METABOLIC PROFILING OF HUMAN SALIVA BEFORE AND AFTER INDUCED PHYSIOLOGICAL STRESS BY ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-ION MOBILITY-MASS SPECTROMETRY.

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ABSTRACT

A method has been developed for metabolite profiling of the salivary metabonome based on protein precipitation and ultra-high performance liquid chromatography coupled with ion mobility-mass spectrometry (UHPLC-IM-MS). The developed method requires 0.5 mL of human saliva, which is easily obtainable by passive drool. Standard protocols have been established for the collection, storage and pre-treatment of saliva. The use of UHPLC allows rapid global metabolic profiling for biomarker discovery with a cycle time of 15 minutes. Mass spectrometry imparts the ability to analyse a diverse number of species reproducibly over a wide dynamic range, which is essential for profiling of biofluids. The combination of UHPLC with IM-MS provides an added dimension enabling complex metabolic samples to be separated on the basis of retention time, ion mobility and mass-to-charge ratio in a single chromatographic run. The developed method has been applied to targeted metabolite identification and untargeted metabolite profiling of saliva samples collected before and after exercise-induced physiological stress. δ-Valerolactam has been identified as a potential biomarker on the basis of retention time, MS/MS spectrum and ion mobility drift time.

Keywords: Metabolite Profiling; UHPLC; Ion mobility; Mass Spectrometry; Saliva; Physiological Stress; Exercise; δ-Valerolactam.

1. INTRODUCTION

Human saliva is a complex biofluid composed of secretions from major and minor salivary glands, gingival crevicular fluid, oral bacteria and food debris. Saliva ensures the stability in the oral cavity and aids in functions such as lubrication, speech, digestion, antibacterial activity, maintaining integrity of tooth enamel and sense of taste. Whole saliva consists of a variety of biologically relevant compounds including hormones, nucleic acids, peptides, proteins, metabolites, immunoglobulins, enzymes (lysozymes, lingual lipase, α-amylase etc.), mucins, electrolytes (Na, K, Ca, Mg, carbonates, phosphates etc.) and nitrogenous products such as urea and ammonia (Humphrey and Williamson, 2001). Saliva is a highly variable and individualised fluid and it's composition and flow is affected by various factors including age, gender, circadian rhythm, circannual cycle, psycho-emotional state, physical exercise, hydration, systemic diseases, medication and nutrition. (De Almeida et al., 2008).

In the past, saliva or oral fluid has been neglected as a diagnostic medium and preference has been given to other more invasively collected biofluids such as blood, serum and plasma. Saliva has the advantage of allowing non-invasive sample collection and substances present in saliva are in their biologically active form (Pink et al., 2009). The pace of research into saliva as a diagnostic medium has accelerated in recent years with the advent of new techniques, which have provided a wealth of information regarding the biochemical and physicochemical properties of saliva. The applications of saliva as a diagnostic medium have been summarised elsewhere and the potential for early detection of systemic diseases or exposure to harmful substances has been demonstrated (Pink et al., 2009; Walsh, 2007). The complexity, biological significance of saliva and the advancements in analytical technology have shown the immense potential of this matrix in metabolite profiling.

Metabolic profiling, sometimes described as metabonomics or metabolomics, refers to the targeted or global assessment of endogenous small-molecule metabolites within a biological system. The term metabonomics was coined by Nicholson et al. and defined as "the quantitative measurement of the dynamic multiparametric response of a living system to pathophysiological stimuli or genetic modification" (Lindon and Nicholson, 2008; Nicholson et al., 1999). The metabolome is regarded as the most predictive phenotype and hence the study of metabolites has been acknowledged as a desirable tool for monitoring the biological state of organisms and for disease diagnosis (Zhang et al., 2012). Metabolite profiling is a technologically driven science and has made significant progress with the advancement of separation science (Issaq et al., 2008; Xiayan and Legido-Quigley, 2008), with the majority of applications centred around global profiling and biomarker detection in biofluids, such as whole blood, serum, plasma, urine, cerebrospinal fluid, synovial fluid, semen etc. (Zhang et al., 2012). Untargeted metabolic profiling of saliva using NMR and LC-MS has shown promise (Aimetti et al., 2011; Álvarez-Sánchez et al., 2012; Takeda et al., 2009; Wei et al., 2010). Liquid chromatography-mass spectrometry (LC-MS) is a widely used technique for the targeted and non-targeted, global metabolic profiling of low molecular mass metabolites and other compounds in biofluids. However, the use of this technique for metabolite profiling of saliva remains under explored.

Ion mobility spectrometry is a technique which separates ionised molecules on the basis of their collision cross sections (i.e. size and shape) in the gas phase and has been extensively reviewed (Creaser et al., 2004; Kanu et al., 2008). The application of ion mobility spectrometry hyphenated with mass spectrometry (IM-MS) to metabolite profiling has been reported (Dwivedi et al., 2007; Harry et al., 2008), but the use of on mobility-mass spectrometry combined with ultra-high performance liquid chromatography (Swartz, 2005) has not been explored.

This study describes the development and validation of a method for the targeted and untargeted metabolic profiling of saliva by ultra-high performance liquid chromatography-ion mobility-mass spectrometry (UHPLC-IM-MS). The method has been applied to the profiling of metabolites and the discovery of potential discriminating biomarker ions of exercise-induced physiological stress.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Acetonitrile (analytical grade), water (analytical grade) and formic acid (>99.9%) were obtained from ThermoFisher Scientific (Loughborough, UK). L-carnitine (>99%) and hydrocortisone (>99%) were obtained from Sigma-Aldrich (Gillingham, UK). A 400 ng/mL stock solution of leucine enkephalin was obtained from Waters Corporation (Manchester, UK) and used to prepare a 2 ng/mL working solution of leucine enkephalin in water:acetonitrile (50:50) (v/v) as a LockMass (m/z 556.2771) solution. A standard mixture of 0.01 mg/mL L-carnitine and hydrocortisone was prepared in water:acetonitrile (95:05) (v/v) with 0.1% formic acid. A standard solution of 0.01 mg/mL L-phenylalanine was prepared in water:acetonitrile (95:05) (v/v) with 0.1% formic acid.

2.2 Sampling and storage

Sampling of saliva for this study was carried out under the ethical approval obtained from the local ethical advisory committee (Ethical Advisory Committee, Loughborough University, Loughborough, LE11 3TU) with reference number G10-P24. Participants were healthy, non-smoking males, aged 18-35 and were recruited from Loughborough University staff and students (n=10).

Saliva was collected by passive drool from participants after an overnight fast. Participants were seated with their head tilted forward, allowing saliva to pool in front of the mouth. Saliva was allowed to be dribbled out of the mouth into a glass collection vial (30 mL Chromacol, UK) at regular intervals. This process was repeated until sufficient amount of saliva (i.e. 1 mL) was obtained (Chiappin et al., 2007). Saliva collected from all the participants was re-aliquoted as 500 µL aliquots into microcentrifuge tubes (2.0 mL, LoBind Eppendorf, UK). 500 µL saliva from all the participants was pooled together and re-aliquoted as 500 µL aliquots to be used for method development and quality control (QC). Samples were aliquoted prior to storage to minimise freeze-thaw cycles. The saliva samples were stored at -80°C immediately after aliquoting to arrest biological activity.

2.3 Sample pre-treatment

Saliva samples stored at -80°C were thawed at room temperature for 30 minutes prior to sample clean-up by protein precipitation. Each thawed saliva sample was vortexed for 30 seconds followed by ultra-sonication for 1 minute to breakdown mucous substances in saliva and to improve homogeneity. Protein precipitation was achieved by the addition of 1 mL acetonitrile to 500 µL of saliva i.e. 2:1 ratio of precipitant to saliva (Polson et al., 2003). The mixture was vortexed for 30 seconds followed by ultra-sonication for 1 minute and then subjected to centrifugation at 10,000 g for 10 minutes at ambient temperature. Precipitated proteins from the sample were removed as a pellet at the bottom of the microcentrifuge tube. The supernatant was transferred to a fresh microcentrifuge tube and evaporated to near dryness (~5 µL) using Turbovap LV concentration workstation (Caliper Life Sciences, Hopkinton, MA, USA) prior to being reconstituted in 50 µL water/acetonitrile (95:05) (v/v)

with 0.1% formic acid. The reconstituted saliva extract was placed in a 200 µL polypropylene micro-insert (Supelco, UK) in an autosampler vial (2 mL) for UHPLC-IM-MS analysis.

2.4 Method development

2.4.1 Instrumentation

The liquid chromatographic separations were performed on a Waters ACQUITY UPLC chromatograph with an in-built auto-sampler (Waters Corporation, Manchester, UK) fitted with a Waters ACQUITY BEH (bridged ethylene hybrid) C18 column (2.1 mm x 100 mm, 1.7 µm; Waters Corporation, Manchester, UK) maintained at 40°C. A VanGuard BEH C18 pre-column (Waters Corporation, Manchester, UK) was attached in series with the analytical column. The UPLC system was coupled with a Waters Synapt HDMS ion-mobility mass spectrometer (Waters Corporation, Manchester, UK). MassLynx 4.1 (Waters Corporation, Manchester, UK) was used for controlling the setup and for data acquisition. A Waters ACQUITY UPLC chromatograph (Waters Corporation, Manchester, UK) coupled with a LTQ Orbitrap Mass Spectrometer (ThermoFisher Scientific, UK) was used to acquire accurate mass measurements on discriminant metabolites.

2.4.2 UHPLC conditions

Mobile phase A consisted of 0.1% aqueous formic acid (v/v) and mobile phase B was 0.1% formic acid in acetonitrile (v/v). The chromatographic gradient programme was optimised using a solution of L-carnitine and hydrocortisone (0.01 mg/mL). The mobile phase flow rate was set to 0.5 mL/min. The saliva extract (10 µL injected) was analysed by the optimised chromatographic gradient: 5% B (0-1 min), increased to 35% B (1-10 min). This was followed by a column clean-up phase built into the method to reduce carry over and condition the column for analysis of subsequent samples, in which the mobile phase was increased to 95% B (10-11 min) and maintained at 95% B (11-12 min) before returning to initial conditions (13-15 min).

2.4.3 MS conditions

Electrospray ionisation conditions for the Synapt HDMS, with the ion source operated in positive ion mode were: capillary voltage 3.0 kV; cone voltage 30 V; source temperature 120°C; desolvation temperature 300°C; desolvation gas, N2 gas flow 600 L/hr; cone gas flow 30 L/hr. Mass spectra were acquired with a scan rate of 1 sec/scan with a 0.02 sec inter-scan delay. Conditions for LTQ-Orbitrap MS were: heated electrospray ionisation (HESI) source operated under positive polarity mode; source voltage 4.5 kV; capillary voltage 35V; capillary temperature 300ºC and sheath gas flow 45 L/hr.

2.4.4 Optimisation of the ion-mobility separation

The tri-wave (T-wave) drift cell conditions were set at 30 mL/min drift gas (N2) with a variable travelling wave height ramp of 7.0-15.0 V and wave velocity of 300 m/s. The mass spectrum acquisition rate was 0.065 ms/scan. Optimisation of tri-wave parameters was carried out on-line by directly infusing 2 ng/mL solution of Leucine Enkephalin (data not

shown). The optimised variable travelling wave height ramp was set to 6.0-13.0 V. Saliva extract was analysed prior to and after optimisation of ion-mobility separation.

2.4.5 Dynamic range determination and reproducibility

Injection volumes (1-20 µL) of pooled saliva extract were analysed by UHPLC-IM-MS. Chromatographic peak areas of selected endogenous metabolite ions eluting at various retention times throughout the chromatogram were extracted and plotted against the injection volume of saliva.

The reproducibility of the analytical method was assessed using the optimised injection volume (10 µL) determined from the dynamic range experiment. Six pooled saliva extract samples were analysed consecutively by UHPLC-IM-MS. Chromatographic retention times and chromatographic peak areas of extracted endogenous metabolite ions were used to establish the reproducibility of the analytical method.

2.5 Analysis of before-exercise and after-exercise saliva samples

Participants exercised for 30 minutes on a cycle ergometer (Lode Corival, Groningen, The Netherlands) at a power output of 2 Watts/Kg of body mass of the participant. Two saliva samples were collected per participant, one before exercise and the other 30 minutes after the end of the exercise.

2.5.1 Analytical protocol

A series of five consecutive injections of a pooled saliva extract were run prior to the sample analysis to condition the chromatographic column. The analysis of replicate injections showed little retention time drift following this conditioning procedure. The sample list was randomised using Microsoft Excel prior to analysis and a pooled saliva extract was analysed after every 5 saliva samples as a quality control sample (QC). The sensitivity of the instrument was monitored on-line using the LockMass signal. A separate method blank study was carried out as a part of initial UHPLC-IM_MS method development for this work. Method blanks demonstrated no contamination due to polypropylene micro-centrifuge tubes or polypropylene micro-inserts used for this study. Method blanks also demonstrated no carry-over between saliva sample runs.

2.5.2 Data mining and multivariate statistical analysis

Data mining was carried out using MarkerLynx XS (Waters Corporation, Manchester, UK). The parameters used were retention time range 0-10 minutes; retention time tolerance was set to 0.1 min; mass range 50-500 Da; mass tolerance 0.05 Da; isotopic peaks were excluded for analysis; minimum spectral peak intensity was set to 50 counts. The resulting three dimensional data matrix containing arbitrarily assigned peak index (consisting of m/z and retention time pairs), sample names (observations) and their normalised chromatographic peak areas (variables) was exported to SIMCA-P+ (Umetrics, Sweden) for further data processing. Data was normalised to the total ion intensity within the particular sample for each feature (i.e. m/z_retention-time pair) using MarkerLynx XS (Waters Corporation,

Manchester, UK) using chromatographic peak area. This method of normalisation adjusts all the ion intensities so that the sum of intensities within each sample is the same. Multivariate statistical analysis, principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), were carried out on normalised data to identify discriminant metabolites in saliva between the two groups of samples i.e. before and after exercise.

3. RESULTS AND DISCUSSION

3.1 Method development

Protein precipitation was the only sample clean-up procedure applied to the saliva prior to UHPLC-IM-MS analysis, in order to minimise the effect of discrimination and the unpredictable behaviour of unknown metabolites during sample preparation (Álvarez-Sánchez et al., 2012). The selection of endogenous metabolites for validating the method ensured that the matrix effects were accounted for during data processing.

[Figure 1]

A typical UHPLC-IM-MS profile for a saliva extract is shown in Figure 1 illustrating the complexity of the three dimensional nested dataset obtained from the analysis. The two dimensional retention time vs ion mobility drift time plot shows multiple species contributing to single chromatographic peaks (Figure 1 c), demonstrating that the combination of ion mobility with UHPLC allows further separation of co-eluting species. Ion mobility spectrum is plotted as bins, where each bin represents an acquired mass spectrum (65 µs/scan), with 200 bins covering the drift time range of 0-13 ms. Spectra were accumulated to yield 200 mass spectra and one ion mobility spectrum per second giving 6-7 data points across a typical UHPLC peak.

The UHPLC chromatographic gradient programme used for the analysis of saliva extract was optimised using 0.01 mg/mL solution of L-carnitine and hydrocortisone such that the highly polar metabolite, L-carnitine, was slightly retained on the column, whereas low polarity hydrocortisone eluted towards the end of the 10 min chromatographic gradient (data not shown). The total cycle time was 15 minutes, which is suitable for high throughput global metabolic profiling workflows.

[Figure 2]

The major parameters that affect the separation of ions in the T-wave drift cell are IMS gas pressure, IMS wave velocity and IMS wave height. The ion mobility separation shown in Figure 1 was improved by adjusting the variable wave height ramp as a wave gradient effectively increases the length of the IMS drift cell (Shvartsburg and Smith, 2008).

Figure 2 (a) shows IM separation prior to optimisation, with a wave height ramp of 7.0-15.0 V. The majority of the components of the saliva extract have a drift time in the first 100 bins/spectra, associated with IM drift times of <2.6 ms. Ideally, only the first 20 bins/spectra and the last 20 bins/spectra should be empty with analytes distributed across the remaining bins/spectrum (0.13-11.7 ms) of an overall drift time plot for optimum separation. This can be seen in Figure 2 (b), with an optimised wave height ramp of 6.0-13.0 V. Under the optimised UHPLC and ion mobility conditions, analytes that are unresolved and co-eluting in the retention time dimension may be resolved in the ion mobility dimension as a result of the increased peak capacity of UHPLC-IM-MS.

The effect of UHPLC injection volume was assessed for injection volumes in the range of 1-20 µL. Four randomly extracted metabolite ions, spread across the chromatographic range were selected to assess the dynamic range of the analytical system. Chromatographic peak areas showed a linear response for injection volumes in the range 1µL to 10µL, with the response levelling out above 15 µL (data shown in supplementary information figure S1). Hence, a 10 µL injection volume of saliva extract was used for subsequent analysis and global metabolic profiling studies as it lies in the dynamic range of the analytical method.

The results of a UHPLC reproducibility study (n=6) of peak area and retention time are summarised in supplementary information Table T1. Data show less than 9% relative standard deviation (RSD) for chromatographic peak areas and less than 1% RSD for retention times across the range of the chromatogram. The data show that the method is consistent and reliable, which is required for measuring small differences in concentration of detectable metabolites in metabolic profiling studies.

[Figure 3]

3.2 Targeted metabolite identification

The nested UHPLC-IM-MS dataset collected from a saliva sample maybe interrogated for the presence of a targeted analyte. Figure 3 shows an example of the extracted ion chromatogram (figure 3 a) and ion mobility data (figure 3 c) for a targeted analyte, L-phenylalanine present in saliva. Both ion mobility and retention times show a close match with a standard indicating positive identification of the L-phenylalanine and that matrix effects do not adversely affect retention times. The shape of the chromatographic and ion mobility peaks (Figure 3 a and c) indicates the presence of a single species. Selection of the appropriate retention time and ion mobility region (retention time 1.21 min, ion mobility bin numbers 64-68) for L-phenylalanine enhances the relative mass spectral response of the protonated amino acid (Figure 3 b and d).

3.3 Global metabolite profiling analysis of before- and after-exercise saliva samples

The UHPLC-IM-MS analysis of saliva extracts generates a complex dataset consisting of mass-to-charge ratio, chromatographic retention time and ion mobility drift time. IM separation being orthogonal to that of liquid chromatographic separation and mass-to-charge ratio increases the peak capacity as a result of the separation of species of the same m/z or retention time in the mobility dimension. The resulting multidimensional UHPLC-IM-MS datasets present a challenge for the multivariate statistical techniques commonly employed for global metabolite profiling studies. The powerful software available for de-convoluting UHPLC-MS data is not equipped to account for ion-mobility dimension while compiling data matrix for multivariate analysis. The complexity of the data was therefore reduced in the initial stages of the analysis by collapsing the IM dimension.

[Figure 4]

Key factors in the analysis of complex biological metabonomic samples include chromatographic column conditioning, number of quality control samples and run order

(Want et al., 2010). These factors were evaluated using pooled saliva samples collected from male volunteers before-exercise and after-exercise. Figure 4 (a) shows unsupervised principal component analysis (PCA) of all the randomised before-exercise and after-exercise saliva samples and replicates of quality control (QC) samples consisting of an extract of a pooled saliva sample, which was analysed after every batch of 5 samples. The model was based on a total of 4078 variables, which were normalised and Pareto scaled it being the preferred option while dealing with metabolomic data obtained by MS (Van den Berg et al., 2006). Clustering of quality control (QC) samples (circled in Figure 4 a) provides evidence of consistent and reliable data quality adequate for metabolic profiling studies.

The unsupervised PCA approach indicates partial separation between the two classes of samples (Figure 4 a) with before-exercise samples having low principle component 2 (PC2) scores while after-exercise samples showing higher PC2 scores. Supervised approaches, such as partial least squares (PLS) and orthogonal partial least squares discriminant analysis (OPLS-DA) models, are often applied in metabonomics to uncover relationships buried in the background of larger inherent effects (Fonville et al., 2010). In biological systems, for instance, it is often the case that several factors contribute to the metabolic state of an organism e.g. age, gender, nutrition, environment etc. Hence, to focus on the biological question of interest, physiological stress in this case, and to disregard other sources of variation an OPLS-DA model was used. A total of 3067 normalised, Pareto scaled variables were used to construct the model, which was able to distinguish between the two classes of samples (Figure 4 b).

[Figure 5]

A loadings S-plot was constructed using the OPLS-DA model to segregate discriminant metabolites (shown in supplementary information Figure S2). A number of variables were selected from extreme ends of the s-plot and their normalised trend plots were compared as initial screening for potential biomarker ions (data not shown). Discriminant features were extracted from the S-plot and were verified by comparing their extracted ion chromatogram (EIC) from raw data. Figure 5 (a) shows an example of the raw data for a selected potential biomarker ion (m/z 100.07 ± 0.02 and a retention time of 1.31 min) showing up-regulation of this ion with a mean fold change of 15. The retention time and m/z data for this molecule were used to extract the selected ion mobility spectrum shown in Figure 5 (b). The single symmetrical IM spectrum peak indicates the presence of a single component. Identification of this component would therefore require the candidate molecule to match the ion mobility as well as the retention time and m/z characteristics.

[Figure 6]

Accurate mass measurement data and isotopic ratio data on the discriminant metabolite ion were obtained by analysing a pooled saliva extract sample using high resolution mass spectrometry. The molecular formula, C5H9NO, was determined from the accurate mass and isotopic pattern. The generated molecular formula was used to search electronic sources including ChemSpider and the Human Metabolome Database (HMDB) (Wishart et al., 2013)

for possible candidate metabolites. The number of candidate molecules was reduced by application of biological knowledge about the candidate metabolites, such as the biological availability of candidate metabolite in human saliva; δ-valerolactam and N-methyl-2-pyrrollidinone were shortlisted as possible matches. Authentic standards of the shortlist of candidate metabolites were analysed using UHPLC-IM-MS and by UHPLC-MS/MS.

The retention time, MS/MS spectrum and ion mobility drift time of the biomarker ion m/z 100.07±0.02 in the pooled saliva samples matched that of δ-valerolactam (Figure 6). This provides strong evidence for the assignment of this molecule as a potential biomarker of exercise induced physiological stress. δ-valerolactam is included in the human metabolome database (reference number HMDB11749) as a known metabolite present in saliva (Wishart et al., 2013). It has been shown in mouse models to result from the metabolism of cadaverine (Callery and Geelhaar, 1984), which is known to be present at part per million levels in human saliva as a potential source of δ-valerolactam (Cooke et al., 2003). The physiological role of δ-valerolactam is not well understood and the effects of exercise and other stresses on its metabolism are at present unknown.

4. CONCLUSIONS

This study demonstrates proof of principle in a pilot study with a small number of participants that mass-to-charge and ion mobility data may be acquired within the timescale of an UHPLC experiment for metabonomic analysis of saliva. The combination of UHPLC with IM-MS provides an added dimension enabling enhanced separation of co-eluting components of complex metabonomic samples on the basis of retention time, ion mobility and mass-to-charge ratio in a single 10-minute chromatographic run.

The reported results show the potential of the UHPLC-IM-MS technique for the targeted and the global metabolic profiling analysis of saliva and other biological fluids, and for the subsequent biomarker discovery process. In this study, δ-valerolactam has been identified as a potential biomarker in saliva of exercise-induced physiological stress on the basis of retention time, MS/MS spectrum and ion mobility drift time compared to a δ-valerolactam standard. The advantages of ion mobility spectrometry with UHPLC-MS for discovery and identification of metabolites from metabolite profiling studies have been demonstrated. This work demonstrates the potential of hyphenated ion mobility mass spectrometry for non-hypothesis driven discovery-based metabolomic studies.

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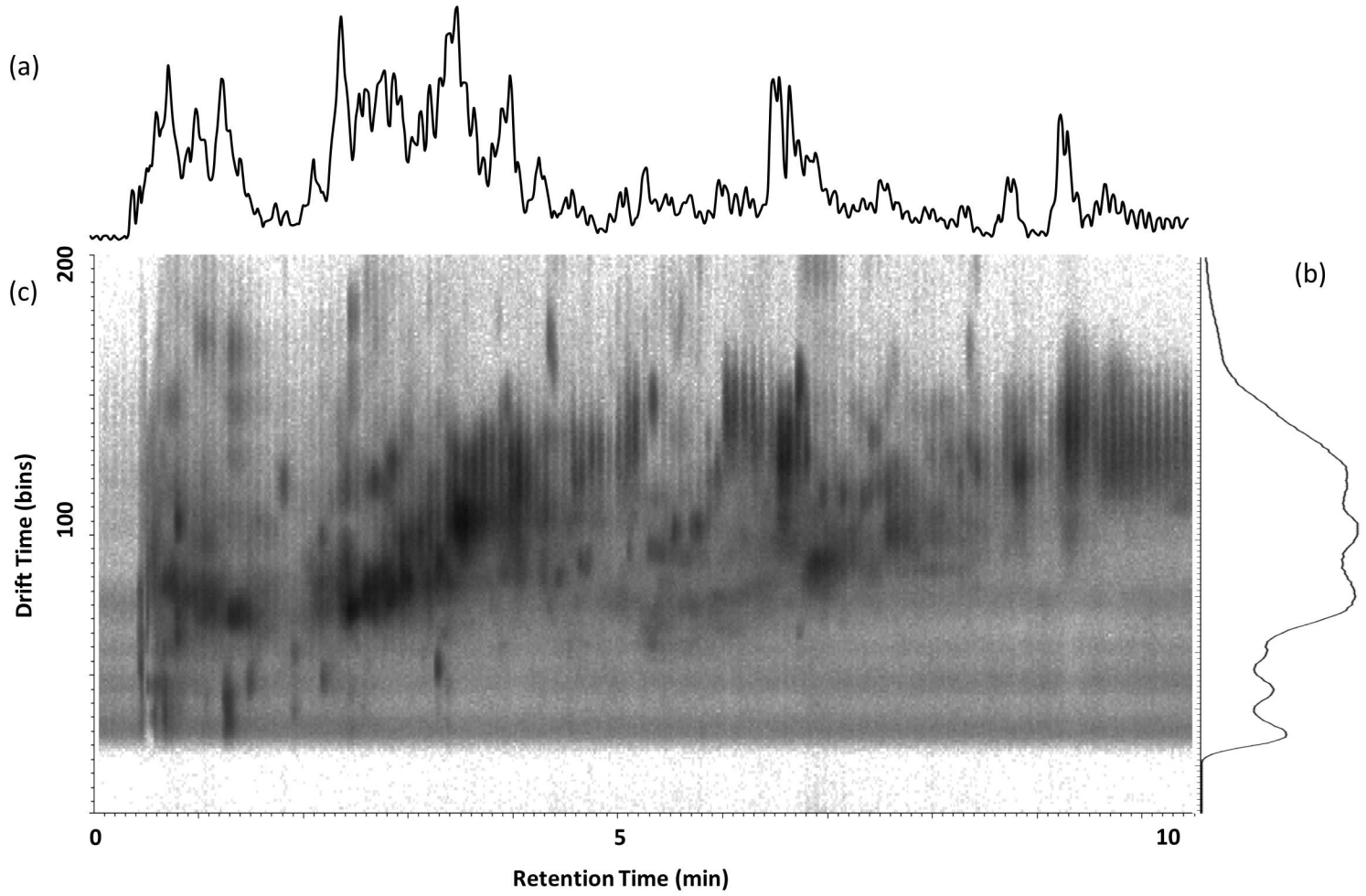
7. LIST OF FIGURES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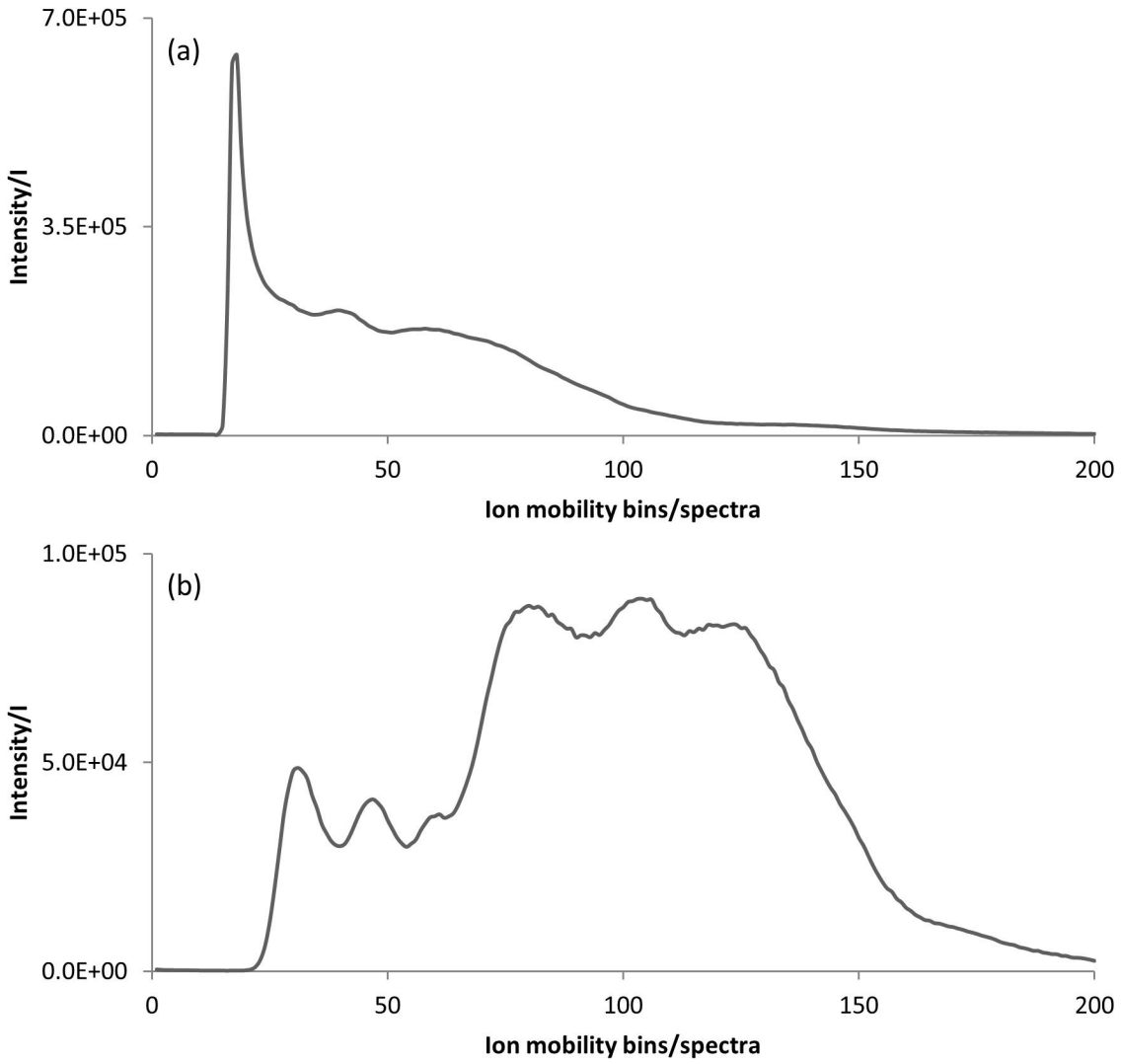
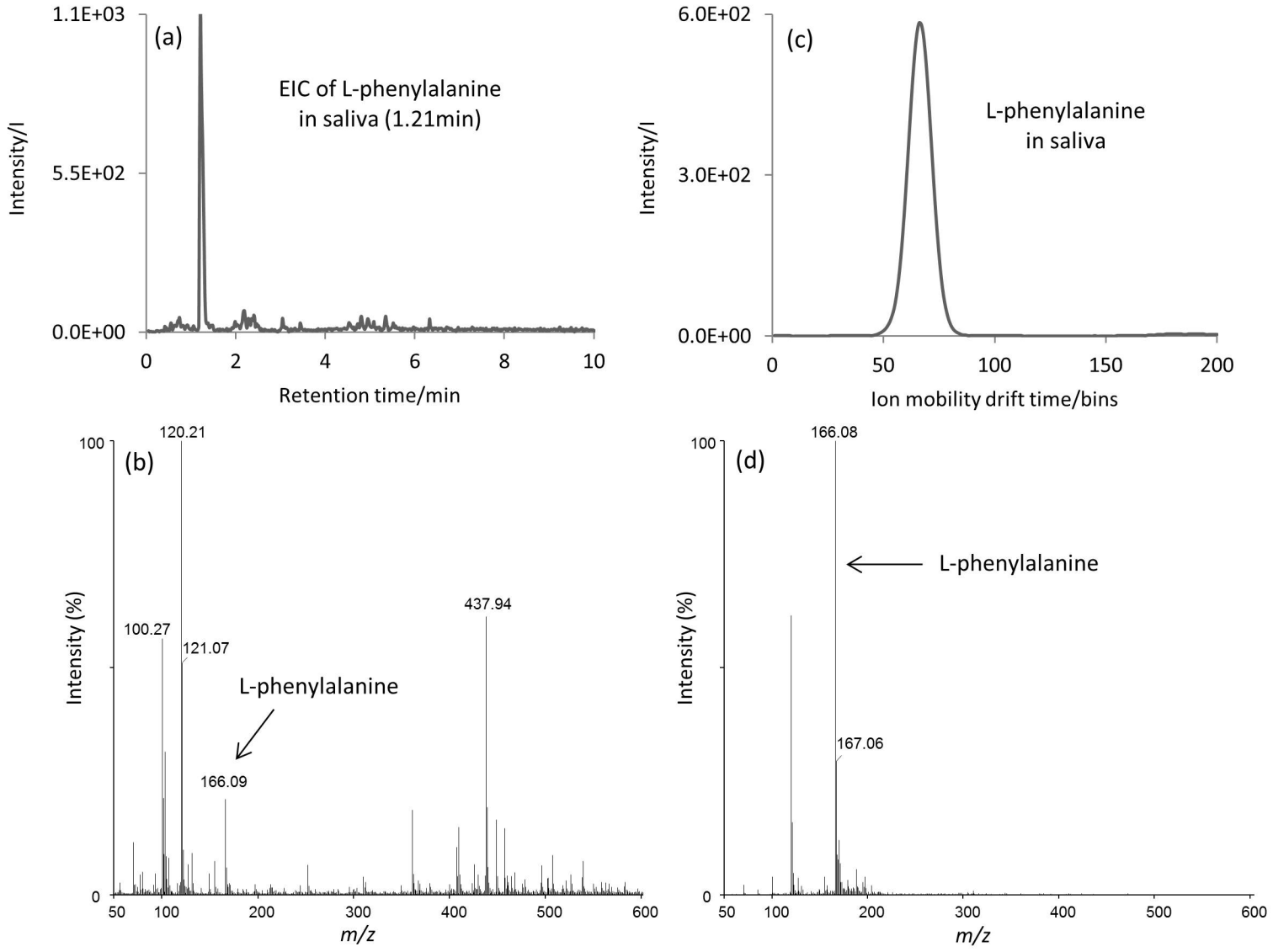
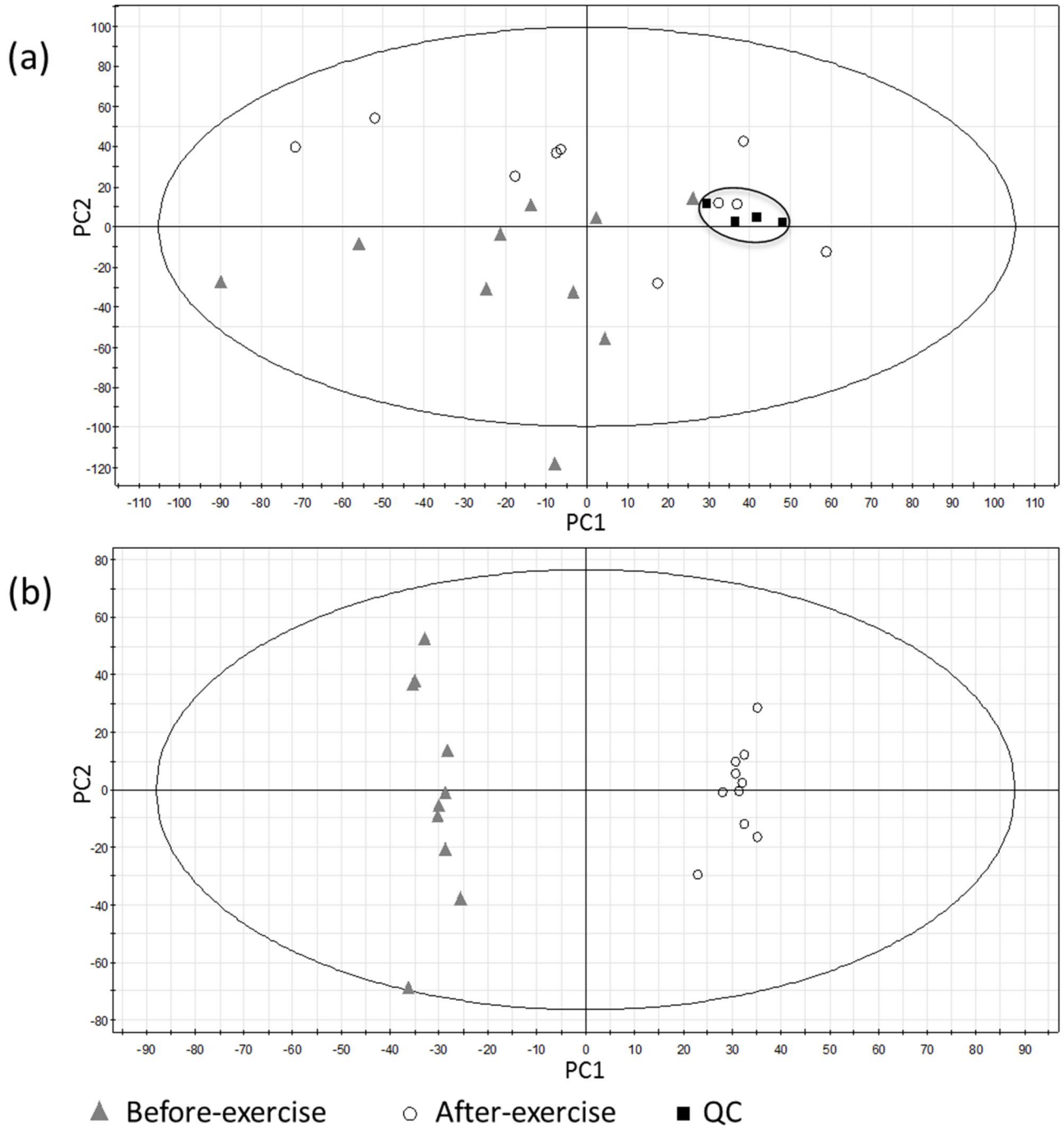
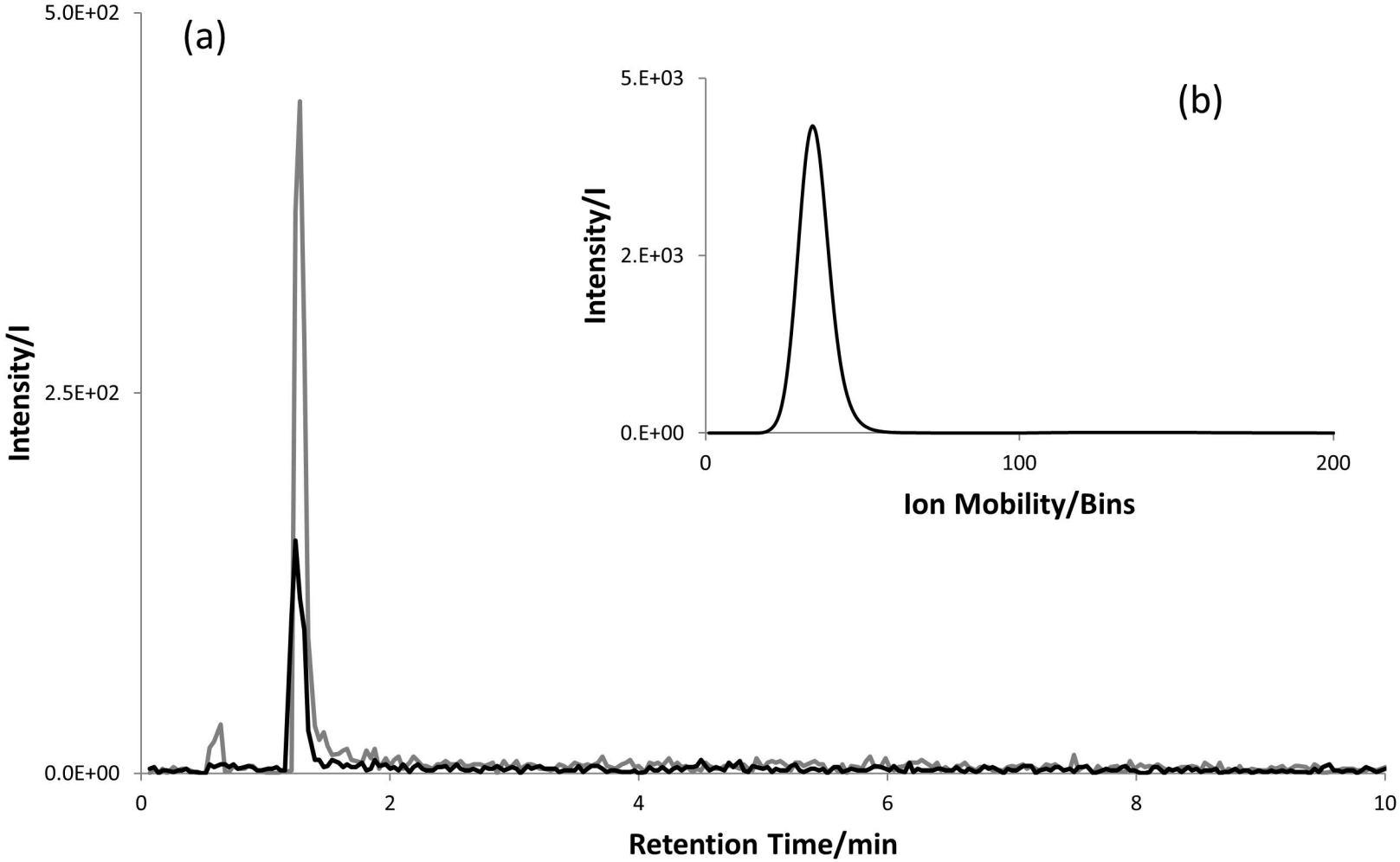
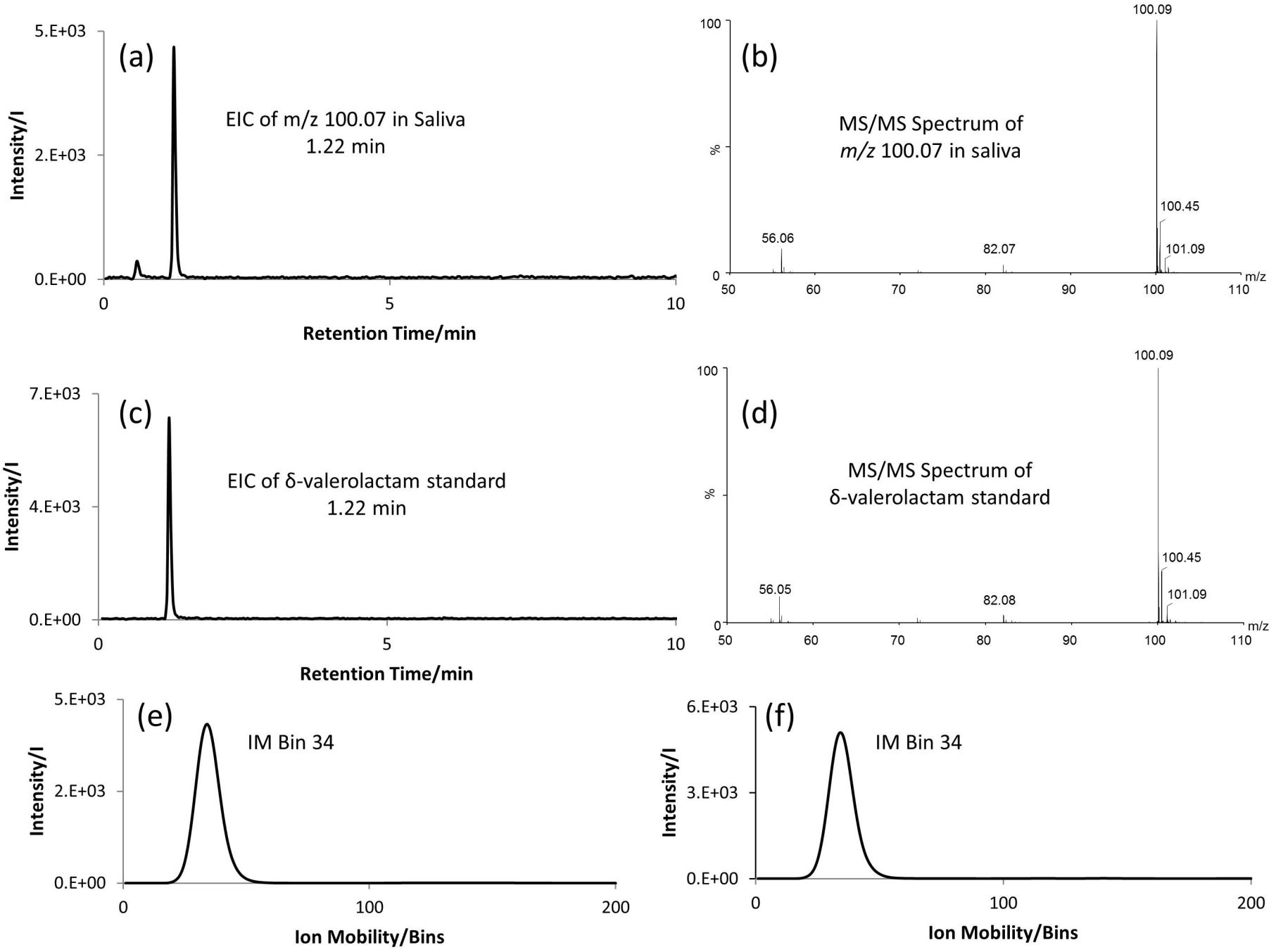
10
Retention Time (min)


(a)
7.0E+05
3.5E+05
0.0E+00
Intensity/I
0
50
100
150
200
Ion mobility bins/spectra
(b)
1.0E+05
5.0E+04
0.0E+00
Intensity/I
0
50
100
150
200
Ion mobility bins/spectra


(a)
EIC of L-phenylalanine
in saliva (1.21min)
Intensity/I
1.1E+03
5.5E+02
0.0E+00
0
2
4
6
8
10
Retention time/min
(c)
L-phenylalanine
in saliva
Intensity/I
6.0E+02
3.0E+02
0.0E+00
0
50
100
150
200
Ion mobility drift time/bins
(b)
120.21
100.27
121.07
L-phenylalanine
166.09
437.94
Intensity (%)
100
0
50
100
200
300
400
500
600
m/z
(d)
166.08
L-phenylalanine
167.06
Intensity (%)
100
0
50
100
200
300
400
500
600
m/z


(a)
PC2
PC1
(b)
PC2
PC1
Before-exercise
After-exercise
QC


(a)
5.0E+02
2.5E+02
0.0E+00
Intensity/I
0
2
4
6
8
10
Retention Time/min
(b)
5.E+03
2.E+03
0.E+00
Intensity/I
0
100
200
Ion Mobility/Bins


(a)
EIC of m/z 100.07 in Saliva
1.22 min
Intensity/I
5.E+03
2.E+03
0.E+00
0
5
10
Retention Time/min
(b)
MS/MS Spectrum of
m/z 100.07 in saliva
100.09
100.45
101.09
56.06
82.07
100
%
0
50
60
70
80
90
100
110
m/z
(c)
EIC of δ-valerolactam standard
1.22 min
Intensity/I
7.E+03
4.E+03
0.E+00
0
5
10
Retention Time/min
(d)
MS/MS Spectrum of
δ-valerolactam standard
100.09
100.45
101.09
56.05
82.08
100
%
0
50
60
70
80
90
100
110
m/z
(e)
IM Bin 34
Intensity/I
5.E+03
2.E+03
0.E+00
0
100
200
Ion Mobility/Bins
(f)
IM Bin 34
Intensity/I
6.E+03
3.E+03
0.E+00
0
100
200
Ion Mobility/Bins