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ORIGINAL PAPER

Real-time 2D separation by LC × differential ion mobility hyphenated to mass spectrometry

Emmanuel Varesio · J. C. Yves Le Blanc ·
Gérard Hopfgartner

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Abstract The liquid chromatography–mass spectrometry (LC-MS) analysis of complex samples such as biological fluid extracts is widespread when searching for new biomarkers as in metabolomics. The success of this hyphenation resides in the orthogonality of both separation techniques. However, there are frequent cases where compounds are co-eluting and the resolving power of mass spectrometry (MS) is not sufficient (e.g., isobaric compounds and interfering isotopic clusters). Different strategies are discussed to solve these cases and a mixture of eight compounds (i.e., bromazepam, chlorprothixene, clonazepam, fendiline, flusilazol, oxfendazole, oxycodone, and pamaquine) with identical nominal mass (i.e., m/z 316) is taken to illustrate them. Among the different approaches, high-resolution mass spectrometry or liquid chromatography (i.e., UHPLC) can easily separate these compounds. Another technique, mostly used with low resolving power MS analyzers, is differential ion mobility spectrometry (DMS), where analytes are gas-phase separated according to their size-to-charge ratio. Detailed investigations of the addition of different polar modifiers (i.e., methanol,

ethanol, and isopropanol) into the transport gas (nitrogen) to enhance the peak capacity of the technique were carried out. Finally, a complex urine sample fortified with 36 compounds of various chemical properties was analyzed by real-time 2D separation LC×DMS-MS(/MS). The addition of this orthogonal gas-phase separation technique in the LC-MS(/MS) hyphenation greatly improved data quality by resolving composite MS/MS spectra, which is mandatory in metabolomics when performing database generation and search.

Keywords Real-time 2D separation · Differential ion mobility spectrometry · Liquid chromatography · Mass spectrometry

Introduction

When complex mixtures of low molecular weight compounds were analyzed, a high peak capacity is mandatory to avoid the loss of relevant analyte information. Very often, liquid chromatography (LC) or gas chromatography (GC) is hyphenated to mass spectrometry (MS) to separate the numerous constituents of such samples. If the separation power is not sufficient, comprehensive two-dimensional (2D) chromatography (e.g., GC×GC and LC×LC) can increase the chromatographic peak capacity, but the second dimension requires very fast separation speed that can be challenging for LC. Holland and Jorgensen have demonstrated that the LC cycle time of the second dimension needs to be around one third of the first dimension peak width in order to have an efficient and comprehensive sampling [1]. Moreover, Giddings showed that the peak capacity of both dimensions could be multiplied only when both retention mechanisms are truly orthogonal [2].

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E. Varesio (✉) · G. Hopfgartner
School of Pharmaceutical Sciences, University of Geneva,
University of Lausanne, Life Sciences Mass Spectrometry,
Quai Ernest-Ansermet 30,
1211 Geneva 4, Switzerland
e-mail: emmanuel.varesio@unige.ch

J. C. Y. Le Blanc
AB Sciex, 71 Four Valley Drive,
Concord, ON L4K 4V8, Canada

Comprehensive LC remains difficult to achieve since there is no generic combination of separation mechanisms due to the analytes and mobile phases properties [3].

From a MS perspective, the co-elution of isobaric compounds or interferences from the isotopic cluster of another compound remains a separation challenge. For decades, ion mobility spectrometry (IMS), which separates ions according to their size-to-charge ratio and their interactions with a gas, has been used as a standalone technique for the detection of chemical warfare agents, explosives or narcotics [4]. However, IMS is also widely hyphenated to mass spectrometry (IMS-MS) and is used for structural studies of small molecules or biopolymers [5]. In addition, the orthogonality of both techniques has also the advantage to separate isomeric compounds (e.g., enantiomers [6] and conformers [7, 8]). Kanu et al. [9] recently reviewed the four methods of performing IMS among which field-asymmetric waveform ion mobility spectrometry (FAIMS), also known as differential ion mobility spectrometry (DMS), is an alternative to the traditional drift tube or the traveling-wave devices [10]. Guevremont reviewed the FAIMS fundamentals and history, as well as its different applications for small and large molecules [11]. Two different device designs exist and both are continuously able to monitor the ions produced by electrospray, unlike the drift tube format that injects pulses of ions (e.g., Bradbury–Nielsen ion gate). FAIMS devices are mainly hyphenated to low resolving power MS analyzers like quadrupoles or linear ion traps. Nowadays, the FAIMS acronym is commonly associated with the concentric cylindrical electrodes design, whereas DMS is related to the parallel planar electrodes device. DMS operates with an asymmetric electric field applied between the two planar electrodes perpendicular to the ions motion. The gap is normally filled with a transport gas at a controlled pressure and temperature. The ion mobility is different when the analyte experiences the low-field portion than for the high-field portion of the oscillating electric field. Thus ions are drifting towards one of the two electrodes and a specific compensation voltage (CoV) is required to avoid their neutralization onto the electrodes. In recent years, the coupling of DMS to MS analysis has mainly focused on the elimination of chemical noise [12]. More recently, it has been demonstrated that the addition of gas-phase modifiers in the vapor state could significantly improve the separation power of DMS device [13, 14] and could clearly be orthogonal to other techniques such as LC.

Here, we discuss the different strategies that can be chosen for complex samples analysis in order to increase the resolving power of either MS or LC and enhance the overall liquid chromatography–mass spectrometry (LC-MS) assay selectivity. When multidimensional separation is required, the gas-phase separation dimension provided by

DMS can be easily implemented. Therefore, this paper also focuses on real-time 2D separation, which can be performed with the first dimension relying on LC and the second dimension relying on DMS. The data obtained during the analysis of complex matrix (e.g., urine) and the benefits of the approach in improving the MS and MS/MS information obtained on a triple quadrupole linear ion trap (QqQ_{LIT}) are illustrated.

Experimental section

Chemicals and sample preparation

Formic acid (FA) was purchased from Merck (Darmstadt, Germany). Acetonitrile, methanol (MeOH), isopropanol (i-PrOH), and ethanol (EtOH) were of HPLC-grade and purchased from VWR International (Nyon, Switzerland). Ultrapure water was provided by a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

The “316 mix” consisted of eight compounds (i.e., bromazepam (BRO), chlorprothixene (CHP), clonazepam (CLO), fendiline (FEN), flusilazol (FLU), oxfendazole (OXF), oxycodone (OXC), pamaquine (PAM)) possessing a nominal mass of m/z 316 when using positive electrospray ionization. All these compounds were part of the low molecular weight compounds (LMWC) mix defined hereafter.

The “LMWC mix” was made of 36 LMWC purchased from Sigma-Aldrich (Oakville, ON, Canada), Cerilliant (Round Rock, TX, USA) or Toronto Research Chemicals (North York, ON, Canada) (see Table S1 in the Electronic supplementary material (ESM)).

As a complex sample, human urine was chosen and diluted 2-fold by spiking the LMWC mix at a final concentration of 10 ng/ml.

High-resolution MS infusion experiments

High-resolution MS experiments were carried out on a APEX III FT-ICR-MS from Bruker Daltonics (Bremen, Germany) equipped with a 9.4-Tesla/160 mm bore actively screened superconducting magnet system from Magnex Scientific (Yarnton, Oxford, UK) and with a cylindrical ICR cell that has equipotential-line-segmented trapping plates (“infinity cell”). Infusion of the 316 mix (1–40 $\mu\text{g}/\text{ml}$ in 0.1% FA water–MeOH (1:1, v/v)) was done at 5 $\mu\text{l}/\text{min}$ with an external syringe pump. Electrospray voltage was of 4.5 kV, nitrogen was used as nebulizing gas, capillary exit voltage was set at 80 V and drying gas temperature was of 200 °C. Hexapole ion accumulation was of 0.4 s and the ions transfer time-of-flight was set to 1.7 ms. MS spectrum was resulting from eight accumulated spectra acquired in narrowband detection centered on m/z 316 with a mass window of 2.36 u. The time

domain (FID) size was of 32 k data points with a transient length of 4.771 s. The FID signal was baseline corrected and Fourier transformed with the power calculation mode to produce a frequency spectrum finally converted into an m/z spectrum.

UHPLC-MS experiments

The ultra-high-pressure liquid chromatography (UHPLC) system was an UltiMate 3000 RSLC system from Dionex (Germering, Germany). Separation was performed on a 100×2.1-mm i.d. (1.7 μm) Kinetex XB-C18 core-shell column from Phenomenex (Torrance, CA, USA). Mobile phase A consisted of 0.1% FA in water:acetonitrile (98:2, v/v) and mobile phase B was made of 0.1% FA in acetonitrile. The flowrate was of 300 $\mu\text{l}/\text{min}$ with a column oven temperature of 50 °C. The gradient was linear from 5% to 95% B in 3 min with a washing step of 1 min before column re-equilibration.

MS experiments were carried out on a TripleTOF 5600 (AB Sciex) equipped with a Turbo-V Duo Ionspray interface and a calibrant delivery system used for the time-of-flight calibration. Electrospray voltage was of 5.5 kV, nitrogen was used for nebulizing, curtain and collision gases. Ion source gases were set at 40 and 50 psi for GS1 and GS2 respectively. Curtain gas was of 25 psi. Ionspray source temperature was of 600 °C. Mass range was monitored from m/z 50 to 1,000 with an accumulation time of 50 ms to ensure accurate sampling of the LC peaks.

DMS-MS/MS infusion experiments

A differential ion mobility device was mounted on a QqQ_{LIT} mass spectrometer (QTRAP 5500, AB Sciex, Concord, ON, Canada) [15]. A Turbo-V Ionspray source was used for positive electrospray ionization (ESI) with a voltage of 5.5 kV and a temperature of 150 °C. The DMS separation voltage (SV; i.e. asymmetric rf peak-to-peak voltage) was ramped from 1.0 to 3.8 kV by steps of 200 V. The CoV was ramped from -65 to +20 V by steps of 0.2 V (laboratory frame). DMS resolution was set to low and the curtain gas (i.e., nitrogen) was of 10 psi. The DMS cell temperature was of 150 °C. In order to enhance the DMS separation power, several gas-phase modifiers (i.e., methanol, ethanol, and isopropanol) were added separately to the curtain gas at a concentration of 1.5% by volume using an external pumping device. The modifier was allowed to evaporate completely in the gas before its introduction into the DMS cell. Infusions of the 316 mix (25–500 ng/ml in 0.1% FA water–MeOH (1:1, v/v)) were done at 7 $\mu\text{l}/\text{min}$ with the MS built-in syringe pump.

Enhanced product ion (EPI) MS/MS experiments were acquired from m/z 80 to 350 at a scanning speed of 1,000 u/s

with a fixed trap fill time of 2 ms (Q_0 trapping active and Q_1 segments duration adjusted). A generic collision energy (CE) of 45 eV with a CE spread of 15 eV was applied, nitrogen was used as collision gas, and Q_1 analyzer was operated at unit resolution for the precursor ion selection.

HPLC×DMS-MS experiments

The binary high-pressure gradient LC system was a Prominence LC-20 AD XR system from Shimadzu (Kyoto, Japan). Separation was performed on a 50×20-mm i.d. (5 μm) Hypersil Gold C18 column from Thermo Fisher Scientific (Waltham, MA, USA). Mobile phase A consisted of 0.1% FA in water/acetonitrile (98:2, v/v) and mobile phase B was made of 0.1% FA in acetonitrile. The flowrate was of 250 $\mu\text{l}/\text{min}$ and the gradient was linear from 5% to 99% B in 10 min with a washing step of 2 min before column re-equilibration.

MS(/MS) experiments were performed with the differential ion mobility device mounted on the QqQ_{LIT} mass spectrometer (QTRAP 5500, AB Sciex). A Turbo-V Ionspray source was used for positive ESI with a voltage of 4 kV and a temperature of 650 °C. When the DMS device was turned on, a continuous SV of 3.5 kV was applied. DMS resolution was set to low and the curtain gas was of 10 psi. The organic gas-phase modifier was isopropanol added at 1.5% in the curtain gas. The CoV was stepped from -32 to +12 V with steps of 1.2 V (laboratory frame).

Enhanced MS scan was performed from m/z 250 to 500 at a scanning speed of 10,000 u/s with a fixed trap fill time of 5 ms. Targeted MS/MS experiments for the 316 mix compounds were acquired in EPI mode from m/z 100 to 320 with collision energy of 35 eV and Q_1 operating at unit resolution.

Software for data acquisition and processing

DMS and MS experiments conducted on the QTRAP 5500 were acquired by a modified Analyst 1.5 software version (AB Sciex). FT-MS experiments were acquired by apex-Control 2.0 software (Bruker Daltonics). Data processing was done by using PeakView 1.1 software (AB Sciex) for QTRAP data or by using DataAnalysis 3.4 software (Bruker) for FT-MS data.

Results and discussion

Strategies to separate compounds with identical nominal mass

A standard mixture of eight low molecular weight compounds with identical nominal mass has been used to

illustrate different strategies based on MS that can be applied for their separation. All these compounds possess different structures and chemical properties, different exact masses and are all ionized by positive electrospray (Fig. 1 and Table 1).

Given that these compounds are of identical nominal mass but are not isobaric, therefore the first MS-based strategy when instrumentation is available is to use high-resolution mass spectrometers such as FT-ICR-MS or Orbitrap to separate the compounds based on their exact mass. In the standard mixture, the exact masses of the protonated molecular ions span from m/z 316.007993 (bromazepam) to m/z 316.238339 (pamaquine) corresponding to a m/z window of 0.230346 u. Figure 2a represents the MS spectrum obtained by infusing the 316 mix solution in a FT-ICR-MS instrument with a resolving power at FWHM of about 1,000,000. All the compounds are baseline resolved with a mass accuracy of 0.3–0.4 ppm. However, in the case of this 316 mix solution, MS instruments with a resolving power of ca. 60,000 would have been sufficient to baseline separate the critical pair of compounds with the smallest mass difference of 15 mmu (i.e., CHP and FLU).

When high resolving power MS is not accessible or when analyzing isobaric compounds like structural isomers [16], a chromatographic or electrophoretic technique hyphenated to MS is generally used, such as GC- or LC-MS. The separation of the compounds in this second strategy mainly is based on their chemical properties, such as hydrophobicity (e.g., logP and logD) or the existence of acidic/basic moieties (e.g., pK_a) and not based on their exact mass. As an example, Fig. 2b shows the UHPLC-MS

separation of the “316 mix” obtained with a generic linear gradient of 3 min performed with a core-shell C18 column. The compounds are separated according to their increasing hydrophobicity (Table 1). In this strategy, the addition of a first chromatographic separation dimension to the MS resolving power increases the overall peak capacity, enabling the unequivocal separation of the eight compounds (Fig. 2b (upper panel)). This additional chromatographic dimension renders accessible the use of low and medium resolving power mass spectrometers, such as quadrupole-based, (2D/3D)-ion traps or time-of-flight analyzers. However, it remains important to mention that with UHPLC separation, LC peak widths are generally of 1–1.5 s at base which requires a MS duty cycle below 100 ms to acquire enough data points for an accurate LC peak definition especially for quantitative analyses [17]. The lower panel of Fig. 2b shows the eight extracted ion chromatograms (XIC) with a tolerance window of 20 mmu. While most of the compounds are baseline resolved, CHP and FEN are nearly co-eluting since they possess a close logD value at this mobile phase pH. These two compounds are, however, resolved in the MS dimension. Nevertheless, if the same UHPLC separation was performed on a quadrupole-based MS instrument, these two compounds would not be separated due to the low MS resolving power of that type of analyzer.

Another strategy based on MS is to perform MS/MS or MSⁿ experiments. The fragmentation of LMWC is quite specific to each analyte and selectivity is increased when compounds are analyzed based on their collision-induced fragments. Few years ago, Leuthold et al. [18] already

Fig. 1 Structures of the “316 mix” compounds

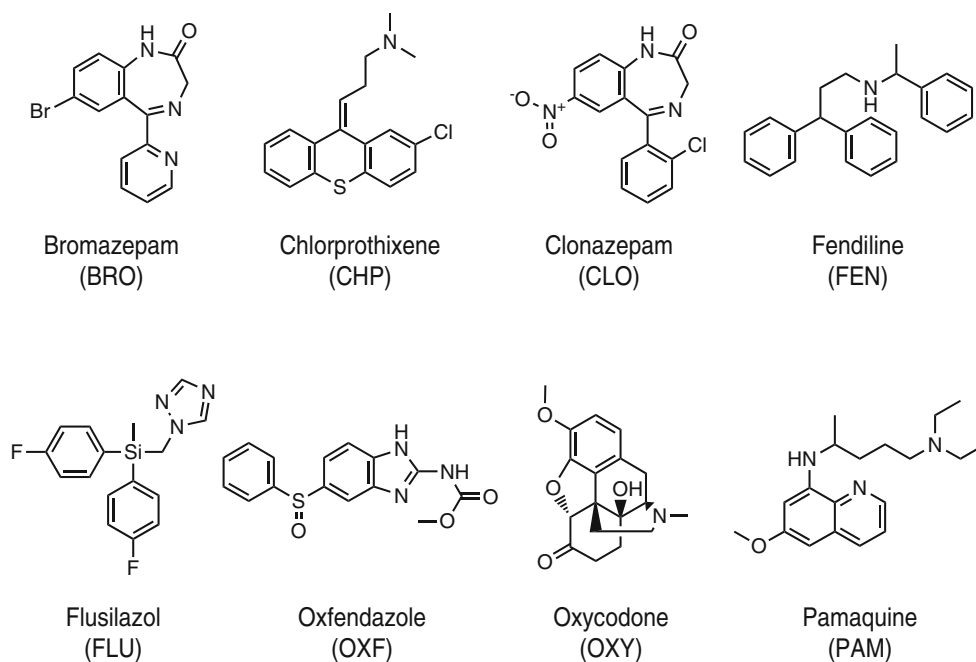


Table 1 Chemical properties of the “316 mix” compounds^a

Name	Formula	MW (g/mol)	Exact mass (MH ⁺)	LogP	LogD (pH 4)	pK _a ^b
Bromazepam	C ₁₄ H ₁₀ BrN ₃ O	316.2	316.007993	2.3	2.3	2.0
Chlorprothixene	C ₁₈ H ₁₈ ClNS	315.9	316.092124	5.2	2.1	9.1
Clonazepam	C ₁₅ H ₁₀ ClN ₃ O ₃	315.7	316.048345	2.8	2.8	1.6
Fendiline	C ₂₃ H ₂₅ N	315.5	316.205976	5.2	2.1	9.5
Flusilazol	C ₁₆ H ₁₅ F ₂ N ₃ Si	315.4	316.107606	3.7	3.6	2.9
Oxfendazole	C ₁₅ H ₁₃ N ₃ O ₃ S	315.3	316.075038	2.0	1.2	4.5
Oxycodone	C ₁₈ H ₂₁ NO ₄	315.4	316.154335	1.6	-1.4	7.6
Pamaquine	C ₁₉ H ₂₉ N ₃ O	315.5	316.238339	4.4	1.0	10.5

^aCalculated properties with ACD/Labs software suite release 12.01 (Toronto, Canada)

^bpK_a values are given for the most basic moieties

showed that the use of innovative MS-based workflows including quantitative SRM and confirmatory MS/MS and MS³ experiments, were adequate to analyze a pharmaceutical drug and its metabolite in plasma without chromatographic step prior to MS detection (i.e., by using a chip-based electrospray infusion device). In the case of the 316 mix compounds, all their MS/MS spectra were acquired individually by infusion on a QqTOF instrument (RP=30,000) with generic collision energy settings (i.e., CE=45 eV with a spread of ±15 eV). Figure S1 in the ESM shows that for nearly all MS/MS spectra, at least one to two intense fragment ions (labeled in bold with asterisk) can be selected to build the set of specific SRM transitions for each compound. However, FEN shares most of its fragment ions with FLU and an additional chromatographic separation step or an MS³ experiment would be required to improve the selectivity of its analysis.

Finally, another strategy based on MS is IMS, which separates ions according to their size-to-charge ratio and their interactions with a buffer gas. IMS can be performed under different principles and in this study FAIMS or DMS has been used.

Differential ion mobility spectrometry on a QqQ_{LIT} MS instrument

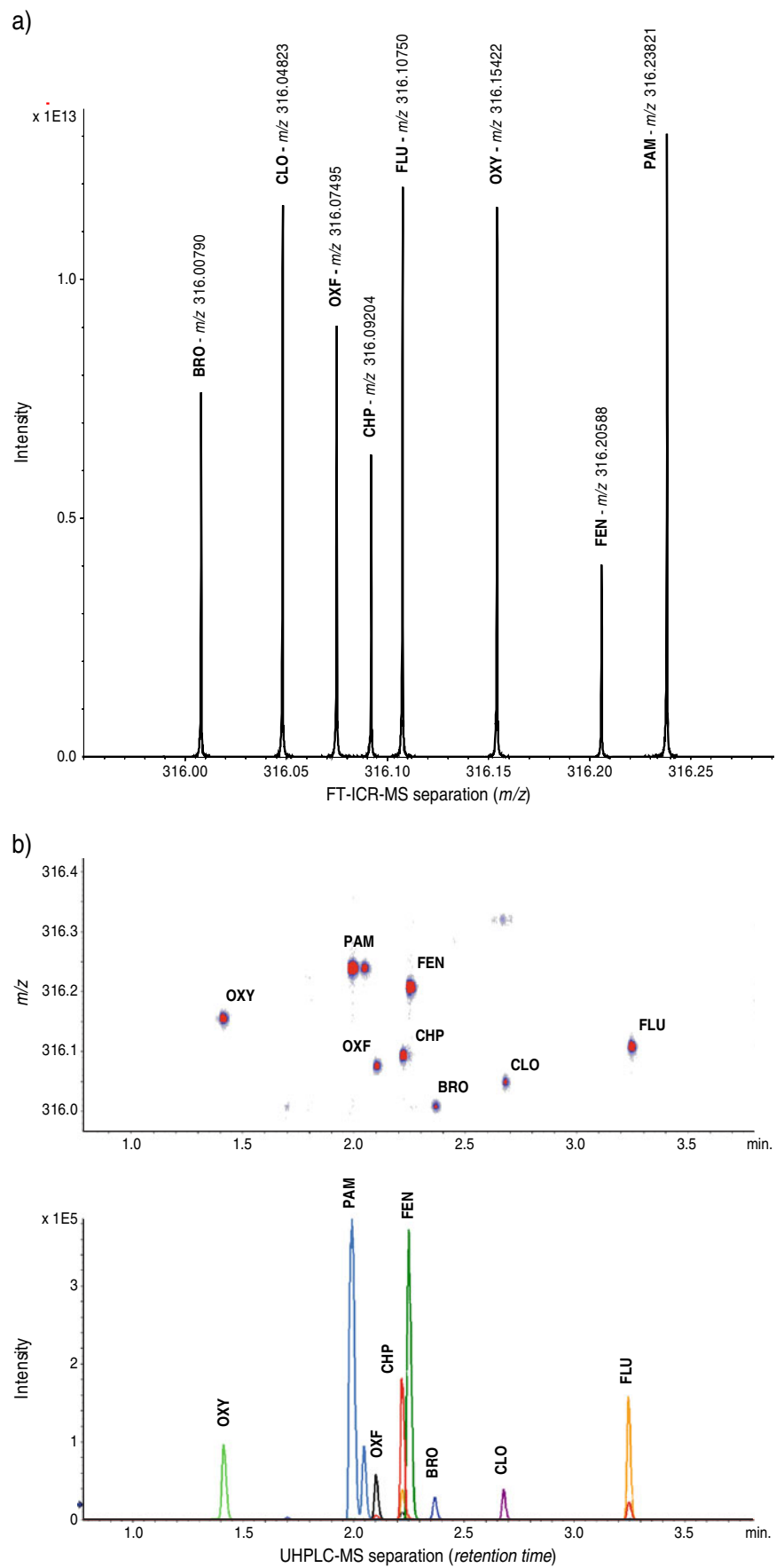
Unlike drift tube IMS where the traveling time of an analyte is measured and related to its size in space (i.e., collision cross-section), DMS separation is based on the ion mobility difference that exists between the high and the low portion of the oscillating electric field applied between the two planar electrodes of the DMS cell. Each analyte possess its own differential mobility and separation occurs by scanning the compensation voltage applied on one electrode to let ions with a specific differential mobility value to go through the DMS cell without being neutralized on the electrodes. Figure 3a shows the DMS separation obtained for the 316 mix at a separation voltage of 3,800 V (i.e., field strength in E/N value of 131 Td). The compounds are barely separated with CoV values spanning from 2.4 to 7.1 V. This indicates that

the eight analytes with identical nominal mass experience a relatively similar differential mobility within a narrow 10 V compensation voltage window.

Nearly a decade ago, Krylova et al. have demonstrated that moisture in air (above 50 ppm) can change the field dependence of ion mobility for organophosphorus compounds because their collision cross-section was dictated by the degree of solvation [19]. Since then, several authors have demonstrated that the addition of an organic modifier into the buffer gas can dramatically improve the separation power of the DMS device [13, 14, 20, 21]. The separation enhancement has been explained by the formation of transient clusters between the analyte ions and the neutral modifier molecules during the low-field portion of the cycle, which alters the analyte's mobility because the collision cross-section of these clusters is larger compared with the high-field portion where these clusters are broken up. In this study, three different polar modifiers (i.e. methanol, ethanol, isopropanol) were added separately into the curtain gas at a concentration of 1.5% by volume. The resulting separations of the 316 mix with each modifier are shown in Fig. 3b–d. For each DMS gas-phase separation, analyte's compensation voltages and peak widths at half height are given in Table 2.

Although the eight compounds possess identical nominal masses, their chemical properties are quite different (Table 1) as well as their interactions with the modifiers. Methanol was found to be the least efficient to enhance the DMS separation for these compounds (CoV span of 23.4 V). On the contrary, isopropanol provides the largest CoV separation from -41.3 V for the bromazepam to +1.6 V for pamaquine. Interestingly, the pamaquine is remaining within a 10-V window from its initial CoV value (i.e., without any modifier added in the curtain gas), and is the least affected by the addition of any of the three polar modifiers, except for MeOH where its signal is severely suppressed (i.e., ca. 100-fold). Schneider et al. [20] have demonstrated that peak capacity can be used to compare the DMS separation power for different polar modifiers. Table 2 shows that the peak capacity increases from 2.7 without gas modifier

Fig. 2 Separation of the “316 mix” compounds **(a)** by infusion on a high-resolution MS instrument (Apex III FT-ICR-MS) with a resolving power of 1,000,000 or **(b)** by UHPLC separation on a core-shell column coupled to a MS instrument (TripleTOF 5600) with a resolving power of 30,000. **b** *Upper panel* corresponds to the LC-MS contour plot and *lower panel* shows the overlaid XIC traces with an extracted window of 20 mmu



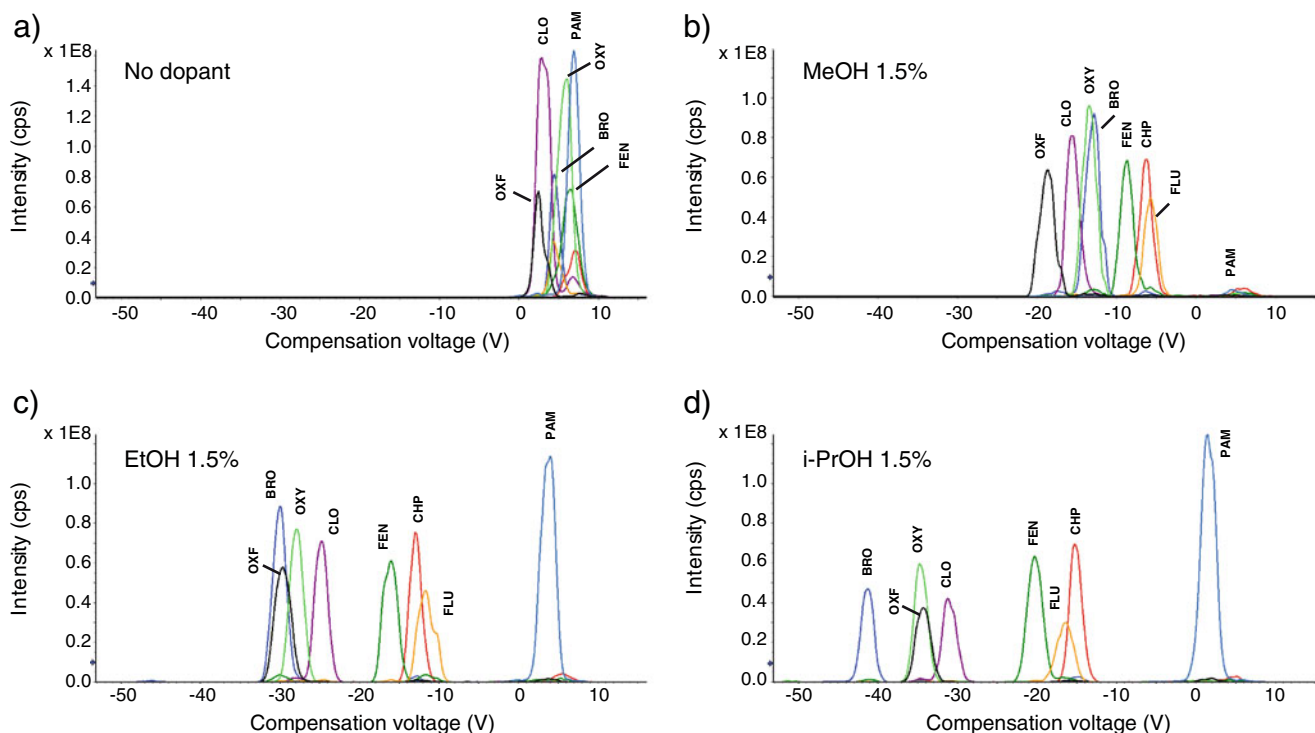


Fig. 3 Separation of the “316 mix” by DMS coupled to a QqQ_{LIT} MS operated in EPI scan mode ($E/N=131$ Td). DMS separation was performed: **(a)** without polar modifier (nitrogen), **(b)** with 1.5% of MeOH as modifier, **(c)** with 1.5% of EtOH as modifier, and **(d)** with

1.5% of i-PrOH as modifier. Fragment ions monitored in EPI mode for the extracted CoV traces were: m/z 209.1 (BRO), m/z 231.1 (CHP), m/z 270.1 (CLO), m/z 105.1 (FEN), m/z 165.1 (FLU), m/z 159.0 (OXF), m/z 298.1 (OXY), and m/z 243.2 (PAM)

to 20.4 when using 1.5% isopropanol corresponding to a 7.5-fold separation enhancement. As a matter of fact, peak capacity is also depending on the field strength and

comparison between the different modifiers has to be performed for the same DMS cell conditions (i.e., separation voltage, molecular density of the gas, and temperature).

Table 2 DMS separation of the “316 mix” without or with organic modifiers added into the curtain gas

Compound	No modifier (N_2)		MeOH at 1.5% (v/v)		EtOH at 1.5% (v/v)		i-PrOH at 1.5% (v/v)	
	CoV	FWHH	CoV	FWHH	CoV	FWHH	CoV	FWHH
Bromazepam	4.5	1.4	-12.9	2.1	-30.0	2.0	-41.3	1.9
Chlorprothixene	7.1	1.8	-6.3	1.7	-12.9	1.8	-15.2	1.8
Clonazepam	3.1	2.0	-15.6	2.0	-24.8	2.0	-31.0	2.0
Fendiline	6.4	2.2	-8.7	1.8	-16.1	2.3	-20.3	2.2
Flusilazol	4.4	1.5	-5.7	1.9	-11.8	2.8	-16.4	2.5
Oxfendazole	2.4	1.3	-18.7	2.2	-29.6	2.5	-34.3	2.2
Oxycodone	5.8	2.1	-13.4	2.2	-27.9	2.0	-34.6	2.0
Pamaquine	6.9	1.7	4.7	2.0	3.8	2.2	1.6	2.2
CoV _{min} (V)	2.4		-18.7		-30.0		-41.3	
CoV _{max} (V)	7.1		4.7		3.8		1.6	
CoVspan (V)	4.7		23.4		33.8		42.9	
FWHH _{average} (V)		1.8		2.0		2.2		2.1
Peak capacity ^a	2.7		11.8		15.4		20.4	

CoV compensation voltage, EtOH ethanol

^a Peak capacity (PC) is calculated according to [20]: $PC = \frac{CoV_{max} - CoV_{min}}{FWHH_{average}}$

Real-time 2D separation by LC×DMS-MS for the analysis of a complex sample

As a case study and in order to generate a complex sample, the eight compounds of the 316 mix were spiked into human urine together with the other 28 compounds constituting the “LMWC mix”. The 2-fold diluted spiked urine sample was then analyzed by LC-MS in Enhanced MS mode with a generic gradient of 10 min and a 5-cm narrow bore column packed with a 5- μm C18 stationary phase. Figure 4a shows the resulting 2D contour plot of the LC-MS analysis where compounds are mainly separated within the first 8 min and are spread over the whole m/z range (m/z 250–500).

Contrary to the UHPLC separation shown in Fig. 2b, the eight compounds are not completely resolved with these LC conditions (i.e., short column packed with 5 μm particles and generic 10 min gradient) and some compounds are co-eluting (cf. contour plot with zoomed region around m/z 316 region in Fig. S2 in the ESM). Moreover, some isotopic contribution from matrix compounds might interfere (yellow circle showing M+2 isotope of m/z 314.3 in Fig. S2 in the ESM). Therefore, for a better understanding and monitoring, the same urine sample was also analyzed in MS/MS mode (Enhanced Product Ion experiment) targeted for the 316 mix compounds. Figure 4b shows the TIC chromatogram of the LC-MS/MS analysis and Fig. 4c shows the overlaid XIC traces of the eight compounds based on their specific fragment (as described for Fig. S1 in the ESM). From the overlaid XIC traces it is obvious that the peaks 1 and 2 eluting at 4.47 and 5.56 min in the TIC

are constituted of two co-eluting compounds that cannot be distinguished in the LC-MS contour plot. As a matter of fact, without the MS/MS dimension artificially added for this case study, one would have missed this type of information since their peak shape does not indicate an underlying second compound at the first glance.

As previously described, the resolving power of the linear ion trap analyzer is not sufficient to differentiate these co-eluting compounds and an additional separation dimension is required. Therefore the DMS cell was mounted in front of the MS instrument to perform a real-time gas-phase separation prior MS dimension. In line with the previous results from Fig. 3, the DMS cell was operated with the addition of modifier in the curtain gas to enhance its separation power. However when hyphenated with LC, the compensation voltage had to be stepped by 1.2 V in order to scan the CoV domain in 4 s to sample ca. 4–5-fold the LC peaks (peak widths at the base of 15–20 s). As expected, the addition of the DMS device in the LC-MS(/MS) analysis could distinguish the co-eluting compounds without ambiguity. This is demonstrated in Fig. 5a that shows the composite MS/MS spectrum of peak 1 without operating the DMS device and in Fig. 5b, c that shows MS/MS spectra taken at the apex CoV values corresponding to bromazepam and pamaquine respectively. Similarly, MS/MS spectra from peak 2 with and without DMS separation are given in Fig. S3 in the ESM.

It is noticeable that the new dimension added to the LC-MS technique by the DMS gas-phase separation enables to clearly identify the two co-eluting compounds from peaks 1 and 2 by their distinct MS/MS spectrum. The real-time

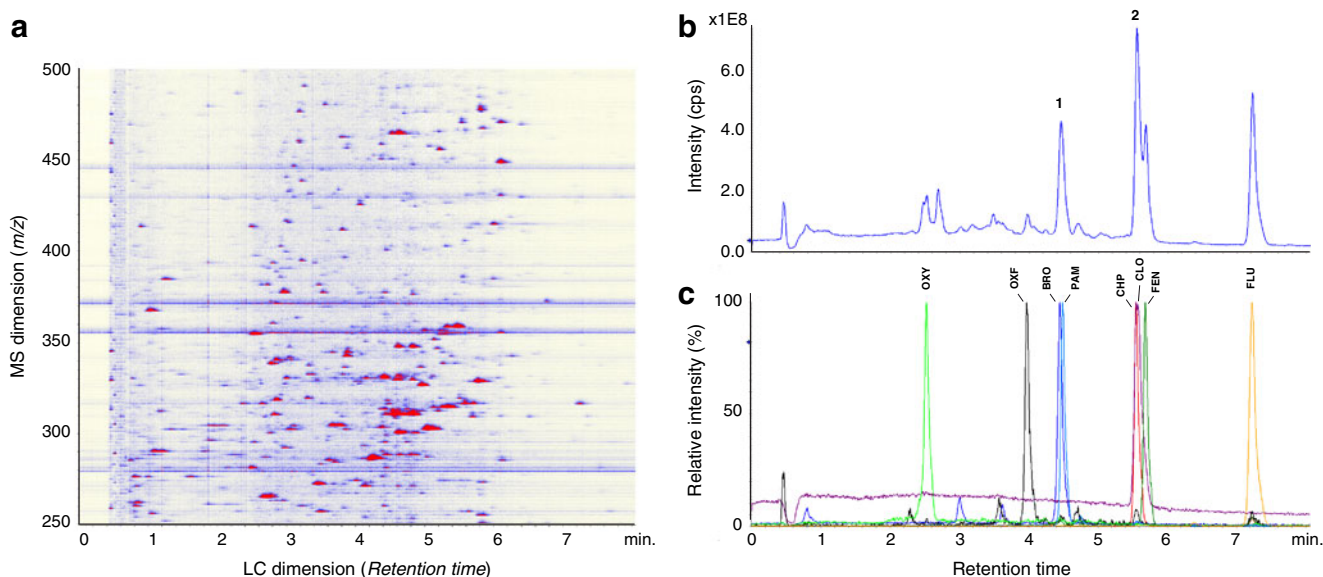
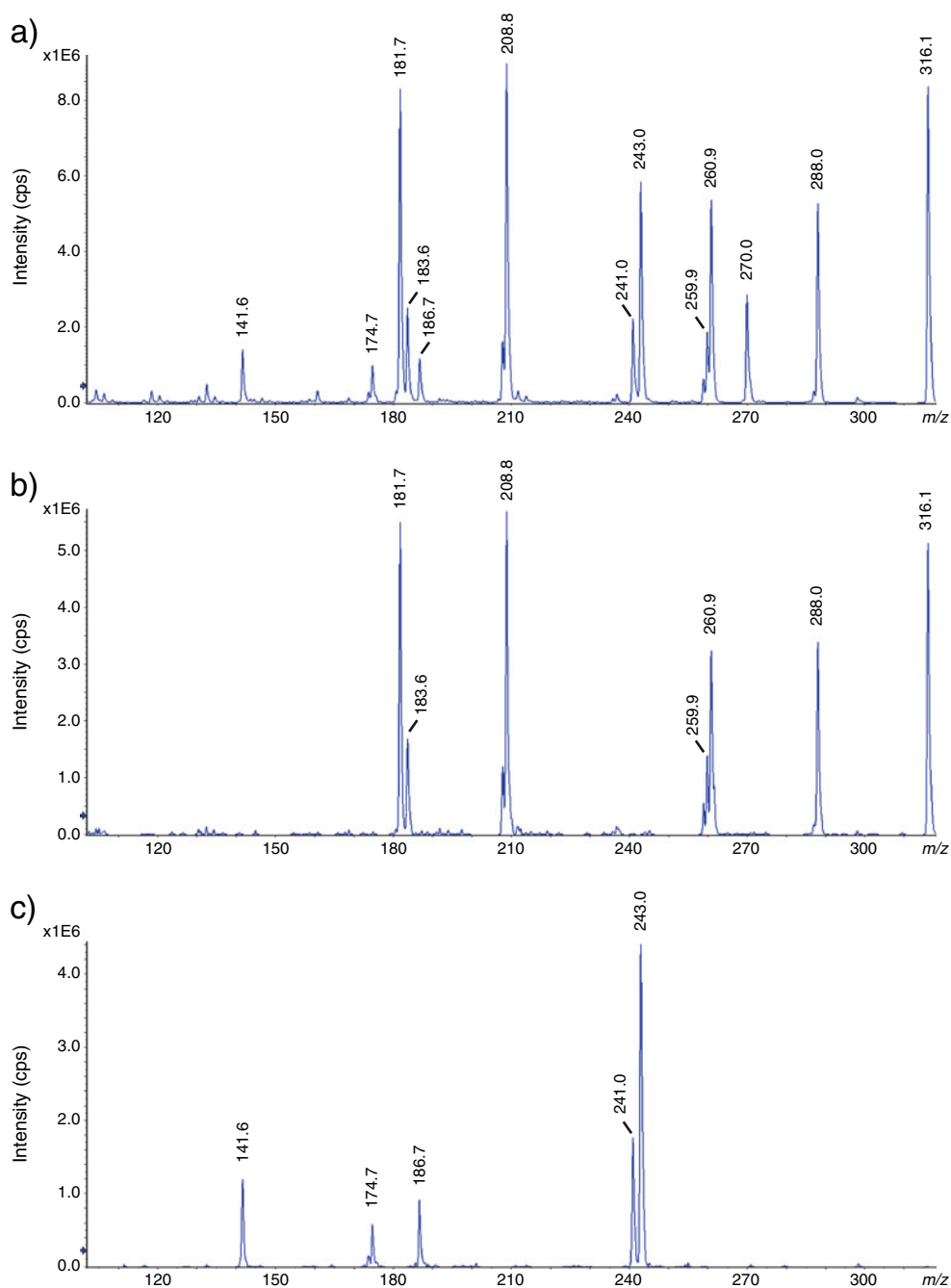


Fig. 4 LC-MS(/MS) separation of the diluted human urine spiked with the “LMWC mix”. **(a)** Contour plot of the LC-MS separation (EMS mode), the intensity threshold (red color) was set at $1\text{e}7$ cps; **(b)** LC-MS/MS separation of the spiked urine targeted for the “316 mix”

compounds (EPI mode—TIC); **(c)** XIC traces from the EPI experiment based on specific fragments for each compound of the “316 mix”, the fragment ions used for the extracted traces were the same as in Fig. 3

Fig. 5 MS/MS spectra (EPI mode) of peak eluting at 4.5 min (peak 1). **(a)** without DMS separation (composite spectrum) and with DMS separation at the apex CoV **(b)** of bromazepam and **(c)** of pamaquine. MS/MS spectra were acquired with a collision energy of 35 eV (nitrogen)



LC×DMS-MS combination expands the peak capacity of the separation without increasing the LC runtime. However, the CoV range screening is relatively slow due to the experiment performed on the QqQ_{LIT} instrument and the CoV domain has to be stepped in order to maintain a sufficient sampling rate of the LC dimension. Therefore, this type of coupling would benefit from a faster MS instrument like the new $QqTOF$ mass spectrometers described previously (e.g., TripleTOF 5600) with duty cycles as low as 10–20 ms for acquiring the full MS domain at high resolution. Such an analytical platform would be of great help in the case of complex samples analysis.

Conclusions

When analyzing biological fluid extracts by LC-MS, a higher peak capacity may be required and the use of either comprehensive LC×LC or high-resolution mass spectrometry such as FT-MS analyzers can fulfill these requirements. However, these techniques do not have a large users base. In addition, for some compounds (e.g., isobaric compounds), an additional separation dimension is required. Differential ion mobility has shown good peak capacity when a polar modifier is vaporized into the buffer gas (e.g., isopropanol). This gas-phase technique separates ions based

on their size-to-charge ratio and is orthogonal to MS. DMS allows the continuous monitoring of ions produced by electrospray and can be easily hyphenated to LC to perform a real-time 2D separation prior to MS detection. Although the DMS peak capacity and scanning speed are reduced compared to LC, a striking point of 2D LC×DMS-MS(/MS) is its ability to resolve composite MS/MS spectra due to different CoV values for co-eluting compounds. This is particularly interesting for metabolomics analyses by LC-MS(/MS) where structural identification of potential biomarkers is performed by MS/MS spectra database search. Finally, when considering the short residence time of ions through the DMS cell in the present configuration [15], a fast acquiring MS instruments such as time-of-flight analyzers would be ideally suited for the LC×DMS hyphenation and would allow to fully exploit this orthogonal coupling in qualitative applications.

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References

- Holland LA, Jorgenson JW (1995) Separation of nanoliter samples of biological amines by a comprehensive two-dimensional microcolumn liquid chromatography system. *Anal Chem* 67(18):3275–3283
- Giddings J (1995) Sample dimensionality—a predictor of order-disorder in component peak distribution in multidimensional separation. *J Chromatogr A* 703(1–2):3–15
- François I, Sandra K, Sandra P (2009) Comprehensive liquid chromatography: fundamental aspects and practical considerations—A review. *Anal Chim Acta* 641:14–31
- Cottingham K (2003) Ion mobility spectrometry rediscovered. *Anal Chem* 75(19):435A–439A
- Creaser CS, Griffiths JR, Bramwell CJ, Noreen S, Hill CA, Thomas CLP (2004) Ion mobility spectrometry: a review. Part 1. Structural analysis by mobility measurement. *Analyst* 129(11):984–994. doi:10.1039/b404531a
- Dwivedi P, Wu C, Matz LM, Clowers BH, Siems WF, Hill HH (2006) Gas-phase chiral separations by ion mobility spectrometry. *Anal Chem* 78(24):8200–8206. doi:10.1021/ac060877z
- Dong L, Shion H, Davis RG, Terry-Penak B, Castro-Perez J, van Breemen RB (2010) Collision cross-section determination and tandem mass spectrometric analysis of isomeric carotenoids using electrospray ion mobility time-of-flight mass spectrometry. *Anal Chem* 82(21):9014–9021. doi:10.1021/ac101974g
- Zhu M, Bendiak B, Clowers B, Hill HH (2009) Ion mobility-mass spectrometry analysis of isomeric carbohydrate precursor ions. *Anal Bioanal Chem* 394(7):1853–1867. doi:10.1007/s00216-009-2865-y
- Kanu AB, Dwivedi P, Tam M, Matz L, Hill HH (2008) Ion mobility-mass spectrometry. *J Mass Spectrom* 43(1):1–22. doi:10.1002/jms.1383
- Shvartsburg AA, Smith RD (2008) Fundamentals of traveling wave ion mobility spectrometry. *Anal Chem* 80(24):9689–9699. doi:10.1021/ac8016295
- Guevremont R (2004) High-field asymmetric waveform ion mobility spectrometry: a new tool for mass spectrometry. *J Chromatogr A* 1058(1–2):3–19. doi:10.1016/j.chroma.2004.08.119
- Coy SL, Krylov EV, Schneider BB, Covey TR, Brenner DJ, Tyburski JB, Patterson AD, Krausz KW, Fornace AJ, Nazarov EG (2010) Detection of radiation-exposure biomarkers by differential mobility prefiltered mass spectrometry (DMS-MS). *Int J Mass Spectrom* 291(3):108–117. doi:10.1016/j.ijms.2010.01.013
- Blagojevic V, Chramow A, Schneider BB, Covey TR, Bohme DK (2011) Differential mobility spectrometry of isomeric protonated dipeptides: modifier and field effects on ion mobility and stability. *Anal Chem* 83(9):3470–3476. doi:10.1021/ac200100s
- Rorrer LC III, Yost RA (2011) Solvent vapor effects on planar high-field asymmetric waveform ion mobility spectrometry. *Int J Mass Spectrom* 300:173–181. doi:10.1016/j.ijms.2010.04.002
- Schneider BB, Covey TR, Coy SL, Krylov EV, Nazarov EG (2010) Planar differential mobility spectrometer as a pre-filter for atmospheric pressure ionization mass spectrometry. *Int J Mass Spectrom* 298(1–3):45–54. doi:10.1016/j.ijms.2010.01.006
- Bieri S, Varesio E, Muñoz O, Veuthey J-L, Christen P (2006) Use of porous graphitic carbon column for the separation of natural isomeric tropane alkaloids by capillary LC and mass spectrometry. *J Pharm Biomed Anal* 40(3):545–551. doi:10.1016/j.jpba.2005.07.007
- Hopfgartner G (2011) Can MS fully exploit the benefits of fast chromatography? *Bioanalysis* 3(2):121–123. doi:10.4155/bio.10.191
- Leuthold LA, Grivet C, Allen M, Baumert M, Gr H (2004) Simultaneous selected reaction monitoring, MS/MS and MS3 quantitation for the analysis of pharmaceutical compounds in human plasma using chip-based infusion. *Rapid Comm Mass Spectrom* 18(17):1995–2000. doi:10.1002/rcm.1587
- Krylova N, Krylov E, Eiceman GA, Stone JA (2003) Effect of moisture on the field dependence of mobility for gas-phase ions of organophosphorus compounds at atmospheric pressure with field asymmetric ion mobility spectrometry. *J Phys Chem A* 107(19):3648–3654
- Schneider BB, Covey TR, Coy SL, Krylov EV, Nazarov EG (2010) Chemical effects in the separation process of a differential mobility/mass spectrometer system. *Anal Chem* 82(5):1867–1880. doi:10.1021/ac902571u
- Schneider BB, Covey TR, Coy SL, Krylov EV, Nazarov EG (2010) Control of chemical effects in the separation process of a differential mobility mass spectrometer system. *Eur J Mass Spectrom* 16(1):57–71. doi:10.1255/ejms.1025