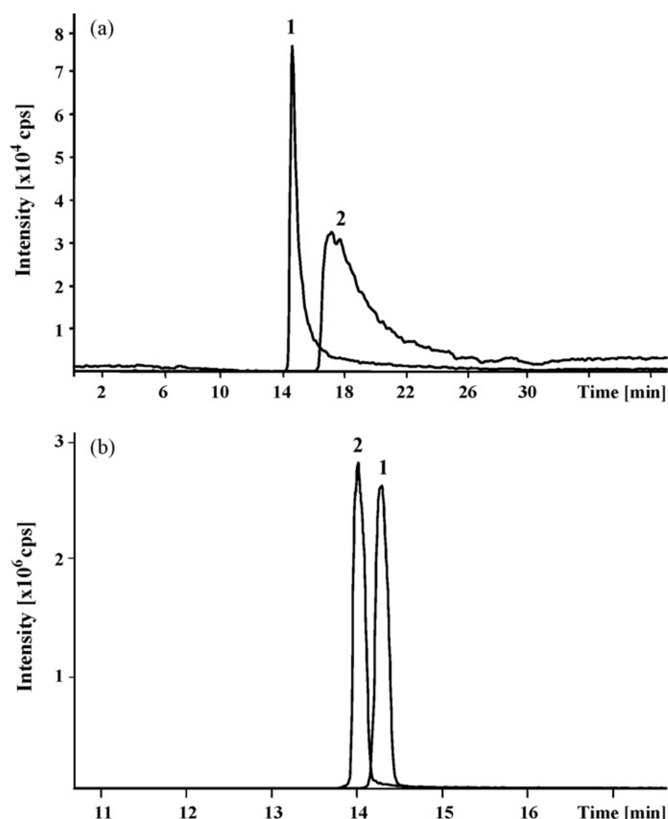


**Fig. 2.** Separation of isocitric acid (peak 1) and citric acid (peak 2). (a) Overlay of XICs of SRM transitions 191 → 73 (isocitrate) and 191 → 87 (citrate) recorded in the negative ionization mode on an Agilent 1200 LC system coupled to a QqQ-MS and (b) negative mode full scan TIC obtained on an Agilent 1100 LC-IT system (*m*/*z* range 85–250). Experimental conditions: acidic HILIC method (pH 3.5) (see Section 2 for details).

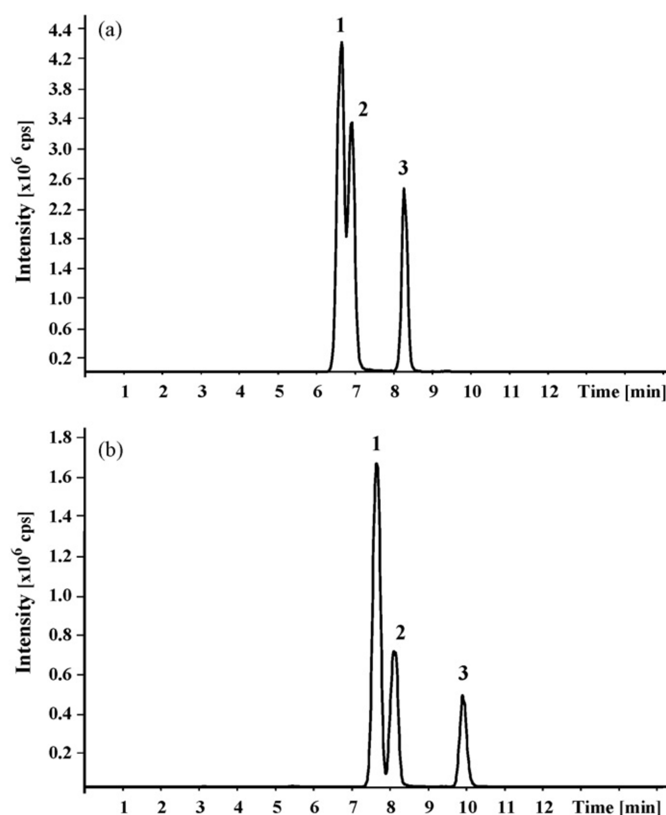


**Fig. 3.** XIC of the SRM transition 137 → 110 (positive ionization mode) of the separation of hypoxanthine (1), 2′-deoxyinosine (2) and inosine (3) on the zwitterionic HILIC stationary phase under (a) acidic conditions (pH 3.5) and (b) neutral conditions (pH 7.5).

assumption was supported by the following findings: when a standard mixture containing the two isomers was injected onto the LC–QTrap-MS system made up of an Agilent 1200 HPLC instrument, isocitrate eluted before citrate and both peaks tailed over more then 10 min elution time (Fig. 2a). The same standard mix was also analyzed on an Agilent 1100 HPLC coupled to an ESI-iontrap (IT) MS instrument where citrate was the first eluted compound and both metabolites showed sharp peaks (Fig. 2b). It is assumed that the surface of stainless steel parts of the older 1100 LC–IT-MS system is already more passivated, i.e. metal surfaces of both the HPLC instrument as well as the ESI source are stronger oxidized due to contact with acidic mobile phases. Compared to the newer 1200 system, adsorptive interactions with analytes are hence weaker on the former. In addition, separation of the two isomeric metabolites as shown in Fig. 2a turned out to be not very well reproducible on our LC–QTrap-MS system showing poor run-to-run repeatability of retention times and detection sensitivity especially for citric acid. As the comparison in Fig. 2 illustrates, this seems to be not a chromatographic problem but rather related to the system.

Similar findings regarding chromatographic behavior were also made for another group of compounds, *viz*. phosphates, in particular multiply phosphorylated metabolites like nucleotides that showed poor peak performance especially under acidic elution conditions. Phosphorylated organic compounds like nucleotides, phosphopeptides, phospholipids or phosphorylated sugars have been reported to interact with stainless steel surfaces of LC systems and the inner wall of electrospray probes leading to partly irreversible adsorption and even loss of analytes [43,44]. However, these detrimental interactions have been shown to be suppressible on the one hand by the addition of chelating compounds like EDTA, tartaric or citric acid as well as carbonate [43], and on the other hand by application of extremely basic mobile phase conditions [44] which, however, can be problematic regarding stability of silica based stationary phases. Our findings were in line with these reports since all phosphorylated metabolites generally showed better peak performance under basic conditions (pH 8.0–9.0 depending on the stability of the tested stationary phase) compared to acidic conditions (pH 3.5).

Based on the information gathered during screening of the metabolites on the various stationary phases, two columns were finally selected for the development and fine-tuning of the LC–MS/MS methods, *viz*. the zwitterionic HILIC phase on which two methods were developed, one employing acidic (pH 3.5), the other neutral eluents (pH 7.5) with ACN as the organic modifier, as well as the PFP modified RP stationary phase which showed excellent peak performance under acidic methanolic conditions (pH 3.5) especially for less polar metabolites exhibiting π–π interactions like aromatic compounds. All three methods were evaluated with respect to possibly interfered MS/MS detection regarding on the one hand isobaric compounds that have to be separated chromatographically due to identical parent masses, at least in cases where no specific SRM transitions are available. On the other hand, in-source decay of metabolites has to be considered which may lead to the formation of compounds that are *per se* not present in the sample or yield other target compounds and thus may affect quantitative results. As an example, in Fig. 3 determination of hypoxanthine is shown for which interferences caused by inosine as well as 2′-deoxyinosine have to be taken into account. As a result of cleavage of the (deoxy)sugar residue which

**Table 2**
Number of metabolites determinable by the three LC–ESI-MS/MS methods grouped according to compound classes.

| Compound class | HPLC method | | |
|---|---|---|---|
| | HILIC pH 3.5 | HILIC pH 7.5 | RP pH 3.5 |
| AAs and small peptides | 44 | 20 | 1 |
| Organic acids | 22 | 14 | 23 |
| Vitamins and biogenic amines | 19 | 16 | 4 |
| Nucleobases | 6 | 7 | 3 |
| Nucleosides | 11 | 10 | 4 |
| Nucleotides | 4 | 38 | – |
| Nucleotide sugars | 12 | 2 | – |
| Coenzymes | 3 | 12 | – |
| Pyrophosphates | 2 | 3 | – |
| Sugar phosphates | – | 16 | – |
| Other phosphates | 3 | 12 | – |
| Sugars and sugar acids | 2 | 11 | – |
| Total | 128 | 161 | 35 |

*Abbreviation*: AAs: amino acids.

may easily occur under the conditions prevailing during ESI process, hypoxanthine is generated from both nucleosides and thus, chromatographic separation of the nucleobase from the two critical metabolites is required to allow its unimpaired detection. In the acidic HILIC method, inosine was well separated whereas 2′-deoxyinosine partly coeluted with hypoxanthine (Fig. 3a), thus quantitative determination of the nucleobase is not possible under these conditions. In comparison, at pH 7.5, hypoxanthine was sufficiently well separated from both nucleosides by HILIC (Fig. 3b).

Chromatographic method(s) with which the metabolites can be determined are listed in Table 1. Corresponding retention times of all metabolites which could reliably and reproducibly be determined with these developed methods are given in Tables S-1, S-2 and S-3 of the Supplementary Material. Table 2 gives a comparison of the three methods regarding separation performance with respect to the different compound classes. In total, 128 and 161 metabolites can be measured with the acidic and the neutral HILIC method, respectively, while the optimized RP method comprises 35 compounds. Under acidic conditions (HILIC and/or RP), in particular amino acids and organic acids are well determinable while at higher pH sugars and sugar acids as well as phosphates (nucleotides, coenzymes, sugar phosphates and other phosphates) are addressed, the latter for the reasons outlined above. Interestingly, chromatographic performance of nucleotide sugars such as ADP-glucose or GDP-fucose was found to be better at acidic pH although both, nucleotides as well as free sugars are better determinable with the neutral HILIC method. Overall, 218 intracellular metabolites can be detected with the presented analytical platform, the majority thereof, *viz.* 197 compounds (90%) under HILIC conditions, the remaining 21 metabolites exclusively with the RP method. Additional 26 isobaric compounds, more precisely sugar derivatives, that could not be differentiated by product ion showed coelution under the optimized (HILIC) conditions, yet quantitation is possible for the collective only. This is the case for pentose- and hexose phosphates, disaccharides as well as several isobaric nucleotide sugars (*cf.* Table 1), the separation of which will be addressed in a future study. It is obvious that improvements in efficiencies, e.g. by use of UHPLC instruments [12–14], may partly relieve some separation and quantitation problems if there is at least some minor selectivity. However, for some groups of metabolites with isomeric compounds such as sugarphosphates more selective methods based on different selectivity principles need to be developed.

### 3.3. Method performance

Regarding detection sensitivity of the three optimized methods for the individual analytes especially with respect to quantitative aspects the following issues shall be mentioned at this point: in all three methods, generally a dwell time of 10 ms was chosen for all SRM transitions addressed, meaning that instrumental parameters (voltages) like DP, CE and CXP for one specific transition were maintained for this time span before the next transition was measured. In addition, to allow the electronics to reach stable conditions a pause of 5 ms was adjusted between two consecutive transitions as well as a settling time of 700 ms when the instrument changed from positive to negative mode and *vice versa*. Depending on the total number of transitions measured in positive and negative mode, respectively, different cycle times resulted for the three methods with one cycle being the time necessary to once measure all transitions including settling times and pauses between the single SRM transitions. Thus, under acidic HILIC conditions (77 and 34 SRM transitions in positive and negative mode, respectively), one cycle took 3.07 s, under neutral HILIC conditions (71 transitions in positive, 68 transitions in negative mode), cycle time was 3.49 s and for the RP method (14 and 13 SRM transitions in positive and negative mode, respectively) cycles of 1.81 s were possible. Depending on the peak width typically obtained under the various chromatographic conditions different numbers of data points resulted to define one specific peak. For instance, under HILIC conditions (both acidic and neutral), 85% of the analytes showed a peak width at baseline between 0.5 and 3 min and thus the peaks were typically defined by approximately 10–60 data points (note, as a rule of thumb 10–20 data points are typically required to adequately describe a peak [45,46]). Under optimized RP conditions, peak shape was generally better, with peak width in the range of 0.5 and 1.5 min for more than 90% of the analytes, resulting in a number of 17–50 data points defining one peak. It is obvious that detection sensitivity for one specific peak is better the higher the number of data points available. Thus, compound specific adjustment of the dwell time would be a possibility to improve detection and hence, e.g. LOQ of critical analytes [47]. However, for the sake of simplicity in the presented methods the same dwell time was adjusted for all SRM transitions measured.

After fine-tuning of the LC–MS/MS conditions, the three methods were validated with respect to LOQ (determined at a signal-to-noise ratio S/N of 10), linearity range (established with 7–9 calibrants), precision (under intra-day repeatability conditions; $n=3$) as well as accuracy (recovery; $n=3$). In Tables S-1, S-2 and S-3 of the Supplementary Material, detailed results regarding LOQ, upper limit of the linear range as well as slope and weight of the linear regression are provided for all metabolites.

Sensitivity of the three methods was very good for the majority of the metabolites under the different chromatographic conditions as illustrated in Fig. 4a. Thus, in any case 20–25% of the metabolites showed a LOQ below 1 μg/L, for further 40% (in the case of the acidic HILIC method) and ~65% (for the neutral HILIC and the RP method), respectively, LOQ was between 1 and 10 μg/L. LOQ above 1 mg/L was observed for just two metabolites in the case of the two acidic methods, respectively, and for only one analyte in the case of the neutral HILIC method. Regarding linear dynamic ranges of the LC–MS/MS methods, it is known that analyte response depends on various instrumental factors, with the linear range of ESI-QqQ-MS instruments being especially limited by concentration-dependent ionization yield in the ESI source on the one hand, and by ion saturation of the channel electron multiplier (CEM) detector on the other hand [48]. The latter, detector saturation, is independent of the analyte and thus predictable, and is for the instrument used in the present study typically in the range of about $2 \times 10^6$ cps. In contrast, ionization in the ESI source depends on the analyte structure

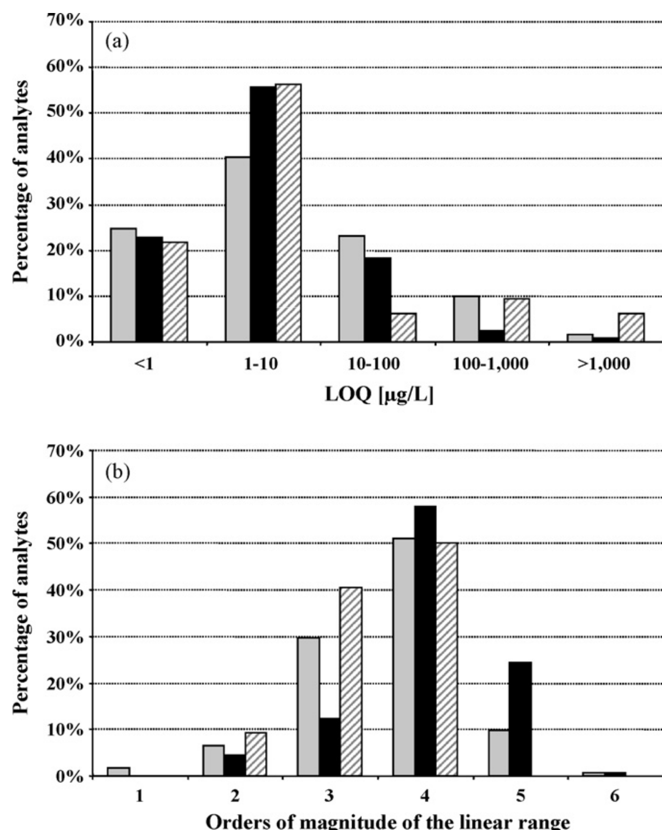


**Fig. 4.** Method characteristics of the acidic HILIC method (grey bars), the neutral HILIC method (black bars) and the RP method (dashed bar) regarding (a) limit of quantification (LOQ) as well as (b) linear range.

and its concentration [49,50], and non-linearity may already occur at signals much lower than $10^6$ cps. For all metabolites, highest concentration still yielding linear response was evaluated (Tables S-1–S-3) and linearity was determined in the range between the analyte's LOQ and this concentration level. As shown in Fig. 4b, for all three methods at least half of the measured metabolites showed a linear range over four orders of magnitude. Under neutral HILIC conditions, further 25% of the compounds exhibited linearity over a still wider range, while under acidic conditions (HILIC as well as RP), the number of analytes showing linearity over less than four orders of magnitude was higher.

Precision as well as accuracy were determined for the three methods by repeated measurement ($n=3$) of standard mixtures containing all analytes at three concentration levels, i.e. close to the LOQ (low), at an intermediate concentration (middle) as well as close to the upper limit of the linear dynamic range of each metabolite (high). For both HILIC methods, at middle as well as high concentration level, precision was below 5% RSD for 70–80% of the metabolites and below 10% RSD for more than 95% of the analytes (Fig. 5a and c). At the concentration close to the LOQ, in both HILIC methods only about half of the metabolites showed precision below 5% RSD and ∼85% below 10% RSD, for the remaining compounds (12–14%) precision was worse than 10% RSD. For the RP method, distribution was somewhat different (Fig. 5e). In the middle and at the high end of the linear range, all but two metabolites, respectively, showed precision better than 10% RSD but the number of analytes with precision <5% RSD was lower compared to the HILIC methods. At the low concentration level, only 25% of the metabolites exhibited precision below 5% RSD, and for 30% precision was >10% RSD.

Regarding accuracy of the measurements, again the two HILIC methods revealed similar behavior (Fig. 5b and d): at the middle concentration level, accuracy was 100 ± 5% for ∼75–80% of the metabolites, and 100 ± 10% for more than 95% of the analytes. Close to the LOQ, only 62% and 75% of the compounds, respectively, showed an accuracy between 95% and 105%, and the number of analytes with a recovery of 100 ± 20% was higher. At the high end of the linear range, only about half of the metabolites exhibited an accuracy of 100 ± 10%, and for more than one-third of the analytes recovery was below 90%. In the RP method, at all three concentration levels about half of the metabolites showed an accuracy of 100 ± 5% (Fig. 5f). In the middle of the linear range, again accuracy for all but two metabolites was between 90% and 110%, while at lower concentration more analytes showed an accuracy below 90%. At the highest concentration level, again tendency for lower recovery was observed similar to the HILIC methods. Potential sources of inaccuracies may originate from inadequately resolved isobars (isomers) (*vide supra*), isotopic contributions [51], and (especially in the presence of matrix) from ion suppression/enhancement, as well as ion adduct formation [51] (*vide infra*).

### 3.4. Qualitative analysis of antibiotics fermentation extracts

To evaluate the applicability of the developed analytical platform, extracts from β-lactam antibiotics fermentation broths were analyzed for the targeted metabolites. Thus, samples from penicillin as well as cephalosporin production were obtained from respective fermentation broths by centrifugation and extraction of the metabolites with cold methanol. Prior to LC–MS/MS analysis, extracts were diluted 1:200 with ACN/$H_2O$ (80:20, v/v) or $H_2O$/MeOH (80:20, v/v) for the HILIC- and RP-LC–MS/MS methods, respectively. Overall, 108 metabolites could be detected and identified in the two samples, 87 thereof in the penicillin extract and 94 in the cephalosporin extract (Table 3
).

Several metabolites addressed in this study, *viz.* in total 80 compounds can be determined by two or even all three of the developed methods (*cf.* Table 1). Thus, six metabolites, i.e. guanine, xanthine, cytidine, 2′-deoxycytidine, uridine as well as *N*-acetylglutamic acid are amenable for analysis by all three methods, 66 compounds can be determined by both HILIC methods, five organic acids (fumaric, methylmalonic, succinic, nicotinic and isonicotinic acid) can be measured under both acidic conditions (HILIC and RP) and three analytes, hypoxanthine, phenylpyruvic acid as well as biotin, are determinable by the neutral HILIC as well as the acidic RP method but not by the acidic HILIC method due to coelution of interfering compounds and poor peak shape in the case of biotin, respectively. Qualitative results obtained for these compounds with the respective two or three different methods were consistent for all but two metabolites, meaning that all compounds present in the two extracts at concentrations above their limit of detection (LOD) were actually detected when the same sample was analyzed under different LC conditions. For instance, xanthine ($M=152$) was determined in the penicillin extract by all three methods, but could not be detected in the cephalosporin sample. *N*-Acetylglutamic acid ($M=189$) was found in the cephalosporin extract under both HILIC as well as the RP conditions, whereas in the penicillin sample with none of the methods this compound could be detected (Table 3). The two exceptions were guanidinoacetic acid ($M=117$) and phenylpyruvic acid ($M=164$), which could both be determined under HILIC conditions at pH 7.5 (the former only in the penicillin extract, the latter in both samples) but not under acidic conditions. For both compounds, sensitivity of the neutral HILIC method was much higher and respective LOQs were significantly lower compared to the acidic methods: in the case of guanidinoacetic acid, slope of the linear regression $k$ was $2.8 \times 10^5$ and LOQ was 170 µg/L

**Table 3**
Metabolites determined in extracts of β-lactam antibiotics fermentation broths from penicillin as well as cephalosporin production. Compounds are listed according to their molecular weight (*M*)[a].

| Metabolite | *M*[b] | Penicillin | | | Cephalosporin | | |
|---|---|---|---|---|---|---|---|
| | | HILIC pH 3.5 | HILIC pH 7.5 | RP pH 3.5 | HILIC pH 3.5 | HILIC pH 7.5 | RP pH 3.5 |
| Ethanolamine | 61.0 | + | | | + | | |
| Glycine | 75.0 | + | | | + | | |
| Putrescine | 88.0 | + | | | + | | |
| L-Alanine + sarcosine | **89.0** | +[c] | | | +[c] | | |
| Lactic acid | 90.0 | – | – | | + | + | |
| Cadaverine | 102.2 | + | | | + | | |
| Choline | 104.2 | | + | | | + | |
| L-Serine | 105.0 | + | | | + | | |
| Hypotaurine | 109.2 | – | – | | + | + | |
| Cytosine | **111.1** | + | | | + | | |
| Histamine | **111.2** | + | | | + | | |
| Uracil | 112.1 | | + | | | + | |
| Creatinine | 113.1 | + | | | – | | |
| Dihydrouracil | 114.1 | | + | | | – | |
| L-Proline | 115.0 | + | | | + | | |
| L-Valine | **117.0** | + | + | | + | + | |
| Guanidinoacetic acid | **117.1** | – | + | | – | – | |
| Betaine | **117.2** | + | + | | + | + | |
| L-Threonine | 119.0 | + | | | + | | |
| Nicotinic acid | 123.0 | + | | + | + | | + |
| 5-Methylcytosine | 125.1 | – | – | | + | + | |
| Thymine | 126.0 | | + | | | – | |
| *N*-Acetylputrescine | 130.2 | | | + | | | + |
| L-Isoleucine | **131.0** | + | + | | + | + | |
| L-Leucine | **131.0** | + | + | | + | + | |
| Creatine | **131.1** | + | + | | – | – | |
| 3-Guanidinopropionic acid | **131.1** | – | – | | + | + | |
| Glutaric acid | **132.0** | | + | | | + | |
| L-Asparagine | **132.0** | + | + | | + | + | |
| L-Ornithine | **132.2** | + | + | | + | + | |
| L-Aspartic acid | 133.0 | + | | | + | | |
| Adenine | 135.1 | + | | | + | | |
| Hypoxanthine | 136.1 | | + | + | | – | – |
| Salicylic acid | **138.1** | | | + | | | + |
| p-Hydroxybenzoic acid | **138.1** | | | – | | | + |
| L-Lysine | **146.0** | + | + | | + | + | |
| L-Glutamine | **146.0** | + | | | + | | |
| Acetylcholine | **146.2** | – | | | + | | |
| L-Glutamic acid | **147.0** | + | + | | + | + | |
| *O*-Acetyl-L-serine | **147.1** | – | | | + | | |
| L-Methionine | 149.0 | + | | | + | | |
| Guanine | 151.1 | + | + | + | + | + | + |
| Xanthine | 152.1 | + | + | + | – | – | – |
| L-Histidine | 155.0 | + | | | + | | |
| Allantoin | 158.1 | – | | | + | | |
| Tryptamine | 160.2 | – | – | | + | + | |
| L-Carnitine | 161.2 | + | + | | + | + | |
| Phenylpyruvic acid | 164.2 | | + | – | | + | – |
| L-Phenylalanine | 165.0 | + | | | + | | |
| Pyridoxal | 167.1 | + | + | | + | + | |
| Pyridoxine | **169.0** | + | +[c] | | + | +[c] | |
| L-Cysteic acid | **169.2** | + | +[c] | | + | +[c] | |
| Glycerol 3-phosphate | 172.1 | | + | | | + | |
| L-Arginine | 174.0 | + | | | + | | |
| L-Citrulline | 175.0 | + | | | + | | |
| Glucosamine | 179.2 | | + | | | – | |
| L-Tyrosine | 181.0 | + | | | + | | |
| *N*-Acetyl-L-glutamine | **188.2** | – | | | + | | |
| Azelaic acid | **188.2** | | | + | | | + |
| *N*-Acetyl-L-glutamic acid | 189.2 | – | – | – | + | + | + |
| *N*-Acetyl-L-methionine | 191.3 | + | + | | + | + | |
| Glucuronic acid | 194.1 | | – | | | + | |
| Gluconic acid | 196.2 | | – | | | + | |
| Sebacic acid | 202.3 | | | + | | | + |
| L-Tryptophan | 204.0 | + | | | + | | |
| α-Lipoamide | 205.3 | | | + | | | + |
| 2-Deoxyribose 5-phosphate | 214.1 | | + | | | – | |
| L-Cystathionine | 222.3 | + | | | + | | |
| L-Carnosine | 226.2 | + | + | | + | + | |
| 2′-Deoxycytidine | 227.2 | + | + | + | + | + | + |
| 2′-Deoxyuridine | 228.2 | | | + | | | + |
| Thymidine | 242.2 | | + | | | + | |

Table 3 (*Continued* )

| Metabolite | $M$[b] | Penicillin | | | Cephalosporin | | |
|---|---|---|---|---|---|---|---|
| | | HILIC pH 3.5 | HILIC pH 7.5 | RP pH 3.5 | HILIC pH 3.5 | HILIC pH 7.5 | RP pH 3.5 |
| Cytidine | 243.2 | + | + | + | + | + | + |
| Uridine | 244.2 | + | + | + | + | + | + |
| 2′-Deoxyadenosine | 251.2 | | + | | | + | |
| 2′-Deoxyinosine | 252.2 | + | + | | + | + | |
| Hexose phosphates[d] | **260.1** | | +[c] | | | +[c] | |
| Adenosine | **267.2** | + | + | | + | + | |
| 2′-Deoxyguanosine | **267.2** | + | + | | + | + | |
| Inosine | 268.2 | + | + | | + | + | |
| Guanosine | 283.2 | + | + | | + | + | |
| Xanthosine | 284.2 | + | | | + | | |
| NANA | 309.3 | | + | | | – | |
| UMP | 324.2 | | – | | | + | |
| dAMP | 331.2 | | + | | | – | |
| Disaccharaides[e] | **342.3** | | +[c] | | | +[c] | |
| Sucrose | **342.3** | | + | | | + | |
| AMP | 347.2 | | – | | | + | |
| XMP | 364.2 | | + | | | – | |
| Riboflavin | 376.0 | | | + | | | + |
| S-(5′-adenosyl)-L-homocysteine | 384.4 | – | | | + | | |
| S-(5′-adenosyl)-L-methionine | 398.5 | – | | | + | | |
| 5-Methyltetrahydrofolic acid | 459.6 | – | | | + | | |
| UDP-Gal + UDP-Glc | **566.3** | +[c] | | | – | | |
| UDP-GlcNAc + UDP-GalNAc | **607.4** | +[c] | | | +[c] | | |
| L-Glutathione oxidized | 612.6 | – | – | | + | + | |
| NAD | 663.4 | – | – | | + | + | |
| FAD | 785.6 | – | | | + | | |

*Abbreviations*: NANA: *N*-acetylneuraminic acid; GlcNAc: *N*-acetyl-D-glucosamine; GalNAc: *N*-acetyl-D-galactosamine.

[a] Empty cells indicate that the metabolite is not determinable with the respective method.

[b] *M* in bold indicates isobaric compounds.

[c] Metabolites coelute under the respective chromatographic conditions.

[d] Including fructose 6-phosphate, galactose 1-phosphate, glucose 1-phosphate, glucose 6-phosphate, mannose 1-phosphate and mannose 6-phosphate.

[e] Including lactose, maltose and trehalose.

under acidic HILIC conditions, while at pH 7.5 $k$ was $2.0 \times 10^7$ and LOQ was 0.7 µg/L. Method characteristics for phenylpyruvic acid were $k = 1.7 \times 10^3$ and $4.0 \times 10^4$ with LOQ = 6000 and 85 µg/L under acidic RP and neutral HILIC conditions, respectively (see also Tables S-1, S-2 and S-3 of the Supplementary Material). Obviously, at the chosen dilution (1:200), the both metabolites could not be detected with the respective less sensitive method.

### 3.5. Quantitative analysis of antibiotics fermentation extracts

In order to ascertain whether the three different methods provide consistent results also in the case of quantitative analyses, concentrations of a number of selected metabolites were determined in the fermentation extracts, although sample preparation and extraction protocol have not been optimized so far. However, twenty-five compounds that can be determined by at least two of the developed LC–MS/MS methods and that were randomly chosen were quantified in the penicillin and the cephalosporin extract, respectively, by external calibration using pure multicomponent standard solutions. In order to evaluate possible matrix effects which may falsify quantitative results especially due to ion suppression, the sample extracts were analyzed at two different dilutions, i.e. at 1:50 as well as 1:200. Additionally, samples were also analyzed by matrix-matched calibration which was realized by a standard addition procedure, in which the extracts were spiked with pure standards at three concentration levels. Thus, standards of each analyte were added at concentrations corresponding to approximately 50%, 75% and 100% of the quantitative results obtained by external calibration. Standard addition procedure was done twice for each extract to end up again at final dilutions of the samples of 1:50 and 1:200. Results obtained for the 25 metabolites with the four different approaches – external calibration 1:50 and 1:200 diluted, and matrix-matched calibration 1:50 and 1:200 diluted – are given for the penicillin extract in Table 4, and for the cephalosporin sample in Table 5. For the former, penicillin, concentrations of five compounds turned out to be below LOQ of the methods also in the less (1:50) diluted sample.

Comparison of the quantitative results obtained with the two and three different LC–MS/MS methods, respectively, for one specific sample, i.e. 1:50 or 1:200 diluted extracts quantified by external or by matrix-matched calibration, showed that concentrations generally agreed very well (deviation of the concentrations obtained with the different methods <20%), with the exception of lower concentrated compounds such as cytidine, 2′-deoxycytidine, guanine or pyridoxal for which somewhat higher deviations were observed particularly in the penicillin extract (Table 4). Regarding the results obtained with the four assays (two different dilutions, two distinct calibrations) employing a specific method, i.e. comparison of the results obtained for differently diluted, spiked and non-spiked samples analyzed by the same method – acidic HILIC, neutral HILIC or acidic RP method – the following observations were made: under acidic as well as neutral HILIC conditions, in both extracts 11–14 metabolites, i.e. about half of the compounds, revealed consistency amongst the four different assays showing a deviation of the individual results less than ±20% from the mean concentration calculated from the four results. Somewhat larger deviations were again found for the lower concentrated compounds in both matrices, penicillin and cephalosporin. In the cephalosporin extract (Table 5), results obtained for several nucleosides like adenosine, guanosine and inosine as well as glutathione and the basic AAs Lys and carnitine revealed less consistent results for the differently diluted samples analyzed under either acidic or neutral conditions. These findings were attributed to the occurrence of slight matrix effects leading to ion suppression for these analytes, which was supported by the fact that in any case con-

**Table 4**
Concentrations [mg/L] of selected metabolites in a penicillin fermentation broth extract determined at different dilutions by external calibration using pure standards and by matrix-matched calibration employing standard addition[a].

| Metabolite | External calibration | | | | | | Matrix-matched calibration[b] | | | | | | Mean | *s* | % RSD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dilution 1:50 | | | Dilution 1:200 | | | Dilution 1:50 | | | Dilution 1:200 | | | | | |
| | HILICpH 3.5 | HILICpH 7.5 | RPpH 3.5 | HILICpH 3.5 | HILICpH 7.5 | RPpH 3.5 | HILICpH 3.5 | HILICpH 7.5 | RPpH 3.5 | HILICpH 3.5 | HILICpH 7.5 | RPpH 3.5 | | | |
| Adenosine | 14.1 | 15.3 | | 16.2 | 13.1 | | 12.2 | 14.2 | | 13.0 | 14.9 | | 14.1 | 1.33 | 9 |
| Cytidine | 0.26 | 0.11 | 0.45 | 0.15 | 0.22 | n.a. | 0.23 | 0.19 | 0.29 | 0.36 | 0.13 | n.a. | 0.24 | 0.11 | 44 |
| 2′-Deoxycytidine | 0.21 | 0.21 | 0.19 | 0.29 | 0.31 | n.a. | 0.41 | 0.22 | 0.30 | 0.58 | 0.40 | n.a. | 0.31 | 0.12 | 39 |
| 2′-Deoxyguanosine | 3.12 | 3.85 | | 3.05 | 3.86 | | 2.65 | 3.02 | | 3.69 | 3.71 | | 3.37 | 0.46 | 14 |
| Glutathione oxid. | <LOQ | <LOQ | | <LOQ | <LOQ | | n.a. | n.a. | | n.a. | n.a. | | | | |
| Guanine | 0.56 | 0.71 | 0.65 | 2.01 | 1.04 | n.a. | 1.88 | 1.94 | 1.96 | 1.61 | 1.32 | n.a. | 1.37 | 0.59 | 43 |
| Guanosine | 123 | 91.9 | | 156 | 110 | | 106 | 140 | | 148 | 121 | | 124 | 22.1 | 18 |
| Hypotaurine | <LOQ | <LOQ | | <LOQ | <LOQ | | n.a. | n.a. | | n.a. | n.a. | | | | |
| Inosine | 95.9 | 88.8 | | 84.7 | 107 | | 89.6 | 82.9 | | 73.6 | 53.2 | | 84.5 | 16.0 | 19 |
| L-Asparagine | 42.5 | 43.8 | | 47.3 | 53.0 | | 40.8 | 46.8 | | 42.1 | 37.2 | | 44.2 | 4.81 | 11 |
| L-Carnitine | 66.1 | 63.0 | | 67.1 | 75.8 | | 87.0[c] | 158[c] | | 81.2 | 86.7 | | 85.6[d] | 30.7[d] | 36[d] |
| L-Glutamic acid | 774 | 673 | | 721 | 747 | | 667 | 729 | | 595 | 647 | | 694 | 59.1 | 9 |
| L-Isoleucine | 104 | 126 | | 108 | 94.5 | | 97.8 | 95.5 | | 109 | 104 | | 105 | 10.2 | 10 |
| L-Leucine | 228 | 289 | | 261 | 257 | | 244 | 215 | | 253 | 205 | | 244 | 27.2 | 11 |
| L-Lysine | 242 | 290 | | 363 | 356 | | 251 | 308 | | 246 | 314 | | 296 | 47.8 | 16 |
| L-Valine | 138 | 119 | | 117 | 120 | | 117 | 106 | | 102 | 110 | | 116 | 11.0 | 10 |
| 5-Methylcytosine | <LOQ | <LOQ | | <LOQ | <LOQ | | n.a. | n.a. | | n.a. | n.a. | | | | |
| NAD | <LOQ | <LOQ | | <LOQ | <LOQ | | n.a. | n.a. | | n.a. | n.a. | | | | |
| Nicotinic acid | 16.5 | | 14.4 | 18.1 | | n.a. | 16.1 | | 16.1 | 16.4 | | n.a. | 16.3 | 1.18 | 7 |
| Pyridoxal | 0.14 | 0.17 | | 0.24 | 0.24 | | 0.15 | 0.28 | | 0.25 | 0.15 | | 0.20 | 0.06 | 27 |
| Pyridoxine | 1.12 | 1.66 | | 1.45 | 1.88 | | 1.95 | 2.22 | | 1.71 | 2.21 | | 1.78 | 0.37 | 21 |
| Tryptamine | <LOQ | <LOQ | | <LOQ | <LOQ | | n.a. | n.a. | | n.a. | n.a. | | | | |
| Uridine | 137 | 123 | 120 | 174 | 135 | n.a. | 119 | 99.2 | 110 | 125 | 120 | n.a. | 126 | 20.0 | 16 |
| Xanthine | 66.4 | 77.2 | 55.9 | 72.7 | 68.3 | n.a. | 53.7 | 53.4 | 45.6 | 57.6 | 65.8 | n.a. | 61.7 | 9.92 | 16 |

n.a.: not analyzed.

[a] Empty cells indicate that the metabolite is not determinable with the respective method. Dilutions in ACN/$H_2O$ (80:20, v/v) for HILIC-LC–MS/MS and in $H_2O$/MeOH (80:20, v/v) for RP-LC–MS/MS.

[b] Standard addition performed by spiking with pure standards at concentrations corresponding to approximately 50%, 75% and 100% of the concentrations determined by external calibration.

[c] Spiked levels outside of the linear range.

[d] Including results obtained by matrix-matched calibration with spiked levels outside of the linear range.

**Table 5**
Concentrations [mg/L] of selected metabolites in a cephalosporin fermentation broth extract determined at different dilutions by external calibration using pure standards and by matrix-matched calibration employing standard addition[a].

| Metabolite | External calibration | | | | | | Matrix-matched calibration[b] | | | | | | Mean | s | % RSD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dilution 1:50 | | | Dilution 1:200 | | | Dilution 1:50 | | | Dilution 1:200 | | | | | |
| | HILICpH 3.5 | HILICpH 7.5 | RPpH 3.5 | HILICpH 3.5 | HILICpH 7.5 | RPpH 3.5 | HILICpH 3.5 | HILICpH 7.5 | RPpH 3.5 | HILICpH 3.5 | HILICpH 7.5 | RPpH 3.5 | | | |
| Adenosine | 102 | 110 | | 205 | 200 | | 387[c] | 363[c] | | 459[c] | 293[c] | | 265[d] | 132[d] | 50[d] |
| Cytidine | 0.30 | 0.31 | 0.38 | 0.39 | 0.48 | n.a. | 0.30 | 0.34 | 0.36 | 0.41 | 0.47 | n.a. | 0.37 | 0.07 | 17 |
| 2′-Deoxycytidine | 0.76 | 0.60 | 0.53 | 0.97 | 0.86 | n.a. | 0.79 | 0.65 | 0.51 | 0.88 | 0.79 | n.a. | 0.73 | 0.16 | 21 |
| 2′-Deoxyguanosine | 3.23 | 3.56 | | 3.56 | 3.45 | | 2.89 | 2.76 | | 3.51 | 3.37 | | 3.29 | 0.31 | 9 |
| Glutathione oxid. | 7.47 | 8.48 | | 34.3 | 28.1 | | 32.6 | 27.8 | | 34.3 | 35.5 | | 26.1 | 11.5 | 44 |
| Guanine | 0.50 | 0.41 | 0.43 | <LOQ | 0.73 | n.a. | 0.62 | 0.46 | 0.48 | 0.45 | 0.53 | n.a. | 0.51 | 0.10 | 20 |
| Guanosine | 115 | 110 | | 149 | 151 | | 226 | 226 | | 266 | 248 | | 186 | 61.9 | 33 |
| Hypotaurine | 7.77 | 6.10 | | 7.52 | 5.62 | | 5.58 | 7.57 | | 6.47 | 5.09 | | 6.47 | 1.04 | 16 |
| Inosine | 3.85 | 3.96 | | 5.12 | 5.53 | | 5.77 | 5.43 | | 5.66 | 5.43 | | 5.09 | 0.76 | 15 |
| L-Asparagine | 169 | 202 | | 188 | 178 | | 175 | 175 | | 161 | 198 | | 181 | 14.1 | 8 |
| L-Carnitine | 87.5 | 72.4 | | 127 | 119 | | 222[c] | 336[c] | | 136 | 149 | | 156[d] | 85.4[d] | 55[d] |
| L-Glutamic acid | 814 | 948 | | 882 | 792 | | 668 | 789 | | 698 | 754 | | 793 | 91.5 | 12 |
| L-Isoleucine | 50.9 | 49.7 | | 41.6 | 44.4 | | 45.0 | 39.4 | | 53.8 | 56.0 | | 47.6 | 5.92 | 12 |
| L-Leucine | 89.1 | 110 | | 102 | 118 | | 70.2 | 75.9 | | 82.2 | 94.4 | | 92.7 | 16.6 | 18 |
| L-Lysine | 374 | 388 | | 510 | 554 | | 443 | 560 | | 444 | 518 | | 474 | 72.0 | 15 |
| L-Valine | 104 | 97.1 | | 113 | 112 | | 89.2 | 86.0 | | 104 | 115 | | 102 | 11.0 | 11 |
| 5-Methylcytosine | 0.59 | 0.67 | | 0.61 | 0.68 | | 0.85 | 0.64 | | 0.53 | 0.65 | | 0.65 | 0.09 | 14 |
| NAD | 30.5 | 32.8 | | 35.6 | 33.8 | | 48.3 | 41.2 | | 40.0 | 37.2 | | 37.4 | 5.67 | 15 |
| Nicotinic acid | 9.63 | | 7.12 | 8.12 | | n.a. | 7.65 | | 6.40 | 6.90 | | n.a. | 7.64 | 1.14 | 15 |
| Pyridoxal | 0.12 | 0.14 | | 0.29 | 0.25 | | 0.18 | 0.23 | | 0.21 | 0.32 | | 0.22 | 0.07 | 32 |
| Pyridoxine | 1.07 | 1.63 | | 1.44 | 1.97 | | 2.45 | 2.62 | | 2.14 | 2.57 | | 1.99 | 0.57 | 29 |
| Tryptamine | 0.91 | 0.84 | | 0.97 | 0.94 | | 0.71 | 0.84 | | 0.90 | 0.77 | | 0.86 | 0.09 | 10 |
| Uridine | 55.4 | 71.7 | 59.6 | 74.1 | 51.5 | n.a. | 84.8 | 91.7 | 71.1 | 65.0 | 78.5 | n.a. | 70.3 | 12.8 | 18 |
| Xanthine | 2.52 | 2.43 | 2.42 | <LOQ | <LOQ | n.a. | n.a. | n.a. | 2.56 | n.a. | n.a. | n.a. | 2.48 | 0.07 | 3 |

n.a.: not analyzed.

[a] Empty cells indicate that the metabolite is not determinable with the respective method. Dilutions in ACN/$H_2O$ (80:20, v/v) for HILIC-LC–MS/MS and in $H_2O$/MeOH (80:20, v/v) for RP-LC–MS/MS.

[b] Standard addition performed by spiking with pure standards at concentrations corresponding to approximately 50%, 75% and 100% of the concentrations determined by external calibration.

[c] Spiked levels outside of the linear range.

[d] Including results obtained by matrix-matched calibration with spiked levels outside of the linear range.

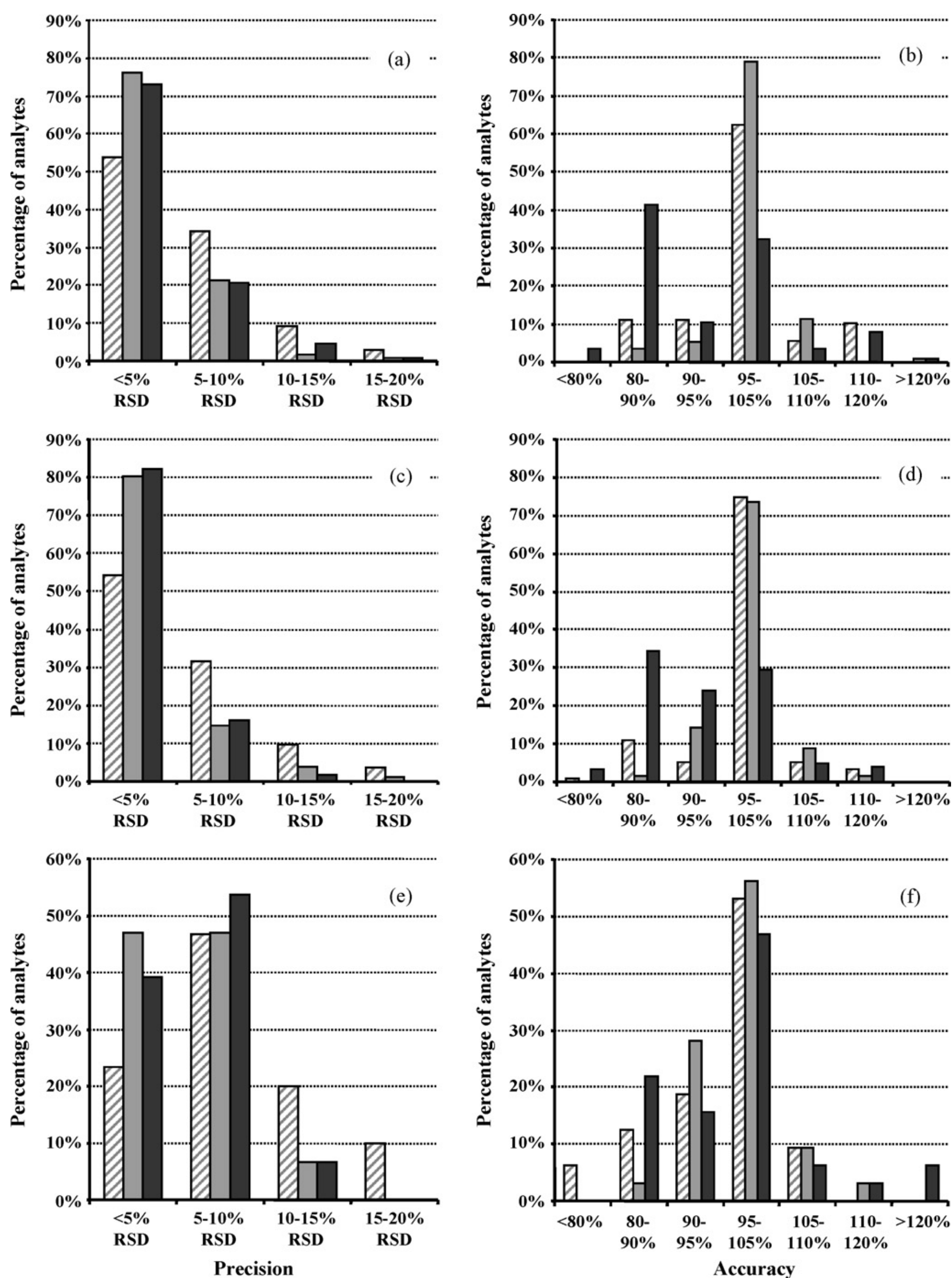


**Fig. 5.** Precision (a, c and e) and accuracy (b, d and f) determined for the acidic HILIC (a and b), the neutral HILIC (c and d) and the RP method (e and f) at low (dashed bars), middle (grey bars) and high concentration levels (black bars) ($n = 3$).

centrations determined in the 1:50 diluted samples were lower compared to the stronger (1:200) dilution. This is in line with the assumption that in less diluted samples ion suppression is more pronounced as a result of the higher overall ion concentration. Interestingly, in the penicillin extract no such effects were observed for the mentioned analytes, whereas guanine showed a similar trend, i.e. lower concentration in the 1:50 diluted sample quantified by external calibration. A more detailed examination of relative and absolute matrix effects was carried out for selected groups of analytes, for instance amino acids, organic acids or amines, under acidic HILIC conditions and results can be found elsewhere [52]. However, for all compounds results determined by matrix-matched calibration in the 1:50 and 1:200 diluted extracts coincided well for both, the acidic as well as the neutral HILIC method, i.e. concentrations deviated by less than 20%. Only exceptions were pyridoxal and deoxycytidine in the penicillin extract for which concentrations in the range of 0.2 and 0.4 mg/L and deviations of about 30% were found.

Under RP conditions, only the 1:50 diluted samples were analyzed quantitatively by external as well as matrix-matched calibration. In the cephalosporin extract concentrations of all metabolites accessible under RP conditions were consistent, while in the samples obtained from the penicillin fermentation broth guanine, cytidine and 2′-deoxycytidine showed deviations above 20% (37–55%) when externally and matrix-matched calibrated results were compared. In any case, however, concentrations obtained with the distinct calibrations by the RP method agreed well with the respective results determined by the HILIC methods.

From the results given in Tables 4 and 5 and discussed above, some intrinsic problems of quantitative metabolomic studies become evident: when attempting to analyze all or numerous metabolites present in a cell one has to be aware of the wide concentration ranges covered by the analytes. Due to the limited linear range of LC–ESI-MS/MS methods, multiple dilutions have to be analyzed in order to reliably detect all compounds in the sample within the optimum range of their linear detector response. Thus, determination of low concentrated metabolites requires analysis of less diluted samples which, due to the extremely high overall ion concentration, however, bear the risk of increased matrix effects such as ion suppression as well as overloading of the column and the MS detector. As an alternative, different sample extraction protocols may be applied to selectively enrich various groups of metabolites of interest. In this context, quantitative extraction of metabolites from biological samples is still a challenge [53]. Sample composition of the cell extract has to be identical to the metabolome present at the time of sample collection. Thus, metabolic (enzymatic) activity has to be quenched immediately while simultaneously, chemically instable compounds have to be stabilized, e.g. by addition of antioxidants. However, no changes in the intrinsic concentration of metabolites should occur as a result of introduction or removal of compounds during sample treatment and/or analysis.

Although several metabolites turned out to be straightforwardly quantifiable by external calibration using pure standards with satisfying accuracy, others require more elaborative strategies such as standard addition procedures including spiking of the samples at multiple concentration levels in order to cope with non-quantitative ionization yields resulting from matrix effects. Such matrix-matched calibration procedures, however, are prone to errors, especially when the spiked concentrations do not suit the intrinsic amount of the analyte in the sample. For instance, as a result of the relatively high endogenous concentration of adenosine in the cephalosporin extract (Table 5) in combination with the low upper limit of the linear range of the HILIC methods for this compound (0.25 and 0.5 mg/L at pH 3.5 and 7.5, respectively, *cf.* Tables S-1 and S-2 of the Supplementary Material), spiked levels were outside of the linear range and consequently, no accurate data were obtained for this metabolite with the chosen dilutions. Similarly, in both matrices spiked concentrations of carnitine turned out to be too high for a 1:50 dilution. Thus, standard addition experiments require some preliminary information about the concentration levels of the analytes of interest in order to be able to adjust the spiking levels. Accordingly, performing matrix-matched calibration for every single sample entails an enormous expenditure of work and measurement time, which *de facto* makes it more or less unsuited for routine applications. As a viable alternative, corrected matrix-matched calibration can be performed, for which purpose standard addition is carried out in a representative matrix at several concentration levels and after correction for the endogenous concentration of the analytes *ab initio* present in this matrix, a suitable calibration function is obtained which can be used for external calibration of non-spiked samples present in the same or a similar matrix [52,54]. That way, matrix effects are also accounted for in the calibration standards although work load is only slightly higher compared to performing external calibration in pure standards. Likewise, an averaged corrected matrix-matched calibration is obtained when several different (but similar) matrices, for instance various extracts taken at different times of the fermentation process, are spiked at distinct levels and the resulting linear regressions are used to yield an averaged equation. Such an averaged corrected matrix-matched calibration can then be used for quantitation of samples present in different matrices since it better accounts for lot-to-lot variations. It may also minimize errors in quantitation due to ion adduct formation. If for example $(M+H)^+$ ions dominate in plain standard solutions while adduct ions are strongly populated in the matrix, different responses are expected from the same analyte concentration in plain standard solution and in the sample [55]. Also use of isotopically labeled internal standards has been shown to improve accuracy especially for critical analytes [52], although availability of such standards may pose a problem. The use of $^{13}C$-labeled cell extracts grown on $^{13}C$-labeled substrate seems to become the currently most adequate solution to this problem in quantitative metabolomics [56].

## 4. Conclusion

With the herein presented analytical platform, qualitative and quantitative analysis of a large number of key metabolites of the endo-metabolome of cell lysates such as amino acids, organic acids, amines, purines and pyrimidines, nucleosides, nucleotides, sugar derivatives or phosphorylated compounds is possible. Using a triple quadrupole MS instrument in the SRM mode, reliable identification of the targeted metabolites is given not only *via* their molecular weight but also based on specific fragmentation patterns. Hyphenation to HPLC further allows discrimination of isobaric and even isomeric compounds with good sensitivity, precision and accuracy. Use of a zwitterionic silica based stationary phase in the HILIC mode turned out to be an effective means for targeted metabolomics covering up to 160 hydrophilic compounds within one single chromatographic run and more than 220 metabolites when used under both, acidic and neutral mobile phase conditions. A crucial point, however, remains quantitation of the metabolites, since matrix-matched calibration procedures are required for a number of analytes in order to circumvent false results caused by relative or absolute matrix effects. As a compromise between work load – which is lower when external calibration is done employing pure standards – and accuracy of quantitative results – which is higher when matrix effects are accounted for by matrix-matched calibration performing standard addition – (averaged) corrected matrix-matched calibration can be used for routine applications.

Powerful analytical methods are of course a prerequisite for comprehensive metabolic studies which are more and more feasible due to the ongoing technical development of sophisticated instruments and techniques. They can however only constitute a small part in the emerging field of systems biology since the best analytical method may fail if adequate sample extraction procedures and reliable data processing tools are not implemented.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.11.051.

## References

[1] S. Rochfort, J. Nat. Prod. 68 (2005) 1813.
[2] S. Bajad, V. Shulaev, Trends Anal. Chem. 26 (2007) 625.
[3] M. Oldiges, S. Luetz, S. Pflug, K. Schroer, N. Stein, C. Wiendahl, Appl. Microbiol. Biotechnol. 76 (2007) 495.
[4] M.J. van der Werf, R.H. Jellema, T. Hankemeier, J. Ind. Microbiol. Biotechnol. 32 (2005) 234.
[5] K. Dettmer, P.A. Aronov, B.D. Hammock, Mass Spectrom. Rev. 26 (2007) 51.

[6] V. Mapelli, L. Olsson, J. Nielsen, Trends Biotechnol. 26 (2008) 490.
[7] M. Arita, Brief. Funct. Genomics Proteomics 3 (2004) 84.
[8] W.B. Dunn, D.I. Ellis, Trends Anal. Chem. 24 (2005) 285.
[9] J.M. Hagel, P.J. Facchini, Phytochem. Rev. 7 (2008) 479.
[10] T. Kind, V. Tolstikov, O. Fiehn, R.H. Weiss, Anal. Biochem. 363 (2007) 185.
[11] K. Morgenthal, S. Wienkoop, M. Scholz, J. Selbig, W. Weckwerth, Metabolomics 1 (2005) 109.
[12] H.G. Gika, G.A. Theodoridis, I.D. Wilson, J. Sep. Sci. 31 (2008) 1598.
[13] R.S. Plumb, P.D. Rainville, W.B. Potts III, K.A. Johnson, E. Gika, I.D. Wilson, J. Proteome Res. 8 (2009) 2495.
[14] L. Jia, J. Chen, P. Yin, X. Lu, G. Xu, Metabolomics 4 (2008) 183.
[15] J.C. Lindon, J.K. Nicholson, Annu. Rev. Anal. Chem. 1 (2008) 45.
[16] G.A.N. Gowda, S. Zhang, H. Gu, V. Asiago, N. Shanaiah, D. Raftery, Expert Rev. Mol. Diagn. 8 (2008) 617.
[17] C.A. Daykin, F. Wulfert, Front. Drug Des. Discov. 2 (2006) 151.
[18] W. Eisenreich, A. Bacher, Phytochemistry 68 (2007) 2799.
[19] I.J. Colquhoun, J. Pestic. Sci. 32 (2007) 200.
[20] J.L. Ward, J.M. Baker, M.H. Beale, FEBS J. 274 (2007) 1126.
[21] D.S. Wishart, Trends Anal. Chem. 27 (2008) 228.
[22] X. Feng, X. Liu, Q. Luo, B.-F. Liu, Mass Spectrom. Rev. 27 (2008) 635.
[23] S.C. Brown, G. Kruppa, J.-L. Dasseux, Mass Spectrom. Rev. 24 (2005) 223.
[24] D. Goodenowe, Metab. Profiling (2003) 125.
[25] R. Ramautar, G.W. Somsen, G.J. de Jong, Electrophoresis 30 (2009) 276.
[26] J. Kopka, J. Biotechnol. 124 (2006) 312.
[27] T.O. Metz, J.S. Page, E.S. Baker, K. Tang, J. Ding, Y. Shen, R.D. Smith, Trends Anal. Chem. 27 (2008) 205.
[28] W. Lu, B.D. Bennett, J.D. Rabinowitz, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 871 (2008) 236.
[29] X. Lu, X. Zhao, C. Bai, C. Zhao, G. Lu, G. Xu, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 866 (2008) 64.
[30] G. Theodoridis, H.G. Gika, I.D. Wilson, Trends Anal. Chem. 27 (2008) 251.
[31] M. Bedair, L.W. Sumner, Trends Anal. Chem. 27 (2008) 238.
[32] S. Cubbon, C. Antonio, J. Wilson, J. Thomas-Oates, Mass Spectrom. Rev. (2009), doi:10.1002/mas.20252.
[33] J. Trygg, E. Holmes, T. Lundstedt, J. Proteome Res. 6 (2007) 469.
[34] J. van der Greef, A.K. Smilde, J. Chemom. 19 (2006) 376.
[35] V. Shulaev, Brief. Bioinf. 7 (2006) 128.
[36] K. Guo, L. Li, Anal. Chem. 81 (2009) 3919.
[37] H. Yoshida, T. Mizukoshi, K. Hirayama, H. Miyano, J. Agric. Food Chem. 55 (2007) 551.
[38] L. Coulier, R. Bas, S. Jespersen, E. Verheij, M.J. Van der Werf, T. Hankemeier, Anal. Chem. 78 (2006) 6573.
[39] W. Lu, E. Kimball, J.D. Rabinowitz, J. Am. Soc. Mass Spectrom. 17 (2006) 37.
[40] T. Uehara, A. Yokoi, K. Aoshima, S. Tanaka, T. Kadowaki, M. Tanaka, Y. Oda, Anal. Chem. 81 (2009) 3836.
[41] S.U. Bajad, W. Lu, E.H. Kimball, J. Yuan, C. Peterson, J.D. Rabinowitz, J. Chromatogr. A 1125 (2006) 76.
[43] Y. Asakawa, N. Tokida, C. Ozawa, M. Ishiba, O. Tagaya, N. Asakawa, J. Chromatog. A 1198–1199 (2008) 80.
[44] R. Tuytten, F. Lemière, E. Witters, W.V. Dongen, H. Slegers, R.P. Newton, H.V. Onckelen, E.L. Esmans, J. Chromatogr. A 1104 (2006) 209.
[45] J.W. Dolan, LC–GC North Am. 22 (2004) 26.
[46] J.V. Hinshaw, LC–GC North Am. 21 (2003) 268.
[47] K. Greulich, L. Alder, Anal. Bioanal. Chem. 391 (2008) 183.
[48] H.E. Fries, M. Aiello, C.A. Evans, Rapid Commun. Mass Spectrom. 21 (2007) 369.
[49] H. Mei, in: W.A. Korfmacher (Ed.), Using Mass Spectrometry for Drug Metabolism Studies, CRC Press, Boca Raton, FL, USA, 2005, p. 103.
[50] H.P. Nguyen, K.A. Schug, J. Sep. Sci. 31 (2008) 1465.
[51] M. Jemal, Y.-Q. Xia, Curr. Drug Metab. 7 (2006) 491.
[52] S. Schiesel, M. Lämmerhofer, W. Lindner, Anal. Bioanal. Chem., submitted for publication.
[53] J.D. Rabinowitz, E. Kimball, Anal. Chem. 79 (2007) 6167.
[54] S. Schiesel, M. Lämmerhofer, W. Lindner, Accepted for publication in Anal, Bioanal. Chem. (2009).
[55] K. Bester, Anal. Bioanal. Chem. 391 (2008) 15.
[56] L. Wu, M.R. Mashego, J.C. Van Dam, A.M. Proell, J.L. Vinke, C. Ras, W.A. Van Winden, W.M. Van Gulik, J.J. Heijnen, Anal. Biochem. 336 (2005) 164.