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Comparison of capillary electrophoresis–mass spectrometry and hydrophilic interaction chromatography–mass spectrometry for anionic metabolic profiling of urine

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ABSTRACT

In order to assess the utility of a recently developed capillary electrophoresis–mass spectrometry (CE–MS) method for the study of anionic metabolites in urine, a comparison was made with hydrophilic interaction chromatography–MS (HILIC–MS) using negative electrospray ionization. After optimization of the HILIC conditions, a gradient employing 10 mM ammonium acetate (pH 6.8) in acetonitrile–water (5 min 90% acetonitrile followed by 90%–50% acetonitrile in 10 min) was selected, providing baseline separation of five representative anionic test metabolites. Relative standard deviations (RSDs) for HILIC retention times and peak areas were below 0.2% and 7.7%, respectively, and detection limits were in the range 0.04–2.21 μM. Metabolites in rat urine could also be analysed in a reproducible way with retention time and peak area RSDs below 0.6% and 13.6%, respectively. The CE–MS and HILIC–MS methods were compared in terms of reproducibility, sensitivity, selectivity and coverage of the anionic urinary metabolome. In general, peak area RSDs were similar whereas HILIC–MS yielded better retention-time repeatability and up to 80 times lower detection limits (expressed in injected concentration) for test metabolites as compared to CE–MS. Rat urine analysis by HILIC–MS provided detection of 1360 molecular features compared to 347 molecular features revealed with CE–MS. Of these, a number of 144 molecular features were found with both HILIC–MS and CE–MS, which showed on average 10 times higher peak areas in HILIC–MS. The HILIC retention and CE migration times of the common features were clearly not correlated. The HILIC and CE behavior of the test metabolites and 16 putatively identified common features were evaluated involving their physicochemical properties, indicating a markedly different separation selectivity, and thus significant degree of orthogonality of HILIC and CE.
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1. Introduction

Elucidation and understanding of biochemical pathways require accurate and specific determination of metabolite levels in biofluids and tissues. Metabolites may exhibit very diverse physicochemical properties and can be present in a wide range of concentrations [1]. Capillary electrophoresis (CE) coupled to mass spectrometry (MS) is particularly suitable for the direct profiling of highly polar and charged metabolites as predominantly present in urine. CE can provide efficient separation of metabolites based on their charge-to-size ratios without the need for analyte derivatization [2]. Recently, we have developed a CE–MS method for the profiling of anionic metabolites applying triethylamine in the background electrolyte (BGE) and sheath liquid [3]. This new method provided significantly higher signal intensities as compared to other CE–MS methods in negative ionization mode. Urine samples from antibiotic-treated rats and control rats could be distinguished, and potential biomarkers were revealed [4]. Having this new method available, the question arises to what extent CE–MS can provide complementary information on the anionic metabolome with respect to more common liquid chromatographic (LC) techniques for metabolic profiling.

LC–MS can provide information on the quantity of low-abundant metabolites without the need for analyte derivatization. Common reversed-phase (RP) LC, however, is less suited for the analysis of highly polar compounds, and for small charged

Abbreviations: BGE, background electrolyte; CE:, capillary electrophoresis; ESI:, electrospray ionization; GC:, gas chromatography; HILIC:, hydrophilic interaction chromatography; LC, liquid chromatography; LOD, limit of detection; MS, mass spectrometry; RP:, reversed-phase; RSD, relative standard deviation; TOF, time-of-flight

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compounds, ion-pairing agents are frequently used to increase their retention [5]. Unfortunately, ion-pairing agents can cause substantial ionization suppression of compounds in MS and source contamination. Hydrophilic interaction chromatography (HILIC) allows profiling of polar compounds, providing complementary information to RPLC, as has been demonstrated by numerous metabolomics studies [6–19]. HILIC utilizes a highly organic mobile phase (often acetonitrile) containing water in combination with a polar stationary phase. Analyte separation is based on partitioning between the mobile phase and a layer of adsorbed water molecules on the stationary phase as well as electrostatic interactions with polar groups on the stationary phase [20–22]. Hence, in order to appreciate the utility of CE–MS for profiling of anionic metabolites, comparison with HILIC–MS seems indicated.

So far, only a few studies have been conducted in which both CE–MS and HILIC–MS were used for comparative and comprehensive profiling [17,23–26]. Sugimoto *et al.* applied HILIC–MS and CE–MS to analyse edamame and Japanese sake [23,24]. However, as HILIC–MS was used to profile sugars only, no conclusions could be drawn on the complementarity of HILIC–MS and CE–MS for untargeted metabolite profiling. Büscher and colleagues extensively compared CE, LC and gas chromatography (GC) methodologies, all in combination with MS [25]. Metabolites were analysed with GC–MS after two different derivatization procedures. Ion-pair RPLC and HILIC were used as LC separation modes and two CE–MS methods were employed for the analysis of cationic and anionic metabolites. A mixture of 91 test metabolites representing central carbon and energy metabolism was used for comparison. Of these test metabolites, 33 compounds could be detected by all three platforms. CE and LC showed the greatest overlap in metabolite coverage (26 compounds) and each analytical technique was capable to measure two compounds which could not be detected with the two other methodologies. The CE–MS, LC–MS and GC–MS methodologies were considered in general, that is, GC, HILIC, ion-pair RPLC and cationic and anionic CE methods were not individually compared. Moreover, the evaluation of the complementarity of the techniques for metabolic profiling was limited since it was based on a confined number of test metabolites in standard solutions [25]. Ibáñez and coworkers studied the effect of dietary polyphenols on the proliferation of colon cancer cells with CE–MS, HILIC–MS and RPLC–MS [26]. Saric *et al.* used the same techniques to analyse the metabolome of the *Fasciola hepatica* worm [17]. In both studies, a small part of the detected metabolite features was identified. There was no or a limited overlap of identified metabolites detected by CE–MS, HILIC–MS and RPLC–MS, indicating the potential complementarity of the analytical techniques [17,26]. It should be noted, however, that Ibáñez *et al.* as well as Saric *et al.* employed positive electrospray ionization (ESI) for HILIC–MS and/or CE–MS, and thus could only compare the methodologies based on detected cationogenic compounds. Still, a significant number of urinary metabolites is acidic and can only be detected using negative ESI. Therefore, in order to achieve comprehensive profiling and to evaluate the complementarity of HILIC–MS and CE–MS, particular attention should also be paid to anionic metabolites.

In the present study, we compared a previously optimized CE–MS method with HILIC–MS for anionic metabolic profiling of urine samples. We first optimized a HILIC–MS method by carrying out infusion experiments to determine and evaluate the effect of different HILIC mobile phase compositions on the signal intensities of representative anionic test metabolites. An efficient gradient HILIC–MS method applying an acetonitrile–water mobile phase containing ammonium acetate was developed and the performance was assessed in terms of sensitivity, linearity and repeatabilities of peak area and retention time. Furthermore, HILIC–MS was applied to urine analysis and the number of detected molecular features of urine components was determined. Outcomes were extensively compared with results obtained with the earlier developed CE–MS method for anionic urinary profiling [3,4]. Numbers of common and unique urinary molecular features were considered and MS responses of common features were compared. Moreover, differences in separation selectivity of CE–MS and HILIC–MS were assessed by comparing migration and retention times of test metabolites as well as putatively identified common features.

2. Materials and methods

2.1. Chemicals

Acetic acid, ammonium formate, ammonium hydroxide (25% solution), acetonitrile, formic acid, methanol, glutaric acid, hippuric acid, DL-pyroglutamic acid and uridine were obtained from Fluka (Steinheim, Germany). Sodium hydroxide and L-proline were purchased from Sigma Aldrich (Steinheim, Germany). Triethylamine was from Fisher Scientific (Loughborough, UK), piperidine was from Alfa Aesar (Karlsruhe, Germany) and ammonium acetate was supplied by Merck (Darmstadt, Germany). Water was deionized and purified with a Milli-Q purification system (Millipore, Bedford, USA) prior to use.

2.2. Test mixture and rat urine sample

Stock solutions (50 mM) of the metabolites glutaric acid, hippuric acid, proline, pyroglutamic acid and uridine were prepared in deionized water. Stock solutions of the metabolites were mixed and diluted to obtain a test mixture in which each metabolite was present at the appropriate concentration (0.25–80 μM). Test metabolite mixtures were prepared in water–acetonitrile (1:4, v/v) and water for HILIC–MS and CE–MS analyses, respectively.

A mixture of aliquots of rat urine samples provided by AstraZeneca (Department of Drug Metabolism and Pharmacokinetics, Macclesfield, UK) [27] was prepared and stored at −80 °C. Prior to analysis, the urine sample was thawed. For HILIC–MS, urine was diluted with acetonitrile in a proportion of 1:4 (v/v) and centrifuged at 10,000 rcf for 10 min in order to attain proper metabolite peak shapes (see Section 3.1). When analysed with CE–MS, dilution of urine with BGE in a proportion of 1:1 (v/v) was sufficient to achieve good CE performance [3,4].

2.3. HILIC–MS

All samples were analysed on an LC system (Shimadzu, Kyoto, Japan) coupled online via an electrospray interface to a time-of-flight (TOF) mass spectrometer (micrOTOF, Bruker Daltonics, Bremen, Germany) using a Waters XBridge™ Amide column (3.5 μm, 3.0 × 100 mm).

In the optimized HILIC–MS method, the test metabolite mixture and urine sample (5 μL; approximately 1.5% of column volume) were injected and analysed under gradient elution with mixing solvent A (10 mM ammonium acetate in water–acetonitrile (1:1, v/v)) and solvent B (10 mM ammonium acetate in water–acetonitrile (1:9, v/v)) in varying ratios at an overall flow rate of 0.5 mL/min. The gradient scheme was as follows: 0.0–5.0 min, 100% B; 5.0–15.0 min, from 100% B to 100% A; 15.0–20.0 min, 100% A; 20.1–30.0 min, 100% B. Column temperature was 45 °C during separation.

Optimal signal intensities for test metabolites were obtained using the following interface conditions: dry gas temperature, 180 °C; dry gas flow, 4 L/min, nebulizer pressure, 50 psi; ESI voltage, 2 kV. Data were acquired in negative ionization mode in the mass range *m/z* 50–800 with a repetition rate of 1 Hz.

Recorded mass spectra were internally calibrated using sodium acetate clusters which were detected within every run.

2.4. CE–MS

The CE–MS experiments were performed and described before [3,4]. Briefly, CE–MS was carried out on a Beckman P/ACE MDQ instrument (Beckman Coulter, Fullerton, USA) coupled to a micrOTOF mass spectrometer using a sheath-liquid electrospray interface from Agilent Technologies (Waldbronn, Germany). A BGE of 25 mM triethylamine (pH 11.7) and a sheath liquid of 5 mM triethylamine in water–methanol (1:1, v/v) were used. Fused-silica capillaries (Polymicro Technologies, Phoenix, USA) had a total length of 100 cm and an internal diameter of 50 μm. Hydrodynamic injections were performed using a pressure of 0.5 psi for 30 s (16 nL; approximately 0.8% of capillary volume). The separation voltage was 30 kV and the capillary temperature was set at 20 °C. The sheath liquid was delivered with a flow rate of 5 μL/min by a 10 mL gas-tight syringe of Hamilton (Reno, USA) using a syringe pump (KD Scientific, Holliston, USA). The following optimized CE–MS interface conditions were applied: dry gas temperature, 180 °C; dry gas flow, 4 L/min; nebulizer pressure, 10 psi; ESI voltage, 4 kV. The negative ionization mode was used and data were acquired in the mass range 50–800 *m/z* with a rate of 1 Hz. Sodium formate clusters were used for internal calibration.

2.5. Data analysis

Data were processed using the DataAnalysis software of Bruker Daltonics. Using the 'Find Molecular Features' function of this software, the number of molecular features obtained during urine analysis was determined. A molecular feature was defined as the unique combination of an *m/z* value and retention time (HILIC–MS) or migration time (CE–MS), present in ten consecutive MS spectra with a signal-to-noise ratio of at least three. Peak areas and peak heights of metabolites were assessed using extracted-ion chromatograms (HILIC–MS) or electropherograms (CE–MS).

3. Results and discussion

3.1. HILIC–MS method

For the optimization of the HILIC–MS method for anionic metabolic profiling, the composition of the mobile phase was studied. Ammonium acetate, sometimes in combination with acetic or formic acid, is often used as mobile phase additive in HILIC–MS for urinary metabolic profiling studies [28]. In a previous CE–MS study, we found triethylamine and piperidine to be more favourable salts with respect to attainable signal intensities for anionic metabolites [3]. Infusion experiments were performed to study the influence of several mobile phase additives on signal intensities of the test metabolites glutaric acid, hippuric acid, proline, pyroglutamic acid and uridine. These metabolites represent the diverse compound classes present in urine. The metabolites (100 μM each) were dissolved in acetonitrile–water (1:1, v/v) with and without 10 mM ammonium acetate, 10 mM ammonium formate, 0.1% formic acid, 0.1% triethylamine or 0.1% piperidine. Using ammonium formate and formic acid, test metabolite signals were lower as obtained with ammonium acetate. With triethylamine, the signal intensities of the tested metabolites were on average 6.5 times higher compared to ammonium acetate. Similar results were obtained using piperidine with 6.3 times higher signals. However, the use of triethylamine and piperidine as mobile phase additives in HILIC–MS resulted in reduced retention times (too fast elution) and broad and asymmetric peaks. Overall this led to reduced plate numbers and poor metabolite resolution. Furthermore, proline and pyroglutamic acid could not be detected. Therefore, ammonium acetate was selected and the influence of its concentration (0–10 mM) in a mobile phase of acetonitrile–water was further evaluated. The separation, peak shapes and signal intensities of the five test metabolites obtained with HILIC–MS were taken into consideration. A concentration of 10 mM ammonium acetate in the eluent yielded more symmetric and narrow peaks as compared to 0 and 5 mM ammonium acetate. This was especially apparent for the compounds uridine and hippuric acid of which the peak widths were about 40% smaller at 10 mM ammonium acetate. Furthermore, only at 10 mM ammonium acetate, baseline separation of proline and pyroglutamic acid was achieved. Still, the increase of ammonium acetate concentration from 0 to 10 mM resulted in up to 59% lower signal intensities, except for proline which showed a remarkable signal increase of 67%. Adjusting the pH of the mobile phase to 9.0 with ammonium hydroxide did not affect the separation of the test metabolites, but decreased their signal intensities up to 60% as compared to pH 6.8 (not adjusted) due to ion suppression.

The acetonitrile–water gradient of the mobile phase containing 10 mM ammonium acetate (pH 6.8) was optimized to obtain a large separation window without deteriorating the peak shape and detection limits (LODs) of metabolites. A high percentage of acetonitrile in the mobile phase at the start of the analysis is desirable in order to maximize the separation of metabolites. Therefore, the start of the gradient was set at 90% acetonitrile and held at 90% for 5 min to enhance the separation of metabolites eluting in the beginning of the chromatogram. Then, the percentage acetonitrile was gradually decreased to 50% and different slopes of the gradient were tested to maximize peak separation and minimize the peak widths. A gradient of 4% acetonitrile per min appeared to be optimum by providing the best resolution of metabolite peaks within a relatively short analysis time. The mobile phase was held at 50% acetonitrile for another 5 min in order to elute all compounds from the column. Thereafter, the percentage acetonitrile in the mobile phase was switched back to 90% and hold for 10 min to allow reequilibration of the HILIC–MS system.

Because of the high percentage of acetonitrile at the start of the gradient, the sample injection solvent had to contain a large amount of acetonitrile to prevent peak splitting and to achieve narrow and symmetric peaks. The maximum allowable acetonitrile percentage in the sample is, however, limited by the solubility of polar and charged compounds. More than 80% acetonitrile in the sample solvent resulted in precipitation of metabolites. Moreover, dilution of the sample with acetonitrile will reduce the concentration sensitivity. Using a sample injection volume of 5 μL containing 80% acetonitrile in combination with a gradient starting at 90% acetonitrile resulted in narrow and symmetric peaks. Injection volumes of 7.5 μL and larger, or a gradient starting at 95% acetonitrile, gave deformed and splitted peaks.

The optimized HILIC-MS method comprised gradient elution employing 10 mM ammonium acetate (pH 6.8) in acetonitrile–water, an injection solvent containing 80% acetonitrile and an injection volume of 5 μL.

3.2. Comparison of CE–MS and HILIC–MS

3.2.1. Reproducibility, sensitivity and metabolite coverage

Using the optimized HILIC–MS method, the test metabolites were baseline separated with retention times ranging from 3.1 to 13.5 min (Fig. 1). The retention times were highly reproducible for all metabolites with relative standard deviations (RSDs) below 0.2%. Also peak area repeatability was acceptable with RSD values below 8% for ten consecutive measurements (Table 1). Mixtures of

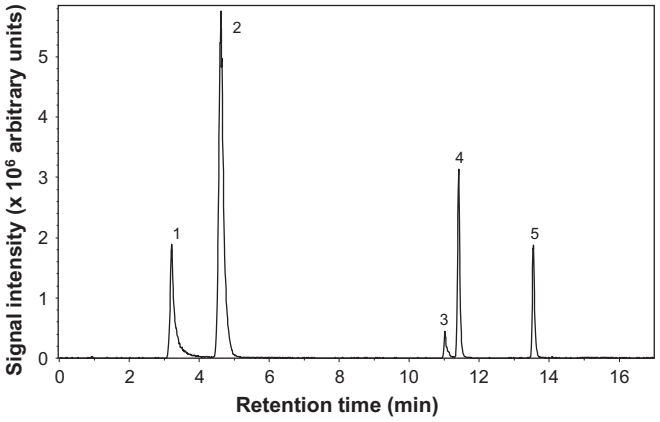

Fig. 1. Multiple extracted-ion chromatogram obtained during HILIC–MS of a test metabolite mixture (50 μM each). Metabolites: 1. uridine (*m/z* 243.06); 2. hippuric acid (*m/z* 178.05); 3. proline (*m/z* 114.06); 4. pyroglutamic acid (*m/z* 128.04); 5. glutaric acid (*m/z* 131.04). Gradient elution with (a) 10 mM ammonium acetate in water–acetonitrile (1:1, v/v) and (b) 10 mM ammonium acetate in water–acetonitrile (1:9, v/v): 0–5 min, 100% b; 5–15 min, from 100% b to 100% a; 15–20 min, 100% a. See Section 2 for other experimental conditions.

Table 1
Retention/migration time and peak area repeatability (RSD (%), n = 10) for five test metabolites analysed using HILIC–MS and CE–MS.

Compound	Retention/migration time		Peak area	
	HILIC–MS	*CE–MS*	*HILIC–MS*	*CE–MS*
Glutaric acid	0.03	0.33	7.70	10.83
Hippuric acid	0.13	0.38	5.26	6.57
Proline	0.03	0.34	2.42	6.08
Pyroglutamic acid	0.04	0.42	6.14	7.25
Uridine	0.16	0.36	4.56	3.97

the five test metabolites in the concentration range 0.25–80 μM were analysed to determine the linearity of the method. For all compounds, a good linearity was observed with coefficients of determination (R^2) above 0.99. LODs (signal-to-noise ratio of 3) for the test metabolites were in the range 0.04–2.21 μM (Table 2).

The anionic test metabolites have previously been analysed with an optimized CE–MS method employing a triethylamine-containing BGE and sheath liquid [3]. In CE–MS, all metabolites were baseline separated with migration times from 7.0 to 17.5 min. RSDs for elution/migration time of the test compounds were lower with HILIC–MS, but repeatability for peak areas was similar for most metabolites (Table 1). Using CE–MS, detection limits for the five test metabolites ranged from 0.92 to 9.08 μM [3]. Thus, the LODs obtained with HILIC–MS were up to 80 times lower for these compounds (Table 2). Most likely, this can be explained by the larger sample injection volume employed in HILIC (5 μL) as compared to CE (16 nL). The injection volumes relative to capillary and column volume are approximately 0.8% and 1.5%, respectively. Notably, the sensitivity difference is not as severe as supposed from the difference in injection volume (factor 300) only. Furthermore, the ionization efficiencies of the test metabolites may be relatively higher under the applied CE–MS conditions. The observed difference in signal-to-noise ratios between HILIC–MS and CE–MS was not the same in magnitude for each test metabolite. Noise levels varied extensively throughout the HILIC chromatograms and CE electropherograms. Furthermore, ionization conditions in HILIC–MS vary due to the gradient elution leading to different ionization efficiencies.

In order to examine the applicability of the optimized HILIC–MS and CE–MS method for the analysis of biological samples, a rat urine sample was repetitively analysed. Notably, in CE–MS urine samples were diluted with BGE in a proportion of 1:1 (v/v), whereas for HILIC–MS a dilution of 1:4 (v/v) with acetonitrile was required for proper HILIC separation. Based on the analysis of rat urine, the CE–MS and the HILIC–MS method were compared in terms of number of observed molecular features, analyte elution/migration order and repeatability. The base-peak chromatograms and electropherograms obtained with the optimum HILIC–MS and CE–MS method showed large numbers of peaks, which were widely spread over the elution/migration time window (Figs. 2A and C). Overall, the absolute signal intensities were higher with HILIC–MS. HILIC–MS provided almost four times more molecular features than CE–MS (1360 vs. 347 features). Five molecular features, which were detected with both systems (same *m/z* value) and which were spread over the elution/migration window (Figs. 2B and 2D), were selected to assess repeatability. Ten consecutive measurements of the urine sample showed that the elution time repeatabilities of these urine metabolites were better for HILIC–MS (0.17-0.55%) with respect to CE–MS (5.93–11.8%), whereas peak area repeatabilities were similar (Table 3). As demonstrated before [4], CE–MS migration time repeatability for the rat urine components could be reduced to $< 0.4\%$ by applying a proper alignment procedure.

Table 2
Limits of detection (μM) for five test metabolites applying HILIC–MS and CE–MS.

Compound	HILIC–MS	CE–MS[a]	Factor difference
Glutaric acid	0.75	9.08	12.1
Hippuric acid	0.05	0.92	18.4
Proline	2.21	3.99	1.8
Pyroglutamic acid	0.04	3.21	80.3
Uridine	0.06	1.61	26.8

Test metabolites were dissolved in acetonitrile-water (4:1, v/v) and water using HILIC–MS and CE–MS, respectively

[a] Previously reported by Kok *et al.* [3].

Taken all molecular features into account, proportionately more molecular features with *m/z* values below 200 were detected with CE–MS, whereas the relative number of features with *m/z* values above 400 was higher in HILIC–MS (Fig. 3). To compare the two analytical methodologies more extensively, molecular features which were detected with both CE–MS and HILIC–MS (common molecular features) were determined. Molecular features were considered to be common if the difference of the respective *m/z* values was less than 10 mDa. An *m/z* value only detected with one of the two methods was regarded as a unique molecular feature for the respective method. Based on these criteria, HILIC–MS and CE–MS revealed 1216 and 203 unique features, respectively, and 144 common molecular features were found with *m/z* values ranging from 108 to 503 of which most had *m/z* values below 300. There were 28 isobaric compounds among the 144 common molecular features. These features could not be assigned unambiguously based on mass only and were therefore dismissed during further evaluation. The ratios of peak areas and peak heights of the remaining 116 common molecular features were calculated to compare the signal intensities obtained with HILIC–MS and CE–MS (Fig. 4). The ratios of peak area and height ranged from 0.20–63.9 and 0.59–130.5, and were on average 10.1 and 16.3, respectively. For 11 compounds, peak abundances in CE–MS were even higher than obtained with HILIC–MS. The large variance in intensity ratio between HILIC–MS and CE–MS might be caused by varying degrees of ionization suppression. As the separation principles of HILIC and CE are so manifestly different, the co-migration of urinary compounds and thus ionization conditions may vary strongly along the elution/migration window.

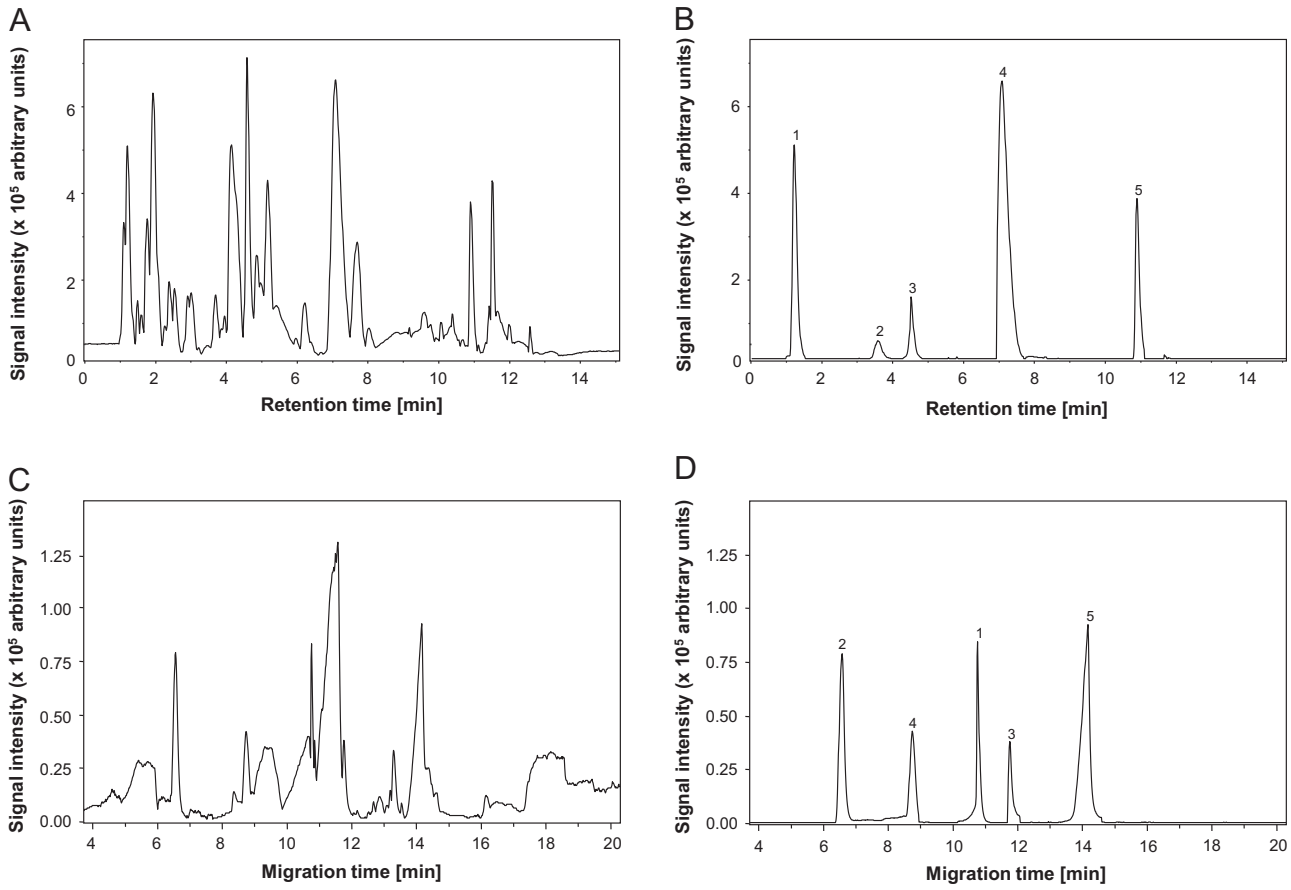

Fig. 2. Base-peak and multiple extracted-ion chromatograms and electropherograms obtained when analysing a rat urine sample by HILIC–MS (A, B) and CE–MS (C, D). Peaks: 1. *m/z* 212.00; 2. *m/z* 112.05; 3. *m/z* 124.99; 4. *m/z* 243.06; 5. *m/z* 167.02. HILIC–MS conditions: gradient elution with (a) 10 mM ammonium acetate in water–acetonitrile (1:1, v/v) and (b) 10 mM ammonium acetate in water–acetonitrile (1:9, v/v): 0–5 min, 100% b; 5–15 min, from 100% b to 100% a; 15–20 min, 100% a. CE–MS conditions: BGE, 25 mM triethylamine (pH 11.7); sheath liquid, 5 mM triethylamine in water–methanol (1:1, v/v). See Section 2 for other experimental conditions.

Table 3
Retention/migration time and peak area repeatability (RSD (%), n=10) for five metabolites in rat urine detected with HILIC–MS and CE–MS.

m/z value	Retention/migration time		Peak area	
	HILIC–MS	*CE–MS*[a]	*HILIC–MS*	*CE–MS*[a]
212.00	0.17	10.8	7.89	13.3
112.05	0.29	5.93	11.6	8.58
124.99	0.55	10.8	13.6	13.2
243.06	0.39	8.09	10.5	13.5
167.02	0.23	11.8	7.68	7.85

[a] Previously reported by Kok *et al.* [4].

3.2.2. Separation selectivity

In order to appreciate the observed differences in selectivity between CE–MS and HILIC–MS, the elution and migration behavior of the test metabolites and common features in rat urine were evaluated, involving – when possible – the molecular structure of the compounds. The observed elution order of the test metabolites in HILIC roughly follows compound polarity (Fig. 1). Uridine is the only test metabolite that is uncharged at the applied pH of 6.8, which may explain its fast elution. The aromatic ring of hippuric acid reduces its overall polarity leading to modest retention in HILIC. Proline and pyroglutamic acid have a rather polar structure providing significant retention. The dicarboxylic glutaric acid is highly polar and strongly retained in HILIC. In CE–MS, proline migrated first, followed by successively uridine, hippuric acid, pyroglutamic acid and glutaric acid [3], exhibiting a different order than observed during HILIC–MS. The CE migration order largely follows the overall negative charge of the compounds at pH 11.7. The amino group of proline is partly protonated, whereas glutaric acid is doubly charged at pH 11.7.

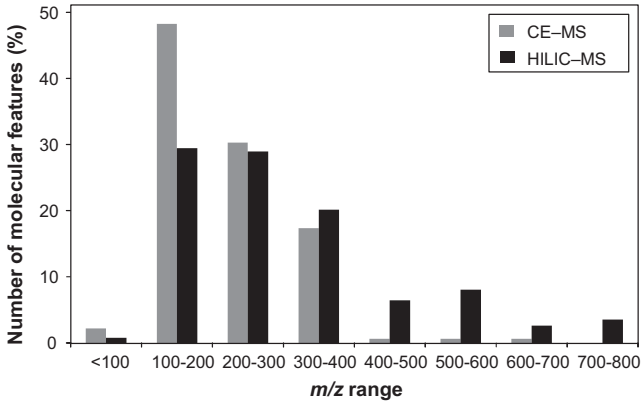

Fig. 3. Distribution of *m/z* values of molecular features detected during CE–MS and HILIC–MS of a rat urine sample. The relative number of features (%) in the various *m/z* ranges is depicted taking the total number of molecular features detected with the respective analytical method as 100%.

The distinctive separation selectivity of CE and HILIC was strongly underlined by plotting the CE–MS migration times and the HILIC–MS retention times of the 116 common features observed during the analysis of rat urine (Fig. 5). Compounds are

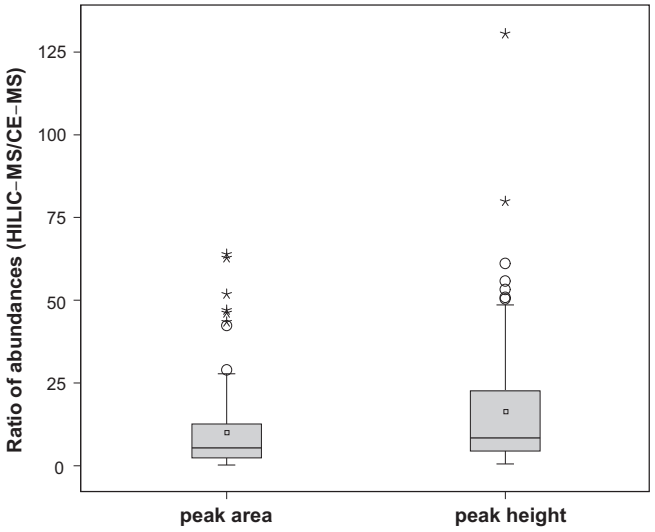

Fig. 4. Box plot of the ratio of peak areas and peak heights of 116 common molecular features in a rat urine sample detected with both HILIC–MS and CE–MS. The 25th percentile, median and 75th percentile are represented by the boxes. The whiskers indicate the 5th and 95th percentile and the squares represent the average ratio of the peak areas and peak heights, respectively. Circles indicate ratios higher than 95% of the observed ratios and asterisks symbolize outliers.

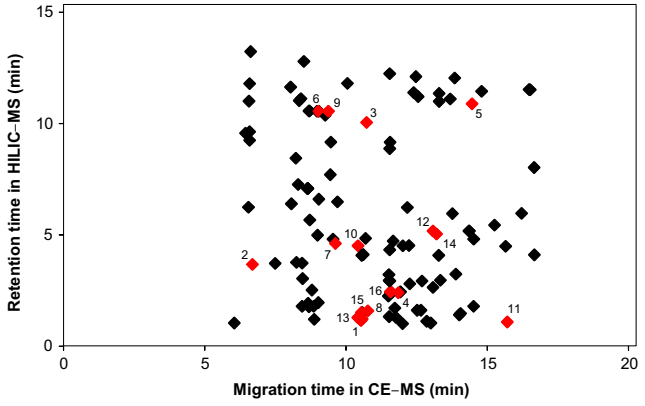

Fig. 5. Relation between migration times and retention times of common molecular features obtained during CE–MS and HILIC–MS of a rat urine sample. The red diamonds with numbers represent metabolites which were putatively identified based on accurate mass. The identities together with the corresponding numbers are given in Table 4.

scattered over the entire plot, indicating a clear lack of correlation between CE migration and HILIC retention. To explore the observed differences in separation selectivity of CE and HILIC in more molecular detail, the *m/z* values of the common features were searched against metabolomics databases [29–31]. This way, 16 molecular features could be assigned based on accurate mass. These features included typical urinary compounds, such as allantoin, glucuronic acid, hippuric acid, indoxyl sulfate, phenol and uric acid (Table 4 and red diamonds in Fig. 5). As can be expected, all identified compounds, except creatinine, have an acidic group allowing their negative ionization.

The CE and HILIC migration and retention data of the identified metabolites in Table 4 confirm the different selectivity of the methods. For example, the sulfonic acids cresol sulfate, benzenediol sulfate, methoxycatechol sulfate and indoxyl sulfate were hardly retained and not separated in HILIC–MS, whereas in CE–MS these compounds showed considerable migration times. Moreover, benzenediol sulfate could be separated from the other sulfonic acids in CE, most probably because it carries relatively more negative charge due to deprotonation of its phenol group. In

Table 4
Retention (RT) and migration times (MT) of putatively identified urinary metabolites detected with both HILIC–MS and CE–MS.

#	*m/z* Value	RT ($_{min}$) HILIC–MS	MT ($_{min}$) CE–MS	Putative identity
1	93.03	1.14	10.67	Phenol
2	112.05	3.59	6.51	Creatinine
3	124.01	10.05	10.73	Taurine
4	157.09	2.39	11.86	Allantoin
5	167.02	10.89	14.47	Uric acid
6	168.08	10.55	8.99	Methylhistidine
7	178.05	4.61	9.64	Hippuric acid
8	187.01	1.48	10.78	Cresol sulfate
9	187.07	10.55	9.38	Glycylhydroxyproline
10	188.03	4.50	10.43	Kynurenic acid
11	188.99	1.08	15.72	Benzenediol sulfate
12	193.05	5.17	13.09	Glucuronic acid
13	203.00	1.20	10.67	Methoxycatechol sulfate
14	204.03	5.03	13.21	Xanthurenic acid
15	212.00	1.23	10.70	Indoxyl sulfate
16	276.14	2.43	11.58	Methionine–lysine

contrast, taurine, also comprising a sulfonic acid group, could only be separated from the other sulfonic acids in HILIC. In CE–MS, uric acid (diprotic) and its oxidation product allantoin (monoprotic) were separated based on their charge-to-size ratios. For these compounds, a large difference in retention times was observed in HILIC–MS, although the molecular structures of allantoin and uric acid are highly similar. Another example is the separation of compounds having a carboxylic group. Methylhistidine, hippuric acid, glycylhydroxyproline, kynurenic acid, glucuronic acid, xanthurenic acid and methionine–lysine had retention times between 2.43 and 10.55 min, and migration times ranged from 8.99 to 13.21 min. The elution order of these metabolites in the CE system differed completely from their elution order in HILIC. Furthermore, despite the apparent larger separation window in the optimized HILIC method, methylhistidine and glycylhydroxyproline coeluted, whereas separation of these compounds was achieved in CE.

4. Concluding remarks

In order to assess the added value of CE–MS for anionic metabolic profiling of urine, a previously developed CE–MS method was compared to an optimized HILIC–MS method using ammonium acetate as mobile phase additive. In general, higher signal intensities were obtained with HILIC–MS compared to CE–MS. This is mainly due to the considerably larger injection volume applied in HILIC–MS. Still, the average difference in analyte response between CE–MS and HILIC–MS is much less than estimated from injection volume only, indicating overall higher absolute sensitivity in CE–MS. Notably, CE–MS can be particularly useful when volume-limited samples have to be analysed. The separation selectivities of CE–MS and HILIC–MS appeared to be considerably different, as demonstrated by the low correlation between CE migration and HILIC retention of urinary metabolites. The unique detection of 203 and 1216 molecular features in rat urine by CE–MS and HILIC-MS, respectively, further demonstrated the different selectivity of the methods. Hence, although HILIC–MS revealed significantly more molecular features than CE–MS, there is a clear added value for CE–MS. In metabolomics studies, CE–MS could aid in finding discriminatory compounds (e.g. potential biomarkers) and help to elucidate metabolic pathways.

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