Anal Bioanal Chem
DOI 10.1007/s00216-012-6701-4

ORIGINAL PAPER

Anionic metabolic profiling of urine from antibiotic-treated rats by capillary electrophoresis–mass spectrometry

Miranda G. M. Kok · Marco M. A. Ruijken · Jonathan R. Swann · Ian D. Wilson · Govert W. Somsen · Gerhardus J. de Jong

Received: 2 November 2012 / Revised: 19 December 2012 / Accepted: 22 December 2012

Abstract A recently developed capillary electrophoresis (CE)-negative-ionisation mass spectrometry (MS) method was used to profile anionic metabolites in a microbial-host co-metabolism study. Urine samples from rats receiving antibiotics (penicillin G and streptomycin sulfate) for 0, 4, or 8 days were analysed. A quality control sample was measured repeatedly to monitor the performance of the applied CE-MS method. After peak alignment, relative standard deviations (RSDs) for migration time of five representative compounds were below 0.4 %, whereas RSDs for peak area were 7.9–13.5 %. Using univariate and principal component analysis of obtained urinary metabolic profiles, groups of rats receiving different antibiotic treatment could be distinguished based on 17 discriminatory compounds, of which 15 were downregulated and 2 were upregulated upon treatment. Eleven compounds remained down- or upregulated after discontinuation of the antibiotics administration, whereas a recovery effect was observed for others. Based on accurate mass, nine compounds were putatively identified; these included the microbial-mammalian co-metabolites hippuric acid and indoxyl sulfate. Some discriminatory compounds were also observed by other analytical techniques, but CE-MS uniquely revealed ten metabolites modulated by antibiotic exposure, including aconitic acid and an oxocholic acid. This clearly demonstrates the added value of CE-MS for nontargeted profiling of small anionic metabolites in biological samples.

Keywords Capillary electrophoresis · Mass spectrometry · Anionic metabolic profiling · Rat urine · Gut microbial depletion

Abbreviations

^{1}H NMR	Proton nuclear magnetic resonance
ABx-Dy	Samples taken at day *y* after the use of antibiotics for *x* days
BGE	Background electrolyte
CE	Capillary electrophoresis
EOF	Electro-osmotic flow
ESI	Electrospray ionisation
GC	Gas chromatography
LC	Liquid chromatography
MS	Mass spectrometry
NaOH	Sodium hydroxide
PC	Principal component
PCA	Principal component analysis

M. G. M. Kok · G. W. Somsen · G. J. de Jong
Biomolecular Analysis, Department of Pharmaceutical Sciences, Utrecht University, P.O. Box 80082, 3508 TB, Utrecht, The Netherlands

M. G. M. Kok (✉) · G. W. Somsen · G. J. de Jong
Research group Analysis Techniques in Life Science, Avans Hogeschool, P.O. Box 90116, 4800 RA, Breda, The Netherlands
e-mail: M.G.M.Kok@uu.nl

M. M. A. Ruijken
MsMetrix, Doornhoecklaan 81, 3601 JT, Maarssen, The Netherlands

J. R. Swann
Department of Food and Nutritional Sciences, School of Chemistry, Food and Pharmacy, University of Reading, Whiteknights, Reading RG6 6AP, UK

I. D. Wilson
Department of Drug Metabolism and Pharmacokinetics IM, AstraZeneca, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

Published online: 12 January 2013

QC	Quality control
RPW	Reference peak warping
RSD	Relative standard deviation
TCA	Tricarboxylic acid
TEA	Triethylamine
TOF	Time-of-flight

Introduction

The gut microbiota, the population of microorganisms that resides in the gastrointestinal tract, contributes significantly to the metabolic phenotype of the host via interactive microbial–host co-metabolism [1, 2]. Global metabolic profiling is the comprehensive analysis of metabolites in a biological system providing a snapshot of the biochemical status of a complex organism. This metabolic profile contains information from endogenous processes encoded in the host genome and from environmental inputs such as nutritional factors and gut microbial activity. Furthermore, information relating to trans-genomic interactions between the host genome and gut microbiome is also captured in the metabolic signature. Metabolic profiling strategies therefore provide a window into the host metabolic system and permit the influence of the gut microbiome and trans-genomic interactions on the host to be studied [3–5]. Germ-free or antibiotic-treated animal models are typically used to study the influence of the gut microbiota on host metabolism [1, 2]. Several analytical techniques have been applied to profile urine and fecal samples of rats and mice after administration of antibiotics, including proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, gas chromatography (GC), and reversed-phase liquid chromatography (LC) [6–11]. In all reported studies, antibiotic exposure modulated the biochemical signatures of animals. In the studies applying ^{1}H NMR spectroscopy, the discrimination was based on a range of compounds, including microbially derived metabolites, amino acids, organic acids and short-chain fatty acids [6, 8–10]. Using GC and LC approaches, oligosaccharides, pyridines, and purines were also found to be discriminatory urinary compounds [7].

Capillary electrophoresis (CE) coupled with mass spectrometry (MS) using a sheath-liquid interface shows good potential for profiling highly polar and charged metabolites and is therefore expected to give complementary information about alterations in polar metabolites after antibiotic treatment with respect to the other applied methodologies [12]. Most of the reported CE-MS methods for global metabolic profiling have been conducted using positive electrospray ionisation (ESI), because of the relatively higher analyte responses as compared with negative ionisation mode CE-MS. However, a significant proportion of the metabolites present in biological samples is acidic in nature and can only be ionised effectively using negative ESI. Therefore, the application of negative ionisation mode CE-MS to metabolic profiling studies may capture additional metabolic information and expand metabolome coverage. So far, CE-MS-based studies performed in negative ionisation mode, have generally followed a targeted approach [13]. In these studies, compound classes are preselected based upon a priori knowledge and their abundance in biological samples is assessed. However, the application of this approach in an untargeted manner may allow the unbiased profiling of a class of metabolites not measured by other metabolomics approaches. Therefore, negative mode CE-MS-based metabolic profiling offers a complementary alternative to NMR and LC-MS.

Recently, we have developed a CE-MS method with improved signal intensities for metabolites in negative ionisation mode [14]. The optimised method employed a background electrolyte (BGE) of 25 mM triethylamine (TEA) and a sheath liquid of 5 mM TEA in water–methanol (1:1, *v*/*v*). It exhibited a considerable increase in coverage of the polar human urine metabolome when compared with commonly used CE-MS methods applying ammonium acetate. Moreover, signal intensities were significantly higher with gain factors of up to seven [14]. In the present study, we evaluated the applicability of this negative mode CE-MS method for global anionic metabolic profiling of urine samples from rats receiving the antibiotics penicillin G and streptomycin sulfate for 0, 4, or 8 days. The resulting profiles were aligned and the compounds present in the various urine samples were determined with a peak picking procedure. Peak areas of the compounds were assessed and individually normalised to the sum of the areas of all compounds. Average peak areas for the compounds detected before and after antibiotic treatment were compared, and principal component analysis (PCA) was also performed to discriminate between the various treatment groups based on differences between the obtained metabolic profiles. In addition, the metabolites with discriminating power were putatively identified. The same rat urine samples have previously been analysed with high-resolution ^{1}H NMR spectroscopy [6]. The ^{1}H NMR spectroscopic urinary profiles revealed metabolic differences following antibiotic treatment. This offered the opportunity to compare the results obtained from the two analytical platforms and appreciate the value of CE-MS for anionic metabolic profiling. The CE-MS results were related to other studies on microbial–host co-metabolism as well.

Materials and methods

Chemicals

Methanol and formic acid were obtained from Fluka (Steinheim, Germany). Sodium hydroxide (NaOH) and creatinine

were purchased from Sigma Aldrich (Steinheim, Germany), and TEA was from Fisher Scientific (Loughborough, UK). Water was deionised and purified with a Milli-Q purification system (Millipore, Bedford) prior to use.

Rat urine samples

Urine samples from 18 rats were provided by AstraZeneca (Department of Drug Metabolism and Pharmacokinetics, Macclesfield, UK) and stored at −80 °C. Rats were divided into three groups (n=6 each), receiving different regimens of antibiotic treatment, as extensively described elsewhere [6]. Briefly, one group (AB4) received antibiotics from days 0 to 4, whereas another group of rats (AB8) received antibiotics for 8 days. AB4 animals allowed the metabolic consequences of short-term bacterial recolonisation to be studied; the period of 4 days was not sufficient for complete recolonisation of the gut microbiota [6]. A control group (AB0) did not receive any antibiotics. The antibiotics, penicillin G (2 mg/mL) and streptomycin sulfate (4 mg/mL), were provided ad libitum in the rats' drinking water. These antibiotics are effective against both Gram-positive and Gram-negative bacteria, and complementary contribute to depletion of the microbiota. Sample collection was performed overnight for 16 hours on days −1 to 0 (D0), 3–4 (D4), and 7–8 (D8). Aliquots (10 μL) of all urine samples were pooled and used as quality control (QC) sample. Prior to analysis, urine samples were mixed with BGE (1:1, v/v). In total, 54 rat urine samples were randomly analysed and the QC sample was measured after every fourth run to assess the stability and repeatability of the CE-MS method.

CE-MS

CE-MS experiments were performed on a Beckman P/ACE MDQ instrument (Beckman Coulter, Fullerton) coupled to a time-of-flight (TOF) mass spectrometer (micrOTOF, Bruker Daltonics, Bremen, Germany) using a sheath-liquid electrospray interface from Agilent Technologies (Waldbronn, Germany). Separations were carried out in a fused-silica capillary (Polymicro Technologies, Phoenix) with an internal diameter of 50 μm and a total length of 100 cm. New fused-silica capillaries were rinsed with 1 M NaOH for 20 min and water for 10 min at 20 psi. Between runs, capillaries were flushed with acetic acid (10 % solution) for 4 min, water for 3 min and BGE for 1 min, applying a pressure of 50 psi. The BGE was 25 mM TEA (pH 11.7). The sheath liquid consisted of 5 mM TEA in water–methanol (1:1, v/v) and was delivered at 5 μL/min using a 10-mL syringe (Hamilton, Reno, NV) and a syringe pump of KD Scientific (Holliston, MA). The use of TEA, which is highly volatile, did not affect the performance of the mass spectrometer and additional cleaning procedures were not needed. Rat urine samples were injected hydrodynamically using a pressure of 0.5 psi for 30 s. The separation voltage was 30 kV for 30 min, and the capillary temperature was set at 20 °C. The following interface conditions were used: dry gas temperature, 180 °C; dry gas flow, 4 L/min; nebuliser pressure, 10 psi; and ESI voltage, 4 kV. Data were acquired in negative ionisation mode in the mass range m/z 50 to 800 with a repetition rate of 1 Hz. At the end of every run, a 10-mM sodium formate plug was injected (1 psi, 30 s), flushed through the capillary, and the sodium formate clusters detected by ESI-MS were subsequently used for internal mass calibration of the recorded mass spectra.

Data analysis

CE–MS data were processed using MsXelerator software from MsMetrix (Maarssen, The Netherlands). The metabolite profiles were aligned based on a reference sample using the reference peak warping (RPW) function with the m/z values 112.05, 243.06, 124.99, 160.04, and 167.02 used as reference compounds allowing a maximum difference in m/z between the various metabolic profiles of 0.01 Da. Using the high-resolution peak picking procedure of the MsXelerator software, compounds present in the QC sample having a migration time between 5 and 30 min and with a signal-to-noise ratio of three for at least ten successive spectra were determined. After removal of isotope peaks, peak matching was carried out to determine peak areas of the common compounds in the rat urine samples. Compounds were considered matching (i.e., the same) when their mass difference and migration time difference was smaller than 0.01 Da and 0.1 min, respectively. Per injected sample, peak areas were normalised to the total peak area obtained in the electropherogram. Average peak areas of detected compounds for each treatment group were calculated and area ratios between the treatment groups were determined to assess differences in metabolite profiles. Paired t tests (AB4-D0 vs. AB4-D4 and AB4-D8, and AB8-D0 vs. AB8-D4 and AB8-D8) were conducted to determine significant changes among individual metabolite concentrations in rat urine upon antibiotic treatment. Independent sample t tests were performed to assess differences in metabolite concentrations between AB4 and AB8 rats at both days 0 and 4. p values$\leq$0.05 were considered to indicate a statistically significant difference. Furthermore, the resulting table of normalised peak areas for detected compounds was used for PCA. The PCA loading plot was used to determine the variables that were responsible for the separation of the different rat urine samples. Discriminatory compounds between the various treatment groups were putatively identified by comparing the observed masses with

masses recorded in the metabolomics databases HMDB, METLIN, and MassBank [15–17].

Results and discussion

CE-MS performance

To assess the reproducibility of the applied CE-MS method, the QC sample was measured repetitively throughout the entire two-week period in which the urine samples were analysed. A typical base peak electropherogram of rat urine is shown in Fig. 1a. Peak areas, migration times, and effective mobilities for five representative rat-urine compounds detected in the QC sample (Fig. 1b) were determined and relative standard deviations (RSDs) were calculated. These five compounds were selected based on their migration times (covering the complete metabolite profile) and abundance (low and high intensities). The RSDs of the obtained peak areas of each of the selected compounds ranged from 7.9 to 13.5 % (Table 1). This is considered acceptable for an ESI-MS based bioanalytical method. The peak areas showed a random variation in time and no significant difference was observed between the first and the last day, indicating good system robustness. The migration times of the selected QC compounds showed a relatively large variability (RSDs, 8.1–11.8 %, Table 1). Therefore, effective electrophoretic mobilities were calculated using creatinine (*m/z* 112.05; detected at circa 6.5 min) as neutral electro-osmotic flow (EOF) marker. The identity of the EOF marker was confirmed by addition of creatinine to the sample. RSD values of the effective electrophoretic mobilities were considerably smaller (factors 2.4–4.7) than the RSD values for migration time (Table 1), indicating that a part of the variability was caused by EOF variation between runs.

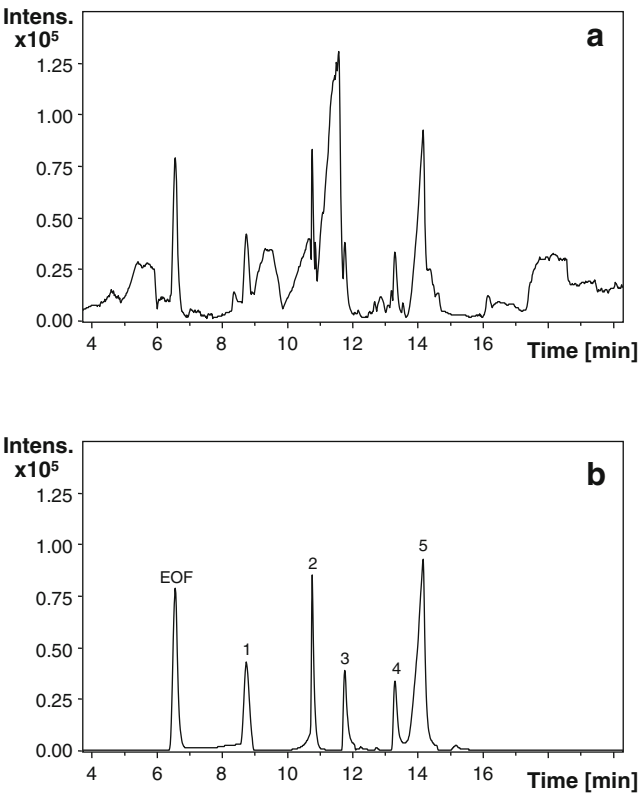

Fig. 1 Base peak electropherogram (**a**) and multiple extracted-ion electropherogram (**b**) obtained during CE-MS of the rat urine QC sample. Compound *m/z* values: *EOF marker*, 112.05; *1*, 243.06; *2*, 212.00; *3* 124.99; *4*, 160.04; and 5, 167.02. Experimental conditions, see "Materials and methods"

The reported RSDs represent a day-to-day repeatability over fourteen days using two different capillaries. The observed variability in migration times is quite commonly observed in CE, especially when bare fused-silica capillaries are used [18, 19]. Still, the observed RSDs for electrophoretic mobility were too large for a reliable comparison of metabolic profiles, indicating the need for an alignment procedure (see "Data preprocessing"). The observed migration time variation might be caused by adsorption of low abundant proteins or other matrix compounds to the capillary wall. Noncovalent coating of the capillary wall with charged polymers may in principle be used to substantially improve the repeatability of migration times of metabolites [20]. However, such a capillary coating appeared to be unstable in combination with a BGE containing TEA [14]. This might be due to a potential displacement of the charged-polymer coating by TEA.

Data preprocessing

Because of the variability in the migration times of compounds in the different samples, peak alignment was necessary in order to reliably compare the metabolite profiles after the various antibiotic treatments. Various peak alignment procedures have been used in CE for metabolomics [18, 19]. In this study, peak alignment was performed with the RPW function of the MsXelerator software. RPW is a three-dimensional alignment method that is based on representative peaks well spread across the migration time axis. Mass was taken into account for the alignment, because a two-dimensional (migration time and intensity) alignment procedure, using base peak electropherograms, was insufficient for aligning the metabolic profiles properly.

Creatinine and the compounds listed in Table 1 (except *m/z* 212.00) were selected as reference compounds (see "Data analysis") to which all metabolite profiles were aligned. The compound with an *m/z* value of 212.00 was used as control to assess the variability in migration time after peak alignment (vide infra). The alignment is based on a spline function to fit a nonlinear curve between the observed migration times of the reference compounds in the

Table 1 Reproducibility of peak areas, migration times and effective electrophoretic mobilities (μ_{eff}) of five representative urinary compounds detected during repetitive CE-MS analysis of the QC sample (n=15, 2-week period)

Compound m/z	Peak area RSD (%)	Migration time RSD (%)	μ_{eff} RSD (%)
243.06	13.5	8.1	3.4
212.00	13.3	10.8	2.3
124.99	13.2	10.8	2.3
160.04	8.9	11.4	2.8
167.02	7.9	11.8	4.6

various samples. Despite the variations in analyte migration times between analysed samples, the reference compounds could be aligned properly (Fig. 2a, b). In order to further check the effectiveness of the profile alignment, the migration-time RSDs of three arbitrary compounds—migrating in the beginning, in the middle, and at the end of the profile—were determined. As can be seen in Fig. 2c, only small variations in the migration times were observed after peak alignment. The largest RSD was observed for m/z value 212.00 with a value of only 0.4 %. This is a tremendous improvement compared with the variability before alignment (10.8 %; Table 1) and will allow the proper application of algorithms used for multivariate analysis.

After alignment, peak picking was performed to determine which compounds were present in the rat urine samples (see "Data analysis" for procedure). This resulted in the detection of 347 compounds with signal-to-noise ratios above three in the QC sample. Then, peak matching was carried out to reveal compounds present in all rat urine samples and the respective peak areas were determined. For a proper comparison, peak area normalisation is necessary due to considerable variation in individual rat urine volumes. Normalisation to creatinine concentration is often used in MS-based urinary metabolomics [21, 22]. However, the ^{1}H NMR spectroscopic study of the same urine samples indicated that creatinine clearance varied with antibiotic treatment and was therefore not considered a suitable normalisation method [6]. Normalisation based on total ion current was also dismissed, because of the relatively high background signals observed in the CE-MS electropherograms. Therefore, we decided to normalise individual peak areas with respect to total peak area, an approach commonly used in NMR-based metabolomics. This was found to improve the peak area variability between urine samples from individual rats. For each treatment group of rats, the common and normalised peak area RSDs of the compounds mentioned in Table 1 were determined. The RSDs of normalised peak areas were up to a factor 4 lower.

Exploring metabolite profiles

Before performing multivariate data analysis, the urinary metabolic profiles were investigated to assess the quality of the data after the various preprocessing steps and to evaluate whether useful information about changes in metabolite levels after antibiotic treatment can be obtained.

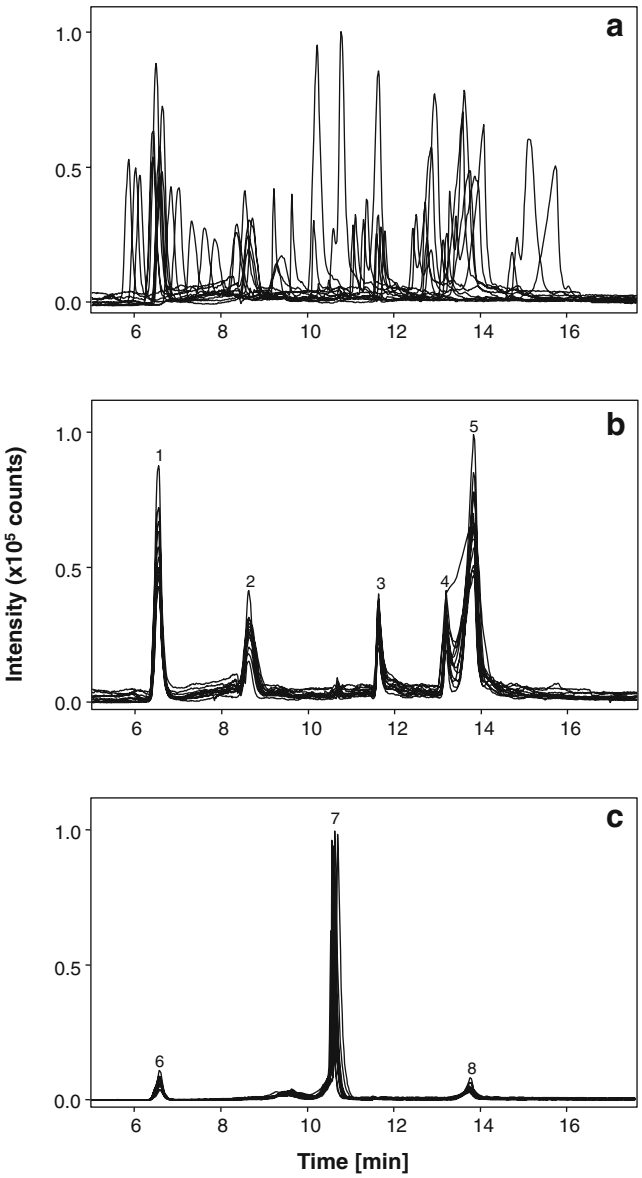

Fig. 2 Multiple extracted-ion electropherograms obtained during repeated CE-MS analysis (n=15) of the QC sample before (**a**) and after (**b**, **c**) peak alignment using the reference peak warping function with peaks 1–5 as reference compounds and peaks 6–8 as verification compounds. Compound m/z values: *1*, 112.05; *2*, 243.06; *3*, 124.99; *4*, 160.04; *5*, 167.02; *6*, 503.16; *7*, 212.00; and *8*, 335.05

Average peak areas of corresponding compounds in urine samples from rats in the same treatment group were calculated for the three different time points (days 0, 4, and 8). Taking a criterion of an average peak area ratio of ≥3 or ≤0.33, compounds were considered to be potentially significantly up- or downregulated upon antibiotic treatment. Some compounds showed striking differences in peak areas before and after the administration of antibiotics. None of those observed compounds could be linked to the administered penicillin G and streptomycin sulfate or metabolites derived from those antibiotics. Both penicillin G and streptomycin sulfate are acidic, and could potentially be detected by CE-MS in negative ionisation mode. However, penicillin G and streptomycin sulfate are poorly absorbed from the gastrointestinal tract and hardly enter the systemic circulation [6]. Therefore, one would indeed not expect to detect these compounds and their metabolites in urine samples after antibiotic treatment. In the ^{1}H NMR study of Swann et al., antibiotic-related metabolites were also not found in urine samples but only in fecal samples of treated rats [6].

At identical study conditions (i.e., at days 0 and 4) for the groups of rats receiving 4 (AB4) and 8 days of antibiotic treatment (AB8), average peak areas of urinary compounds were similar ($p>0.05$), indicating a proper normalisation procedure. This is depicted for three metabolites in Fig. 3, where the grey (AB4 rats) and black bars (AB8 rats) are equal at days 0 and 4. Furthermore, these three metabolites show a large fluctuation upon antibiotic treatment. The compounds with *m/z* values 178.05 and 462.97 show a large decrease of a factor of 5.9 and 87.3, respectively after 4 days, whereas the peak area for *m/z* 503.16 was a factor of 4.9 higher. After discontinuation of the antibiotic treatment, two different effects were observed for the initially decreased compounds. Peak areas for *m/z* 178.05 remained significantly decreased at day 8 with respect to day 0 ($p\leq0.01$), whereas a recovery effect (i.e., increasing peak areas) was observed for *m/z* 462.97 ($p>0.05$) (Fig. 3, grey bars). Particularly apparent after the use of antibiotics for 8 days was a significant increase (factor of 26.6) of the metabolite with an *m/z* of 503.16 (Fig. 3, black bars). Overall, the results indicated that the quality of the processed data allowed the determination of differences in compound abundances among the various treatment groups.

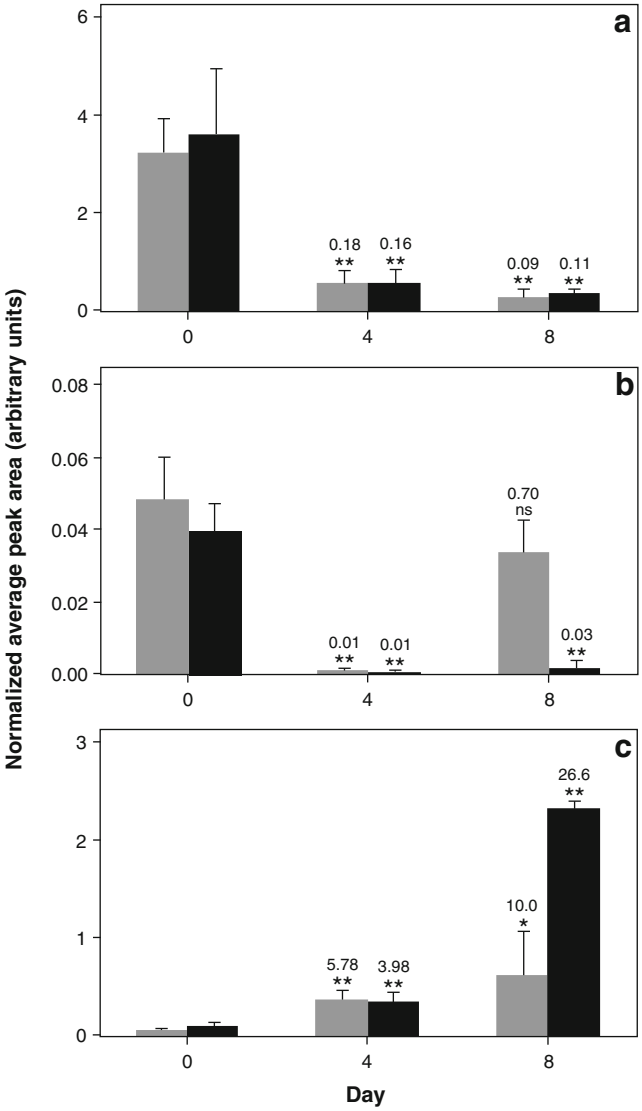

Fig. 3 Normalised average peak areas (± standard deviation) at days 0, 4, and 8 of three urinary compounds with **a** *m/z* 178.05, **b** *m/z* 462.97, and **c** *m/z* 503.16 upon antibiotic treatment. *Grey bars* represent rats ($n=6$) receiving 4 days of antibiotic treatment (AB4), and *black bars* represent rats ($n=6$) receiving 8 days of antibiotics (AB8). Peak areas of the respective compounds at day 0 were compared with the areas at days 4 and 8 for both the AB4 and AB8 animals. Fold changes in peak area are indicated *above the bars* including the statistical significance of the observed differences: *$p\leq0.05$; **$p\leq0.01$; *ns* $p>0.05$

PCA of metabolite profiles

PCA of the urinary metabolic profiles was performed to observe whether the differently treated rats could be discerned on the basis of CE-MS and to reveal discriminating compounds in an unsupervised way. The PCA score plot of the metabolic profiles of all rat urine samples is shown in Fig. 4a. The first principal component (PC) accounted for 33 % of the variance, whereas PC2 explained 21 % of the variance. The QC replicates are closely clustered in the center of the PCA score plot. This confirms the stability and reproducibility of the CE–MS method (including alignment), and thus also its suitability for metabolic profiling studies. Samples of untreated rats (AB0-D0, AB0-D4, AB0-D8, AB4-D0, and AB8-D0) are more widespread, with four samples as outliers. This spread represents the variation of the composition of urine originating from various individual rats and collected at different days. However, as indicated by PCA, the urinary metabolic profiles from nontreated rats and antibiotic-treated rats were markedly different (Fig. 4a). Swann et al. also distinguished

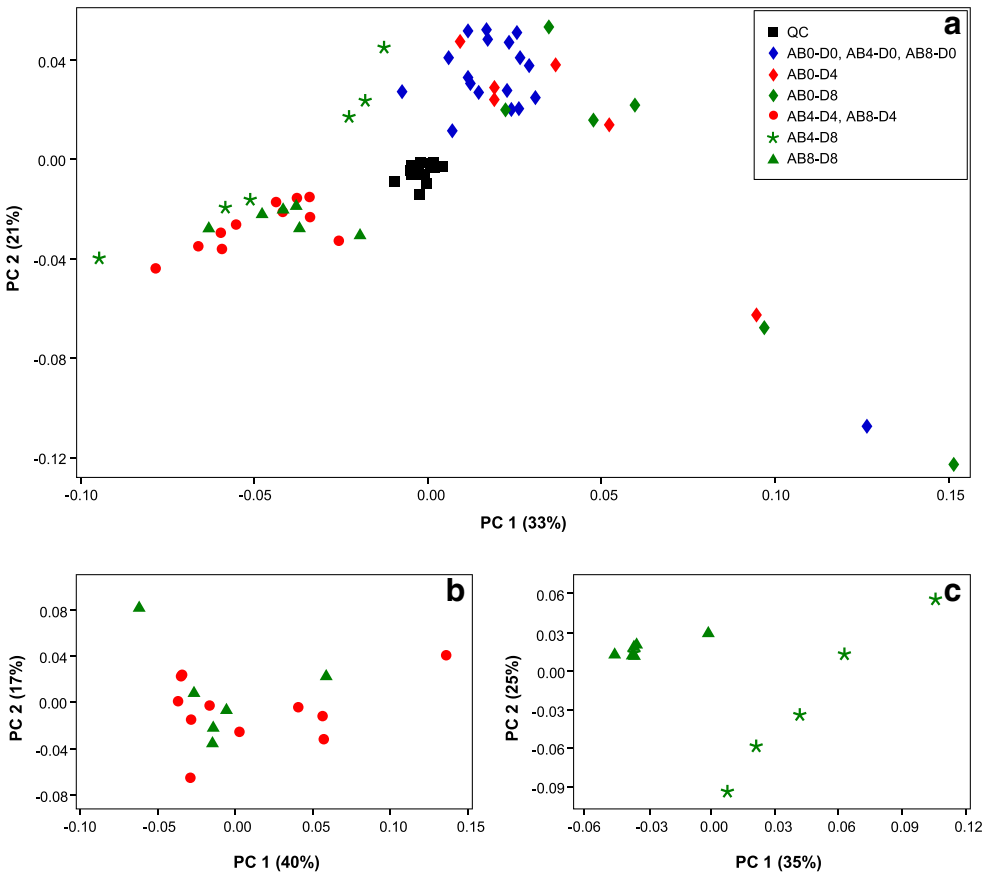

Fig. 4 PCA score plots of metabolic profiles of rat urine samples analysed by CE-MS. PCA was performed on all samples (**a**), on urine samples of rats receiving continuous antibiotic treatment for 4 and 8 days (**b**) and on urine samples at day 8 after 4 and 8 days of antibiotic treatment (**c**). *Squares* represent QC samples; *diamonds* represent untreated rats (AB0-D0, AB0-D4, AB0-D8, AB4-D0, and AB8-D0); *dots* represent rats after 4 days of continuous antibiotic treatment (AB4-D4 and AB8-D4); *stars* represent rats at day 8 after 4 days of antibiotic treatment (AB4-D8); *triangles* represent rats after 8 days of continuous administration of antibiotics (AB8-D8). The colors indicate the various time points: *blue* represents the samples at day 0, *red* the samples at day 4 and *green* the samples at day 8

samples originating from nontreated and treated rats, using ^{1}H NMR spectroscopy [6]. The samples after 4 (AB4-D4 and AB8-D4) and 8 days (AB8-D8) of continuous intake of the antibiotics showed no separation in the overall PCA plot. PCA performed on only the metabolic profiles of urine samples after continuous antibiotic treatment for 4 (AB4-D4+AB8-D4) and 8 days (AB8-D8) also showed no distinction (Fig. 4b). This result suggests the duration of antibiotic treatment does not lead to major differences in the metabolic profiles measured by CE-MS. It should be noted that during exploration of the metabolic profiles ("Exploring metabolite profiles") the compound with *m/z* 503.16 showed a higher increase in signal after 8 days than after 4 days of antibiotic treatment (Fig. 3c). The subtle variation in the concentration of *m/z* 503.16 may be too small to cause discrimination of the two groups by PCA.

Discrimination of the samples from the AB4-D8 and AB8-D8 groups was less pronounced in the overall PCA plot (Fig. 4a). However, when PCA was performed on the metabolic profiles of only these samples, there was a much better separation with noticeable differences in urinary metabolites (Fig. 4c), except for one sample (sitting in the cluster of triangles). Similar results were obtained within the NMR study [6]. PCA of samples from the groups AB8-D4 and AB8-D8 showed a similar separation as in Fig. 4c. This could be expected as no differences were observed between AB4-D4 and AB8-D4.

Interestingly, PCA of all metabolic profiles revealed the emergence of two subgroups within the recolonising AB4-D8 group consistent with animal housing. The metabolic profiles of three rats showed near resemblance to untreated rats, whereas the profiles of the other three rats with the same antibiotic regimen were similar to the profiles of rat urine samples during treatment (Fig. 4a). The same subgroups emerged in the study by Swann et al. when these urinary metabolic profiles were measured by NMR [6]. In addition, the microbial populations were also enumerated in the fecal samples of these animals and clear differences were observed in the bacterial profiles of these two AB4-D8 subgroups. The subgroup with the closest resemblance to nontreated rats had a substantially higher bacterial load compared with the other subgroup.

Discriminating compounds related to antibiotic treatment

The PCA loading plot indicated the variables (metabolites) responsible for the discrimination of the various rat urine samples (see Fig. 5 for *m/z* values). After antibiotic treatment for 4 days (AB4-D4 and AB8-D4), 15 compounds appeared to be downregulated and 2 compounds were upregulated. The three compounds that showed large ratios of peak areas before and after 4-day treatment ("Exploring metabolite profiles") were also indicated by PCA as discriminatory between the

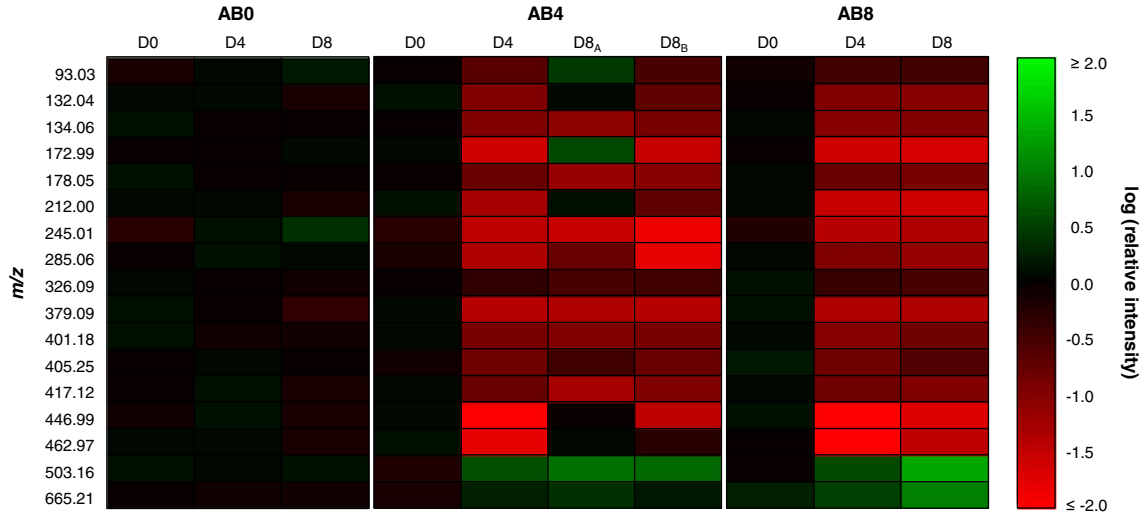

Fig. 5 Heat map of average peak areas in time (D0, D4, and D8) for discriminating compounds in urine from rats receiving 0 (AB0), 4 (AB4), or 8 days (AB8) of antibiotic treatment. The two subgroups observed in the AB4-D8 group are indicated with A and B. Each block represents the average peak area of the indicated *m/z* value for a specific rat group, relative to the average peak area observed for the indicated *m/z* value for nontreated rats. Peak areas are depicted as color-coded log values with *red* indicating decreased and *green* increased peak areas. *Black* represents no changes in urine concentration. Putative identities of the *m/z* values are listed in Table 2

various treatment groups. The abundances of the compounds in the different urine samples, relative to the average peak areas observed for nontreated rats are depicted in a heat map (Fig. 5). The red color indicates decreased urine concentrations for the particular compounds upon antibiotic treatment, whereas green represents increased abundances. The cells in the heat map representing urine samples from nontreated rats are predominantly very dark or black, which indicates that the concentration of the 17 discriminatory compounds did not vary significantly over time when rats were not exposed to antibiotic treatment. Establishing reference values by averaging the peak areas of untreated rats per *m/z* value appears valid.

Following cessation of antibiotic administration at day 4, some metabolic modulations persisted at day 8, while in some compounds a recovery effect occurred (Fig. 5). Ambiguous results were obtained for the *m/z* values 93.03, 132.04, 172.99, 212.00, and 446.99, leading to the appearance of two subgroups. For subgroup A, the peak areas at day 8 were similar to nontreated rats, whereas for subgroup B, the effect of the antibiotics on the metabolite profile was still present at day 8 (Fig. 5). As indicated above, this probably reflects the cage-related differences in bacterial recolonisation [6].

Based on the exact masses, nine of the discriminatory compounds could be putatively identified (Table 2). The compounds with *m/z* value 178.05 and 212.00 are most probably hippuric acid and indoxyl sulfate, respectively. The appearance of ESI fragments at *m/z* values 134.05 and 132.04 in the mass spectra [15–17], underlines the presence of hippuric acid and indoxyl sulfate, respectively. Both hippuric acid and indoxyl sulfate were downregulated upon antibiotic treatment. The concentration of hippuric acid remained significantly lower posttreatment, whereas a recovery effect was observed for indoxyl sulfate in one subgroup of AB4. This is consistent with the findings of Swann et al. with ^{1}H NMR spectroscopic analysis of the same urine samples [6]. Moreover, the decrease in concentration of hippuric acid and indoxyl sulfate after gut microbial depletion has been observed in other studies as well [7, 9, 10]. Hippuric acid and indoxyl sulfate have been found to be mammalian-microbial co-metabolites since their production is dependent on the presence of microorganisms in the gastrointestinal tract [1, 6, 7, 9]. It is expected therefore, that administration of antibiotics will lead to downregulation of both hippuric acid and indoxyl sulfate.

The mass of 93.03 could be attributed to phenol, a compound that is synthesised from tyrosine by some bacteria. The concentration of phenol in urine was decreased after the use of antibiotics for 4 days. As mentioned above, two subgroups of rats were observed based on the obtained metabolic profiles after discontinuation of the antibiotics. The phenol concentration increased for three rats in the AB4-D8 group; the concentration for the other three rats in the same group remained lower compared with day 0 (Fig. 5). Zheng et al. also found a decrease in phenol in rat urine after the oral administration of the antibiotics imipenem and cilastatin. Moreover, a recovery effect was observed after discontinuation of treatment [7].

The *m/z* values 172.99 and 405.25 were putatively identified as aconitic acid and an oxocholic acid, respectively. Aconitic acid plays a role in the tricarboxylic acid (TCA) cycle, being the intermediate in the conversion of citric acid to isocitric acid. Aconitic acid has not been found before as a discriminative compound for urinary metabolic profiles of untreated and antibiotic-treated rats. However, other compounds of the TCA

Table 2 Putatively identified discriminatory compounds (up- or downregulated) revealed by PCA of CE-MS data obtained for urine of nontreated and antibiotic-treated rats

Putative identity	MT (min)[a]	Observed *m/z*	Calculated *m/z*	Error (mDa)
Phenol	11.49	93.032	93.034	−1.84
Indoxyl	10.70	132.044	132.045	−0.55
Fragment hippuric acid	9.40	134.060	134.061	−1.60
Aconitic acid	11.65	172.991	173.009	−17.7
Hippuric acid	9.40	178.050	178.050	−0.02
Indoxyl sulfate	10.70	212.002	212.002	0.24
Oxocholic acid	7.22	405.246	405.264	−17.9
Trisaccharide	6.69	503.157	503.161	−3.92
Tetrasaccharide	6.62	665.207	665.214	−6.74

[a]Migration time after alignment

cycle were altered upon antibiotic treatment. Swann et al. found an upregulation of citric acid and fumaric acid [6]. In contrast, Zheng et al. observed a decrease in the urinary concentration of citric acid and fumaric acid after the intake of antibiotics [7]. In the present study, citric acid was also detected with the applied CE-MS method, but PCA did not reveal this compound to differentiate between the various rat urine samples. Because of the relatively high urinary concentration of citric acid and the multiple carboxylic acid groups, this compound showed distorted peaks in the CE-MS profiles, which complicated the comparison of peak areas between the urine samples.

3-oxocholic acid, 3,7-dihydroxy-12-oxocholanoic acid, and 7-ketodeoxycholic acid are dihydroxy bile acids with a molecular weight of 406.27, which are secreted via the urinary tract. These bile acids differ in the position (3, 7, and 12) of two hydroxyl groups. To the best of our knowledge, this is the first time that such a bile acid has been found to be elevated in rat urine after gut microbial depletion with antibiotic treatment. Other bile acids have shown to be altered in various tissue samples of rats and mice after microbial manipulation [23, 24].

The compounds detected with *m/z* 503.16 and 665.21 are most probably a tri- and a tetrasaccharide, respectively. These compounds were up-regulated in rat urine after the administration of penicillin G and streptomycin sulfate (Fig. 5). In the ^{1}H NMR study of Swann et al. on the same sample set, there was an increase in urine concentration of glycoproteins and an increase in oligosaccharides in analysed fecal samples [6]. Degradation of glycoproteins will lead to more oligosaccharides, and thereby results in elevated concentrations of oligosaccharides, which was also observed in fecal samples of mice after vancomycin-induced depletion of gut microorganisms [9].

Eight of the detected compounds responsible for discrimination of the various groups of rats could not be assigned based on accurate mass only, because there were multiple metabolite candidates in the databases with equal masses. These compounds all had different migration times, indicating they are unique discriminatory compounds (i.e., no ESI-induced adducts or fragments). Interestingly, compounds that correspond with these *m/z* values were not reported in other antibiotic treatment studies using ^{1}H NMR, GC and LC methodologies. This indicates that the CE-MS method in negative ionisation mode provides complementary information to the other metabolite profiling platforms, thereby increasing the coverage of the urinary metabolome, which is important in global metabolic profiling studies. In order to extend the knowledge of microbial-host co-metabolism, identification of the unknown compounds will be required. This could be achieved, for instance, by conducting MS/MS experiments using a quadrupole TOF-MS instrument and/or by spiking the urine samples with reference standards.

Concluding remarks

A new CE-MS method for the global profiling of anionic metabolites, requiring minimal sample pretreatment (only 1:1 (*v/v*) dilution with BGE), was applied to urine samples from rats receiving various antibiotic treatments. Repeated analysis of a QC sample throughout the study showed that the method enabled acquisition of reproducible peak areas for representative urinary compounds over a two-week period. Moreover, a dedicated peak alignment procedure resulted in CE-MS profiles with low migration-time variability, allowing reliable comparison of the urinary sample data. Rats differentially treated with antibiotics could be distinguished based on their urinary metabolic profiles obtained with CE-MS. Seventeen discriminatory compounds were revealed, including several acidic or neutral compounds, such as indoxyl sulfate, phenol, an oxocholic acid, oligosaccharides, and aconitic acid. The latter cannot be assessed by CE-MS applying a low-pH BGE and positive ESI. Employment of a CE-MS method particularly suited to profile cationogenic metabolites [20] for the analysis of the same rat urine samples, revealed eleven discriminating compounds. Based on *m/z* values, only two of the observed discriminatory compounds could be linked to compounds found with negative mode CE-MS. This indicates the added value of methods allowing global profiling of biological samples in negative ionisation mode. Some of the putatively

identified compounds were also observed in other metabolic profiling studies on gut microbial depletion, including a ^{1}H NMR spectroscopic study performed on the same sample set. However, CE-MS also indicated at least ten discriminating compounds which were not detected in other related studies, thereby clearly demonstrating the potential of CE-MS in negative ionisation mode for metabolic profiling. Identification of the observed discriminatory compounds by MS/MS and standard addition is still required to increase insights in the related metabolic processes. Currently, we are comparing the presented CE-MS method to ion-pair reversed-phase LC-MS and hydrophilic interaction chromatography-MS for the detection of anionic metabolites.

References

1. Nicholson JK, Holmes E, Wilson ID (2005) Nat Rev Microbiol 3:431–438
2. Willing BP, Russell SL, Finlay BB (2011) Nat Rev Microbiol 9:233–243
3. van der Greef J, Stroobant P, van der Heijden R (2004) Curr Opin Chem Biol 8:559–565
4. Fiehn O (2002) Plant Mol Biol 48:155–171
5. Nicholson JK, Wilson ID (2003) Nat Rev Drug Discov 2:668–676
6. Swann JR, Tuohy KM, Lindfors P, Brown DT, Wilson ID, Sidaway J, Nicholson JK, Holmes E (2011) J Proteome Res 10:3590–3603
7. Zheng X, Xie G, Zhao A, Zhao L, Yao C, Chiu NHL, Zhou Z, Bao Y, Jia W, Nicholson JK, Jia W (2011) J Proteome Res 10:5512–5522
8. Romick-Rosendale LE, Goodpaster AM, Hanwright PJ, Patel NB, Wheeler ET, Chona DL, Kennedy MA (2009) Magn Reson Chem 47:S36–S46
9. Yap IKS, Li JV, Saric J, Martin F, Davies H, Wang Y, Wilson ID, Nicholson JK, Utzinger J, Marchesi JR, Holmes E (2008) J Proteome Res 7:3718–3728
10. Williams RE, Eyton-Jones HW, Farnworth MJ, Gallagher R, Provan WM (2002) Xenobiotica 32:783–794
11. Antunes LCM, Han J, Ferreira RBR, Lolić P, Borchers CH, Finlay BB (2011) Antimicrob Agents Chemother 55:1494–1503
12. Ramautar R, Somsen GW, de Jong GJ (2009) Electrophoresis 30:276–291
13. Ramautar R, Mayboroda OA, Somsen GW, de Jong GJ (2011) Electrophoresis 32:52–65
14. Kok MGM, de Jong GJ, Somsen GW (2011) Electrophoresis 32:3016–3024
15. Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, Hau DD, Psychogios N, Dong E, Bouatra S, Mandal R, Sinelnikov I, Xia J, Jia L, Cruz JA, Lim E, Sobsey CA, Shrivastava S, Huang P, Liu P, Fang L, Peng J, Fradette R, Cheng D, Tzur D, Clements M, Lewis A, De Souza A, Zuniga A, Dawe M, Xiong Y, Clive D, Greiner R, Nazyrova A, Shaykhutdinov R, Li L, Vogel HJ, Forsythei I (2009) Nucleic Acids Res 37:D603–D610
16. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G (2005) Ther Drug Monit 27:747–751
17. Horai H, Arita M, Kanaya S, Nihei Y, Ikeda T, Suwa K, Ojima Y, Tanaka K, Tanaka S, Aoshima K, Oda Y, Kakazu Y, Kusano M, Tohge T, Matsuda F, Sawada Y, Hirai MY, Nakanishi H, Ikeda K, Akimoto N, Maoka T, Takahashi H, Ara T, Sakurai N, Suzuki H, Shibata D, Neumann S, Iida T, Tanaka K, Funatsu K, Matsuura F, Soga T, Taguchi R, Saito K, Nishioka T (2010) J Mass Spectrom 45:703–714
18. Nevedomskaya E, Derks R, Deelder AM, Mayboroda OA, Palmblad M (2009) Anal Bioanal Chem 395:2527–2533
19. Garcia-Perez I, Vallejo M, Garcia A, Legido-Quigley C, Barbas C (2008) J Chromatogr A 1204:130–139
20. Ramautar R, Sastre Toraño J, Somsen GW, de Jong GJ (2010) Electrophoresis 31:2319–2327
21. Warrack BM, Hnatyshyn S, Ott K, Reily MD, Sanders M, Zhang H, Drexler DM (2009) J Chromatogr B 877:547–552
22. Ryan D, Robards K, Prenzler PD, Kendall M (2011) Anal Chim Acta 684:17–29
23. Swann JR, Want EJ, Geier FM, Spagou K, Wilson ID, Sidaway JE, Nicholson JK, Holmes E (2011) Proc Natl Acad Sci U S A 108:4523–4530
24. Martin FJ, Dumas M, Wang Y, Legido-Quigley C, Yap IKS, Tang H, Zirah S, Murphy GM, Cloarec O, Lindon JC, Sprenger N, Fay LB, Kochhar S, van Bladeren P, Holmes E, Nicholson JK (2007) Mol Syst Biol 3:112