Research Article

Rapid Communications in Mass Spectrometry

Received: 14 September 2016 Revised: 17 February 2017 Accepted: 5 March 2017 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2017**, *31*, 842–850
(wileyonlinelibrary.com) DOI: 10.1002/rcm.7853

Analysis of 17β-estradiol, estriol and estrone in American eel (*Anguilla rostrata*) tissue samples using liquid chromatography coupled to electrospray differential ion mobility tandem mass spectrometry

Alejandro Cohen[1]*, Neil W. Ross[2,3], Paul M. Smith[2] and James P. Fawcett[1,4,5]*

[1]Proteomics and Mass Spectrometry Core Facility, Faculty of Medicine, Dalhousie University, 5850 College Street, Halifax, Nova Scotia, Canada, B3H 4R2
[2]NovaEel Inc., 2161 Armcrescent E Dr., Halifax, Nova Scotia, Canada, B3H 4R2
[3]Department of Biology, Dalhousie University, 1355 Oxford St., Halifax, Nova Scotia, Canada, B3H 4R2
[4]Department of Pharmacology, Faculty of Medicine, Dalhousie University, 5850 College Street, Halifax, Nova Scotia, Canada, B3H 4R2
[5]Department of Surgery, Faculty of Medicine, Dalhousie University, 5850 College Street, Halifax, Nova Scotia, Canada, B3H 4R2

RATIONALE: 17β-Estradiol (E2), estrone (E1) and estriol (E3) are steroid hormones responsible for the regulation of the female reproductive system. Estradiol is planned to be used to feminize eels in aquaculture in order to improve their size and marketability. The residual levels of these hormones in fish tissue must be monitored to meet the requirements of food regulatory agencies. Few studies have studied these hormones in complex biological matrices such as fish tissue.
METHODS: We developed a method to analyze E1, E2 and E3 in fish tissue using liquid chromatography in combination with differential ion mobility spectrometry (DMS) and tandem mass spectrometry (MS/MS). The mass spectrometer was operated in negative polarity selected reaction monitoring (SRM) mode. To test the performance of this method, residual levels of E1, E2 and E3 were measured in the muscle tissue of juvenile eels subjected to feminization treatment with E2.
RESULTS: We report that following 17β-estradiol treatment, E2 is rapidly metabolized from the eel tissue, with a 50% depletion rate per day. Five days post-treatment, E2 returned to the level found in non-treated controls, similar to levels found in wild mature female eels.
CONCLUSIONS: The method presented herein allows the quantitative analysis of E1, E2 and E3 in fish tissue samples. Under the experimental conditions, E2 in fish tissue samples returned to physiological levels post hormonal treatment.
Copyright © 2017 John Wiley & Sons, Ltd.

The female reproductive organs and secondary sex characteristics of vertebrates, and some insects, are physiologically regulated by endogenous estrogenic hormones. The biosynthesis of these estrogenic compounds has been studied extensively.[1] The three major naturally occurring estrogens in female species are 17β-estradiol (E2), estrone (E1) and estriol (E3); their chemical structures are shown in Fig. 1. 17β-Estradiol has also been used as an exogenous hormone to induce the feminization of certain fish species in aquaculture, including eels.[2]

A number of eel species in the genus Anguilla are caught commercially at the glass eel stage, shipped to grow-out facilities (primarily in Asia) and grown to a market size of >330 g. With the decline in the Japanese eel (*Anguilla japonica*) fishery combined with the recent ban of export of live European eels (*Anguilla anguilla*), the value of the American eel (*Anguilla rostrata*) fishery has increased significantly. One major factor in the farming of wild-caught eels is that, in crowded conditions, the eels tend to develop into males.[3,4] Since male eels fail to grow as large as females, reaching sizes of less than 200 g,[5] feminization is used in commercial eel farming to allow for a larger product. Indeed, a number of reports have examined the feminization of European and Japanese eels (*Anguilla Anguilla* and *A. japonica*, respectively), and found estrogenic compounds to be efficacious in increasing the percentage of females.[6] Currently, there are no commercial eel hatcheries using broodstock despite years of effort.[7] Thus, all farmed eels originate in the wild, limiting the ability to select broodstock that can produce all-female young such as has been studied for other species.[2,8] As such, farming requires the need to add E2 to maintain feminization; thus, the need to quantify and monitor the levels of steroid hormones is critical for commercial growth and regulatory (FDA & Health Canada) purposes.

* *Correspondence to:* A. Cohen or J. P. Fawcett, Proteomics and Mass Spectrometry Core Facility, Faculty of Medicine, Dalhousie University, 5850 College Street, Halifax, Nova Scotia, Canada B3H 4R2.
E-mail: alejandro.cohen@dal.ca; jim.fawcett@dal.ca

Copyright © 2017 John Wiley & Sons, Ltd.

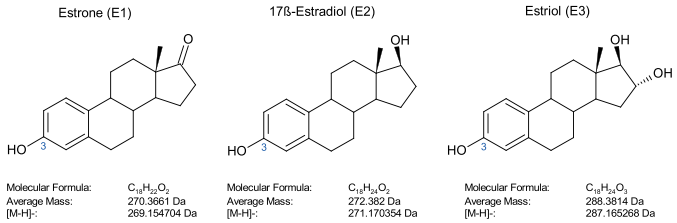

Figure 1. Chemical structures of E1, E2 and E3. The monoisotopic masses of the negatively charged, deprotonated pseudo-molecular ions are labeled as $[M–H]^-$. [Color figure can be viewed at wileyonlinelibrary.com]

The detection and quantification of estrogenic hormones has been achieved using a variety of methods including immunoassays (ELISA),[9,10] gas chromatography (GC)[11–15] and liquid chromatography (LC) coupled to mass spectrometry (MS).[16–26] GC/MS generally requires chemical derivatization of the estrogens prior to analysis, while LC/MS allows the direct analysis of the estrogens without derivatization. When using LC/MS, both atmospheric pressure chemical ionization (APCI)[22,27] and electrospray ionization (ESI) sources have been used. Depending on the analytical LC/MS setup (chromatographic conditions and the ionization source), these type of compounds have successfully been studied in either positive (post-derivatization)[16] or negative ion mode.[17,18,21,28] The introduction of tandem mass spectrometry (MS/MS) has further improved the qualitative and quantitative analysis of these compounds. Within the MS/MS techniques, selected reaction monitoring (SRM, also known as multiple reaction monitoring, MRM) has become the acquisition mode of choice due to its enhanced specificity and sensitivity.[16,18,21,28–30]

The analytical power of mass spectrometry has been further enhanced by the introduction of ion mobility spectrometry.[31,32] Within the many ion mobility techniques, differential ion mobility spectrometry (DMS) has become an important addition to LC/MS/MS systems. The DMS source is located between the LC system and the entrance of the mass spectrometer. DMS is a method of separating ions based on the differences in ion mobility at high and low electric fields in the gas phase, at or near atmospheric pressure. The ion mobility is dependent on the collisional cross section of the analytes in the gas phase. This setup has successfully been applied to the separation of endogenous steroids and other biomolecules such as lipids and peptides.[28,33–35] DMS has the added ability to separate structural isomers, increasing the resolving power of LC/MS when isobaric compounds cannot be separated chromatographically.[34,36] DMS has also proven to be an effective tool to reduce background noise and increase the signal-to-noise ratio in conjunction with SRM acquisitions.[35,37–40] Currently, very few studies have been published demonstrating the benefits of DMS for the separation of steroid hormones.[35,38]

Mass spectrometry has effectively been used to acquire steroid profiles in complex biological matrices; however there is a great discrepancy in the analytical setups and parameters chosen for their detection. In the absence of methods specifically targeting E1, E2 and E3 in fish muscle, we decided to adapt previously published steroid extraction techniques using liquid chromatography, ion mobility and SRM-tandem mass spectrometry for detection purposes. Our methodology was used to quantify the residual amounts of E1, E2 and E3 in eel tissue samples after juvenile specimens had been treated with E2.

EXPERIMENTAL

Chemicals and reagents

17β-Estradiol (98%) was obtained from Fisher Canada (Acros # 436320).

Sample preparation

17β-Estradiol-coated feed preparation, Nutrafry XP feed (Skretting), was used as the base diet for this study. To make the E2-treated feed, 17β-estradiol was dissolved in 95% ethanol at a concentration of 1 mg/mL and sprayed onto the feed with constant mixing at a concentration of 25 mg E2/kg feed. The coated feed was allowed to air dry overnight at room temperature to remove ethanol. Feed was stored at –20°C until day of use.

Eels (*Anguilla rostrata*) of 8 g starting size (9 months post-glass eel) were fed either the control or E2-treated diet at between 3 and 3.5% of body weight per day for 84 days. Following treatment, muscle tissue was rapidly isolated at different time pints and E2 levels were measured. In addition, flesh from wild mature female eels (South Shore Trading Co., Port Elgin, NB, Canada) was also obtained. All animal protocols were approved by the Dalhousie University Committee on Laboratory Animals (Protocol #14-101).

For sample preparation, eels were anaesthetized with 0.2 g/L MS222 (Syndel Laboratories, Vancouver, BC, Canada). Sex of the eels was determined macroscopically.[41] After emptying the abdominal cavity, the mid-section of the fish were cut into 1–2 cm long pieces and frozen at –20°C.

Extraction of 17β-estradiol, estrone and estriol from the frozen eel tissue was based on a method for extraction of estradiol from fish and shellfish.[12] Briefly, muscle was removed from the bone and skin, weighed and placed in a small tube with one volume of 150 mM sodium acetate buffer (pH 5.2) (e.g. 1 mL for each g of tissue). Homogenization was carried out using a Tissue Master 125 homogenizer (Omni International) using a 5 mm probe. The tissue was treated with four cycles of 15–30 s each approaching maximum speed for the last 2 cycles. Homogenized muscle was stored in 0.5 g aliquots at –20°C. Acetonitrile (0.5 mL) was added to 0.5 g of the muscle homogenate, vortexed for 2 min and sonicated for 15 min. Insoluble material was centrifuged at 11,000 *g* for

 Copyright © 2017 John Wiley & Sons, Ltd.

10 min and supernatant removed. The pellet was re-extracted with 0.5 mL of acetonitrile, vortexed, sonicated and centrifuged. The supernatants were combined and back-extracted twice with 1 mL of hexane. The acetone fraction was evaporated to dryness and stored at –20°C. Immediately prior to LC/MS/MS analysis, the dried acetonitrile extract was resuspended twice with 0.05 mL of acetonitrile along with sonication for 1 min. The resuspended fraction was filtered through a 0.45 µm membrane centrifugation device and 50 µL of the filtrate was diluted 1:5 with water (200 µL) and placed in samples vials for analysis.

Efficiency of extraction was assessed by spiking a homogenized eel muscle extract from a non-treated eel with multiple concentrations of E2 (0, 10, 20, 40, 100 and 200 ng E2/mL of extract). After vortexing on high speed for 30 s, samples were stored at –20°C. Extraction was carried out as described above and samples resuspended to theoretical concentrations of 0, 10, 20, 40, 100 and 200 ng/mL in LC loading buffer.

LC/ESI-DMS MS/MS and SRM data analysis

Tissue extracts were analyzed by LC/ESI-MS/MS. The liquid chromatograph (Ultimate 3000 RSLCnano; ThermoScientific, Rochester, USA) was coupled to a triple quadrupole linear ion trap mass spectrometer (QTRAP 5500; Sciex) via a heated assisted electrospray ionization source (Turboionspray; Sciex) using a 25 micron ESI electrode. The chromatographic separation was carried out on a reversed-phase C18 capillary column (Luna 3 µm C18, 100 A, 150 × 0.3 mm; Phenomenex, Torrance, CA, USA) placed in a column oven at 50°C. A guard column (Luna 3 µm C18, 100 A; Phenomenex) was placed to protect and extend the life of the column. The chromatographic conditions are shown in Supplementary Table S1 (Supporting Information).

Differential ion mobility (DMS) was carried out on a SelexION® source (Sciex, Concord, ON, Canada) using isopropanol as the modifier. The ESI, DMS and SRM parameters were optimized infusing 100 ng/mL E1, E2 and E3 standards prepared in 30% ACN with 0.05% NH_4OH. The optimization process is described in the Results and Discussion section. The final method details are shown in Supplementary Table S1 (Supporting Information). Data were acquired with Analyst 1.6.2 software (Sciex), and SRM results analyzed using SKYLINE software (version 2.0).[42]

RESULTS AND DISCUSSION

Determination of MS parameters

Electrospray ionization (ESI) has become one of the most ubiquitous ionization sources in LC/MS/MS setups, thanks to its versatility and ease of use. Nevertheless, ESI still poses a challenge when choosing the appropriate chromatographic and MS detection parameters. During ESI, analytes typically acquire a charge either by deprotonation or protonation of weak acid and basic functional groups, respectively. This process is strongly influenced by the pH of solvents or chromatographic mobile phases. For example, a study by Yamashita *et al.* successfully measured steroid hormones post chemical derivatization using acidified mobile phases in conjunction with reversed-phase LC/ESI-MS, while operating the mass spectrometer in positive ion mode.[16] Other studies have also reported LC/MS data for estrogenic hormones using negative ion mode MS using different mobile phase compositions.[17,18,21,28]

Given the disparity of published data for measuring steroid hormones by LC/ESI-MS/MS, we set out to determine the ideal pH and MS polarity for our experimental conditions. First, we directly infused into the mass spectrometer 100 ng/mL standards containing E1, E2 or E3 dissolved in either acidic (30% ACN with 0.1% formic acid) or basic (30% ACN with 0.05% NH_4OH) solvents. We then acquired spectra for these standards in either positive or negative MS mode. We were unable to produce a stable signal for any of the standards in positive ion mode, regardless of the solvent used. However, when operated in negative ion mode, a strong signal was obtained for each of the three standards dissolved in the basic solvent. The $[M–H]^-$ pseudo-molecular ions for E1, E2 and E3 were detected at *m/z* 269.2, 271.3 and 287.2, respectively (see Supplementary Fig. S1, Supporting Information). In view of these results, we proceeded with our method development using a basic pH mobile phase and negative mode MS.

ESI optimization

Having detected the three standards in negative ion mode, we next optimized our detection method by adjusting the ESI conditions. The optimal ESI conditions were established by infusing a 100 ng/mL E2 standard using a heated assisted electrospray source. Data for ESI optimization was acquired using selected ion monitoring (SIM) at *m/z* 271.3 for the E2 precursor. First, we examined the effect of the source temperature on the analyte signal. This is shown in Supplementary Fig. S2 (Supporting Information). We determined the optimum temperature to be 500°C, based on a compromise between signal strength and stability. The declustering potential, entrance potential, ionization voltage and auxiliary nebulizing gas (Gas 1 on the TurbolonSpray® source) were each ramped to determine their optimum values (Supplementary Fig. S3, Supporting Information). Finally, the auxiliary heater gas, which increases desolvation and the ionization of the sample (Gas 2 on the TurbolonSpray® source), was also optimized; no effect was observed on the signal when ramped (data not shown). Together, these conditions were used to adjust the SRM parameters.

Selected reaction monitoring optimization

Selected reaction monitoring (SRM) allows the tandem mass spectrometer to specifically target a list of selected compounds enabling their detection in complex samples. In order to select the proper transitions for SRM analysis, the E1, E2 or E3 100 ng/mL standards were individually infused and subjected to collisionally activated dissociation (CAD, also referred to as CID, collision-induced dissociation) MS/MS. Figure 2 shows the product ion spectra for each of the standards. Except for the diagnostic precursor ions, most of the product ions are common to the three compounds. This observation suggests that the product ions all share the conserved phenolic end region of the molecule, common to the three steroids. This

Copyright © 2017 John Wiley & Sons, Ltd.

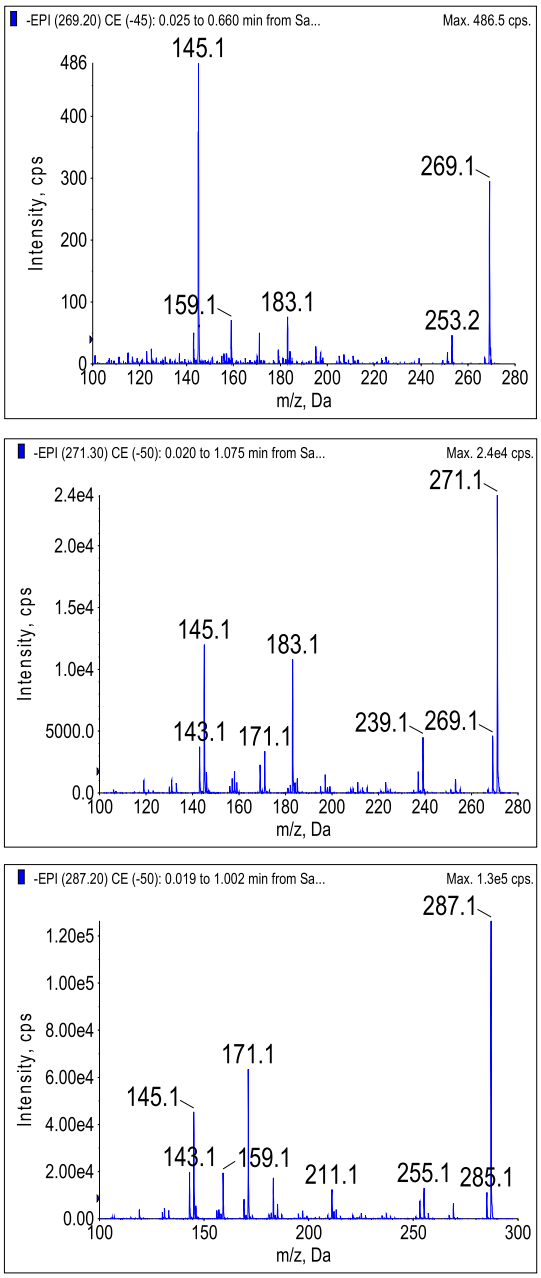

Figure 2. ESI-CAD-MS/MS product ion spectra of E1 (top), E2 (middle) and E3 (bottom). The data was acquired while infusing 100 ng/mL concentration standards prepared in 30% ACN with 0.05% NH_4OH. [Color figure can be viewed at wileyonlinelibrary.com]

would indicate that the negative charge on the $[M–H]^-$ precursor ions likely resides on position 3 of the steroid core structure. This is in agreement with the pKa values of the phenolic (pKa ~10) and alcohol (pKa ~16–19) groups. The product ions found in these spectra share many common peaks as those found in other publications[18] and repository online MS/MS spectral databases, such as mzCloud,[43] an online MS/MS spectral database. Figure 3 shows some tentative structures of the fragment ions found in the product ion spectra. It should be noted that the resolving power of the instrument used and the data acquired do not allow us to establish unequivocal fragment structures.

An automated collision energy optimization test (Compound Optimization option on Analyst software) was performed on the most intense precursor to produce transitions using Skyline's software guidelines adapted to small molecules.[44] The results for these tests are shown in Supplementary Fig. S4 (Supporting Information). The collision gas pressure (CAD value on Analyst Software) was also ramped using the E2 standard based on the SRM traces for each transition. The result of this test is shown in Supplementary Fig. S5 (Supporting Information). The collision energy (CE) voltages shown in Supplementary Table S1 and a CAD value of 8 (arbitrary units) produced the highest signals. These parameters were chosen upon visual inspection of Supplementary Figs. S4 and S5, respectively (Supporting Information). These parameter values were set in the acquisition methods used to tune the LC and DMS separations.

Differential ion mobility spectrometry (DMS) optimization

DMS provides an additional orthogonal separation step when interfaced between the liquid chromatograph and the mass spectrometer. Initially, the DMS parameters were individually tuned for each standard when infused directly into the mass spectrometer. The Separation Voltage (SV) was first ramped to determine the value that produced a significant loss of signal (approximately 3900 V). The Compensation Voltage (CoV) was then ramped at the preset SV to determine the value that recovered the signal. Both tests for E2 can be seen in Supplementary Fig. S6 (Supporting Information). The data was acquired using SIM of the E2 precursor at *m/z* 271.3.

DMS can be further enhanced with the use of modifiers introduced directly into the ion mobility source. Isopropanol improved the separation of the analytes while infusing a mixture of the three standards, as observed by the diagnostic SRM traces for each analyte while ramping the CoV (Figs. 4(A) and 4(B)). We did not evaluate any other modifiers typically used in DSM (e.g. methanol or acetonitrile). Whether these modifiers produce better separation than isopropanol remains to be tested. The DMS traces were further resolved when we introduced nitrogen as a throttle gas, an option controlled by the DMS Resolution Enhancement (DR) parameter (Figs. 4(C) and 4(D)).[45] The increase in the DR came at the expense of signal intensity. Therefore, a compromise (Modifier: On and DR: Low) was reached in which the three standards were completely separated by DMS without sacrificing their SRM signal significantly. When the DR was set to High, the intensity of the signal was almost completely lost (data not shown). The use of modifier and setting of DR should be evaluated individually by users based on the compromise between signal intensity and selectivity. As discussed below, the case for DMS becomes less critical when analytes are separated chromatographically and/or when their precursor and product ions are resolved in the mass-to-charge (*m/z*) dimension.

Liquid chromatography optimization

A chromatographic method was developed using an acetonitrile gradient with 0.05% NH_4OH added in the mobile phase to enhance the formation of $[M–H]^-$ ions during the ESI process. The separation was carried out on a C18 reversed-

 Copyright © 2017 John Wiley & Sons, Ltd.

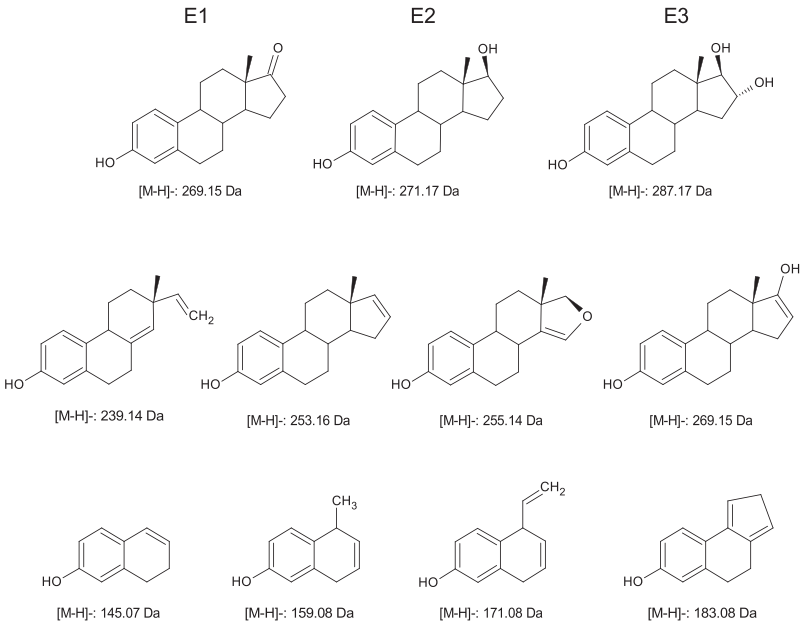

Figure 3. Tentative product ion structures derived from E1, E2 and E3 precursors. Pseudo-molecular $[M–H]^-$ ions are generated by removal of the hydrogen H^+ ion.

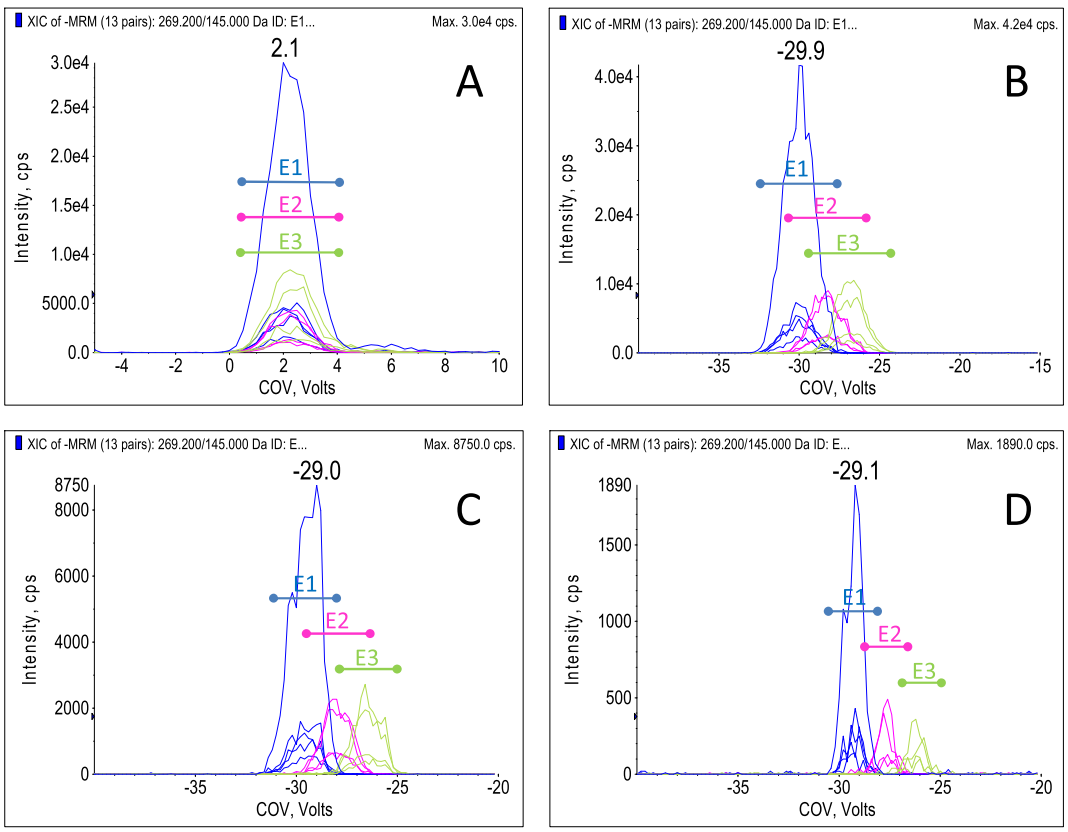

Figure 4. DMS optimization. In absence of modifier, no separation was observed between the E1, E2 and E3 standards when ramping the COV (A) parameter. When a modifier (isopropanol) was introduced, a partial separation of the three standards was observed (B). The ion mobility peaks were further resolved when the DMS Resolution Enhancement was set to Low (C) or Medium (D). Blue, pink and green correspond to the SRM signals for E1, E2 and E3. The horizontal bars labelled E1 (blue), E2 (pink) and E3 (green) indicate the approximate boundaries for the DMS peaks. [Color figure can be viewed at wileyonlinelibrary.com]

Copyright © 2017 John Wiley & Sons, Ltd.

phase column. After a few attempts adjusting the chromatographic gradient, a mixture containing the three standards prepared in mobile phase was chromatographically resolved, as shown in Supplementary Fig. 7(A) (Supporting Information). The effect of turning DMS 'ON' during an LC/SRM acquisition on a mixture of E1, E2 and E3 is shown

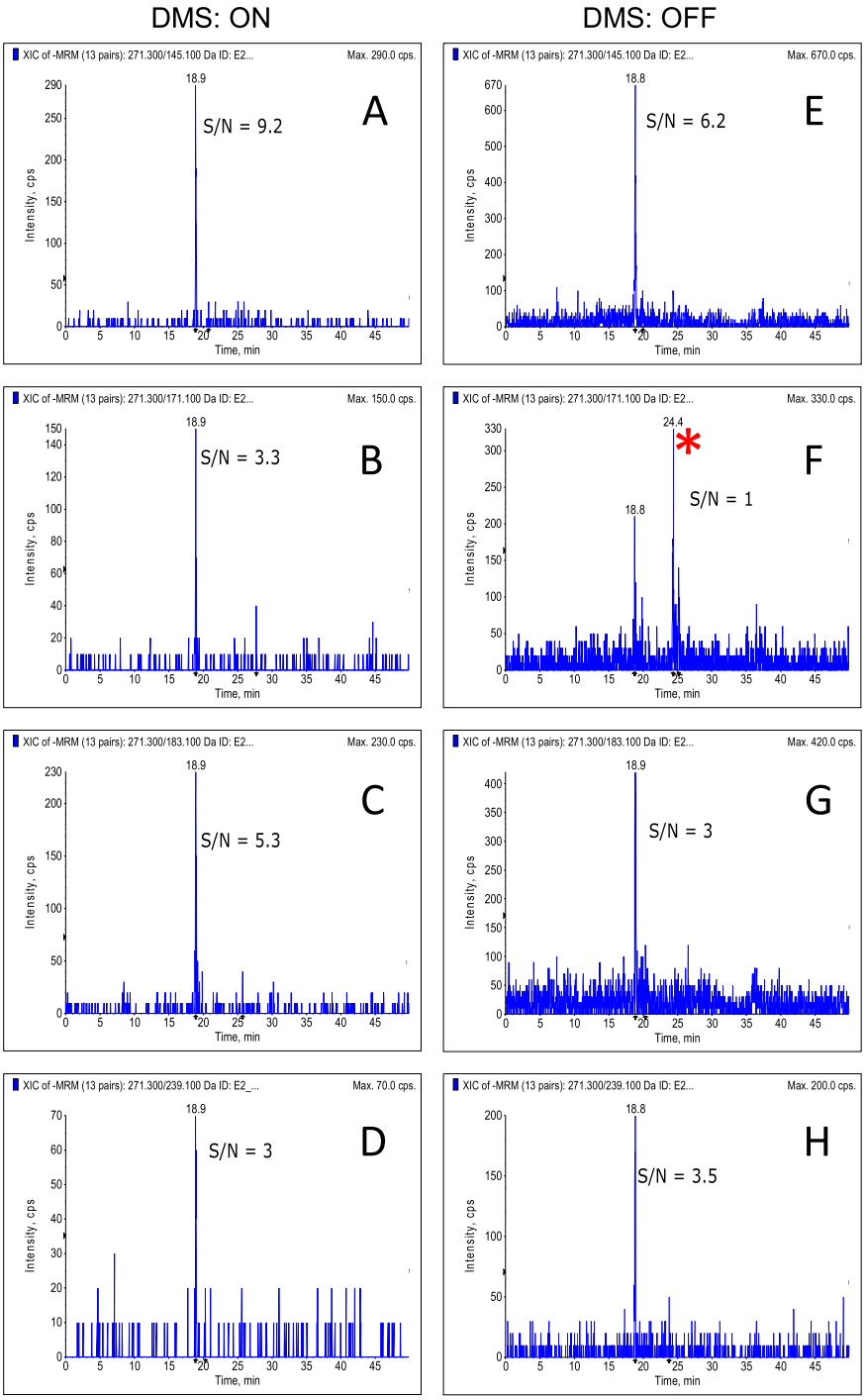

Figure 5. Analysis of a mature female eel muscle tissue by LC/ESI-MS/MS. Left and right columns correspond to DMS 'ON' or 'OFF'. In order from top to bottom, the graphs show the SRM extracted ion chromatograms of E2 for transitions: 271 > 145, 271 > 171, 271 > 183 and 271 > 239. Signal-to-noise ratio (S/N) was calculated using S/N script in Analyst software. Asterisk (*) in (F) shows an additional peak eluting at 24.4 min. The presence of this additional peak at 24.4 min introduces an error in the calculated S/N value by Analyst software for the target peak at 18.9 min, which clearly is greater than one. [Color figure can be viewed at wileyonlinelibrary.com]

 Copyright © 2017 John Wiley & Sons, Ltd.

in Supplementary Fig. S7(B). The SRM chromatograms with DMS turned 'ON' showed an approximate threefold decrease compared to DMS turned 'OFF' (also referred to as 'Transparent mode'), as shown in Supplementary Fig. S7(A). A calibration curve ranging from 1 pg/mL to $1e^6$ pg/mL of a combined E1, E2 and E3 standard was prepared; the results of these standards are shown in Supplementary Fig. S8 (Supporting Information).

Although our LC separation provided effective resolving power on three standards, we decided to proceed with our analyses using DMS anticipating complex biological backgrounds in tissue extract.

It should be noted that the signal produced with the DMS source connected, but turned 'OFF', is lower than that compared to the same setup in the absence of the DMS source (as reported in the vendor's Instruction Manuals). In our setup, DMS was disabled by turning the DMS 'OFF' (or Transparent Mode) through settings on Analyst Software with the DMS source still connected to the mass spectrometer. Thus, the comparison we are making is DMS 'ON' to DMS 'OFF', and not 'no' DMS. A more comprehensive and fair comparison to study the advantages (and disadvantages) of DMS in this scenario should technically be done by removing the DMS source. These types of studies have been performed by others and have shown an average signal loss of 30% for various compounds due to ion diffusion in the planar electrodes of the DMS instrument.[45,46]

Sample analysis

We next tested the capabilities of our analytical setup to detect the steroid hormones in biologically relevant samples. As a proof of concept, we extracted the hormones from muscle tissue of a wild mature female eel specimen. This sample was analyzed in both DMS 'ON' and 'OFF' (Transparent) modes. The purpose of this comparison was to observe changes in S/N, and whether DMS was able to achieve orthogonal separation by 'cleaning' potential interfering peaks, as previously shown in the literature for different compounds.[35,38–40] The results are shown in Fig. 5. In accordance with previous results, the overall SRM signal intensities were reduced by approximately one-third when DMS was turned 'ON'. However, the appearance of additional peaks in the chromatograms when DMS was turned 'OFF' (Fig. 5(F)) supports that DMS can prevent potential coeluting interfering signals in complex biological samples. Additionally, the overall S/N in this particular example appears to improve some transitions upon activation of DMS.

E2 withdrawal study

We next determined the effectiveness of our protocol by measuring and quantifying the decrease of E1, E2 and E3 levels in eel muscle tissue following the end of an E2 treatment cycle. First we calculated the efficiency of E2 extraction from control eel muscle. Here we extracted E2 from non-treated muscle spiked with multiple concentrations of E2 (0, 10, 20, 40, 100 and 200 ng E2/mL of extract). The results for these samples analyzed using the LC/DMS-SRM described above for each spiked level are plotted in Supplementary Fig. S9 (Supporting Information). A strong linearity of LC/MS signal to concentration was observed, with an R^2 value of 0.9957. Extraction efficiency was calculated to be between 84.4 ± 10.9% based on comparison with a 10 ng/mL standard solution of E2 in LC loading buffer. Our limit of detection (LOD) of E2 in muscle tissue was 72 pg E2/g tissue.

To quantify E2 in muscle following the end of treatment, muscle homogenates were obtained from eels removed from tanks over a series of days following the end of treatment. Feminization was determined to be over 90% in the study on day 84, and all eels sampled in the withdrawal study from treated tanks were identified as females, as were the wild eels. The sampled fish from the control tanks were all males. Figure 6 shows the average (± SD) estradiol levels in muscle samples from treated eels prior to and following removal of estradiol from the feed, along with non-treated eels and wild female eels. The estradiol concentration was reduced from 11.2 ± 6.0 ng E2/g tissue to 0.43 ± 0.53 ng E2/g tissue by day 5, equivalent to levels in non-treated eels (0.42 ± 0.28 ng E2/g tissue). Given these extinction values, we calculated the half-life of the E2 in muscle tissue was approximately 1 day. In comparison, the level of E2 in wild eels sampled was 0.95 ng E2/g tissue, or double that of our non-treated eels or treated eels, 5 and 8 days following withdrawal of treatment. We were able to detect E1 in eels just after their last feeding of the estradiol diet – 74.2 pg E1/g tissue. However, in all other samples assayed, the E1 levels were below the LOD (7 pg E1/g tissue), suggesting a rapid removal of this metabolite from eels following the end of treatment. In contrast, wild eel had 34.2 pg E1/g tissue. Finally, the E3 levels of all samples (treated, control and wild) were below the LOD for eel tissue (i.e. 210 pg E3/g tissue).

In this study we expected, and observed, significant changes (a few orders of magnitude) in the levels of hormone levels, especially in E2, as this hormone drug was administered exogenously to our experimental fish. If the analytical setup described herein were applied for other purposes, in which small differences (within the same order of magnitude) in hormone levels are expected, the use of internal standards is strongly recommended to improve the accuracy and reproducibility of the results.

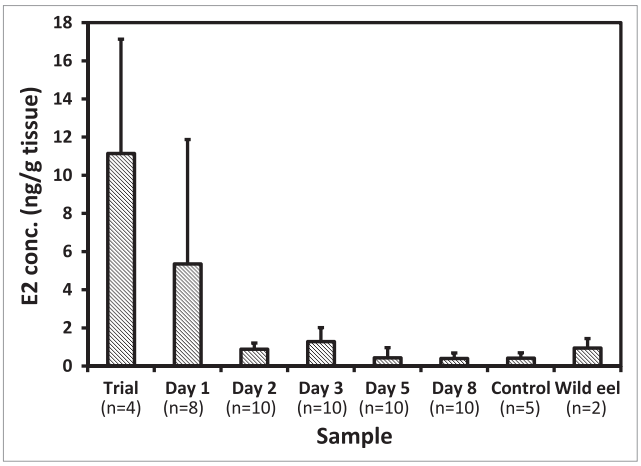

Figure 6. 17β-Estradiol levels in eel muscle tissue over time following withdrawal of E2 feed. Samples size were: Trial, n = 4; Day 1, n = 8; Day 2, n = 10; Day 3, n = 10; Day 5, n = 10; Day 8, n = 10; Control, n = 5; Wild eel, n = 2. LOD = 72 pg E2/g tissue.

Copyright © 2017 John Wiley & Sons, Ltd.

Rapid Communications in Mass Spectrometry

CONCLUSIONS

We have used liquid chromatography in combination with DMS and SRM to measure E1, E2 and E3 levels in eel muscle. Our data suggests that the LC/MS/MS method has a linear response to a broad range of E2 concentrations within eel tissue. As well, we show E2 and its metabolite, E1, are rapidly removed from eel muscle tissue reaching background levels within a week after initial treatment with E2. This information could become valuable in the commercial industry for the approval of fish destined for the food market.

Acknowledgements

This project was funded in part by an NSERC Discovery Grant (JPF) and an NSERC ENGAGE (JPF & NR). Equipment was purchased through a Canadian Foundation for Innovation and Nova Scotia Innovation Trust program (JPF). JPF was supported by a Tier II CRC in Brain Repair during the course of this work. We appreciate the support of Dalhousie University's Cores (Centralized Operation of Research Equipment and Support) program which provided the mass spectrometry expertise and services. The authors would like to thank Rebecca White, Perryn Kruth, Matthew Jacques and other student assistants for their assistance with fish handling and sampling.

REFERENCES

[1] C. J. Gruber, W. Tschugguel, C. Schneeberger, J. C. Huber. Production and actions of estrogens. *N. Engl. J. Med.* **2002**, *346*, 340.
[2] F. Piferrer. Endocrine sex control strategies for the feminization of teleost fish. *Aquaculture* **2001**, *197*, 229.
[3] I. Tzchori, G. Degani, R. Elisha, R. Eliyahu, A. Hurvitz, J. Vaya, B. Moav. The influence of phytoestrogens and oestradiol-17beta on growth and sex determination in the European eel (*Anguilla anguilla*). *Aquac. Res.* **2004**, *35*, 1213.
[4] A. Roncarati, P. Melotti, O. Mordenti, L. Gennari. Influence of stocking density of European eel (*Anguilla anguilla* L.) elvers on sex differentiation and zootechnical performances. *J. Appl. Ichthyol.* **1997**, *13*, 131.
[5] B. Jonsson, B. M. Jessop. Geographic effects on American eel (*Anguilla rostrata*) life history characteristics and strategies. *Can. J. Fish. Aquat. Sci.* **2010**, *67*, 326.
[6] A. J. H. Davey, D. J. Jellyman. Sex determination in freshwater eels and management options for manipulation of sex. *Rev. Fish Biol. Fish.* **2005**, *15*, 37.
[7] Y. Masuda, H. Imaizumi, K. Oda. Artificial completion of the Japanese eel, *Anguilla japonica*, life cycle : Challenge to mass production (The 39th Scientific Symposium of UJNR Aquaculture Panel : The Present and future of the Aquaculture Industry). *Bull. Fish. Res. Agency* **2012**, *111*.
[8] C. I. Hendry, D. J. Martin-Robichaud, T. J. Benfey. Hormonal sex reversal of Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture* **2003**, *219*, 769.
[9] J. P. Nash, B. D.- Cuisset, S. Bhattacharyya, H. C. Suter, F. Le Menn, D. E. Kime. An enzyme linked immunosorbant assay (ELISA) for testosterone, estradiol, and 17,20β-dihydroxy-4-pregenen-3-one using acetylcholinesterase as tracer: application to measurement of diel patterns in rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol. Biochem.* **2000**, *22*, 355.
[10] G. Silván, J. C. Illera, M. Illera. Determination of follicular fluid estradiol levels by enzyme-linked immunosorbent assay. *Steroids* **1993**, *58*, 324.
[11] V. Papadopoulos, S. Carreau, E. Szerman-Joly, M. A. Drosdowsky, L. Dehennin, R. Scholler. Rat testis 17β-estradiol: Identification by gas chromatography-mass spectrometry and age related cellular distribution. *J. Steroid Biochem.* **1986**, *24*, 1211.
[12] L. Zou, H. Lin, J. Jiang. Determination of β-estradiol residues in fish/shellfish muscle by gas chromatography-mass spectrometry. *Chin. J. Anal. Chem.* **2007**, *35*, 983.
[13] S. H. G. Andersson, J. Sjövall. Analysis of profiles of unconjugated steroids in rat testicular tissue by gas chromatography-mass spectrometry. *J. Steroid Biochem.* **1985**, *23*, 469.
[14] L. C. Zacharia, R. K. Dubey, E. K. Jackson. A gas chromatography/mass spectrometry assay to measure estradiol, catecholestradiols, and methoxyestradiols in plasma. *Steroids* **2004**, *69*, 255.
[15] R. J. Santen, L. Demers, S. Ohorodnik, J. Settlage, P. Langecker, D. Blanchett, P. E. Goss, S. Wang. Superiority of gas chromatography/tandem mass spectrometry assay (GC/MS/MS) for estradiol for monitoring of aromatase inhibitor therapy. *Steroids* **2007**, *72*, 666.
[16] K. Yamashita, M. Okuyama, Y. Watanabe, S. Honma, S. Kobayashi, M. Numazawa. Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography–electrospray ionization tandem mass spectrometry. *Steroids* **2007**, *72*, 819.
[17] J. Hu, H. Zhang, H. Chang. Improved method for analyzing estrogens in water by liquid chromatography–electrospray mass spectrometry. *J. Chromatogr. A* **2005**, *1070*, 221.
[18] T. Guo, J. Gu, O. P. Soldin, R. J. Singh, S. J. Soldin. Rapid measurement of estrogens and their metabolites in human serum by liquid chromatography-tandem mass spectrometry without derivatization. *Clin. Biochem.* **2008**, *41*, 736.
[19] S. Pedreira, M. Lolo, B. I. Vázquez, C. M. Franco, A. Cepeda, C. Fente. Liquid chromatography–electrospray ionization–mass spectrometry method in multiple reaction monitoring mode to determine 17α-ethynylestradiol residues in cattle hair without previous digestion. *J. Agric. Food Chem.* **2007**, *55*, 9325.
[20] P. Labadie, E. M. Hill. Analysis of estrogens in river sediments by liquid chromatography–electrospray ionisation mass spectrometry: Comparison of tandem mass spectrometry and time-of-flight mass spectrometry. *J. Chromatogr. A* **2007**, *1141*, 174.
[21] M. Pedrouzo, F. Borrull, E. Pocurull, R. M. Marcé. Estrogens and their conjugates: Determination in water samples by solid-phase extraction and liquid chromatography–tandem mass spectrometry. *Talanta* **2009**, *78*, 1327.
[22] R. Draisci, L. Palleschi, E. Ferretti, C. Marchiafava, L. Lucentini, P. Cammarata. Quantification of 17β-estradiol residues in bovine serum by liquid chromatography-tandem mass spectrometry with atmospheric pressure chemical ionization. *Analyst* **1998**, *123*, 2605.
[23] R. E. Nelson, S. K. Grebe, D. J. O'Kane, R. J. Singh. Liquid chromatography-tandem mass spectrometry assay for simultaneous measurement of estradiol and estrone in human plasma. *Clin. Chem.* **2004**, *50*, 373.
[24] X. Xu, J. M. Roman, H. J. Issaq, L. K. Keefer, T. D. Veenstra, R. G. Ziegler. Quantitative measurement of endogenous estrogens and estrogen metabolites in human serum by liquid chromatography–tandem mass spectrometry. *Anal. Chem.* **2007**, *79*, 7813.
[25] S. S.-C. Tai, M. J. Welch. Development and evaluation of a reference measurement procedure for the determination of estradiol-17β in human serum using isotope-dilution liquid chromatography–tandem mass spectrometry. **2005**. *77*, 6359.

 Copyright © 2017 John Wiley & Sons, Ltd.

[26] M. M. Kushnir, A. L. Rockwood, J. Bergquist, M. Varshavsky , W. L. Roberts, B. Yue, A. M. Bunker, A. W. Meikle. High-sensitivity tandem mass spectrometry assay for serum estrone and estradiol. *Am. J. Clin. Pathol.* **2008**, *129*, 530.
[27] S. Rodriguez-Mozaz, M. J. López de Alda, D. Barceló. Monitoring of estrogens, pesticides and bisphenol A in natural waters and drinking water treatment plants by solid-phase extraction–liquid chromatography–mass spectrometry. *J. Chromatogr. A* **2004**, *1045*, 85.
[28] Y. Fu, Y.-Q. Xia, J. Flarakos, F. L. S. Tse, J. D. Miller, E. B. Jones, W. Li. Differential mobility spectrometry coupled with multiple ion monitoring in regulated LC-MS/MS bioanalysis of a therapeutic cyclic peptide in human plasma. *Anal. Chem.* **2016**, *88*, 3655.
[29] G. D'Ascenzo, A. Di Corcia, A. Gentili, R. Mancini, R. Mastropasqua, M. Nazzari, R. Samperi. Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. *Sci. Total Environ.* **2003**, *302*, 199.
[30] C. E. Ramirez, M. Nouzova, P. Benigni, J. M. E. Quirke, F. G. Noriega, F. Fernandez-Lima. Fast, ultra-trace detection of juvenile hormone III from mosquitoes using mass spectrometry. *Talanta* **2016**, *159*, 371.
[31] R. W. Purves. Enhancement of biological mass spectrometry by using separations based on changes in ion mobility (FAIMS and DMS). *Anal. Bioanal. Chem.* **2013**, *405*, 35.
[32] R. W. Purves, R. Guevremont. Electrospray ionization high-field asymmetric waveform ion mobility spectrometry–mass spectrometry. *Anal. Chem.* **1999**, *71*, 2346.
[33] D. G. Beach, E. S. Kerrin, M. A. Quilliam. Selective quantitation of the neurotoxin BMAA by use of hydrophilic-interaction liquid chromatography–differential mobility spectrometry–tandem mass spectrometry (HILIC–DMS–MS/MS). *Anal. Bioanal. Chem.* **2015**, *407*, 8397.
[34] H. S. Jónasdóttir, C. Papan, S. Fabritz, L. Balas, T. Durand, I. Hardardottir, J. Freysdottir, M. Giera. Differential mobility separation of leukotrienes and protectins. *Anal. Chem.* **2015**, *87*, 5036.
[35] P. R. S. Baker, A. M. Armando, J. L. Campbell, O. Quehenberger, E. A. Dennis. Three-dimensional enhanced lipidomics analysis combining UPLC, differential ion mobility spectrometry, and mass spectrometric separation strategies. *J. Lipid Res.* **2014**, *55*, 2432.
[36] Y. Qi, T. Geib, P. Schorr, F. Meier, D. A. Volmer. On the isobaric space of 25-hydroxyvitamin D in human serum: potential for interferences in liquid chromatography/tandem mass spectrometry, systematic errors and accuracy issues. *Rapid Commun. Mass Spectrom.* **2015**, *29*, 1.
[37] F. Li, M. Ewles, M. Pelzer, T. Brus, A. Ledvina, N. Gray, M. Koupaei-Abyazani, M. Blackburn. Case studies: the impact of nonanalyte components on LC–MS/MS-based bioanalysis: strategies for identifying and overcoming matrix effects. *Bioanalysis* **2013**, *5*, 2409.
[38] J. A. Ray, M. M. Kushnir, R. A. Yost, A. L. Rockwood, A. Wayne Meikle. Performance enhancement in the measurement of 5 endogenous steroids by LC–MS/MS combined with differential ion mobility spectrometry. *Clin. Chim. Acta* **2015**, *438*, 330.
[39] W. Jin, M. Jarvis, M. Star-Weinstock, M. Altemus. A sensitive and selective LC-differential mobility-mass spectrometric analysis of allopregnanolone and pregnanolone in human plasma. *Anal. Bioanal. Chem.* **2013**, *405*, 9497.
[40] A. Kafle, J. Klaene, A. B. Hall, J. Glick, S. L. Coy, P. Vouros. A differential mobility spectrometry/mass spectrometry platform for the rapid detection and quantitation of DNA adduct dG-ABP. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 1473.
[41] K. Beullens, E. H. Eding, P. Gilson, F. Ollevier, J. Komen, C. J. J. Richter. Gonadal differentiation, intersexuality and sex ratios of European eel (Anguilla anguill L.) maintained in captivity. *Aquaculture* **1997**, *153*, 135.
[42] B. Maclean, D. M. Tomazela, N. Shulman, M. Chambers, G. L. Finney, B. Frewen, R. Kern, D. L. Tabb, D. C. Liebler, M. J. Maccoss. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics. Appl. Note* **2010**, *26*, 966.
[43] R. Mistrik, J. Lutisan, Y. Huang, M. Suchy, J. Wang, M. Raab. mzCloud: a key conceptual shift to understand "Who's Who" in untargeted metabolomics, in *Metabolomics Society 2013 Conference*, Glasgow, July, *2013*, pp. 1–4.
[44] B. MacLean, D. M. Tomazela, S. E. Abbatiello, S. Zhang, J. R. Whiteaker, A. G. Paulovich, S. A. Carr, M. J. MacCoss. Effect of collision energy optimization on the measurement of peptides by selected reaction monitoring (SRM) mass spectrometry. *Anal. Chem.* **2010**, *82*, 10116.
[45] B. B. Schneider, T. R. Covey, S. L. Coy, E. V. Krylov, E. G. Nazarov. Planar differential mobility spectrometer as a pre-filter for atmospheric pressure ionization mass spectrometry. *Int. J. Mass Spectrom.* **2010**, *298*, 45.
[46] B. B. Schneider, T. R. Covey, S. L. Coy, E. V. Krylov, E. G. Nazarov. Control of chemical effects in the separation process of a differential mobility mass spectrometer system. *Eur. J. Mass Spectrom. (Chichester, Eng).* **2010**, *16*, 57.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

 Copyright © 2017 John Wiley & Sons, Ltd.