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# Analysis of Paralytic Shellfish Toxins Using High Field Asymmetric Waveform Ion Mobility Spectrometry with Liquid Chromatography – Mass Spectrometry

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## Abstract

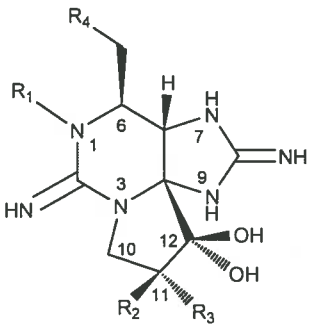
The analysis of paralytic shellfish toxins (PSTs) by LC-MS remains a challenge because of their high polarity, large number of analogues, and complex matrix in which they occur. Here we investigate the potential utility of high field asymmetric waveform ion mobility spectrometry (FAIMS) as a gas phase ion separation tool for analysis of PSTs by mass spectrometry. We investigate the separation of PSTs using FAIMS with two divergent goals; using FAIMS as a primary separation tool for rapid screening by ESI-FAIMS-MS or combined with LC in a multidimensional LC-ESI-FAIMS-MS separation. First, a survey of the parameters that affect the sensitivity and selectivity of PST analysis by FAIMS was carried out using ESI-FAIMS-MS. In particular, the use of acetonitrile as a gas additive in the carrier gas flow offered very acceptable separation of all PST epimeric pairs. A second set of FAIMS conditions was also identified, which focussed PSTs to a relatively narrow CV range allowing development of an LC-ESI-FAIMS-MS method for analysis of PST toxins in a complex mussel tissue extracts. The quantitative capabilities of this method were evaluated by analysis of a PST containing mussel tissue matrix material. Results compared favourably with analysis by the established LC- post column oxidation- fluorescence methodology with recoveries ranging from 70% to 106%, although sensitivity was somewhat reduced. The current work shows the promise of FAIMS as a tool for analysis of algal biotoxins in complex samples and outlines some critical requirements for its future improvement.



**Keywords:** Differential Mobility Spectrometry (DMS), Paralytic Shellfish Poisoning (PSP), Algal Toxins

## Introduction

Paralytic shellfish toxins (PSTs) are potent neurotoxins produced by several marine dinoflagellates and freshwater cyanobacteria that are bio-accumulated by bivalves and lead to cases of paralytic shellfish poisoning (PSP) worldwide [1, 2]. The parent toxin of the class, saxitoxin, is a Schedule 1 chemical weapon and has many structural analogues, which have been identified in algae and shellfish (Figure 1). In most regulatory laboratories, the AOAC mouse bioassay (MBA) has been used for routine analysis [3], but this method suffers from low sensitivity and poor reproducibility [4]. Recently, the replacement of mouse bioassays in Europe for the monitoring of lipophilic toxins [5] has led to with the adoption/use of instrumental analytical methods, capable of more sensitive and reliable quantification as well as confirmation of the chemical nature of the detected toxins. The most commonly used chemical analytical methods for PSTs are those based on liquid chromatography (LC) coupled with fluorescence detection and using pre- or post- column oxidation reactions producing fluorescent PST derivatives [6-8]. These methods have some drawbacks related to system upkeep and lack of absolute structural confirmation, which is only possible using mass spectrometry detection. In general, shellfish toxin analysis over the last several years has been moving towards the use of liquid chromatography coupled to tandem mass spectrometry detection (LC-MS/MS) with electrospray ionization (ESI), which has been successfully implemented for the regulatory monitoring of lipophilic shellfish toxins [9-11].



| $R_1$ | $R_2$ | $R_3$ | $R_4$ | Toxin[a] | $[M+H]^+$, $[M+H-SO_3]^+$ |
|---|---|---|---|---|---|
| H | H | H | | STX | 300 |
| H | H | $OSO_3^-$ | | GTX2 | 396, 316 |
| H | $OSO_3^-$ | H | —O—C(=O)—$NH_2$ | GTX3 | 396, 316 |
| OH | H | H | | NEO | 316 |
| OH | H | $OSO_3^-$ | | GTX1 | 412, 332 |
| OH | $OSO_3^-$ | H | | GTX4 | 412, 332 |
| H | H | $OSO_3^-$ | —OH | dcGTX2 | 353, 273 |
| H | $OSO_3^-$ | H | | dcGTX3 | 353, 273 |

[a] STX = saxitoxin, NEO = neosaxitoxin, GTX = gonyautoxins, dc = decarbamoyl

Fig. 1 structures of PSTs analysed

Compared with lipophilic classes of algal toxins, the analysis of PSTs by LC-MS remains a significant instrumental challenge for several reasons including (a) their highly polar character limiting their retention in reverse phase LC, (b) the large number of structurally similar analogues including several epimeric pairs (Fig. 1), (c) labile precursor ions whose in-source fragments share *m/z* values with precursors or product ions of other analogues and (d) chemical interference and ionization suppression resulting from the complex shellfish tissue extract matrix. Despite these challenges, a number of LC-MS methods have been reported for the analysis of PSTs using hydrophilic interaction liquid chromatography (HILIC) mode of LC separation, which allows for retention and separation of highly polar PSTs [12-15]. This previous work has demonstrated the potential of the technique as well as some of its current limitations, including the presence of ESI signal suppression and matrix interferences [12].

High Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS), also known as Differential Mobility Spectrometry (DMS), is a relatively new mode of analytical separation that was developed for use with mass spectrometry by Guevremont *et al.* [16]. Since that time, several devices based on the same principles of separation have been made commercially

74 available by MS instrument manufacturers [17-20]. The FAIMS device acts as a continuous ion
75 filter to separate ions in the gas phase, at atmospheric pressure after they are produced by ESI but
76 prior to entering the MS. The mechanism of separation in FAIMS is based on differences in the
77 mobility characteristics of ions as they are subjected to an electric field oscillating between high
78 and low field strengths [17, 21-24]. The main advantage of using FAIMS in analytical mass
79 spectrometry lies in the orthogonality of the separation (based on the size, charge, shape and
80 polarisability of an ion), to the separations in both liquid chromatography and mass
81 spectrometry. This orthogonality results in increased selectivity for isolation of a targeted analyte
82 from interfering species such as other isobaric analytes or matrix components. Thus, analytical
83 MS methods incorporating a FAIMS filter step prior to MS analysis are able to selectively
84 resolve and quantify species that otherwise cannot be selectively analysed by ESI-MS or LC-
85 ESI-MS alone. When used as a primary separation tool, ESI-FAIMS-MS can provide sufficient
86 selectivity to perform very rapid analyses (ca. 30 seconds/sample) of samples without the need
87 for LC separation [24, 25]. For more complex samples that cannot be analysed by direct infusion
88 electrospray, FAIMS can be combined with LC (LC-ESI-FAIMS-MS) to perform a highly
89 selective multidimensional separation [22].

90 The current work aims to evaluate FAIMS as a possible means of improving selectivity in
91 LC-MS analysis of PSTs, where interference from matrix compounds has been identified as a
92 limitation of current LC-MS methodology [12]. We begin by investigating the FAIMS separation
93 of PST isomers from one another by direct ESI-FAIMS-MS, with the secondary goal of focusing
94 PSTs to similar CV values suitable for LC-ESI-FAIMS-MS. Recent literature has highlighted the
95 significant benefits that use of polar solvent gas modifiers can impart to differential mobility
96 separations [17, 20, 26, 27]. Here we investigate the impact of acetonitrile vapour on FAIMS

97 separation of PSTs. An LC-ESI-FAIMS-MS method was then developed for analysis of PSTs in
98 mussel tissue extracts. Finally, in order to evaluate the quantitative capabilities of this technique,
99 a PST mussel tissue reference material was analysed and results were compared with those
100 obtained using LC-FLD with post-column oxidation.

101 **Experimental**

102 Optima LC-MS grade Acetonitrile ($CH_3CN$) was purchased from Fisher (Ottawa, ON,
103 Canada). Ammonium Formate (98%) was purchased from Fisher Chemicals (Fairlawn, NJ,
104 USA). Formic Acid (98%) was purchased from EMD (Darmstadt, Germany). Deionized water
105 (DIW) was produced by passing distilled water through a Milli-Q Gradient A10 de-ionized water
106 system (Millipore, USA).

107 All PST standards and matrix materials were provided by the National Research Council
108 Canada (Halifax, NS, Canada). These included the certified reference material calibration
109 solutions for the toxins in Fig. 1: CRM-STX-f, CRM-NEO-c, CRM-GTX2&3-c, CRM-
110 GTX1&4-c and CRM-dcGTX2&3-b. Tissue matrix materials analyzed included CRM-Zero-
111 Mus, a mussel tissue homogenate certified to be free of PSTs as well as two different pilot scale
112 mussel tissue reference materials [28] whose PST concentrations, homogeneity and stability have
113 been well characterized by an established analytical method, LC-pCox-FLD [8]. Extractions of
114 the blended mussel tissues were prepared by using a modified version of a previously published
115 AOAC extraction procedure [6]. Briefly, 4 g of mussel tissue homogenate were mixed with 4 mL
116 of 0.1 M HCl and boiled for 10 min. After centrifugation, the supernatant was passed through a
117 60 mg OASIS HLB solid phase extraction cartridge (Waters, Milford, MA), de-proteinated by
118 adding one volume of acetonitrile and passed through a 4.5 μm pore size filter. Quantification of
119 PSTs in tissue reference material was carried out by spiking 100 μL of deionized water or of

120 mixed toxin standard into separate 400 µL aliquots of extract to construct a three point standard
121 addition calibration curve used to determine the concentration of the PSTs in the tissue.

122 *ESI-FAIMS-MS and LC-ESI-FAIMS-MS - System 1*

123 A Thermo Fisher LTQ-Orbitrap (Bremen, Germany) equipped with a Thermo Fisher FAIMS
124 system was used for both infusion and LC-based analysis. ESI-FAIMS-MS was carried out by
125 infusing toxin CRMs diluted to approximately 1 - 10 µM in 1:1 Acetonitrile:Water with 0.1%
126 acetic acid at 5 µL/min. MS source parameters for these low-flow infusions included a source
127 temperature of 100 °C, capillary temp of 100 °C, capillary voltage of 10 V, a tube lens voltage of
128 40 V, spray voltage of + 3500 V, sheath gas of 10, auxiliary gas of 5 and sweep gas of 0 (all
129 arbitrary units). Mass spectral data was collected in full scan mode from *m/z* 250 - 450 or in
130 selected ion monitoring mode, all with an IT fill time of 100 ms unless otherwise noted. FAIMS
131 outer bias voltage was 10 V and gas flows, dispersion voltage and electrode temperatures are
132 cited for individual experiments throughout. For flow injection analysis and LC-ESI-FAIMS-MS
133 experiments carried out at a flow rate of 200 µL/min, harsher source parameters were used to
134 ensure better desolvation prior to introduction into FAIMS. These included a source temperature
135 of 300 °C, capillary temp of 350 °C, capillary voltage of 10 V, a tube lens voltage of 40 V, spray
136 voltage of + 3500 V, sheath gas of 35, auxiliary gas of 30 and sweep gas of 0 (all arbitrary units).

137 Liquid chromatography was performed using an Agilent 1200 LC system with an Acquity UPLC
138 1.7 um BEH Amide 2.1 x 100 mm column (Waters, Milford, MA, USA) operated in HILIC
139 mode, similar to conditions reported previously [14]. LC flow rate was 250 µL/min and injection
140 volume was 10 µL. Mobile phase used was 2 mM ammonium formate pH 3.5 (A) and 0.1%
141 formic acid in acetonitrile (B) using a linear gradient from 75% to 55% B in 10 min, held for 2
142 min, before returning to the initial conditions.

*System 2: ESI-FAIMS-MS Investigation of gas additives*

A FAIMS-equipped Thermo Fisher TSQ Quantum (San Jose, CA) was used for ESI-FAIMS-MS studies using gas modifiers. The standard setup was modified as described previously to enable the use of gas additives [20]. Briefly, a Waters UPLC system was used to deliver a reproducible flow of solvent into a mixing T installed in-line with the buffer gas flow being delivered to the FAIMS electrodes. System 2 also used a modified electrode set having a 15 mm inner electrode and an improved desolvation region [18]. This resulted in a narrower electrode gap and overall higher electric field strength at a given applied dispersion voltage than system 1, which used a 13 mm inner electrode. A Harvard apparatus syringe pump was used to deliver PST standard solutions to the ESI needle tip at a flow rate of 15 μL/min. The ESI needle was operated in positive ion mode and MS detection was carried out in selected reaction monitoring (SRM) scan mode using the conditions shown in Table 1. Optimized conditions for analysis of the mixture of PSTs in Fig. 1 included a dispersion voltage of 4400 V, a gas flow of 4 L/min $N_2$ with 1.25% acetonitrile as the modifier and inner/outer electrode temperatures of 35/55 °C and CV values and SRM conditions in Table 1. Alternative FAIMS parameters for separation of specific epimeric pairs are described in figure captions and text in Results and Discussion.

160 **Table 1**: FAIMS and MS/MS conditions used for the separation of PSTs using 1.25%
161 acetonitrile as a carrier gas modifier on System 2.

| Analyte | Precursor Ion *m/z* | Product Ion *m/z* | Collision Energy | Compensation Voltage |
|---|---|---|---|---|
| STX | 300 | 204 | 21 | -70 |
| NEO | 316 | 298 | 15 | -70 |
| GTX1 | 412 | 332 | 15 | -38 |
| GTX2 | 396 | 316 | 7 | -36 |
| GTX3 | 396 | 298 | 15 | -33 |
| GTX4 | 412 | 314 | 15 | -30 |
| dcGTX2 | 353 | 273 | 7 | -33 |
| dcGTX3 | 353 | 255 | 15 | -37 |

162

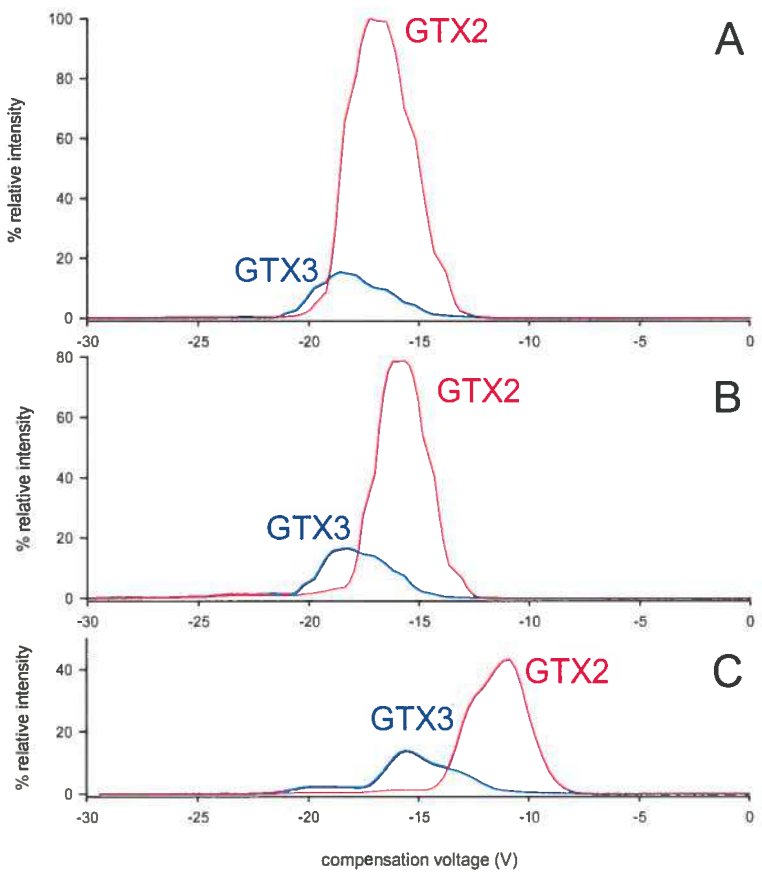
163 **Results and Discussion**

164 Depending on the analytical method being developed, FAIMS optimization for PSTs was carried
165 out towards one of two divergent goals. For selective direct analysis of PSTs by ESI-FAIMS-
166 MS, the epimeric pairs GTX2/GTX3, GTX1/GTX4, and dcGTX2/dcGTX3 (Figure 1) must be
167 separated from one another since they are not resolved in mass spectrometry. Further, the
168 abundant product ions and in-source fragments of GTX2 and GTX3 are sulphate loss product
169 ions identical in composition to protonated NEO (Figure 1), requiring complete separation of
170 these compounds prior to MS analysis. On the other hand, in LC-ESI-FAIMS-MS, isolation of
171 PSTs from interfering matrix components rather than from one another is the primary goal. The
172 LC separation of PST epimers from one another is possible because of significant differences in
173 molecular conformation [12, 29]. Since FAIMS acts as an ion filter, the number of simultaneous
174 CV values that must be monitored at any one time during an LC separation must be minimized in
175 order to minimize the impact on the overall duty cycle of the analysis. Thus, it becomes desirable
176 to focus co-eluting analytes to the same CV values away from interfering matrix components,
177 which have previously been identified as a limitation to LC-MS analysis of PSTs [12].

178 *Separation of PSTs by FAIMS on System 1*

179 The FAIMS separation properties of PSTs were investigated by infusing toxin standards at low
180 flow rates for direct ESI-FAIMS-MS analysis. Each FAIMS parameter was investigated by
181 scanning compensation voltage (CV), initially from -50 V to 50 V. The parameters that were
182 investigated included dispersion voltage (DV), carrier gas composition and flow rate, and
183 electrode temperature. Depending on the differential mobility behaviour of the analytes they can
184 be more effectively transmitted and/or separated through FAIMS with either positive or negative
185 dispersion voltages, referred to as mode P2 and P1, respectively [30]. Under all sets of conditions
186 examined on system 1, PST transmission was around 30-fold higher in P2 mode than in P1
187 mode, limiting the practical utility of the latter for PST separation. In mode P2, an exponential
188 decline in sensitivity was observed below the maximum operating value for dispersion voltage of
189 - 5000 V, which was then used for all subsequent analyses.

190 Increased electrode temperature had the benefit of improving transmission of the un-sulphated
191 PSTs, STX and NEO but the un-desirable effect of increasing in-source fragmentation of the
192 labile GTXs. A decrease in electrode temperature also had a favourable impact on separation
193 between GTX epimeric pairs, as can be seen in the selected ion compensation voltage (SICV)
194 spectra in Figure 2. The behaviour of α- and β- sulphated PST epimers during collisional induced
195 dissociation has been well studied and these compounds differ greatly in lability both in the MS
196 source and during MS/MS experiments [12, 29]. In Figure 2, SICV spectra of the *m/z* 396
197 $[M+H]^+$ ion and its *m/z* 316 sulphate loss source fragment can be used as an approximation for
198 the less labile GTX3 and the more labile GTX2, respectively. At high values of 70/90 °C
199 inner/outer electrode temperature, little separation was observed between the epimers but better
200 separation is observed at the lowest value of 35/45 °C.




201

202 **Figure 2**: Extracted ion compensation voltage spectra showing the effect of electrode
203 temperature on GTX2 (*m/z* 316) and GTX3 (*m/z* 396) separation at (A) 70/90 °C, (B) 50/70 °C
204 and (C) 35/45 °C inner/outer electrode temperatures.

205

206 At sample infusion flow rates, the FAIMS carrier gas flow rate did not have a significant
207 impact on either sensitivity or epimer resolution. A gas flow rate of 3.5 L/min gave improved
208 sensitivity and separation compared with lower flow rates, while higher flow rates resulted in
209 instability of the ion signal. Gas composition proved to have a significant impact on sensitivity
210 and CV of transmission for PSTs, but less impact on separation of epimeric pairs. In P2 mode,
211 the addition of He to the $N_2$ buffer gas resulted in a significant increase in signal intensity as
212 shown in Figure 3 for STX, and a shift to more negative CV values up to 50% He, which is the

maximum the instrument allows due to the risk of electrical discharge. An alternative carrier gas modifier, $CO_2$, has previously been reported to provide more gentle conditions for the detection of fragile ions as well as superior separation for small polar analytes [24, 25]. For PSTs, the addition of $CO_2$ resulted in a significant loss of overall intensity in P2 mode without any improvement in epimer separation of epimeric pairs. In P1 mode, where $CO_2$ has previously been shown to be the most beneficial, addition of $CO_2$ gave a modest improvement in sensitivity, but without enhancement of epimer separation. The relative intensity of the labile $[M+H]^+$ ion of GTX 1 at *m/z* 412 did however increase with the addition of $CO_2$ to the point where it was nearly equal in intensity with the sulphate loss in-source fragment ion at *m/z* 332, supporting a gentler introduction into the mass spectrometer than without $CO_2$. However, compared with 50 % He in $N_2$ as buffer gasses, the absolute intensity was significantly lower when $CO_2$ was added.

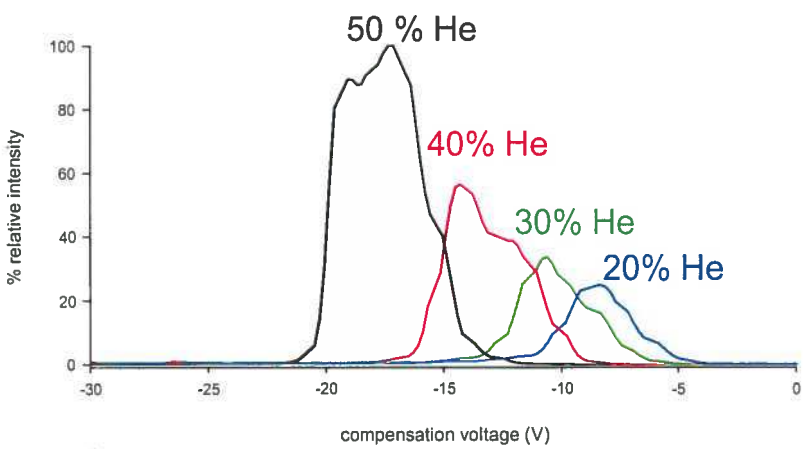



**Figure 3**: Extracted ion compensation voltage spectra showing the impact of increasing % He in $N_2$ for the analysis of protonated saxitoxin at *m/z* 300 by ESI-FAIMS-MS.

The ion filtering effect of FAIMS significantly reduces the total ion current introduced into the mass spectrometer at any one time. This has a beneficial impact for ion trap mass analysers, which can be operated at much higher fill times than when all ions produced by ESI

are introduced into MS [31]. For PST toxin standards at 5 μM, ion trap fill times could be increased from the default value of 10 msec to above 1000 msec without trap saturation. Provided the IT fill time is set to below the time spent at each CV step, it is possible to increase S/N in ESI-FAIMS-MS at no cost to analysis time or FAIMS resolution. Greater care must be taken in LC-ESI-FAIMS-MS experiments where achieving an acceptable number of data points across an LC peak becomes an important consideration.

*ESI-FAIMS-MS/MS Analysis of PSTs Using Gas Additives on System 2*

The recent FAIMS and DMS literature has been dominated by the use of polar solvent vapours as dopants in low % amounts in the buffer gas to enhance selectivity of the FAIMS separation [17, 20, 26, 27]. We investigated this possibility for PSTs using a second ESI-FAIMS-MS system (System 2), which was modified to introduce solvent into the buffer gas flow. As reported previously for a broad range of other analytes [20], acetonitrile was found to have a favourable impact on both the sensitivity of PST analysis and on epimer separation in FAIMS. Without the use of acetonitrile, GTX1 and GTX4 showed no separation using 100 % $N_2$ and were detected in P2 mode (DV = - 4000 V) on system 2, as shown in trace (a) of Figure 4A. When 0.6 % acetonitrile was added to the carrier gas (trace (b) of Figure 4A) baseline separation of the two epimers was observed in P1 mode (DV = + 4000 V). The broad peak at CV = - 41 V can be attributed to species transmitted through FAIMS as dimers or multimers at different CV values than the $[M + H]^+$ ions detected at CV = - 32 V (GTX1) and - 27 V (GTX4), but which then decompose to monomers upon introduction into mass spectrometry. This assignment is supported by the fact that the CV = - 41 V peak increases to 80% relative intensity at higher analyte concentrations (8 μM). Using the more sensitive mass spectrometer in single reaction

253 monitoring (SRM) mode in system 2, toxin standards were analysed at lower analyte
254 concentrations (as low as 0.1 μM) where the formation of multimeric species was minimized.
255 The baseline separation of GTX1 and GTX4 epimers achieved using ESI-FAIMS-MS with 0.6%
256 acetonitrile as a gas additive at a dispersion voltage (DV) of 4000 V allowed for full scan MS
257 spectra of each toxin to be acquired selectively without the need for liquid separation as shown in
258 Figure 4B and 4C. Since the behaviour of PSTs during collision induced dissociation has been
259 well characterized [12, 29], these spectra serve as additional evidence that the CV peaks in Fig. 4
260 represent the true separation of GTX1 and GTX4 epimers rather than of different gas phase
261 conformations of a mixture of isomers resulting from solvation or protonation.

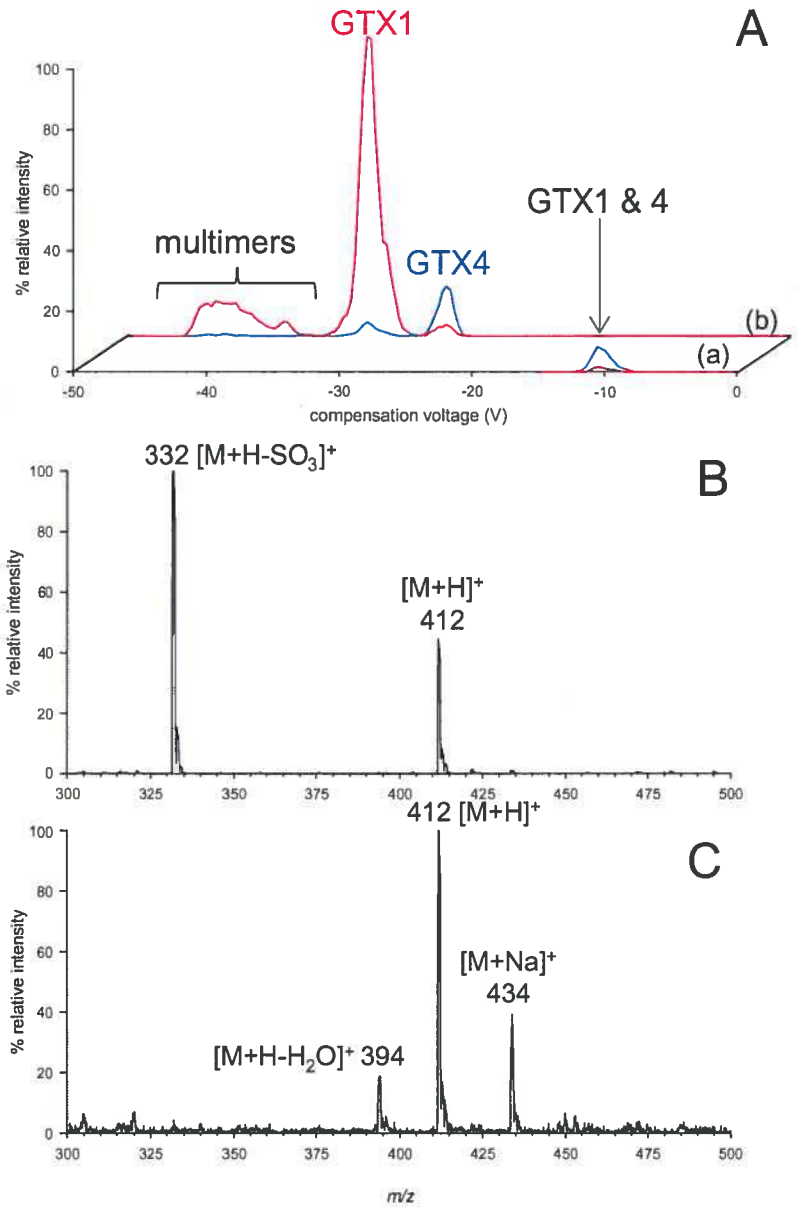



**Fig. 4**: The effect of acetonitrile vapour dopant on the FAIMS separation of GTX1 and GTX4 using system 2. (A) 100% $N_2$ at DV = -4000 Vis shown in trace (a) and 0.6% acetonitrile in $N_2$ at DV = 4000 V is shown in trace (b) using SRM parameters in Table 1. Full scan MS spectra collected using 0.6% acetonitrile at CV = -32 V for GTX1 (B) and at CV = - 26 V for GTX4 (C).

The temperature gradient between the inner and outer FAIMS electrodes was found to be a critical variable in the separation of PST epimers. The optimal 20 °C gradient for separation of

GTX epimers used an inner/outer electrode temperature of 35/55 °C. When this gradient was decreased, the separation deteriorated. For example, GTX2 and GTX3, which were completely separated at 20 °C were only partially separated at 10 °C, and with equal electrode temperatures showed only minimal separation. However, the improved separation using an electrode temperature gradient was achieved at the cost of sensitivity of analysis of the sulphated PSTs with a decrease of between 2 and 10 fold, depending on the analogue. It was therefore not possible to achieve optimal separation and sensitivity for all PSTs under a single set of FAIMS conditions. Conditions optimized for separation of one pair of GTX epimers were not completely optimal for another. In general, improved GTX separation at increased DV and % acetonitrile dopant gave a broadening of the STX peak and pushed it to higher CV values. Of the epimer pairs, GTX 2 and GTX 3 were the most difficult to separate from one another. Figure 5 shows all PSTs analysed at DV = 4400 V with 1.25 % acetonitrile additive, optimal conditions for GTX2 and GTX3 separation, which gave acceptable separation of all epimeric pairs. Under the conditions described in Table 1, all GTX epimer pairs are separated with a resolution of at least 0.6 (GTX2 and GTX3) and baseline resolution for other pairs. Minor peaks observed between ~CV = - 45 V and - 60 V can be attributed to multimeric species of PST ions, which under these conditions are minimized compared with the protonated species. The more gentle conditions for introduction of labile GTX ions into MS afforded by the use of gas additives allowed for all species to be detected as their $[M+H]^+$ ions in SRM scan mode.

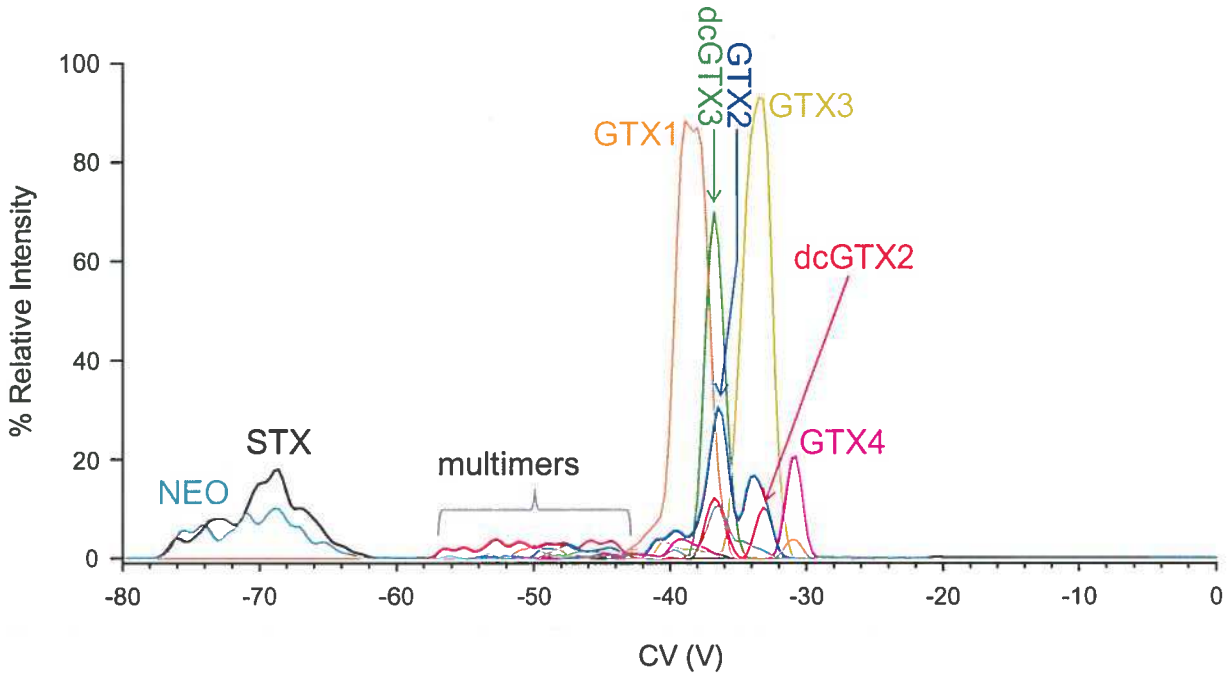



**Figure 5**: Analysis of a PST standard mixture using 1.25% acetonitrile as an additive in $N_2$ gas at a dispersion voltage of 4400 V with an inner/outer electrode temperature of 35/55 °C on System 2. SRM detection conditions for each compound are given in Table 1.

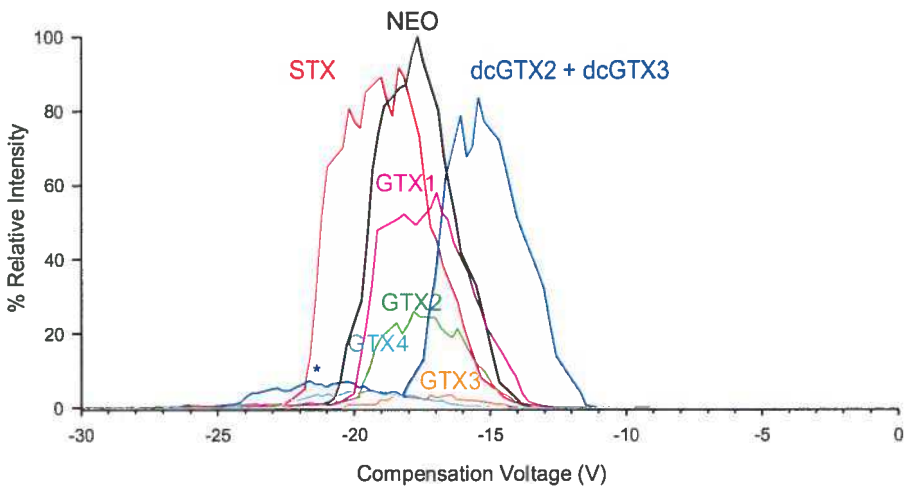
*FAIMS Optimization for LC-ESI-FAIMS-MS on System 1*

It is evident that for selective analysis of PSTs by direct ESI-FAIMS-MS(/MS) the use of gas additives is highly desirable as baseline separation between epimers was not otherwise achieved. However, other more significant challenges related to ionization exist that currently limit the possibility of doing direct analysis of PSTs in complex environmental samples by this technique. In particular, the complex shellfish tissue extracts in which PSTs must typically be quantified cannot currently be analysed by direct electrospray ionization due to severe ionization suppression from matrix components. Analysis of PSTs in these complex samples must instead

303 be done using LC-ESI-FAIMS-MS. In this instrumental configuration, separation of PST
304 epimers is not required as these can readily be separated by LC. However, the CV switching time
305 of FAIMS limits the number of different CV values that can be simultaneously monitored at any
306 point in a chromatographic run. It therefore became desirable to focus PSTs to a narrower CV
307 space, but separated from potential matrix interference. The need to monitor multiple CVs
308 simultaneously during an LC run also has a significant negative impact on analytical sensitivity
309 and limits of detection. For this purpose, the use of 50% He in $N_2$ carrier gas without solvent
310 vapour dopant was favoured as it gave the best sensitivity observed on system 1 while also
311 transmitting the examined PSTs at a smaller range of CV values than with dopant on system 2.

312 In order to scale-up the MS source and FAIMS conditions from the 5 µL/min flow rate
313 used for infusion to the 200 µL/min flow rate of the LC method, several parameters needed to be
314 re-optimized. This was done by carrying out CV scans in single-ion monitoring mode for
315 $[M+H]^+$ and $[M+H\text{-}SO_3]^+$ ions using flow injection analysis of toxin standards. Initially, source
316 parameters including spray voltage, temperature and capillary voltage needed to be optimized in
317 order to achieve acceptable desolvation at the higher flow rate, but FAIMS parameters were
318 observed to be dependent on flow rate and source parameters as well. In particular, the use of
319 higher inner/outer electrode temperature (90/105 °C) resulted in roughly a 4-fold increase in
320 sensitivity at the higher flow rate. This temperature change caused an increase in-source
321 fragmentation to the point where all sulphated toxins were much more sensitively detected as
322 their $[M+H\text{-}SO_3]^+$ source fragments than as $[M+H]^+$ ions. Under these optimized high-flow
323 conditions, little separation was observed between PST epimers as shown in Figure 6. This has
324 the added benefit of reducing the number of CVs that need to be monitored in an LC-ESI-
325 FAIMS-MS experiment, which is important for sensitivity of the analysis. The additional peak

326 detected in the dcGTX2/dcGTX3 standard was ruled out as being separated dcGTX3 by LC-ESI-
327 FAIMS-MS experiment, which showed that no toxin was detected at CV = - 22.5 V. This peak
328 can instead be assigned as a buffer or system contaminant which is effectively filtered by FAIMS
329 during analysis of dcGTX2 and dcGTX3 at CV = - 15.6 V.




330

331 **Fig. 6**: CV Optimization for transmission of PSTs standards through FAIMS on system 1 at LC
332 flow rate (200 µL/min) using flow injection analysis. STX, NEO, GTX3 and GTX4 were
333 detected as their $[M+H]^+$ ions at *m/z* 300, 316, 396 and 412, respectively while GTX1, GTX2,
334 dcGTX2 and dcGTX3 were detected as their $[M+H-SO_3]^+$ source fragments at *m/z* 332, 316, 273
335 and 273, respectively, all in single ion monitoring mode. * labelled peak at - 21 V in dcGTX2/3
336 trace is a system contaminant separated from dcGTXs by FAIMS.

337

338 The limiting factor for analytical sensitivity is the duty cycle of the FAIMS device, which has a
339 switching time between different CV values of about 100 ms, the time required to empty the
340 device of ions that experienced a particular CV. This means that limiting the number of CV
341 values that must be monitored simultaneously is an effective way of limiting any sensitivity
342 losses observed when using FAIMS in combination with LC. Two approaches were investigated
343 for limiting the number of monitored CVs. These included using time periods with a limited
344 number of optimized CV values at a given retention time or reducing the number of monitored

345 CVs to three values which provided coverage of all analytes close to, but not at their CV
346 maxima. Table 2 shows optimized CV values for each analyte under the high flow conditions
347 described in the previous sections, as well as their retention time in HILIC-LC. There are three
348 distinct groupings of PSTs by retention time in the HILIC separation, (a) GTX1 and GTX2 prior
349 to 5 min, (b) GTX3, GTX4 and dcGTX3 between 5 and 6.3 min and (c) STX and NEO after 6.3
350 minutes, while dcGTX2 and dcGTX3 span the first two periods eluting between 4.8 and 5.2 min.
351 It was therefore possible to create a method where a maximum of 3 CV values were monitored at
352 any one time as seen for PST standards in Fig. 7A and PSTs in a mussel tissue reference material
353 in Fig. 7B. The relatively poor detection of GTX4 in Fig. 7B is due primarily to the lower
354 concentration of this analyte in the reference material (0.2 μM in the analysed extract). The
355 primary limitation of this time-binned CV method was that small drifts in retention time over the
356 course of an LC sequence were problematic, particularly the change at 6.3 min which either
357 risked moving onto the front of the STX peak or the tail of the dcGTX3 peak.

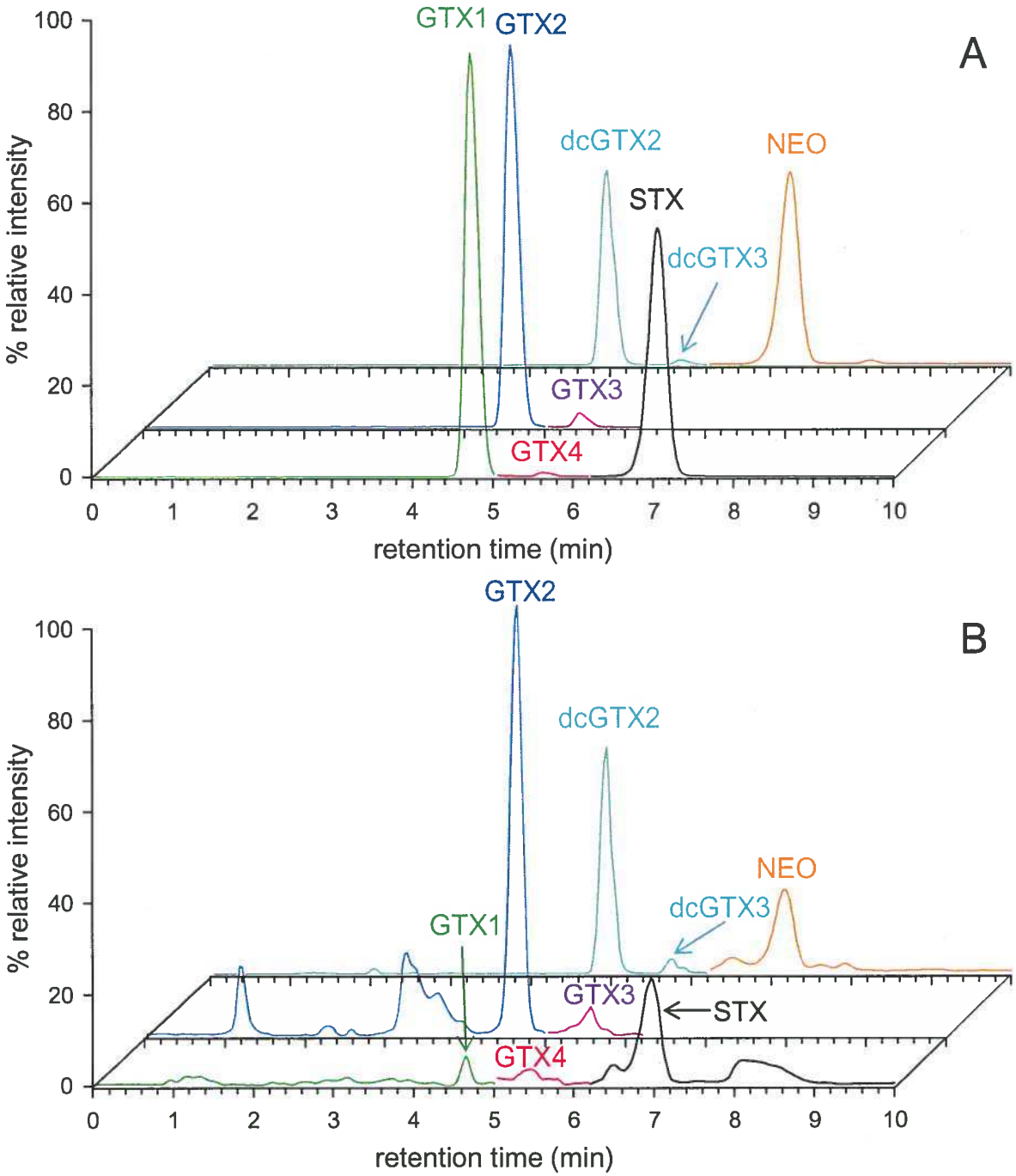



**Fig 7**: LC-ESI-FAIMS-MS analysis of PSTs (A) in a mixed standard and (B) in mussel tissue extracts using retention time bins with optimized CV values for each analyte (details in text) in single ion monitoring mode.

In order to evaluate the quantitative capabilities of LC-ESI-FAIMS-MS for the analysis of PSTs in mussel tissue a more robust method needed to be developed that was not as sensitive to small variations in retention time, which are not uncommon in the analysis of tissue extracts by LC. For this purpose, close approximations to the maximum CV values of each toxin was used to limit the number of CV values which needed to be monitored to 3, but each of these was monitored continuously over the course of the analysis. These individual channels included GTX1 and GTX4 and NEO detected at CV= - 17 V, dcGTX2 and dcGTX3 detected at CV = - 14.5 and STX detected at CV = - 19 V. This more robust method was less sensitive due to the fact that toxins were not detected at their optimum CV values, but was more suitable for analysis of large sample sets where retention time could be expected to drift slightly.

**Table 2**: FAIMS, LC and MS parameters used to detect PSTs in a mussel tissue reference material as well as values determined by LC-ESI-FAIMS-MS and those determined using an established technique, LC-pcox-FLD

| PSP-Mus Pilot | CV (V) | r.t. (min) | $[M+H]^+$ (*m/z*) | *m/z* monitored by LC-ESI-FAIMS-MS | LC-pcox-FLD (µmol/kg tissue ± SD)[a] | LC-ESI-FAIMS-MS (µmol /kg tissue ± SD[b]) |
|---|---|---|---|---|---|---|
| **GTX-1** | - 17.7 | 4.7 | 412 | 332[c] | 2.7 ± 0.3 | 1.9 ± 0.3 |
| **GTX-2** | - 17.8 | 4.6 | 396 | 316[c] | 5.6 ± 0.6 | 4.3 ± 0.1 |
| **GTX-3** | - 17.8 | 5.4 | 396 | 316[c] | 1.6 ± 0.2 | 1.7 ± 0.2 |
| **GTX-4** | - 19.7 | 5.7 | 412 | 332[c] | 0.69 ± 0.07 | < LOQ |
| **dcGTX-2** | - 15.8 | 4.9 | 335 | 273[c] | 8.3 ± 0.8 | 6.5 ± 0.3 |
| **dcGTX-3** | - 15.8 | 5.9 | 335 | 273[c] | 2.2 ± 0.2 | < LOQ |
| **STX** | - 19.1 | 7.0 | 300 | 300 | 2.6 ± 0.3 | 2.68 ± 0.07 |
| **NEO** | - 17.5 | 7.2 | 316 | 316 | 1.4 ± 0.2 | 1.5 ± 0.1 |

[a] Results from an established analytical technique, LC-post column oxidation - fluorescence [8] used previously to characterize the mussel tissue reference material [28].
[b] Standard deviation of three replicate standard addition experiments
[c] $[M+H-SO_3]^+$ product ions formed as the result of in source fragmentation of protonated PSTs

382 The quantitative capabilities of LC-ESI-FAIMS-MS for the analysis of PSTs were
383 evaluated by analysing a second mussel tissue reference material using a standard addition
384 method and the continuous CV monitoring approach described above. This reference material
385 consisted of a mixture of contaminated mussel tissue and toxin-containing algae previously
386 produced on a pilot scale for the standardization of PST measurements [28]. For the most part,
387 quantitative results from LC-ESI-FAIMS-MS agreed well with those from an established method
388 for PST analysis, LC-post column oxidation-FLD [8]. The biggest limitation to these results is
389 the relatively poor sensitivity of the current instrumental setup, which was not able to detect
390 GTX4 or dcGTX3 in the reference material. Both the selectivity and sensitivity of the analysis
391 could be improved by use of a triple quadrupole instrument in single reaction monitoring (SRM)
392 scan mode, which itself would be much more suitable for quantitative analysis in complex tissue
393 extracts.

394 **Conclusions**

395 Our work has outlined the two ways in which FAIMS separation can be used for the analysis of
396 PSTs, either by separation of PST analogues for direct ESI-FAIMS-MS or by focussing of
397 analytes to a narrow CV range away from matrix interference for multidimensional LC-ESI-
398 FAIMS-MS/MS analysis. For direct analysis, the use of low % levels of acetonitrile (~ 1%) as an
399 additive in the carrier gas was necessary to achieve adequate separation of all PST epimeric pairs
400 not resolved by mass spectrometry. The significant positive impact of solvent vapour dopants is
401 consistent with the recent literature in planar DMS devices [17, 27] as well as the cylindrical
402 FAIMS device used here [20]. For LC-ESI-FAIMS-MS, 50 % He in $N_2$ without the use of gas
403 additives provided the best balance of sensitivity while limiting the number of CV values which
404 needed to be simultaneously monitored at a given LC retention time. Faster CV switching time

would allow for simultaneous monitoring of a larger number of CV values which would allow for the superior selectivity of the FAIMS device operated with gas additives to be exploited to a greater extent in the future. For improved analysis of highly labile compounds like PSTs by LC-ESI-FAIMS-MS, better desolvation without inducing in-source fragmentation than could be achieved here with System 1 would also be highly advantageous. This limitation was less evident with the alternative source design on System 2, and should have less of an impact going forward. These highlighted limitations represent practical aspects to the implementation of FAIMS technology rather than fundamental limitations of the technique, which we have shown here to be promising for the analysis of challenging classes of analytes, such as PSTs, in complex samples such as mussel tissue extracts. Future work will involve implementing LC-ESI-FAIMS-MS/MS with gas additives for improved selectivity and an investigation of direct PST quantification using ESI-FAIMS-MS/MS.

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