Journal of Chromatography B, 871 (2008) 357–361

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

An approach to enhancing coverage of the urinary metabonome using liquid chromatography–ion mobility–mass spectrometry☆

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ARTICLE INFO

Article history:
Received 21 February 2008
Accepted 28 April 2008
Available online 8 May 2008

Keywords:
Ion mobility–mass spectrometry
Reversed-phase HPLC
Metabonomics
Urinary metabolome

ABSTRACT

The potential of drift tube ion mobility (IM) spectrometry in combination with high performance liquid chromatography (LC) and mass spectrometry (MS) for the metabonomic analysis of rat urine is reported. The combined LC–IM–MS approach using quadrupole/time-of-flight mass spectrometry with electrospray ionisation, uses gas-phase analyte characterisation based on both mass-to-charge (m/z) ratio and relative gas-phase mobility (drift time) following LC separation. The technique allowed the acquisition of nested data sets, with mass spectra acquired at regular intervals (65 μs) during each IMS separation (~13 ms) and several IMS spectra acquired during the elution of a single LC peak, without increasing the overall analysis time compared to LC–MS. Preliminary results indicate that spectral quality is improved when using LC–IM–MS, compared to direct injection IM–MS, for which significant ion suppression effects were observed in the electrospray ion source. The use of reversed-phase LC employing fast gradient elution reduced sample preparation to a minimum, whilst maintaining the potential for high throughput analysis. Data mining allowed information on specific analytes to be extracted from the complex metabonomic data set. LC–IM–MS based approaches may have a useful role in metabonomic analyses by introducing an additional discriminatory dimension of ion mobility (drift time).

1. Introduction

A number of analytical methods have been employed to produce metabolic signatures of biomaterials. The main techniques currently used in metabonomic studies are high resolution nuclear magnetic resonance (NMR) spectroscopy [1–4], gas chromatography–mass spectrometry (GC–MS) [4–6], liquid chromatography–mass spectrometry (LC–MS) [4,7–9], including ultra performance liquid chromatography (UPLC) [10], and capillary electrophoresis–mass spectrometry (CE–MS) [11] (for a recent review of analytical strategies in this area see [12]). All these techniques result in complex multivariate data sets, which in turn require bioinformatic methods for visualisation and interpretation. There are various advantages in employing NMR in metabonomic/metabolomic studies because the technique is non-destructive and applicable to the direct analysis of biofluid samples and intact biomaterials, with minimal or no sample preparation, for example the direct analysis of tissue employing magic angle spinning NMR [13]. NMR spectroscopy also has good potential for structure determination. However, there are limitations for NMR-based metabonomic studies including issues around sensitivity (>1 nmol metabolite for ^{1}H NMR detection) and the inability to detect some nuclei (e.g. O and S). MS is inherently more sensitive than NMR (though sensitivity is compound dependant) and also has the potential for molecular identification using tandem MS fragmentation or accurate mass determination.

Ion mobility (IM) spectrometry is a gas-phase electrophoretic technique which allows ionised analytes to be characterised on the basis of their ion mobility (K) in the presence of a buffer gas and under the influence of a weak electric field. K is defined by the reduced mass (μ), charge (e) and collision cross section (Ω) (i.e. size and shape) of the ion Eq. (1) [14].

$$K = \left(\frac{3ze}{16N}\right)\left(\frac{2\pi}{\mu k_B T}\right)^{1/2}\left(\frac{1}{\Omega_D}\right) \quad (1)$$

where N is the number density of the buffer gas, k_B is the Boltzmann constant and T is the temperature. The drift time (t_d) of an ion is the time taken to traverse a drift cell (of length l), under the influence

☆ This paper is part of a special volume entitled "Hyphenated Techniques for Global Metabolite Profiling", guest edited by Georgios Theodoridis and Ian D. Wilson.

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doi:10.1016/j.jchromb.2008.04.043

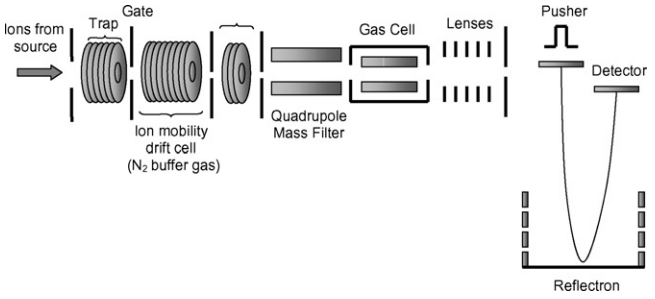

Fig. 1. Schematic diagram of the prototype IM–Q–TOF-MS spectrometer.

of the electric field (E) and in the presence of the buffer gas, and is determined by the mobility of the ion, so mixtures of analyte ions may be separated on the basis of their relative drift times.

$$t_d = \frac{l}{KE} \quad (2)$$

The principles and applications of IM have been the subject of several recent reviews [15–17].

The combination of ion mobility spectrometry with mass spectrometry (IM–MS), as shown in Fig. 1, allows ions generated in the ion source to be separated initially by their mobility in the low pressure drift cell and then by mass-to-charge ratio in the fast scanning time-of-flight (TOF) mass analyser. The coupling of these complementary techniques therefore provides a multidimensional separation of gas-phase ions.

IM is a separation technique that has not until recently been used in bioanalytical applications, but preliminary studies have shown that using IM can aid the analysis of small molecules in complex systems [18] and IM–MS has proved to be a valuable tool for proteomic research [19–21]. The application of IM–MS using an atmospheric pressure drift tube to the analysis of metabolic mixtures has recently been reported for extracts of bacterial cell cultures (*E. coli*) infused directly into the ESI ion source of the spectrometer [22]. IM–MS analysis of the human glycourinome using pre-fractionated urine has also been reported [23]. These studies suggest that LC hyphenated with IM–MS, may have potential for enhancing metabonomic studies without the requirement for additional sample clean-up. In this communication, we describe a preliminary evaluation of the potential of LC–IM–MS using a low pressure IM drift cell for the analysis of the urinary metabolome without prior extraction. Fast gradient reversed-phase liquid chromatography was used for the rapid elution of endogenous metabolites, prior to electrophoretic separation and m/z measurement by IM–MS.

2. Experimental

2.1. Chemicals

Methanol (HPLC grade), acetonitrile (HPLC gradient grade) and formic acid (99.5% Puriss grade) were purchased from Thermo Fisher Scientific (Loughborough, UK). Distilled and deionised water was obtained in-house using a Triple red water purification system (Triple red, Long Crendon, UK).

2.2. IM–MS and LC–IM–MS method

All experiments were carried out employing a prototype IM–Q–TOF-MS (Waters Corporation, Manchester), which is shown schematically in Fig. 1 and has been described in detail elsewhere [18]. Ions from the ESI source were directed into the trap region at the head of the ion mobility drift cell, which was operated in the pressure range 1.0–3.0 Torr N_2. Ions were gated into the drift cell using a gate electrode pulse (3.50 V, 200 μs pulse width and 15 ms pulse period). The IM drift tube consisted of a multi-plate ion guide (15.2 cm in length) to which a voltage gradient (14.24 V/cm) and a supplement RF voltage (3.8 V) were applied to facilitate separation of ion species by relative mobility. Ions passing through the drift region were then directed into the reflectron TOF mass analyses. Ion mobility spectra were acquired by collecting data from 200 TOF pushes (65 μs per bin) and plotting drift time (scan number) against mass-to-charge ratio (m/z). IM–MS data were typically accumulated for 5 s, with a 2 s interscan delay. Initial studies using direct introduction of urine samples into the IM–Q–TOF-MS spectrometer without chromatographic separation were preformed by infusing the aliquots of the prepared urine into the ESI ion source at 10 μl/min using the integrated syringe pump.

Liquid chromatography was performed on a Waters Alliance 2790 chromatograph (Waters Corporation, Manchester, UK) fitted with a Symmetry® (Waters Corporation, Manchester, UK) C18 column (2.1 mm × 50 mm, 5 μm). The LC system was coupled to the ESI ion source of the IM–Q–TOF-MS spectrometer. Urine samples (50 μl injected) were eluted with the following gradient: 100% A (0–2 min), increased to 100% B (2-5 min) and then to 100% A (5–8 min), where A: 0.1% aqueous formic acid and B: 0.1% formic acid in acetonitrile. The mobile phase flow rate was set to 0.2 ml/min. Electrospray ionisation conditions for the MS, with the ion source operated in positive ion mode were: capillary voltage, 3.5 kV; cone voltage 60 V; source temperature 120 °C; desolvation gas, N_2 gas flow 250 l/h; desolvation gas temperature, 180 °C. Masslynx version 4.1 (Waters Corporation, Manchester, UK) was used to control the LC and IM–MS instrument and for data acquisition. Data mining was carried out using DriftScope version 1.0 (Waters Corporation, Manchester, UK).

2.3. Urine sample preparation

Urine samples from male Wistar-derived rats were provided by AstraZeneca (Alderley Park, Macclesfield, UK). Aliquots of a pooled urine ($n = 4$) were used for IM–MS and LC–IM–MS studies. The urine was centrifuged at 13,000 rpm for 5 min to remove particulates and then frozen to −80 °C prior to analysis.

3. Results and discussion

Initial experiments were carried out by directly infusing aliquots of urine into the ESI–IM–Q–TOF-MS spectrometer as described in Section 2. However, the salt component of the urine caused significant ion suppression in the electrospray ion source (data not shown) and hence liquid chromatography was employed to reduce these suppression effects. The urinary metabolic profile is composed mainly of relatively polar/ionic substances, which must be retained on the LC column whilst the salts elute prior to mass spectrometry analysis. In order to obtain the best possible retention of these polar metabolites, we therefore used a gradient separation where the initial segment (0–2 min) was entirely aqueous formic acid, followed by a rapid increase (over 5 min) to 100% acetonitrile. The effectiveness of the LC column for improving spectral quality is shown in the LC–ESI–IM–MS data for a pooled urine sample obtained from the Wistar-derived rats, shown in Fig. 2. The peaks in the LC chromatogram correspond to the IM spectra accumulated during the LC run (Fig. 2(a)). The salt component was eluted in less than 1 min (Fig. 2(a)) showing ion suppression effects and an increased noise in the mass spectrum (Fig. 2(b)), leaving the metabolites and other components of the urine to be eluted in the region 3–8 min, free from salt interferences (Fig. 2(c)).

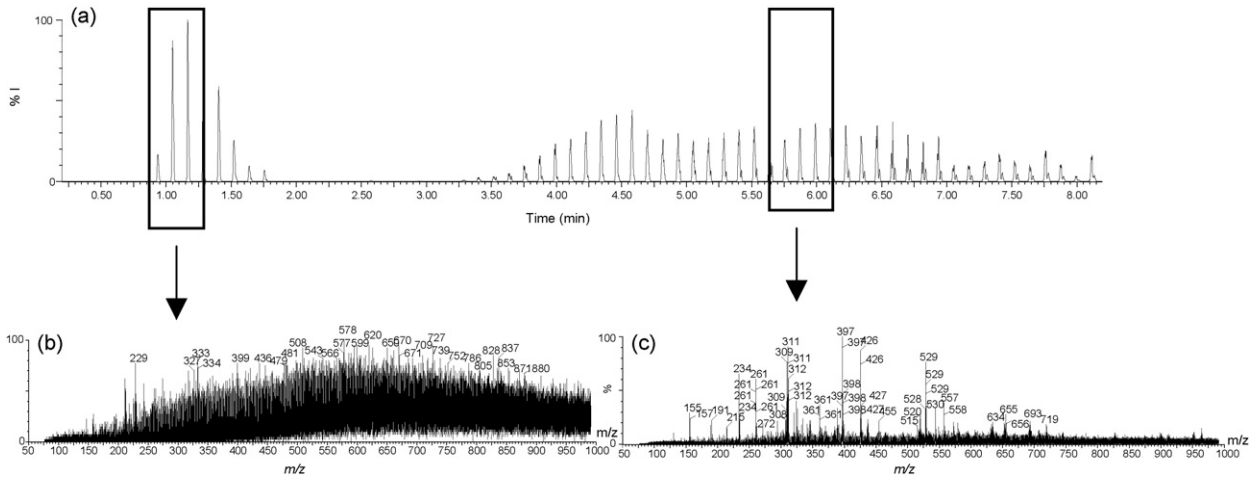

Fig. 2. (a) LC chromatogram showing accumulated IM spectra derived from the LC–IM–MS analysis of urine obtained from male Wistar-derived rats, (b) mass spectrum corresponding to retention time window 1.0–1.3 min, (c) mass spectrum corresponding to retention window 5.5–6.0 min.

The effect of introducing an IM separation in tandem with LC and MS analyses of urinary metabolites is shown in Fig. 3. In this experiment, data were acquired as nested data sets, with mass spectra (65 μs/scan) and IM spectra (~13 ms) scanned repetitively throughout the LC run. Spectra were accumulated to yield 200 mass spectra and one ion mobility spectrum every 7 s. The ion mobility drift time is plotted as 'bins', where each bin corresponds to an acquired mass spectrum. The nested data acquisition of IM and MS data results in an analysis incorporating a gas-phase electrophoretic separation of the ESI generated ions on the basis of charge state and collision cross section (i.e. size and shape), between the reversed-phase chromatography and mass analysis, all within the timescale of the LC–MS run (Fig. 3(a)). IM has a relatively poor resolving power, with a typical full width at half height (FWHH) resolution of 10, corresponding to ~500 theoretical plates in total. However, peak capacity is increased in LC–IM–MS, because the IM separation is orthogonal to that of LC retention and mass-to-charge ratio.

The mass spectrum shown in Fig. 3(b) was generated by summing all 200 mass spectra in each IM scan and is therefore equivalent to the mass spectrum for LC–MS analysis without IM separation. Fig. 3(c) shows the IM spectrum summed over all *m*/*z* values and corresponds to LC–IM analysis without mass separation. The enhanced separation afforded by the combined IM–MS analysis is shown in the 2D plot of ion drift time (bins) vs *m*/*z*, which

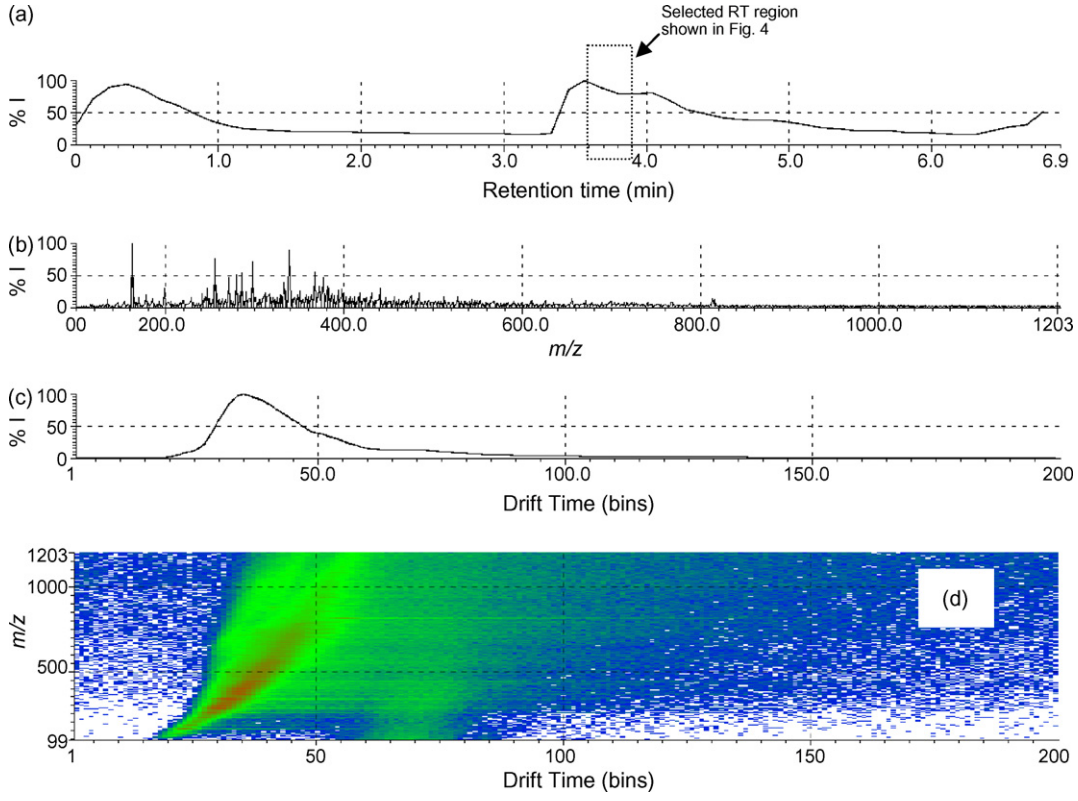

Fig. 3. LC–IM–MS analysis of urine obtained from male Wistar-derived rats, (a) LC chromatogram, (b) the mass spectrum generated by summing all 200 mass spectra in each IM scan during the LC run, (c) total ion mobility spectrum summed over all *m*/*z* values, (d) 2D plot of drift time (bins) vs *m*/*z* plot for the full data set.

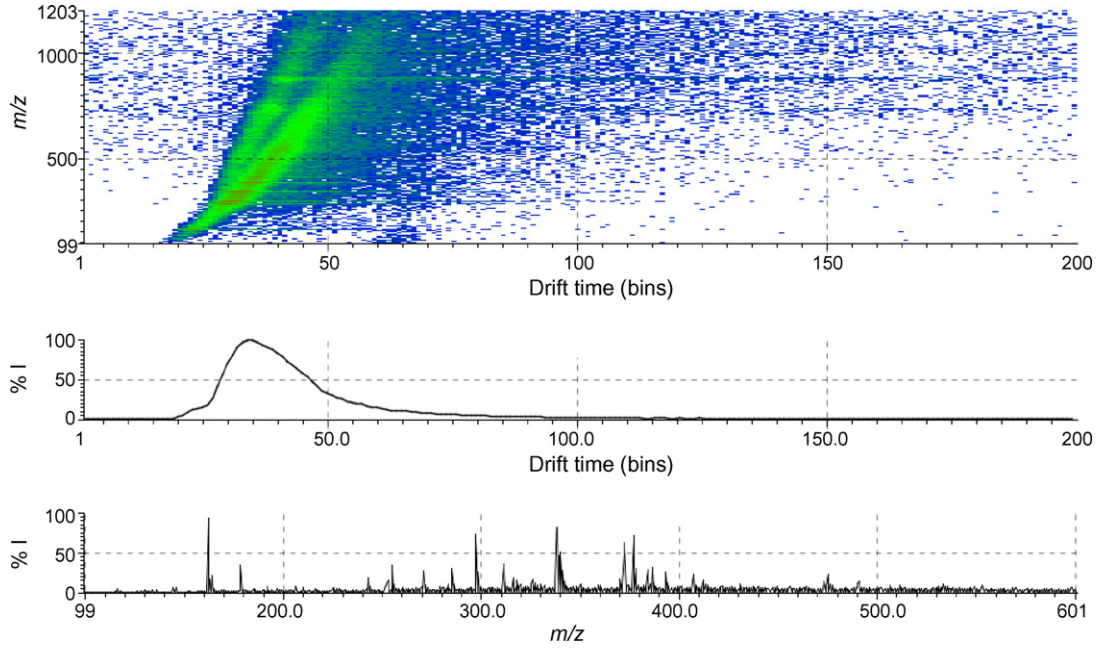

Fig. 4. (a) Drift time (bins) vs *m*/*z* plot of selected retention window 3.6–3.9 min derived from the LC–IM–MS analysis of rat urine, (b) total ion mobility spectrum corresponding to retention time window 3.6–3.9 min, (c) mass spectrum corresponding to total ion mobility spectrum of retention time window 3.6–3.9 min.

is presented in Fig. 3(d) for data averaged over the whole LC run (0–7 min). The bands of colour reflect the intensity of the ions with red representing the highest intensity and blue/white the lowest intensity.

Analysing these multidimensional LC–IM–MS data sets presents a challenge for multivariate statistical techniques commonly used in comparative metabonomics, since conventional statistical theory requires at least twice as many sample replicates as the number of dimensions in the data. Advanced bioinformatic techniques are required, such as artificial neural networks, which are capable of handling complex, multidimensional and non-linear data sets [24]. An alternative approach is to reduce the complexity of the data by selecting retention time or drift time windows for analysis (Fig. 4), or by other pre-treatment strategies [25]. A retention time region (3.6–3.9 min) from the LC run was selected because of the many metabolites eluting in this time window and Fig. 4 shows the IM–MS data associated with this region. There is a significant reduction in background noise (indicated by an increase in blue/white shading) and an overall simplification of the drift time vs *m*/*z* analytical space. The IM and MS spectra shown in Fig. 4(b) and (c) were obtained by averaging the drift time and *m*/*z* data shown in Fig. 4(a). The resulting spectra (Fig. 4(b) and 4(c)) there-

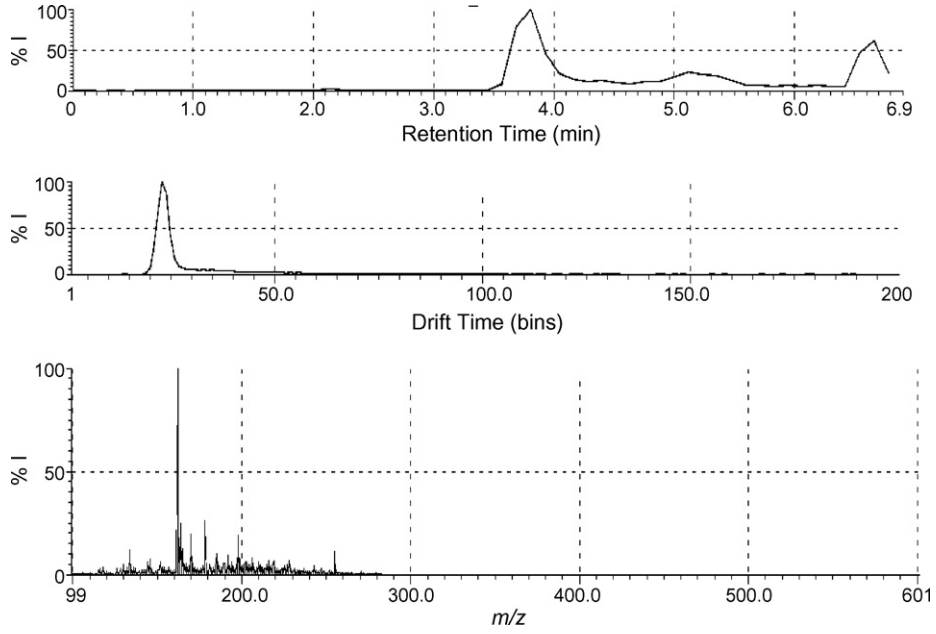

Fig. 5. (a) Extracted ion chromatogram (XIC) for *m*/*z* 162.1 obtained from the LC–IM–MS analysis of male Wistar-derived rat urine, (b) selected ion mobility spectrum of *m*/*z* 162.1, corresponding to the selected retention time window 3.6–3.9 min, (c) mass spectrum corresponding to selected ion mobility spectrum for *m*/*z* 162.1 (IM bins 20–25 combined).

fore correspond to those expected for LC–IM (Fig. 4(b)) and LC–MS (Fig. 4(c)) separations respectively.

It is possible to enhance selectivity for target analytes by utilising the orthogonal separation of the LC, IM and MS dimensions. This is illustrated in Fig. 5 for the m/z 162.1 ion, assigned to the endogenous metabolite carnitine ($C_7H_{15}NO_3$) [21]. The extracted ion chromatogram for this ion is shown in Fig. 5(a). The ion intensity reaches a maximum at a retention time of 3.8 min and the selected ion mobility response for the m/z 162.1 ion in the ion mobility spectra acquired in the retention time window 3.6–3.9 min is shown in Fig. 5(b). The sharp peak observed in the drift time region corresponding to bins 20–25 contrasts with broad total ion mobility response observed in the same retention time window when all 200 bins were averaged, seen in Fig. 4(b). The mass spectrum obtained by averaging the spectra in the region 20–25 bins (Fig. 5(c)) has m/z 162 as the base peak. Comparing this spectrum with that shown in Fig. 4(c), which is equivalent to the LC–MS analysis without mobility selection, demonstrates the effectiveness of the orthogonal LC, IM and MS analysis in simplifying and improving spectral quality. Confidence in the assignment of targeted or unknown species is increased by the presence of an ion of the correct m/z at the expected IM drift time and the LC retention time associated with a metabolite.

4. Conclusions

This study demonstrates that nested LC–IM–MS data may be acquired on directly injected urine samples within the timescale of an LC–MS experiment. The combined LC–IM–MS approach has the potential to enhance the metabonomic coverage by the introduction of a gas-phase electrophoretic separation that is orthogonal to the reversed-phase LC and mass-to-charge MS separations. Data mining for the detection of a targeted analyte is also demonstrated yielding improved spectral quality and confidence in assignment.

Acknowledgements

We thank AstraZeneca, UK for financial support and Waters Corporation, Manchester, for operational support for the IM–Q–TOF-MS.

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