# Targeted Metabolomics and Lipidomics

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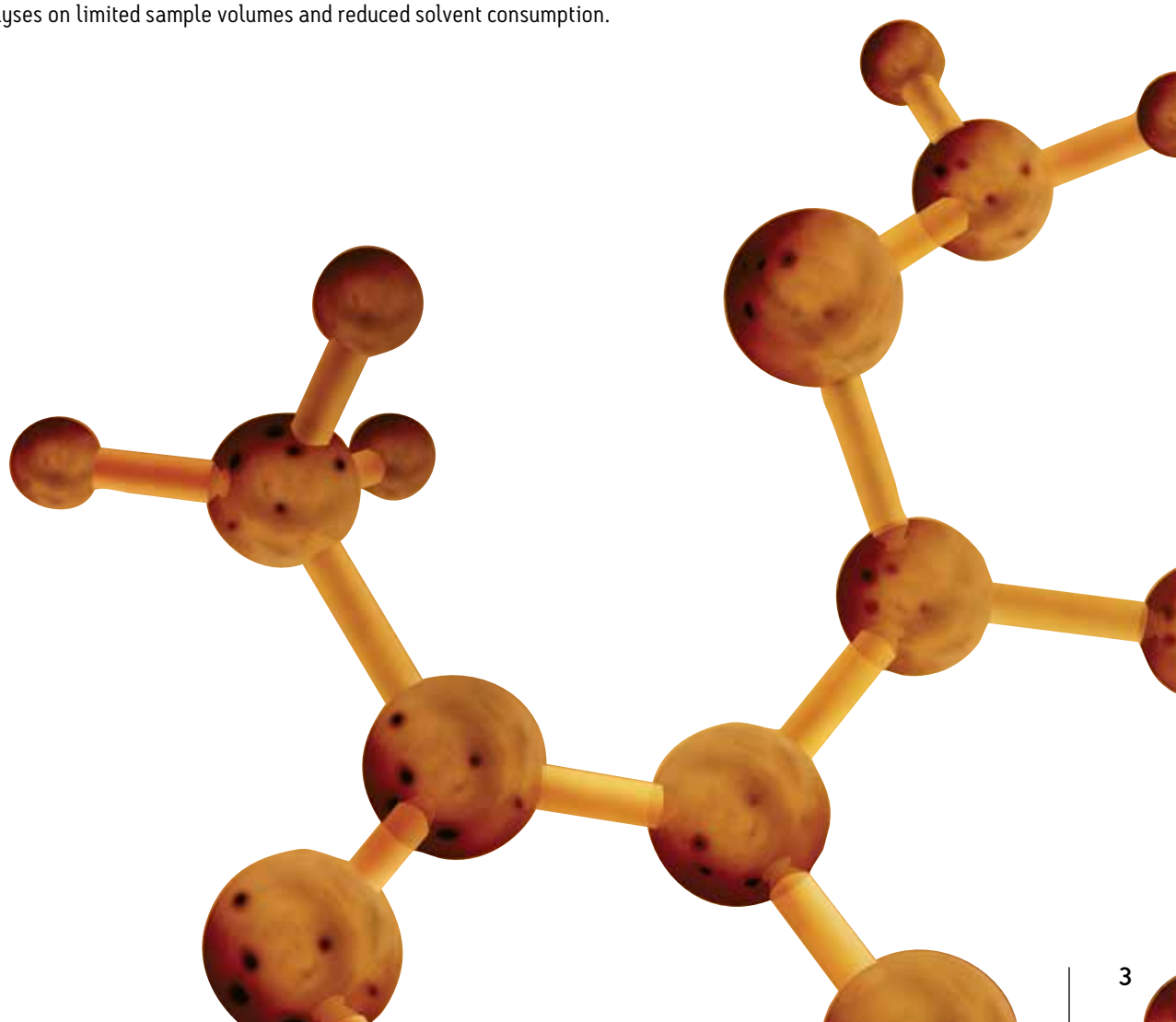
# TARGETED METABOLOMICS AND LIPIDOMICS

Targeted metabolomics and lipidomics are hypothesis-driven approaches that focus on analyzing a selected group of metabolites or lipids. Such approaches are generally used either for validation of initial discoveries or routine analysis for clinical research. The specific metabolites/lipids that will undergo analysis are selected according to the questions asked, and ad hoc analytical methods are developed for their quantification.

Waters innovative chromatographic solutions together with multiple-reaction monitoring (MRM) transitions on the MS instruments help to increase the number of analytes that can be simultaneously quantified in a single acquisition without losses in sensitivity.

- Advanced chromatographic separations of lipids and metabolites coupled with a range of ionization capabilities (UPLC, ionKey, UPC$^2$, APGC)
- High sensitivity and robustness enabling reproducible detection of metabolites and lipids at low levels in complex biological matrices
- Multiple analyses on limited sample volumes and reduced solvent consumption.



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# A Validated Assay for the Quantification of Amino Acids in Mammalian Urine

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## APPLICATION BENEFITS

- A simple, robust, LC/MS/MS assay for the absolute quantification of 20 amino acids in human urine has been developed over the range of 0.2–200.0 µMol.
- Fast analytical throughput: two, microtiter plates (192 samples) per day.

## WATERS SOLUTIONS

Xevo® TQ-S micro

ACQUITY® HSS Columns

ACQUITY UPLC® I-Class

AccQ•Tag™ Ultra

MassLynx® Software

TargetLynx™ Application Manager

StepWave™ ion guide

## KEY WORDS

TQS micro, amino acid, quantification, urine, LC/MS

## INTRODUCTION

Amino acids play a critical role in mammalian biochemistry, forming the simple building blocks of proteins, acting as neurotransmitters in biosynthesis and are essential in lipid transports, to name but a few. The rapid and accurate quantification of amino acids is critical to understanding the underlying biochemistry of multiple physiological and disease states. Previous methodologies have employed either ion exchange chromatography followed by derivatization with fluorescence detection or sample derivatization followed by analysis by LC/UV or LC-Fluorescence. However, both of these approaches are time consuming and require complete chromatographic resolution of the amino acids from other amine-containing compounds, so are not always suitable for the analysis of biological fluids. Here we present a method for the rapid, simple, quantification of amino acids by UPLC/MS/MS.

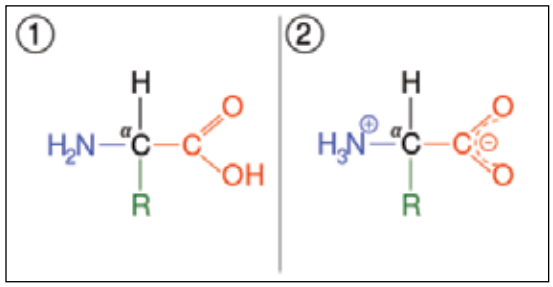


*Figure 1. Amino acid structure.*

## EXPERIMENTAL

### LC conditions

| | |
|---|---|
| LC system: | ACQUITY UPLC I-Class |
| Detector: | Xevo TQ-S micro |
| Vials: | Maximum Recovery vials |
| Column: | ACQUITY UPLC HSS T3 1.8 µm, 150 x 2.1 mm |
| Column temp.: | 45 °C |
| Sample temp.: | Room temperature |
| Injection vol.: | 2-µL |
| Flow rate: | 0.6 mL/min |
| Mobile phase A: | water + 0.1% formic acid |
| Mobile phase B: | acetonitrile + 0.1 % formic acid |
| Gradient: | Maintained at 4% B for 0.5 min; increasing to 10% B at 2.5 min; increasing to 28% B at 5 min, increasing to 95% B at 5 .1 min; reverting to 4% B at 6.2 min for a 1.3 min re-equilibration see Table 1 |

### MS conditions

| | |
|---|---|
| MS system: | Xevo TQ-S micro |
| Ionization mode: | Positive |
| Acquisition range: | MRM mode |
| Capillary voltage: | 1.5 kV |
| Collision energy: | Compound specific see Table 2 |
| Cone voltage: | Compound specific see Table 2 |

### Sample preparation

The sample preparation procedure employed for the analysis of the amino acids is shown below in Figure 2. A 50-µL aliquot of the samples and standards was vortex mixed with 150-µL of methanol, to precipitate protein. A 10-µL aliquot of the resultant sample was then transferred to a sample tube for derivatization according to the process defined in Figure 2.

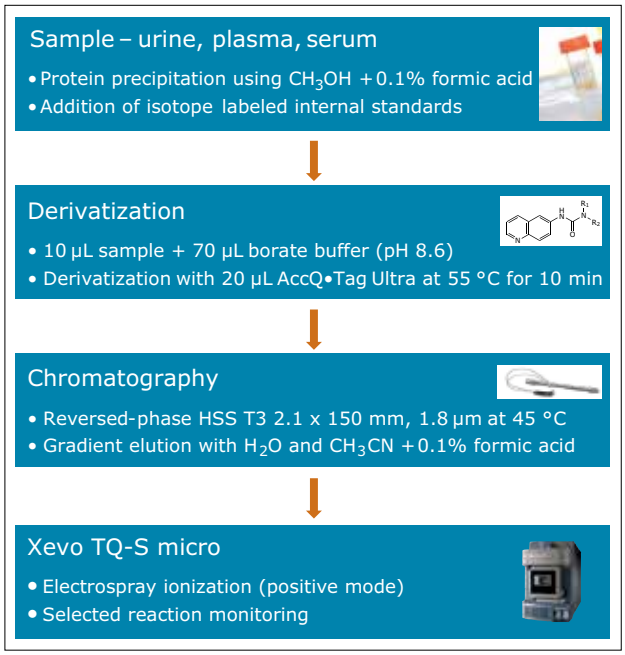


*Figure 2. Sample preparation scheme for amino acid analysis.*

| Time (min) | Flow rate (mL/min) | %B |
|---|---|---|
| 0.0 | 0.6 | 4 |
| 0.5 | 0.6 | 4 |
| 2.5 | 0.6 | 10 |
| 5.0 | 0.6 | 28 |
| 5.1 | 0.6 | 95 |
| 6.1 | 0.6 | 95 |
| 6.2 | 0.6 | 4 |
| 7.5 | 0.6 | 4 |

*Table 1. Chromatographic gradient table.*

| Compound | Parent | Fragment | Window (min) | Dwell time (s) | CV (V) | CE (eV) | RT (min) |
|---|---|---|---|---|---|---|---|
| Histidine-d3 | 329.1 | 159.1 | 0.0–2.0 | 0.026 | 20 | 10 | 1.51 |
| Histidine | 326.1 | 156.1 | 0.0–2.0 | 0.026 | 20 | 10 | 1.52 |
| 1-methylhistidine | 340.1 | 170.1 | 0.0–2.0 | 0.026 | 30 | 18 | 1.70 |
| 4-hydroxyproline | 302.0 | 171.1 | 1.5–2.0 | 0.026 | 10 | 22 | 1.78 |
| 3-methylhistidine | 340.1 | 124.2 | 0.0–2.0 | 0.026 | 30 | 28 | 1.78 |
| Carnosine | 397.1 | 227.2 | 1.8–2.2 | 0.026 | 30 | 14 | 1.99 |
| Asparagine | 303.1 | 171.1 | 1.8–2.1 | 0.026 | 30 | 22 | 2.00 |
| Arginine | 345.1 | 70.1 | 1.8–2.2 | 0.026 | 30 | 36 | 2.01 |
| Taurine | 296.1 | 116.3 | 2.0–2.5 | 0.026 | 30 | 60 | 2.22 |
| Glutamine-d5 | 322.1 | 171.1 | 2.0–2.5 | 0.026 | 30 | 24 | 2.27 |
| Glutamine | 317.1 | 171.1 | 2.0–2.5 | 0.026 | 30 | 24 | 2.29 |
| Serine-d3 | 279.1 | 171.1 | 2.0–2.5 | 0.026 | 30 | 20 | 2.30 |
| Serine | 276.1 | 171.1 | 2.0–2.5 | 0.026 | 30 | 20 | 2.32 |
| Homoserine | 290.1 | 171.1 | 2.2–2.6 | 0.030 | 10 | 18 | 2.46 |
| Ethanolamine | 232.1 | 171.1 | 2.2–2.7 | 0.030 | 10 | 20 | 2.47 |
| Glycine | 246.1 | 116.1 | 2.2–2.8 | 0.013 | 30 | 14 | 2.54 |
| Aspartic acid-d3 | 307.0 | 171.1 | 2.5–2.8 | 0.030 | 30 | 20 | 2.67 |
| Aspartic acid | 304.1 | 171.1 | 2.5–2.8 | 0.030 | 30 | 22 | 2.69 |
| Citrulline | 346.2 | 171.1 | 2.5–2.9 | 0.030 | 30 | 26 | 2.77 |
| Sarcosine | 260.1 | 116.1 | 2.6–3.6 | 0.024 | 30 | 44 | 2.85 |
| Glutamic acid-d3 | 321.0 | 171.0 | 2.5–3.2 | 0.030 | 30 | 20 | 2.90 |
| Glutamic acid | 318.1 | 171.1 | 2.5–3.2 | 0.030 | 30 | 22 | 2.91 |
| β-alanine | 260.1 | 116.1 | 2.6–3.6 | 0.024 | 30 | 44 | 3.06 |
| Threonine | 290.1 | 171.1 | 2.9–3.2 | 0.062 | 30 | 20 | 3.11 |
| Threonine-13C4 15N | 295.1 | 171.0 | 2.9–3.2 | 0.062 | 30 | 26 | 3.11 |
| Alanine-d3 | 263.1 | 171.0 | 3.2–3.6 | 0.024 | 30 | 16 | 3.45 |
| Alanine | 260.1 | 116.1 | 2.6–3.6 | 0.024 | 30 | 44 | 3.47 |
| γ-amino-n-butyric acid | 274.1 | 171.1 | 3.2–4.2 | 0.013 | 10 | 20 | 3.51 |
| Hydroxylysine | 333.2 | 171.1 | 3.4–4.0 | 0.013 | 16 | 16 | 3.56 |
| Aminoadipic acid | 332.1 | 171.1 | 3.2–3.8 | 0.024 | 30 | 18 | 3.61 |
| Proline-d7 | 293.0 | 171.1 | 3.5–4.0 | 0.013 | 30 | 24 | 3.74 |
| Proline | 286.1 | 116.1 | 3.5–4.0 | 0.013 | 30 | 50 | 3.77 |
| β-aminoisobutyric acid | 274.1 | 171.1 | 3.2–4.2 | 0.013 | 10 | 20 | 3.78 |
| Ornithine | 303.1 | 171.1 | 3.6–4.2 | 0.013 | 54 | 22 | 3.85 |
| Cystathionine | 393.2 | 171.1 | 3.5–4.1 | 0.013 | 66 | 18 | 3.86 |
| Phosphoserine | 356.1 | 171.1 | 3.8–4.2 | 0.013 | 20 | 18 | 4.05 |
| Lysine 224-d4 | 246.1 | 171.1 | 3.8–4.3 | 0.013 | 30 | 14 | 4.07 |
| Cystine 291 | 291.1 | 171.1 | 3.8–4.2 | 0.013 | 10 | 12 | 4.07 |
| Cystine 411 | 411.0 | 171.1 | 3.8–4.2 | 0.013 | 60 | 18 | 4.07 |
| Cystine 581 | 581.0 | 171.1 | 3.8–4.2 | 0.013 | 34 | 26 | 4.07 |
| Lysine 487-d4 | 491.2 | 171.0 | 3.8–4.3 | 0.013 | 30 | 32 | 4.07 |
| Lysine 317-d4 | 321.1 | 171.0 | 3.8–4.3 | 0.013 | 50 | 24 | 4.07 |
| Lysine 244 | 244.2 | 171.1 | 3.8–4.3 | 0.030 | 30 | 12 | 4.08 |
| Lysine 317 | 317.1 | 171.1 | 3.8–4.3 | 0.013 | 56 | 16 | 4.08 |
| Lysine 487 | 487.1 | 171.0 | 3.8–4.3 | 0.013 | 18 | 34 | 4.08 |
| α-amino-n-butyric acid | 274.1 | 171.1 | 3.2–4.2 | 0.013 | 10 | 20 | 4.09 |
| Tyrosine | 352.1 | 171.1 | 4.2–4.8 | 0.046 | 30 | 24 | 4.45 |
| Methionine | 320.1 | 171.1 | 4.3–4.8 | 0.046 | 30 | 22 | 4.58 |
| Valine-d8 | 296.1 | 171.1 | 4.4–4.8 | 0.046 | 30 | 20 | 4.61 |
| Valine | 288.1 | 171.1 | 4.4–4.8 | 0.046 | 30 | 16 | 4.64 |
| Isoleucine-d10 | 312.1 | 171.0 | 5.0–5.5 | 0.048 | 30 | 20 | 5.21 |
| Isoleucine | 302.1 | 171.1 | 5.0–5.5 | 0.048 | 30 | 20 | 5.24 |
| Leucine-d10 | 312.1 | 171.0 | 5.0–5.5 | 0.048 | 30 | 20 | 5.27 |
| Leucine | 302.1 | 171.1 | 5.0–5.5 | 0.048 | 30 | 20 | 5.31 |
| Phenylalanine-d5 | 341.1 | 171.1 | 5.2–5.7 | 0.048 | 30 | 22 | 5.43 |
| Phenylalanine | 336.1 | 171.1 | 5.2–5.7 | 0.048 | 30 | 22 | 5.45 |
| Tryptophan-d3 | 378.1 | 171.1 | 5.2–5.8 | 0.048 | 6 | 24 | 5.54 |
| Tryptophan | 375.1 | 171.1 | 5.2–5.8 | 0.048 | 30 | 26 | 5.55 |

*Table 2. Mass spectrometric analysis conditions for amino acids.*

### Data management

- MassLynx Mass Spectrometry Software
- TargetLynx Application Manager

### One day validation

The assay was subjected to a one day validation according to the FDA guidelines for bioanalytical assay validation. The samples employed are shown below:

- Double blank (with no analyte or internal standard)
- Single blank (with analyte but no internal standard)
- Eight point calibration curve (performed at the beginning and end of run)
- Six replicates of:
  - LLOQQC (0.2 µM)
  - LQC (0.6 µM)
  - MQC (30 µM)
  - HQC (160 µM)
  - ULOQQC (20 µM)
- Twenty rat urine samples from a toxicology study ([4-chloro-6-(2, 3-xylidino)-2-pyrimidinylthio]acetic acid) were used to test the robustness of the assay.

### Amino acids quantified in this assay

The amino acids analyzed in this study are listed in Table 3. The twenty proteinogenic amino acids were subjected to validation for absolute quantification using stable isotope labeled internal standards. Those additional eighteen amino acids, for which no stable isotope labeled internal standard was used, were subjected to relative quantification. The stable isotopes employed for each amino acid is listed in Table 4.

| Validated For Absolute Quantification | Monitored For Relative Quantification |
|---|---|
| L-Alanine | 3-Methyl-L-histidine |
| L-Arginine | 1-Methyl-L-histidine |
| L-Asparagine | Cystathionine |
| L-Aspartic acid | DL-β-Aminoisobutryic acid |
| L-Cystine | Ethanolamine |
| L-Glutamic acid | Homoserine |
| L-Glutamine | Hydroxy-L-proline |
| Glycine | Hydroxylysine |
| L-Histidine | L-Carnosine |
| L-Isoleucine | L-Citrulline |
| L-Leucine | L-Ornithine |
| L-Lysine | L-α-aminoadipic acid |
| L-Methionine | L-α-Amino-n-butyric acid |
| L-Phenylalanine | Phosphoserine |
| L-Proline | Sarcosine |
| L-Serine | Taurine |
| L-Threonine | β-Alanine |
| L-Tryptophan | γ-Amino-n-butyric acid |
| L-Tyrosine | |
| L-Valine | |

*Table 3. Amino acids subjected to absolute and relative quantification.*

| Compound | Labeled internal standard |
|---|---|
| L-Alanine | Alanine-d3 |
| L-Arginine | Serine-d3 |
| L-Asparagine | Serine-d3 |
| L-Aspartic acid | Aspartic acid-d3 |
| L-Cystine | Proline-d7 |
| L-Glutamic acid | Glutamic acid-d3 |
| L-Glutamine | Glutamine-d5 |
| Glycine | Serine-d3 |
| L-Histidine | Histidine-d3 |
| L-Isoleucine | Isoleucine-d10 |
| L-Leucine | Leucine-d10 |
| L-Lysine | Lysine-d4 |
| L-Methionine | Valine-d8 |
| L-Phenylalanine | Phenylalanine-d5 |
| L-Proline | Proline-d7 |
| L-Serine | Serine-d3 |
| L-Threonine | Threonine-13C4 |
| L-Tryptophan | Tryptophan-d5 |
| L-Tyrosine | Tyrosine-d7 |
| L-Valine | Valine-d8 |

*Table 4. Amino acids quantified using stable isotope labeled internal standards.*

### Calibration Curves

The calibration line was prepared from a Sigma Aldrich physiological, amino acid standard (acidics, basics, neutrals) by spiking into 50/50 $CH_3OH/H_2O$. The calibration curve was generated over three orders of magnitude, covering the physiological range of 0.2-200.0 µM. The QC samples were prepared from a separate Sigma Aldrich physiological amino acid standard.

## RESULTS AND DISCUSSION

A typical separation obtained from the amino acid standard mix is shown in Figure 3. The data displayed illustrates the separation obtained for the amino acids and the throughput of the assay. The peak shape obtained from the chromatography was excellent with a peak width at the base of approximately 3 seconds. The assay was shown to be reproducible and reliable with no retention time drift. The Xevo TQ-S micro is equipped with a new novel, state of the art, ion transfer optics which allows more ions to be sampled from the source and transferred to the analyser. The StepWave ion guide in the Xevo TQ-S micro is designed to cope with the challenges of the modern laboratory that are produced by high sample throughput and difficult matrices. Neutrals and gas load are passively removed for enhanced transmission with the ions actively transferred into the mass analyzer, improving sensitivity and robustness.

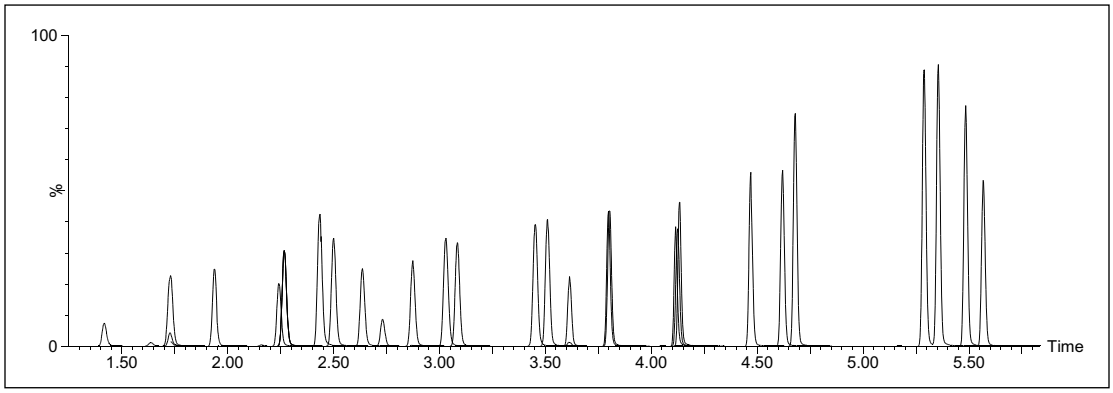


*Figure 3. Amino acid LC/MS QC chromatogram 200 µM.*

## Validation results

The assay validation was carried out according to FDA guidelines. The resulting amino acid data was processed and quantified using Waters® MassLynx Software with TargetLynx Application Manager employing internal standard calibration and 1/x weighting. A summary of the QC data for each amino acid is listed below in Table 5. The data obtained for the quantification of each amino acid was acceptable for routine quantification.

| Amino Acid | 0.2 µM | 0.6 µM | 6 µM | 30 µM | 160 µM | 200 µM |
|---|---|---|---|---|---|---|
| Aspartic Acid | 7.26 | 3.40 | 2.02 | 0.91 | 0.80 | 0.83 |
| Asparagine | 6.30 | 4.13 | 1.01 | 1.71 | 1.74 | 2.58 |
| Cystine | 5.73 | 3.17 | 3.00 | 4.72 | 2.13 | 1.70 |
| Glutamic Acid | 9.81 | 3.02 | 1.28 | 0.88 | 0.88 | 3.31 |
| Glutamine | 3.23 | 3.86 | 0.49 | 1.18 | 0.73 | 1.11 |
| Glycine | 17.92 | 8.48 | 2.32 | 2.31 | 2.27 | 3.21 |
| Histidine | 2.68 | 5.30 | 3.26 | 3.95 | 2.06 | 3.37 |
| Isoleucine | 5.29 | 0.57 | 3.06 | 1.57 | 0.63 | 0.75 |
| Leucine | 5.09 | 1.19 | 1.09 | 3.07 | 0.31 | 0.38 |
| Lysine | 7.46 | 3.11 | 2.48 | 1.10 | 1.07 | 1.29 |
| Methionine | 0.91 | 2.25 | 1.59 | 1.16 | 2.12 | 1.40 |
| Phenylalanine | 4.85 | 1.15 | 0.95 | 0.77 | 5.13 | 0.83 |
| Proline | 8.56 | 3.31 | 1.68 | 1.58 | 0.95 | 1.09 |
| Serine | 23.57 | 6.47 | 2.46 | 1.85 | 1.61 | 2.33 |
| Threonine | 4.30 | 2.45 | 1.25 | 1.24 | 1.06 | 0.68 |
| Tryptophan | 1.88 | 2.14 | 1.74 | 1.00 | 5.86 | 1.38 |
| Tyrosine | 5.32 | 2.52 | 2.96 | 2.32 | 4.43 | 2.79 |
| Valine | 3.55 | 1.12 | 1.65 | 0.44 | 1.01 | 0.84 |

*Table 5. Coefficient of variation (%) for QCs at various concentrations.*

The validation results as well as example chromatograms and calibration lines for the amino acids glutamic acid and aspartic acid are shown in Figures 4–7 and Tables 6 and 7. Here we can see that the methodology demonstrated acceptable peak shape and signal to noise ratio at the lowest level of quantification. The assay demonstrated excellent bias and precision for every amino acid.

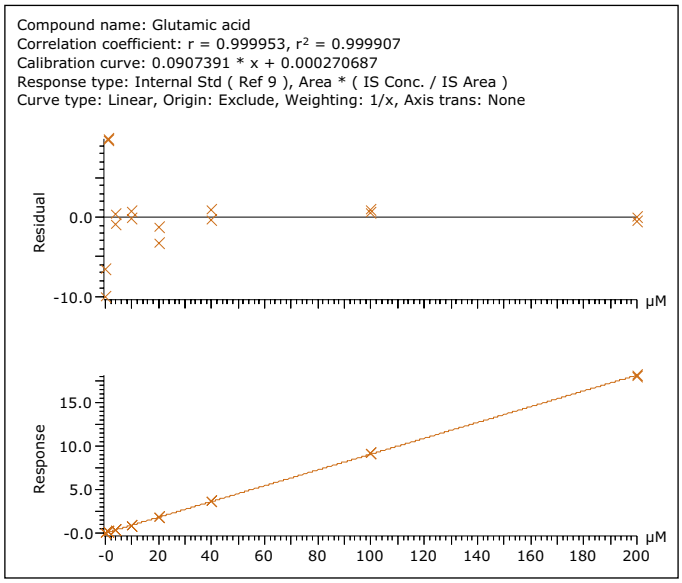


*Figure 4. Calibration line for glutamic acid.*

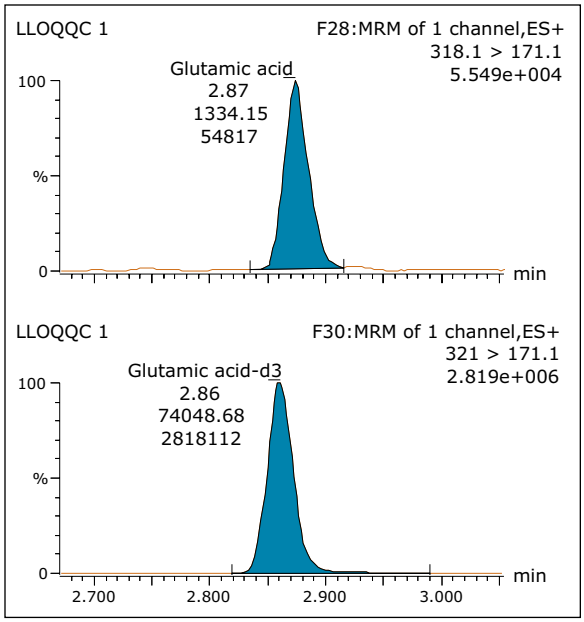


*Figure 5. Lower limit of quantification QC (0.2 µM) LC/MS chromatogram for glutamic acid.*

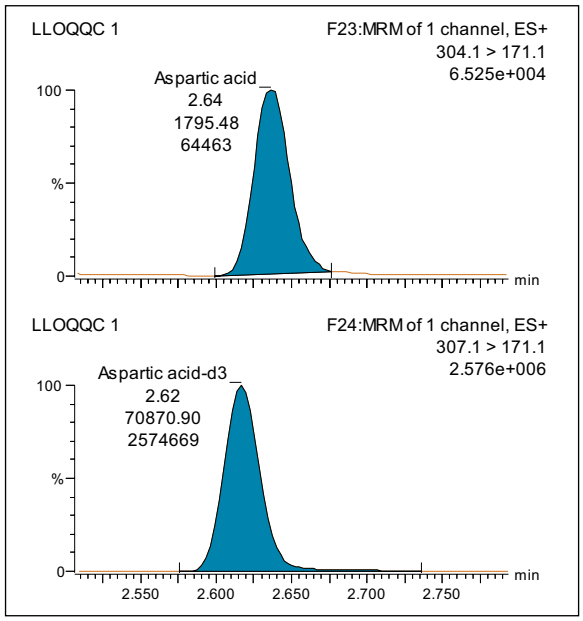


*Figure 6. Lower limit of quantification QC (0.2 µM) LC/MS chromatogram for aspartic acid.*

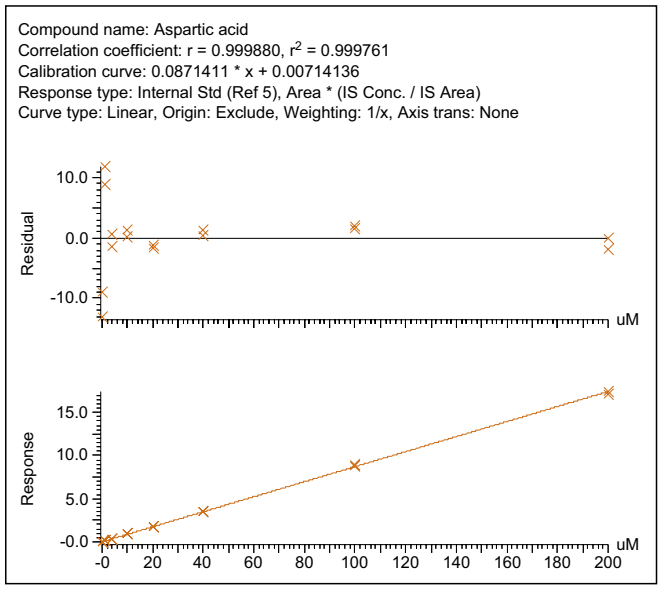


*Figure 7. Calibration line for aspartic acid.*

| Occasion | QC Nominal Concentration (µM) Glutamic acid | | | | |
|---|---|---|---|---|---|
| | 0.2 | 0.6 | 30 | 160 | 200 |
| 1 | 0.20 | 0.58 | 28.6 | 154 | 211 |
| 2 | 0.22 | 0.58 | 29.3 | 158 | 194 |
| 3 | 0.20 | 0.58 | 29.2 | 155 | 198 |
| 4 | 0.20 | 0.58 | 28.8 | 156 | 194 |
| 5 | 0.25 | 0.62 | 28.9 | 156 | 197 |
| 6 | 0.19 | 0.57 | 29.0 | 158 | 194 |
| Mean | 0.21 | 0.58 | 29.0 | 156 | 198 |
| STDEV | 0.02 | 0.02 | 0.25 | 1.37 | 6.56 |
| %CV | 9.81 | 3.02 | 0.88 | 0.88 | 3.31 |
| Bias | 4.08 | -2.56 | -3.36 | -2.37 | -1.05 |

*Table 6. QC validation data for glutamic acid.*

| Occasion | QC Nominal Concentration (µM) Aspartic acid | | | | |
|---|---|---|---|---|---|
| | 0.2 | 0.6 | 30 | 160 | 200 |
| 1 | 0.21 | 0.58 | 28.7 | 154 | 194 |
| 2 | 0.24 | 0.60 | 29.3 | 157 | 191 |
| 3 | 0.21 | 0.62 | 29.4 | 157 | 192 |
| 4 | 0.21 | 0.56 | 29.2 | 155 | 195 |
| 5 | 0.24 | 0.59 | 29.3 | 155 | 195 |
| 6 | 0.20 | 0.60 | 29.2 | 158 | 195 |
| Mean | 0.22 | 0.59 | 29.2 | 156 | 194 |
| STDEV | 0.02 | 0.02 | 0.27 | 1.24 | 1.61 |
| %CV | 7.26 | 3.40 | 0.91 | 0.80 | 0.83 |
| Bias | 9.17 | -1.22 | -2.66 | -2.50 | -3.19 |

*Table 7. QC validation data for aspartic acid.*

## CONCLUSION

A robust, reliable method for the absolute quantification of twenty amino acids and the relative quantification of a further eighteen amino acids in human urine has been developed and evaluated. The assay had an analysis time of 7.5 minutes per sample. This allows the analysis of two, 96-well, microtitre plates of samples in a 24 hour time period. The assay was found to be valid over the physiologically important range of 0.2–200.0 µMol. The chromatography was reproducible and reliable with no retention time drift detected for any of the amino acids. This data demonstrates that LC/MS/MS provides an attractive and viable alternative to traditional modes of amino acid analysis, providing fast and accurate quantification.

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# Targeted Metabolomics Using the UPLC/MS-based Absolute*IDQ* p180 Kit

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## APPLICATION BENEFITS

Waters® ACQUITY UPLC® System with Xevo® TQ and Xevo TQ-S mass spectrometers combines with the commercially available Absolute*IDQ* p180 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) to allow for the rapid identification and highly sensitive quantitative analyses of more than 180 endogenous metabolites from six different biochemical classes (biogenic amines, amino acids, glycerophospholipids, sphingolipids, sugars, and acylcarnitines). The assay is performed using MS-based flow injection and liquid chromatography analyses, which were validated on Waters' tandem quadrupole instruments.

## WATERS SOLUTIONS

ACQUITY UPLC System

ACQUITY UPLC BEH Columns

Xevo TQ Mass Spectrometer

Xevo TQ-S Mass Spectrometer

TargetLynx™ Application Manager

## KEY WORDS

Absolute*IDQ* p180 Kit, flow injection analysis (shotgun), targeted metabolomics, targeted lipidomics, MetIDQ software

## INTRODUCTION

Global metabolic profiling (untargeted metabolomics) is used for the identification of metabolic pathways that are altered following perturbations of biological systems, as shown in Figure 1. The analysis, however, encompasses significant statistical processing that leads to a low rate of successful identification of biomarkers. Additionally, a tedious marker validation process using pure standards is often required for the identification of a particular metabolite, unless an in-house database has been previously generated. Furthermore, the sample preparation required for the extraction of metabolites is a multi-step process that, without a standardization of the operating procedures, likely contributes to the intra- and inter-laboratory variations in the measurements.

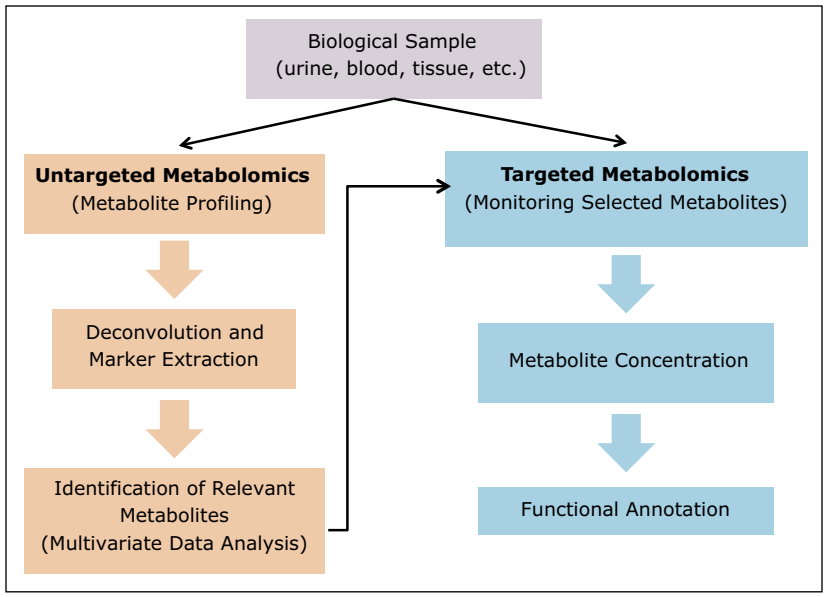


*Figure 1. Workflows illustrating both untargeted and targeted metabolomics approaches.*

To alleviate many of these limiting issues, another approach involves the application of targeted metabolomics assay, seen in Figure 1. The Absolute*IDQ* p180 (BIOCRATES Life Sciences AG) Kit is an MS-based assay for targeted metabolomics allowing the simultaneous identification and quantification of over 180 endogenous metabolites in biological samples.[1-2] MS-based flow injection analysis (FIA) for acylcarnitines, hexoses, glycerophospholipids, and sphingolipids as well as an MS-based LC method for amino acids and biogenic amines are used to provide a robust, high-throughput identification of preselected metabolites, as shown in Figure 2. Here, we applied this targeted metabolomics strategy to identify biochemical alterations and potential biomarkers in serum from mice exposed to 8 Gy of gamma radiation. Significant differences allowed for the identification of metabolites that could be used to develop a signature of radiation exposure in mice.

| Metabolite group | No. of metabolites | FIA-MS/MS | LC-MS/MS |
|---|---|---|---|
| Amino acids and biogenic amines | 40 | | X |
| Acylcarnitines | 40 | X | |
| Lyso-phosphatidylcholines | 14 | X | |
| Phosphatidylcholines | 74 | X | |
| Sphingomyelins | 14 | X | |
| Hexose | 1 | X | |
| Total | 183 | | |

*Figure 2. List of metabolite classes and total metabolites covered by the kit.*

## EXPERIMENTAL

### Mouse irradiation and sample collection

Male C57Bl/6 mice (8 to 10 weeks old) were irradiated at Georgetown University with 8 Gy of gamma rays ($^{137}$Cs source, 1.67 Gy/min). Blood was obtained by cardiac puncture 24 h post-irradiation, and serum was collected with serum separators (BD Biosciences, CA). All experimental conditions and animal handling were in accordance with animal protocols approved by the Georgetown University Animal Care and Use Committee (GUACUC).

### Sample preparation and data analysis

Metabolites were extracted from mouse sera using a specific 96-well plate system for protein-removal, internal standard normalization and derivatization (Absolute*IDQ* p180 Kit). The preparation was performed according to the Kit User Manual. Briefly, 10 samples (n=5 sham irradiated group and n=5 irradiated group) were added to the center of the filter on the upper 96-well plate kit at 10 µL per well, and dried using a nitrogen evaporator. Subsequently, 50 µL of a 5% solution of phenylisothiocyanate was added for derivatization of the amino acids and biogenic amines. After incubation, the filter spots were dried again using a nitrogen evaporator. The metabolites were extracted using 300 µL of a 5-mM ammonium acetate solution in methanol, and transferred by centrifugation into the lower 96-deep well plate. The extracts were diluted with 600 µL of the MS running solvent for further MS analysis using Waters tandem quadrupole mass spectrometers. One blank sample (no internal standards and no sample added), three water-based zero samples (phosphate buffered saline), and three quality control samples were also added to the Kit plate. The quality controls were comprised of human plasma samples containing metabolites, at several concentration

levels, used to verify the performance of the assay and mass spectrometer. A seven-points serial dilution of calibrators was added to the kit's 96-well plate to generate calibration curves for the quantification of biogenic amines and amino acids. The kit included a mixture of internal standards for the quantification of the natural metabolites as follows: chemical homologous internal standards were used for the quantification of glycerophospholipid and sphingomyelin species; whereas, stable isotopes-labeled internal standards were used to quantify the other compound classes. The amount of internal standards was identical in each well, and the internal standard intensities of zero sample and sample wells were compared to allow conclusions on ion suppression effects.

Acylcarnitines, glycerophospholipids, and sphingolipids were analyzed using the Waters tandem quadrupole mass spectrometers (Xevo TQ and Xevo TQ-S MS) by flow injection analysis (FIA) in positive mode, as shown in Figure 3. Hexose was analyzed using a subsequent FIA acquisition in negative mode. Amino acids and biogenic amines were analyzed using an ACQUITY UPLC System connected to the Xevo tandem quadrupole and Xevo TQ-S mass spectrometers in positive mode, as shown in Figure 4.

Identification and quantification of the metabolites was achieved using internal standards and multiple reaction monitoring (MRM) detection. Data analysis and calculation of the metabolite concentrations analyzed by FIA (acylcarnitines, glycerophospholipids, sphingolipids, and hexoses) is automated using Met*IDQ* software (BIOCRATES Life Sciences AG), an integral part of the kit that imports Waters' raw data files. Analysis of peaks obtained by HPLC/UPLC® (amino acids and biogenic amines) was performed using TargetLynx Application Manager, and the results were imported into Met*IDQ* software for further processing and statistical analysis.

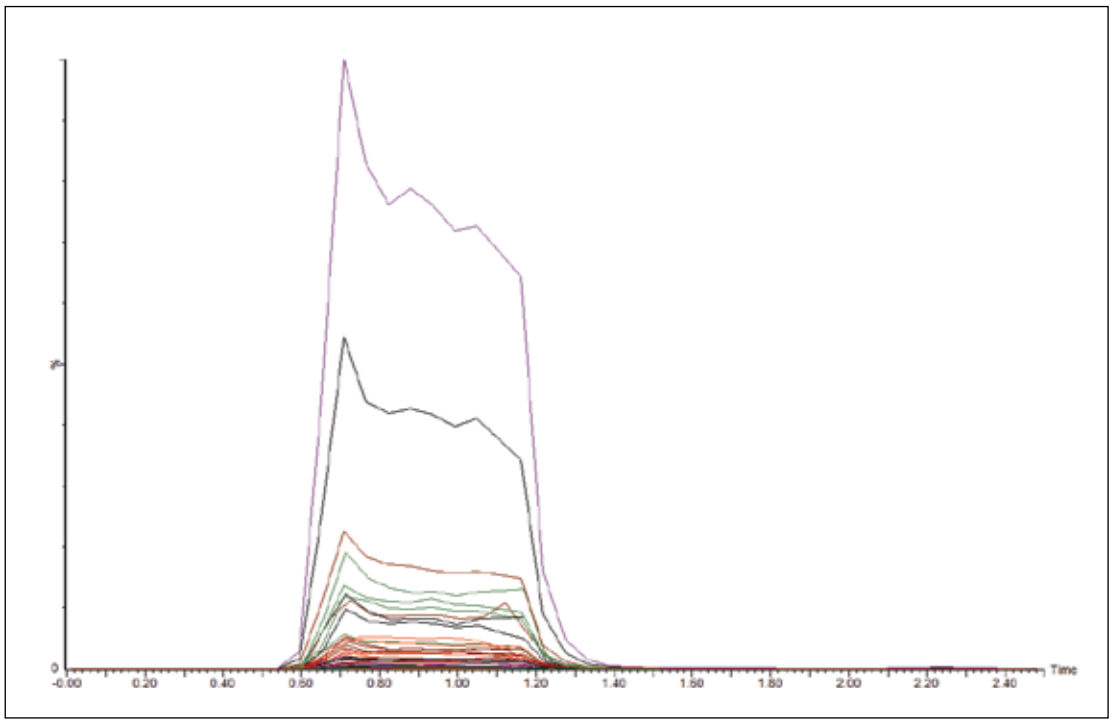


*Figure 3. Representative FIA chromatogram.*

### LC pump settings

Mobile phase A: water and 0.2% formic acid

Mobile phase B: ACN and 0.2% formic acid

### HPLC column

Column: Agilent Zorbax Eclipse XDB $C_{18}$, 3.0 x 100 mm, 3.5 µm

Pre-Column: SecurityGuard, Phenomenex, $C_{18}$, 4 x 3 mm

| Step | Time (min) | Flow (mL/min) | % A | % B | Curve |
|---|---|---|---|---|---|
| 0 | 0.00 | 0.5 | 100.0 | 0.0 | Initial |
| 1 | 0.50 | 0.5 | 100.0 | 0.0 | 6 |
| 2 | 4.00 | 0.5 | 30.0 | 70.0 | 6 |
| 3 | 5.30 | 0.5 | 30.0 | 70.0 | 6 |
| 4 | 5.40 | 0.5 | 100.0 | 0.0 | 6 |
| 5 | 7.30 | 0.5 | 100.0 | 0.0 | 6 |

### UPLC column

Column: Waters ACQUITY UPLC BEH $C_{18}$ 2.1 x 50 mm, 1.7 µm

Pre-Column: Waters ACQUITY UPLC BEH $C_{18}$ VanGuard,™ 1.7 µm

| Step | Time (min) | Flow (mL/min) | % A | % B | Curve |
|---|---|---|---|---|---|
| 0 | Initial | 0.9 | 100.0 | 0.0 | Initial |
| 1 | 0.25 | 0.9 | 100.0 | 0.0 | 6 |
| 2 | 3.75 | 0.9 | 40.0 | 60.0 | 6 |
| 3 | 3.95 | 0.9 | 40.0 | 60.0 | 6 |
| 4 | 4.25 | 0.9 | 100.0 | 0.0 | 6 |
| 5 | 4.35 | 0.9 | 100.0 | 0.0 | 6 |

### Flow injection analysis (FIA) pump settings

| Step | Time (min) | Flow (µL/min) | % A | % B |
|---|---|---|---|---|
| 0 | Initial | 30 | 0.0 | 100.0 |
| 1 | 1.60 | 30 | 0.0 | 100.0 |
| 2 | 2.40 | 200 | 0.0 | 100.0 |
| 3 | 2.80 | 200 | 0.0 | 100.0 |
| 4 | 3.00 | 30 | 0.0 | 100.0 |

### Other systems settings

| Instrument | Parameter | Method | | |
|---|---|---|---|---|
| | | HPLC | UPLC | FIA |
| Autosampler | Injection volume | 10 | 5 | 20 |
| Column Oven | Temp. | 50 °C | 50 °C | No column |
| MS | Capillary voltage | 3.2 | 3.2 | 3.9 |
| | Cone voltage | 27 | 27 | 22 |
| | Source temp. | 150 °C | 150 °C | 150 °C |
| | Desolvation temp. | 600 °C | 600 °C | 350 °C |
| | Cone gas | 50 | 250 | 0 |
| | Desolvation gas | 720 | 1000 | 650 |
| | Collision gas | 0.15 | 0.15 | 0.15 |
| | Collision | 2 | 2 | 2 |

## RESULTS AND DISCUSSION

The extraction of metabolites from biological samples is a key delicate step for an accurate MS analysis. A multi-step sample preparation procedure could contribute to the variation and errors in the measurements of the natural metabolites. In order to minimize these issues, step-by-step operating procedures were followed as described in the Kit User Manual and detailed in the Experimental section of this application note.

The Absolute*IDQ* p180 Kit was tested with both HPLC (Agilent Zorbax Eclipse XDB $C_{18}$, 3.0 x 100 mm, 3.5 µm) and UPLC (Waters ACQUITY UPLC BEH $C_{18}$ 2.1 x 50 mm, 1.7 µm) columns coupled with Xevo TQ and Xevo TQ-S mass spectrometers, as shown in Figure 4. The UPLC-based assay at a flow rate of 0.9 mL/min allowed for a high-throughput separation of the selected metabolites in less than 5 min, which was considerably shorter than the HPLC-based assay at a flow rate of 0.5 mL/min, as shown in Figure 4.

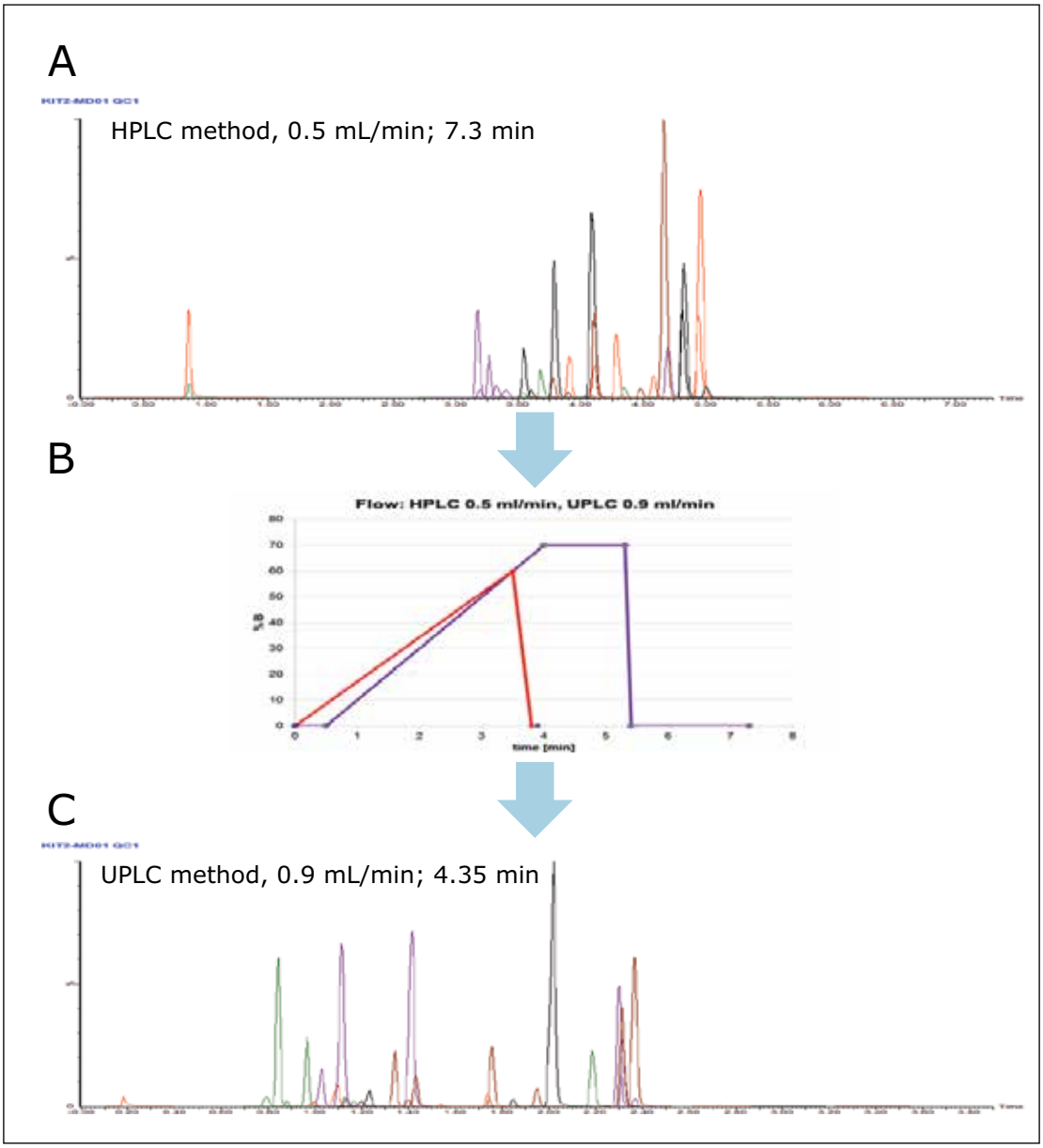


*Figure 4. A.) Representative HPLC/MS chromatogram illustrating the total run time of 7.3 min. B.) Optimization of the chromatographic gradient from HPLC-based method (violet) to UPLC-based method (red). C.) Representative UPLC/MS chromatogram showing a total run time of 4.3 min, which represents a significant gain in speed compared to HPLC/MS.*

The Absolute*IDQ* p180 Kit was utilized to determine differences in the serum metabolome between irradiated and non-irradiated mice. The identification of potential alterations in the levels of metabolites in the serum of mice exposed to gamma radiation is particularly significant because it could lead to the following: 1) a better understanding of the biochemical pathways involved in the response to gamma radiation; and 2) the discovery of biochemical indicators (biomarkers) of acute exposure to ionizing radiation. Rapid identification of biomarkers will be of particular importance in the case of accidental exposures and terrorist acts,[3,4] as classic cytogenetic methods available for biodosimetry are laborious and time-consuming. Using the Absolute*IDQ* p180 Kit, we were able to rapidly measure the serum levels of both polar and non-polar metabolites belonging to major biochemical pathways, as shown in Table 1.

| **Acylcarnitines (40)** | | | |
|---|---|---|---|
| C0 Carnitine | C10:1 Decenoylcarnitine | C5:1-DC Glutaconylcarnitine | C16 Hexadecanoylcarnitine |
| C2 Acetylcarnitine | C10:2 Decadienylcarnitine | C5-DC (C6-OH) Glutarylcarnitine* (Hydroxyhexanoylcarnitine) | C16:1 Hexadecenoylcarnitine |
| C3 Propionylcarnitine | C12 Dodecanoylcarnitine | C5-M-DC Methylglutarylcarnitine | C16:1-OH Hydroxyhexadecenoylcarnitine |
| C3:1 Propenoylcarnitine | C12:1 Dodecenoylcarnitine | C5-OH (C3-DC-M) Hydroxyvalerylcarnitine (Methylmalonylcarnitine) | C16:2 Hexadecadienylcarnitine |
| C3-OH Hydroxypropionylcarnitine | C12-DC Dodecanedioylcarnitine | C6 (C4:1-DC) Hexanoylcarnitine (Fumarylcarnitine) | C16:2-OH Hydroxyhexadecadienylcarnitine |
| C4 Butyrylcarnitine | C14 Tetradecanoylcarnitine | C6:1 Hexenoylcarnitine | C16-OH Hydroxyhexadecanoylcarnitine |
| C4:1 Butenylcarnitine | C14:1 Tetradecenoylcarnitine | C7-DC Pimelylcarnitine | C18 Octadecanoylcarnitine |
| C4-OH (C3-DC) Hydroxybutyrylcarnitine | C14:1-OH Hydroxytetradecenoylcarnitine | C8 Octanoylcarnitine | C18:1 Octadecenoylcarnitine |
| C5 Valerylcarnitine | C14:2 Tetradecadienylcarnitine | C9 Nonaylcarnitine | C18:1-OH Hydroxyoctadecenoylcarnitine |
| C5:1 Tiglylcarnitine | C14:2-OH Hydroxytetradecadienylcarnitine | C10 Decanoylcarnitine | C18:2 Octadecadienylcarnitine |
| **Amino Acids and Biogenic Amines (40)** | | | |
| Alanine | Leucine | Valine | Methioninesulfoxide |
| Arginine | Lysine | Acetylyornithine | Nitrotyrosine |
| Asparagine | Methionine | Asymmetric dimethylarginine | Hydroxyproline |
| Aspartate | Ornithine | Symmetric dimethylarginine | Phenylethylamine |
| Citrulline | Phenylalanine | Total dimethylarginine | Putrescine |
| Glutamine | Proline | alpha-Aminoadipic acid | Sarcosine |
| Glutamate | Serine | Carnosine | Serotonin |
| Glycine | Threonine | Creatinine | Spermidine |
| Histidine | Tryptophan | Histamine | Spermine |
| Isoleucine | Tyrosine | Kynurenine | Taurine |
| **Sphingolipids (14)** | | | |
| SM (OH) C14:1 | SM C18:0 | SM (OH) C22:2 | SM C26:0 |
| SM C16:0 | SM C18:1 | SM C24:0 | SM C26:1 |
| SM C16:1 | SM C20:2 | SM C24:1 | |
| SM (OH) C16:1 | SM (OH) C22:1 | SM (OH) C24:1 | |

The Absolute*IDQ* p180 Kit was utilized to determine differences in the serum metabolome between irradiated and non-irradiated mice. The identification of potential alterations in the levels of metabolites in the serum of mice exposed to gamma radiation is particularly significant because it could lead to the following: 1) a better understanding of the biochemical pathways involved in the response to gamma radiation; and 2) the discovery of biochemical indicators (biomarkers) of acute exposure to ionizing radiation. Rapid identification of biomarkers will be of particular importance in the case of accidental exposures and terrorist acts,[3,4] as classic cytogenetic methods available for biodosimetry are laborious and time-consuming. Using the Absolute*IDQ* p180 Kit, we were able to rapidly measure the serum levels of both polar and non-polar metabolites belonging to major biochemical pathways, as shown in Table 1.

Principal Component Analysis showed that the gamma irradiated group was well separated from the control

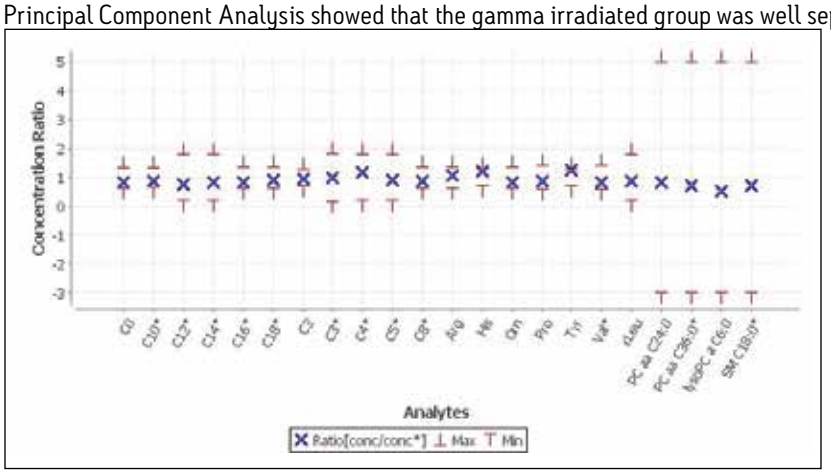


*Figure 5. Quality control samples. Measured concentration/expected concentration ratios are displayed in the MetIDQ software, which is an integral part of the kit. Representative values for acylcarnitines (CO-$C_{18}$), amino acids, and lipids.*

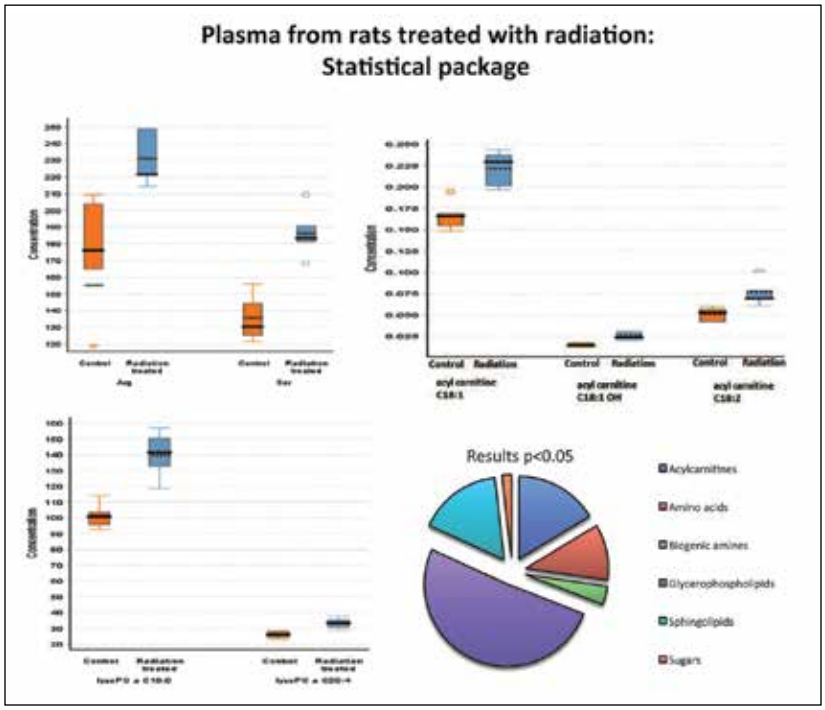


*Figure 6. The box plots show examples of altered metabolites in the serum samples of gamma irradiated mice. The pie chart illustrates the kit metabolite panel separated into metabolite classes. Results of the statistically significant ions are presented as a percentage in each metabolic class.*

## CONCLUSIONS

By combining the ACQUITY UPLC System with the Xevo TQ or Xevo TQ-S Mass Spectrometers and the commercially available Absolute*IDQ* p180 Kit, rapid identification and quantification of more than 180 metabolites in murine serum were successfully attained. Similar applications could lead to novel mechanistic insight and biomarker discovery in drug development, diagnostics, and systems biology research.

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# Targeted Lipidomics of Oxylipins (Oxygenated Fatty Acids)

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## APPLICATION BENEFITS

Here, we present a high-throughput approach for profiling bioactive oxylipins (oxidized fatty acids) in plasma. The combination of mixed mode solid-phase extraction (Oasis® MAX SPE) and UPLC®-ESI-MRM mass spectrometry (Xevo® TQ-S) provides a comprehensive analysis of oxylipins in a targeted analytical workflow. Retention times and transitions of 107 oxylipins (including prostaglandins, prostacyclines, thromboxanes, dihydroprostaglandins, and isoprostanes) were annotated for routine high-throughput analysis of plasma samples. Considering the prominent roles played by oxylipins in health and disease (*e.g.*, inflammation), such a UPLC-based assay could become important in nutritional research, clinical research, and drug discovery and development.

## WATERS SOLUTIONS

Xevo TQ-S Mass Spectrometer

Oasis MAX SPE Cartridges

TargetLynx™ Application Manager

## KEY WORDS

UPLC-MS/MS, fatty acids, metabolomics, lipidomics, triple quadrupole, oxylipins, multiple reaction monitoring, MRM, Xevo TQ-S

## INTRODUCTION

Oxylipins are signaling lipids that play prominent roles in the physiological regulation of many key biological processes, such as the relaxation and contraction of smooth muscle tissue, blood coagulation, and most notably inflammation. Alterations in oxylipin pathways have been associated with response to cardiovascular diseases, host defense, tissue injury and surgical intervention. The ability to semi-quantitatively profile a wide range of oxylipin in plasma samples could help our understanding of their roles in health and disease, as well as serve as biomarkers for disease diagnosis or prognosis.

Oxylipins are produced via enzymatic (e.g., mono- or dioxygenase-catalyzed) or non enzymatic oxygenation of an array of both omega-6 polyunsaturated fatty acid substrates (e.g., linoleic acid, dihomo-γ-linolenic acid, adrenic acid and arachidonic acid) and omega-3 polyunsaturated fatty acid substrates (α-linolenic acid, acid, eicosapentaenoic acid, and docosahexaenoic acid) (Figure 1A and 1B). Three major enzymatic pathways are involved in their generation: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP). These pathways are important drug targets for multiple diseases (Figure 1A and 1B).

The main challenge for the measurement of oxylipins is the extremely low endogenous concentration of such lipid species and their limited stability. Furthermore, oxylipins are not stored in tissues but are formed on demand by liberation of precursor fatty acids from esterified forms. Lastly, the same fatty acid can be oxidized in different positions of its acyl chain leading to many isomeric species, each with specific metabolic actions. As a consequence, this requires a rapid, highly-sensitive, and specific analytical method.

Historically, measurements of oxylipins have been performed using radiometric and enzymatic immunoassays, which often lacked specificity and targeted only few compounds. GC-MS methodology has also been used, but this still requires multi-step procedures involving derivatization of the oxylipins to increase their volatility and stability.

Recently, various LC-MS methodologies have been described to monitor a broad range of low abundance oxylipins.[1-5] In particular the method by Strassburg et al.[2] reports on a wide range of oxylipins produced both enzymatically and non-enzymatically in human plasma. Although such methods are both sensitive and specific, there is an increasing demand for a comprehensive and high-throughput screening method to enable wide-ranging lipidomic studies.

Here we report a high-throughput assay for the profiling of over 100 oxylipins, including prostaglandins, prostacyclines, thromboxanes, dihydroprostaglandins, and isoprostanes, in plasma samples.

| Internal standard | Cayman #number | MRM transition | RT (min) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|---|
| d4-6-Keto PGF1α | 315210 | 373.20 >167.20 | 2.28 | 35 | 15 |
| d4-TBX2 | 319030 | 373.20 >173.10 | 2.86 | 35 | 15 |
| d4-PGF2α | 316010 | 357.30 >197.20 | 3.12 | 35 | 20 |
| d4-PGE2 | 314010 | 355.20 >275.20 | 3.19 | 40 | 16 |
| d4-PGD2 | 312010 | 355.20 >275.20 | 3.31 | 10 | 16 |
| d5-LTE4 | 10007858 | 443.10 >338.00 | 4.11 | 35 | 20 |
| d4-LTB4 | 320110 | 339.20 >197.10 | 4.48 | 35 | 15 |
| d4-12,13-DiHOME | 10009994 | 317.30 >185.20 | 4.56 | 35 | 15 |
| d4-9,10-DiHOME | 10009993 | 317.30 >203.20 | 4.69 | 35 | 15 |
| d11-14,15-DiHETrE | 10008040 | 348.30 >207.10 | 4.77 | 35 | 15 |
| d4-15-deoxy-Δ12,14-PGJ2 | 318570 | 319.20 >275.30 | 5.20 | 35 | 15 |
| d6-20-HETE | 390030 | 325.20 >281.10 | 5.24 | 20 | 18 |
| d4-9-HODE | 338410 | 299.20 >172.10 | 5.53 | 35 | 20 |
| d8-12-HETE | 334570 | 327.30 >184.20 | 5.78 | 35 | 20 |
| d8-5-HETE | 334230 | 327.30 >116.10 | 5.97 | 35 | 20 |

*Table 1. Internal standards used for profiling natural oxylipins in plasma and optimal UPLC-ESI-MS settings.*

## EXPERIMENTAL

### Sample preparation

#### Materials

All chemicals were purchased from Sigma-Aldrich (Germany) and were of analytical grade or higher purity. Oxylipins standards were purchased from Cayman Chemicals (Ann Arbor, MI), Biomol (Plymouth Meeting, PA), and Larodan (Malmö, Sweden). For mixed mode solid phase extraction we used Waters Oasis MAX 3 cc Vac Cartridge, 60 mg Sorbent per Cartridge, 30 µm Particle Size (p/n 186000367). An internal standard mixture containing 16 isotopically labeled compounds was used (Table 1).

### Sample pre-treatment

**(dilution, performed in borosilicate glass tubes 13 x 100 mm):**

1. Add 200 µL of 10% glycerol in water to a glass tube
2. Add 50 – 250 µL of plasma (maximum sample volume available) sample to the tube and mix thoroughly
3. Add 5 µL of 10 mg/mL BHT in ethanol and mix thoroughly
4. Add 5 µL of internal standard solution (400 ng/mL) and mix
5. Make up the total sample volume to 3 mL with 25% MeCN(aq) and mix thoroughly

### MAX mixed mode solid phase extraction

1. Condition Oasis MAX SPE Cartridge with 3 mL of MeCN
2. Condition Oasis MAX SPE Cartridge with 3 mL of 25% MeCN(aq)
3. Load the entire pre-treated sample onto the Oasis MAX SPE Cartridge
4. Wash Oasis MAX SPE Cartridge with 3 mL of 25% MeCN(aq)
5. Wash Oasis MAX SPE Cartridge with 3 mL of MeCN
6. Elute analytes with 1.3 mL of 1% Formic in MeCN8*
7. Transfer eluate to a glass HPLC vial (TruView™ Max Recovery Vial)
8. Evaporate eluate down until only the glycerol remains (under nitrogen at 40 °C)
9. Add 60 µL of 50/50 MeOH/MeCN and mix thoroughly
10. Inject 3 µL onto the UPLC-MS/MS System

**Sample eluted into a glass tube containing 200 µL of 10% glycerol in methanol*

### UPLC conditions

| | |
|---|---|
| System: | ACQUITY UPLC® System in negative ESI mode |
| Column: | ACQUITY UPLC BEH $C_{18,}$ 1.7 µm, 2.1 x 100 mm |
| Mobile phase A: | $H_2O$ + 0.1% acetic acid |
| Mobile phase B: | ACN/IPA (90/10 v/v) |
| Flow rate: | 0.6 mL/min |
| Column temp.: | 40 °C |
| Volume: | 3.0 µL |

Elution gradient:

| Min | A% | B% | Curve |
|---|---|---|---|
| 0.0 | 75 | 25 | |
| 1.0 | 75 | 25 | 6 |
| 8.0 | 5 | 95 | 6 |
| 8.50 | 5 | 95 | 6 |
| 8.51 | 75 | 25 | 6 |
| 10.00 | 75 | 25 | 6 |

### MS conditions

For optimum reproducibility of retention times we recommend the following tubing to connect UPLC analytical column to ESI probe: PEEK Tubing, 1/16 in. (1.6 mm) O.D. X 0.004 in. (0.100 mm) I.D. X 5 ft (1.5 m) length, cut to 400 mm in length.

| | |
|---|---|
| MS system: | Xevo TQ-S in negative ESI mode |
| Acquisition mode: | MRM |
| Capillary voltage: | 2.5 kV |
| Cone voltage: | 10-40 V (compound Specific, default = 35 V) |
| Source temp.: | 150 °C |
| Desolvation gas temp.: | 600 °C |
| Desolvation gas flow: | 1000 L/h |
| Cone gas flow: | 150 L/h |
| Collision energy: | 15-20 V (compound Specific, default = 15 V) |

### Data management

TargetLynx Application Manager

## RESULTS AND DISCUSSION

The primary focus of this work was to provide a high-throughput method to profile bioactive oxylipins in plasma samples.

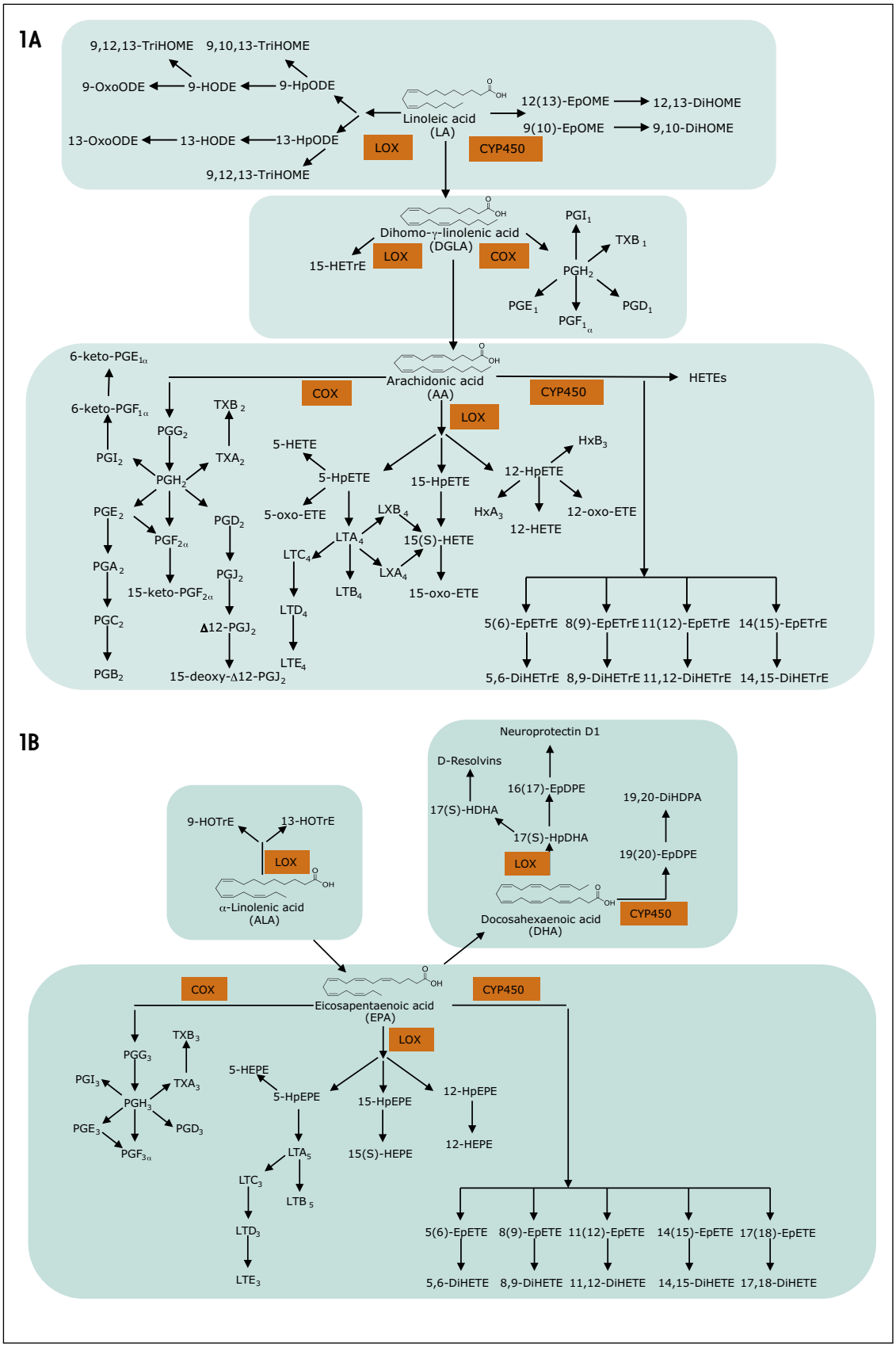



*Figure 1. A. Schematic outline of the oxylipins of the omega-6 series produced by linoleic acid $C_{18}$:2 (LA), dihomo-γ-linoleic acid C20:3 (DHGLA), and arachidonic acid C20:4 (AA), via the cyclooxygenase (COX), lipoxygenase (LOX), CYP-450, or free radical catalyzed pathways.*

*B. Schematic outline of the oxylipins of the omega-3 series produced by α-linolenic acid $C_{18}$:3 (ALA), eicosapentaenoic acid C20:5 (EPA), and docosahexaenoic acid C22:6 (DHA), via the COX, LOX, CYP-450, or free radical catalyzed pathways.*

*Abbreviations: dihydroxyeicosatetraenoic acid (DiHETE), epoxy-octadecenoic acid (EpOME), hydroxy-eicosatrienoic acid (HETrE), hydroxy-eicosatetraenoic acid (HETE), hydroxy-heptadecatrienoic acid (HHTrE), hydroxy-octadecadienoic acid (HODE), hydroxy-eicosapentaenoic acid (HEPE), oxo-eicosatetraenoic acid (KETE), oxo-octadecadienoic acid (KODE), prostaglandin (PG), thromboxane (TX).*

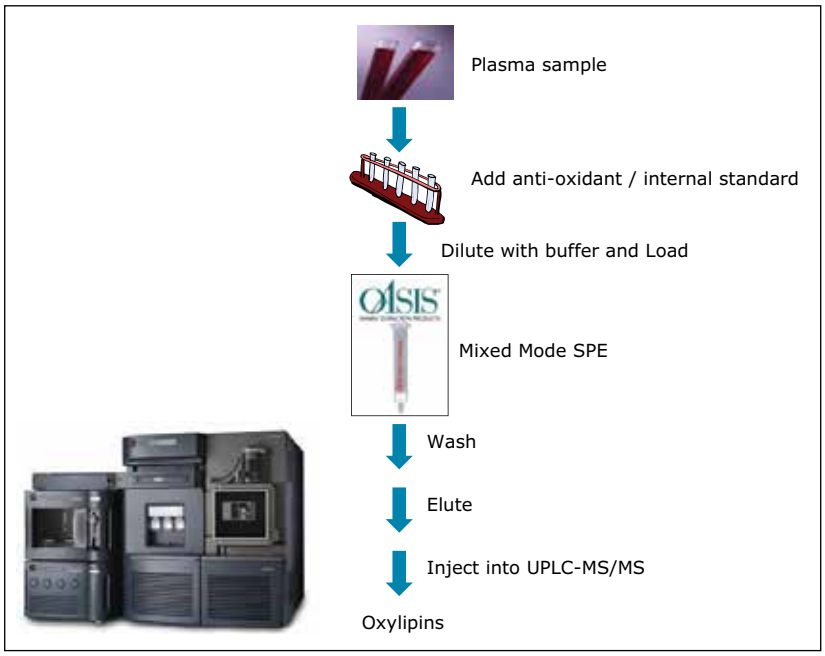
| | Compound name | M1 | M2 | RT | I | Precursor | Class | Pathway |
|---|---|---|---|---|---|---|---|---|
| 1 | Tetranor-PGFM | 329.2 | 311.2 | 0.48 | (d4) PGF2α | AA | Prostanoid | COX |
| 2 | Tetranor-PGEM | 327.1 | 309.2 | 0.53 | (d4) PGE2 | AA | Prostanoid | COX |
| 3 | 20-hydroxy PGE2 | 367.2 | 287.2 | 1.01 | (d4) PGE2 | AA | Prostanoid | COX |
| 4 | Δ17-6-keto PGF1α | 367.2 | 163.1 | 1.76 | (d4) 6-keto PGF1α | AA | Prostanoid | COX |
| 5 | 6-keto PGF1α | 369.2 | 163.1 | 2.27 | (d4) 6-keto PGF1α | AA | Prostanoid | COX |
| 6 | 2,3-dinor-11b PGF2α | 325.2 | 145.1 | 2.27 | (d4) PGF2α | AA | Prostanoid | COX |
| 7 | (d4) 6-keto PGF1α | 373.2 | 167.2 | 2.28 | ISTD | | | |
| 8 | 20-carboxy LTB4 | 365.2 | 347.2 | 2.35 | (d4) LTB4 | AA | Leukotriene | LOX |
| 9 | 6-keto PGE1 | 367.2 | 143.1 | 2.37 | (d4) PGE2 | AA | Prostanoid | COX |
| 10 | 20-hydroxy LTB4 | 351.2 | 195.1 | 2.46 | (d4) LTB4 | AA | Leukotriene | LOX |
| 11 | TXB3 | 367.2 | 169.1 | 2.48 | (d4) TXB2 | EPA | Thromboxane | COX |
| 12 | PGF3α | 351.2 | 193.2 | 2.75 | (d4) PGF2α | EPA | Prostanoid | COX |
| 13 | TXB1 | 371.2 | 171.1 | 2.79 | (d4) TXB2 | DGLA | Thromboxane | COX |
| 14 | PGE3 | 349.2 | 269.2 | 2.83 | (d4) PGE2 | EPA | Prostanoid | COX |
| 15 | (d4) TXB2 | 373.2 | 173.1 | 2.86 | ISTD | | | |
| 16 | 8-iso PGF2α | 353.2 | 193.2 | 2.87 | (d4) PGF2α | AA | Isoprostane | non enzymatic |
| 17 | TXB2 | 369.2 | 169.1 | 2.88 | (d4) TXB2 | AA | Thromboxane | COX |
| 18 | PGD3 | 349.2 | 269.2 | 2.92 | (d4) PGD2 | EPA | Prostanoid | COX |
| 19 | 11β-PGF2α | 353.2 | 193.2 | 2.93 | (d4) PGF2α | AA | Prostanoid | COX |
| 20 | (+/-) 5-iPF2α-VI | 353.2 | 115.1 | 3.04 | (d4) PGF2α | AA | Isoprostane | non enzymatic |
| 21 | 9,12,13-TriHOME | 329.2 | 211.2 | 3.07 | (d4) 9(S)-HODE | LA | Triol | LOX |
| 22 | 9,10,13-TriHOME | 329.2 | 171.1 | 3.12 | (d4) 9(S)-HODE | LA | Triol | LOX |
| 23 | (d4) PGF2α | 357.3 | 197.2 | 3.12 | ISTD | | | |
| 24 | PGF2α | 353.2 | 193.2 | 3.14 | (d4) PGF2α | AA | Prostanoid | COX |
| 25 | PGF1α | 355.2 | 293.2 | 3.14 | (d4) PGF2α | DGLA | Prostanoid | COX |
| 26 | (d4) PGE2 | 355.2 | 275.2 | 3.19 | ISTD | | | |
| 27 | PGE2 | 351.2 | 271.2 | 3.2 | (d4) PGE2 | AA | Prostanoid | COX |
| 28 | 11β-PGE2 | 351.2 | 271.2 | 3.25 | (d4) PGE2 | AA | Prostanoid | COX |
| 29 | PGK2 | 349.2 | 205.1 | 3.28 | (d4) PGE2 | AA | Prostanoid | COX |
| 30 | 15-keto PGF2α | 351.2 | 219.1 | 3.28 | (d4) PGF2α | AA | Prostanoid | COX |
| 31 | 5(S),14(R)-Lipoxin B4 | 351.2 | 221.2 | 3.29 | (d4) LTB4 | AA | Lipoxin | LOX |
| 32 | PGE1 | 353.2 | 273.2 | 3.29 | (d4) PGE2 | DGLA | Prostanoid | COX |
| 33 | (d4) PGD2 | 355.2 | 275.2 | 3.31 | ISTD | | | |
| 34 | PGD2 | 351.2 | 271.2 | 3.32 | (d4) PGD2 | AA | Prostanoid | COX |
| 35 | PGD1 | 353.2 | 273.2 | 3.32 | (d4) PGD2 | DGLA | Prostanoid | COX |
| 36 | 11β-13,14-dihydro-15-keto PGF2α | 353.2 | 113.2 | 3.35 | (d4) PGF2α | AA | Prostanoid | COX |
| 37 | 15-keto PGF1α | 353.2 | 221.1 | 3.37 | (d4) 6-keto PGF1α | DGLA | Prostanoid | COX |
| 38 | 13,14-dihydro PGF2α | 355.2 | 275.2 | 3.39 | (d4) PGF2α | AA | Prostanoid | COX |
| 39 | 13,14-dihydro-15-keto PGE2 | 351.2 | 175.2 | 3.54 | (d4) PGE2 | AA | Prostanoid | COX |
| 40 | 13,14-dihydro-15-keto PGF2α | 353.2 | 183.1 | 3.56 | (d4) PGF2α | AA | Prostanoid | COX |
| 41 | 5(S),6(R)-Lipoxin A4 | 351.2 | 115.1 | 3.58 | (d4) LTB4 | AA | Lipoxin | LOX |
| 42 | 5(S),6(S)-Lipoxin A4 | 351.2 | 115.1 | 3.68 | (d4) LTB4 | AA | Lipoxin | LOX |
| 43 | 13,14-dihydro-15-keto PGF1α | 355.2 | 193.2 | 3.72 | (d4) PGF2α | AA | Prostanoid | COX |
| 44 | 13,14-dihydro-15-keto PGD2 | 351.2 | 175.2 | 3.77 | (d4) PGD2 | AA | Prostanoid | COX |

| | Compound name | M1 | M2 | RT | I | Precursor | Class | Pathway |
|---|---|---|---|---|---|---|---|---|
| 45 | 1α,1b-dihomo PGF2α | 381.3 | 337.2 | 3.77 | (d4) PGF2α | ADA | Prostanoid | COX |
| 46 | 14,15-LTE4 | 438.2 | 333.2 | 3.78 | (d3) LTE4 | AA | Leukotriene | LOX |
| 47 | LTD4 | 495.2 | 177.1 | 3.9 | (d3) LTE4 | AA | Leukotriene | LOX |
| 48 | Resolvin D1 | 375.2 | 141 | 3.9 | (d11) 14,15-DiHETrE | DHA | rRsolving | LOX |
| 49 | Resolvin E1 | 349.2 | 195 | 3.9 | (d11) 14,15-DiHETrE | EPA | Resolving | LOX |
| 50 | 13,14-dihydro-15-keto PGD1 | 353.2 | 209.1 | 3.91 | (d4) PGD2 | AA | Prostanoid | COX |
| 51 | PGA2 | 333.2 | 271.2 | 3.91 | (d4) PGE2 | AA | Prostanoid | COX |
| 52 | Δ12-PGJ2 | 333.2 | 233.1 | 3.97 | (d4) 15-deoxy-Δ12,14-PGJ2 | AA | Prostanoid | COX |
| 53 | PGJ2 | 333.2 | 233.1 | 3.97 | (d4) PGD2 | AA | Prostanoid | COX |
| 54 | LTB5 | 333.2 | 195.1 | 4.03 | (d4) LTB4 | EPA | Leukotriene | LOX |
| 55 | 11-trans LTD4 | 495.2 | 177.1 | 4.05 | (d3) LTE4 | AA | Leukotriene | LOX |
| 56 | (d3) LTE4 | 441.2 | 336.2 | 4.12 | ISTD | | | |
| 57 | LTE4 | 438.2 | 333.2 | 4.13 | (d3) LTE4 | AA | Leukotriene | LOX |
| 58 | 8(S),15(S)-DiHETE | 335.2 | 235.2 | 4.23 | (d4) LTB4 | AA | Diol | CYP450 |
| 59 | 12,13-DiHODE | 311.2 | 293 | 4.23 | (d4) 9,10-DiHOME | ALA | Diol | CYP450 |
| 60 | bicyclo-PGE2 | 333.2 | 113.2 | 4.25 | (d4) PGE2 | AA | Prostanoid | CYP450 |
| 61 | 11-trans LTE4 | 438.2 | 333.2 | 4.26 | (d3) LTE4 | AA | Leukotriene | LOX |
| 62 | 10(S),17(S)-DiHDoHE | 359.2 | 153.2 | 4.34 | (d8) 12(S)-HETE | DHA | Protectin | LOX |
| 63 | Neuroprotectin D1 | 359.2 | 206 | 4.34 | (d8) 12(S)-HETE | DHA | Protectin | LOX |
| 64 | 17,18-DiHETE | 335.2 | 247.2 | 4.34 | (d11) 14,15-DiHETrE | EPA | Diol | CYP450 |
| 65 | 5(S),15(S)-DiHETE | 335.2 | 115.2 | 4.37 | (d4) LTB4 | AA | Diol | CYP450 |
| 66 | 6-trans-LTB4 | 335.2 | 195.1 | 4.4 | (d4) LTB4 | AA | Leukotriene | LOX |
| 67 | 14,15-DiHETE | 335.2 | 207.1 | 4.46 | (d11) 14,15-DiHETrE | EPA | Diol | CYP450 |
| 68 | (d4) LTB4 | 339.2 | 197.1 | 4.48 | ISTD | | | |
| 69 | 15-deoxy-Δ12,14-PGD2 | 333.2 | 271.2 | 4.49 | (d4) 15-deoxy-Δ12,14-PGJ2 | AA | Prostanoid | COX |
| 70 | Hepoxilin A3 | 335.2 | 273.2 | 4.5 | (d8) 12(S)-HETE | AA | Hepoxilin | LOX |
| 71 | LTB4 | 335.2 | 195.1 | 4.5 | (d4) LTB4 | AA | Leukotriene | LOX |
| 72 | (d4)(±)12,13-DiHOME | 317.3 | 185.2 | 4.56 | ISTD | | | |
| 73 | 12,13-DiHOME | 313.2 | 183.2 | 4.58 | (d4) 12,13-DiHOME | LA | Diol | CYP450 |
| 74 | (d4)-(±)9,10-DiHOME | 317.3 | 203.2 | 4.69 | ISTD | | | |
| 75 | 9,10-DiHOME | 313.2 | 201.1 | 4.71 | (d4) 9,10-DiHOME | LA | Diol | CYP450 |
| 76 | (d11) 14,15-DiHETrE | 348.3 | 207.1 | 4.77 | ISTD | | | |
| 77 | 19,20-DiHDPA | 361.2 | 273.3 | 4.79 | (d11) 14,15-DiHETrE | DHA | Diol | CYP450 |
| 78 | 14,15-DiHETrE | 337.2 | 207.2 | 4.8 | (d11) 14,15-DiHETrE | AA | Diol | CYP450 |
| 79 | 12S-HHTrE | 279.2 | 179.2 | 4.84 | (d8) 12(S)-HETE | AA | Alcohol | COX |
| 80 | 11,12-DiHETrE | 337.2 | 167.2 | 4.98 | (d11) 14,15-DiHETrE | AA | Diol | CYP450 |
| 81 | 5,6-DiHETrE | 337.2 | 145.1 | 4.99 | (d11) 14,15-DiHETrE | AA | Diol | CYP450 |
| 82 | 9-HOTrE | 293.2 | 171.1 | 5.07 | (d4) 9(S)-HODE | ALA | Alcohol | LOX |
| 83 | 17(18)-EpETE | 317.2 | 259.2 | 5.16 | (d11) 14,15-DiHETrE | EPA | Epoxide | CYP450 |
| 84 | (d4) 15-deoxy-Δ12,14-PGJ2 | 319.2 | 275.3 | 5.2 | ISTD | | | |
| 85 | (d6) 20-HETE | 325.3 | 279.2 | 5.24 | ISTD | | | |
| 86 | 20-HETE | 319.2 | 289.2 | 5.25 | d6-20-HETE | AA | Alcohol | CYP450 |
| 87 | 15(S)-HEPE | 317.2 | 219.2 | 5.25 | (d8) 5(S)-HETE | EPA | Alcohol | LOX |
| 88 | 12(S)-HpETE | 317.1 | 153.0 | 5.34 | (d8) 12(S)-HETE | AA | Hydroxyperoxide | LOX |

| | Compound name | M1 | M2 | RT | I | Precursor | Class | Pathway |
|---|---|---|---|---|---|---|---|---|
| 89 | 8,9-DiHETrE | 337.2 | 127 | 5.35 | (d11) 14,15-DiHETrE | AA | Diol | CYP450 |
| 90 | 5(S),6(S)-DiHETE | 335.2 | 115.1 | 5.35 | (d4) LTB4 | AA | Diol | CYP450 |
| 91 | 12(S)-HEPE | 317.2 | 179.1 | 5.35 | (d8) 12(S)-HETE | EPA | Alcohol | LOX |
| 92 | 13-HODE | 295.2 | 195.2 | 5.5 | (d4) 9(S)-HODE | LA | Alcohol | LOX |
| 93 | 5(S)-HEPE | 317.2 | 115.1 | 5.51 | (d8) 5(S)-HETE | EPA | Alcohol | LOX |
| 94 | (d4) 9(S)-HODE | 299.2 | 172.1 | 5.53 | ISTD | | | |
| 95 | 9-HODE | 295.2 | 171.1 | 5.56 | (d4) 9(S)-HODE | LA | Alcohol | LOX |
| 96 | 15-HETE | 319.2 | 219.2 | 5.62 | (d8) 5(S)-HETE | AA | Alcohol | LOX |
| 97 | 16(17)-EpDPE | 343.2 | 233.2 | 5.62 | (d11) 14,15-DiHETrE | DHA | Epoxide | CYP450 |
| 98 | 13-HpODE | 293.1 | 113.0 | 5.63 | (d4) 9(S)-HODE | LA | Hydroxyperoxide | LOX |
| 99 | 13-KODE | 293.2 | 113.1 | 5.64 | (d4) 9(S)-HODE | LA | Ketone | LOX |
| 100 | 17-HDoHE | 343.2 | 281.3 | 5.67 | (d8) 5(S)-HETE | DHA | Alcohol | LOX |
| 101 | 9-HpODE | 293.1 | 185.0 | 5.68 | (d4) 9(S)-HODE | LA | Hydroxyperoxide | LOX |
| 102 | 15-HpETE | 317. | 113.0 | 5.71 | (d8) 5(S)-HETE | AA | Hydroxyperoxide | LOX |
| 103 | 15-KETE | 317.2 | 113.2 | 5.72 | (d8) 5(S)-HETE | AA | Ketone | LOX |
| 104 | 11-HETE | 319.2 | 167.1 | 5.74 | (d8) 12(S)-HETE | AA | Alcohol | COX |
| 105 | 14(15)-EpETE | 317.2 | 207.1 | 5.74 | (d11) 14,15-DiHETrE | EPA | Epoxide | CYP450 |
| 106 | 9-KODE | 293.2 | 185.2 | 5.77 | (d4) 9(S)-HODE | LA | Ketone | LOX |
| 107 | (d8) 12(S)-HETE | 327.3 | 184.2 | 5.78 | ISTD | | | |
| 108 | 12-HETE | 319.2 | 179.2 | 5.81 | (d8) 12(S)-HETE | AA | Alcohol | LOX |
| 109 | 8-HETE | 319.2 | 155.1 | 5.85 | (d8) 5(S)-HETE | AA | Alcohol | LOX |
| 110 | 15(S)-HETrE | 321.2 | 221.2 | 5.88 | (d8) 5(S)-HETE | DGLA | Alcohol | LOX |
| 111 | 9-HETE | 319.2 | 167.1 | 5.91 | (d8) 12(S)-HETE | AA | Alcohol | non-enzymatic |
| 112 | (d8) 5(S)-HETE | 327.3 | 116.1 | 5.97 | ISTD | | | |
| 113 | 5-HETE | 319.2 | 115.1 | 6.00 | (d8) 5(S)-HETE | AA | Alcohol | LOX |
| 114 | 19(20)-EpDPE | 343.2 | 281.3 | 6.09 | (d11) 14,15-DiHETrE | DHA | Epoxide | CYP450 |
| 115 | 12(13)-EpOME | 295.2 | 195.2 | 6.09 | (d4) 12,13-DiHOME | LA | Epoxide | CYP450 |
| 116 | 14(15)-EpETrE | 319.2 | 219.2 | 6.11 | (d11) 14,15-DiHETrE | AA | Epoxide | CYP450 |
| 117 | 5(S)-HpETE | 317.1 | 203.1 | 6.11 | (d8) 5(S)-HETE | AA | Hydroxyperoxide | LOX |
| 118 | 9(10)-EpOME | 295.2 | 171.2 | 6.15 | (d4) 9,10-DiHOME | LA | Epoxide | CYP450 |
| 119 | 12-KETE | 317.2 | 273.3 | 6.25 | (d8) 12(S)-HETE | AA | Ketone | LOX |
| 120 | 5-KETE | 317.2 | 203.2 | 6.26 | (d8) 5(S)-HETE | AA | Ketone | LOX |
| 121 | 11(12)-EpETrE | 319.2 | 167.1 | 6.27 | (d11) 14,15-DiHETrE | AA | Epoxide | CYP450 |
| 122 | 8(9)-EpETrE | 319.2 | 155.1 | 6.33 | (d11) 14,15-DiHETrE | AA | Epoxide | CYP450 |
| 123 | 5(6)-EpETrE | 319.2 | 191.2 | 6.42 | (d11) 14,15-DiHETrE | AA | Epoxide | CYP450 |

*Table 2. List of MRM transitions (M1=precursor; M2= fragment) and retention times (RT) for oxylipins.*

Oxylipins are present at very low abundance in biological samples, and as such the quality of sample preparation is an important factor for successful analyses. To eliminate non-lipid contaminants and highly abundant species like phospholipids, we used mixed mode solid-phase extraction (SPE) prior to UPLC-MS analysis. Normalization of the extraction efficiency was achieved by adding stable isotope labeled compounds (internal standards), prior to the extraction procedure (Table 1 and 2, and Figure 2).




To optimize the chromatographic separation of our analytes, we used a mixture of a wide chemical variety of commercially available oxylipins. Using reversed-phase UPLC (see Experimental), oxylipins eluted in order of decreasing polarity, numbers of double bonds and increasing acyl chain length, allowing the separation of most isomeric and isobaric species (e.g., PGE2 and PGD2) in less than 10 minutes (Figure 3). Using a Xevo TQ-S in negative ESI-mode, retention times and optimal MRM transitions (compound specific precursor ⟹ product ion transitions) were determined for all individual oxylipins (Table 2).

To enhance the sensitivity of detection, these MRM transitions were monitored in defined retention time windows, maximizing dwell times by reducing overlapping transitions. In the case of co-eluting metabolites, compound specific precursor ions and their corresponding fragment ions allowed selective profiling of those compounds. Calibration curves for the majority of the analytes were produced and displayed a linear coefficient (Pearson's correlation, $R^2$) higher than 0.99. (Figure 4). Using this UPLC-MS/MS assay, we rapidly profiled 107 oxylipins in human plasma samples (Figure 5).

With minor modifications in the sample preparation protocol, this assay could be extended to the measure of oxylipins in other biological matrices.

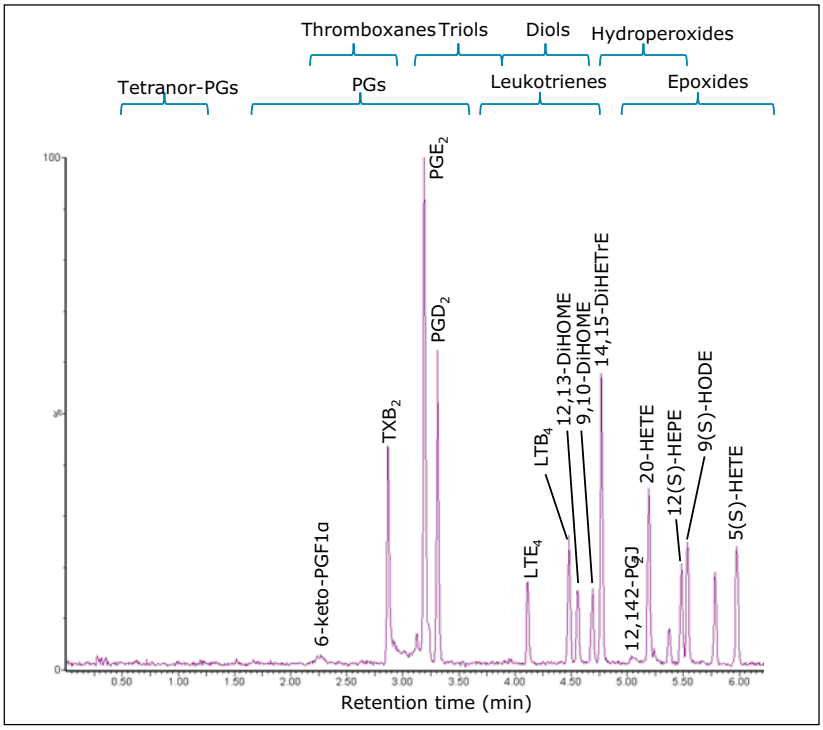



*Figure 3. Representative UPLC-MS/MS chromatogram of a wide chemical variety of oxylipin species.*

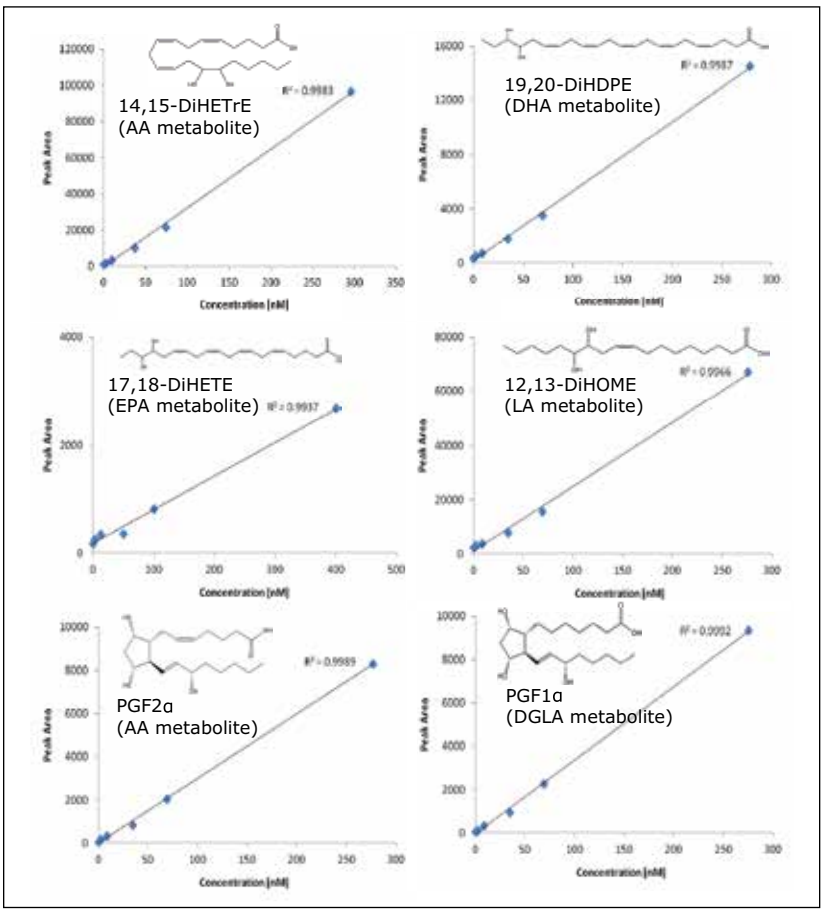



*Figure 4. Linearity of response for representative endogenous oxylipin species present in the plasma samples.*

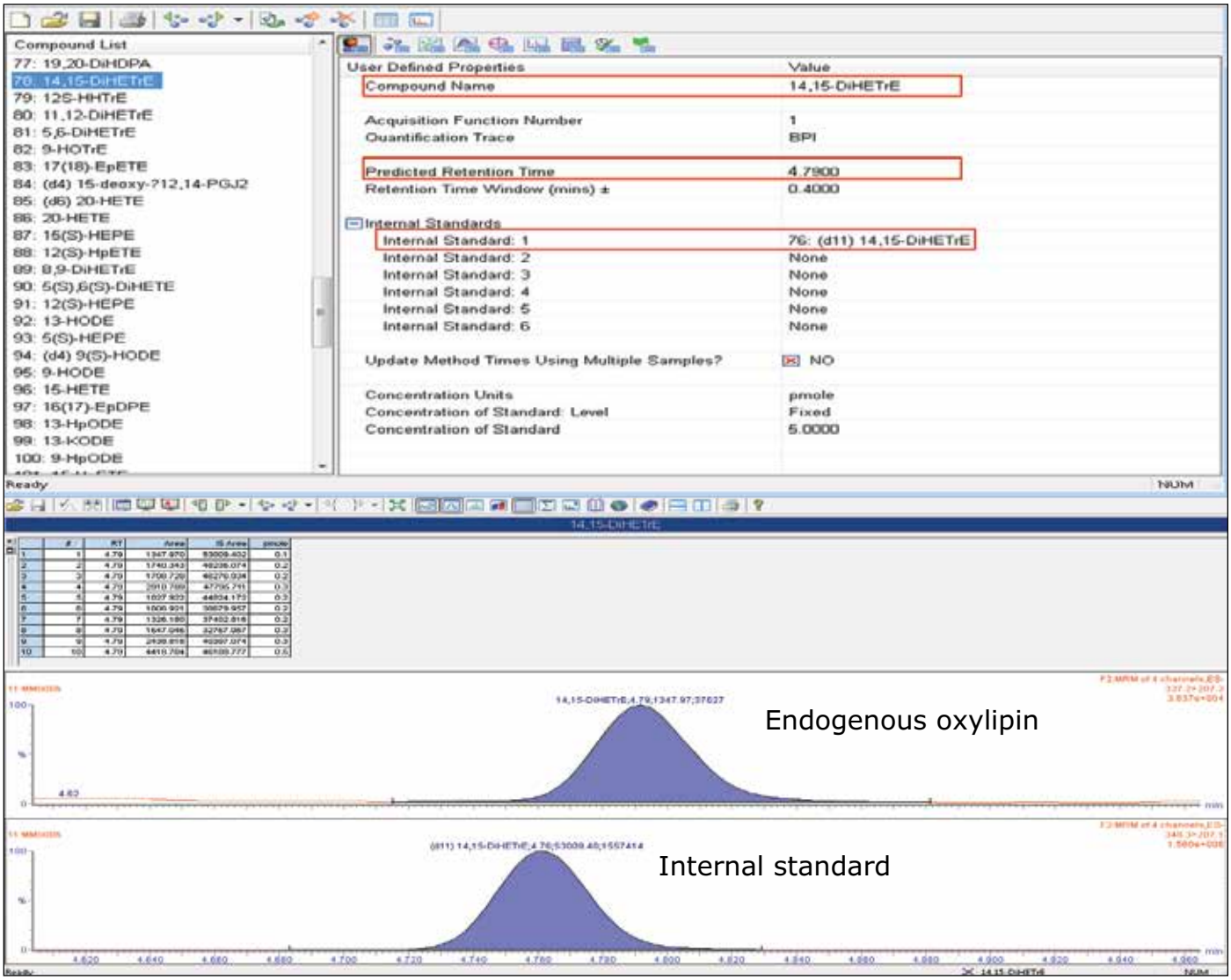



*Figure 5. An example of oxylipin quantification in plasma using TargetLynx, showing the use of a specified retention time, MRM transitions and internal standard for the identification and quantification of a selected oxylipin.*

## CONCLUSIONS

We have presented a routine high-throughput MRM method to profile over 100 oxylipins in plasma. These targets include a wide array of both pro- and anti-inflammatory lipid mediators. This SPE-UPLC-MRM assay could find applications in basic research to facilitate our understanding of the role of these lipid mediators in health and disease, nutritional research, clinical research, and drug discovery and development.

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# Profiling and Quantitation of Metabolomic "Signatures" for Breast Cancer Cell Progression

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## APPLICATION BENEFITS

- Quantify known small molecule biomarkers in targeted analysis with Xevo® TQ MS.
- Confirm known – and identify unknown – small molecule biomarkers in untargeted global profinlin analysis with SYNAPT® G2 HDMS.™
- Targeted and untargeted approaches are complementary to each other; new pathways can be discovered using the untargeted approach.

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## KEY WORDS

Breast cancer cell analysis, biomarker discovery, metabolomics, small molecule metabolites, glycolysis, TCA cycle, metabolism pathway, exact mass, $MS^E$

## INTRODUCTION

Breast cancer is one of the top five cancers that affect human lives seriously. Therefore, it is of great importance to discover the best ways to study this disease. Metabolic reprogramming is required both during the initial breast cancer transformation process (primary tumor) and during the acquisition of metastatic potential (metastases), shown in Figure 1. The reprogramming process includes altered flux through glycolysis and the pentose phosphate pathway (PPP), resulting in increased fatty acid synthesis needed for proliferation.

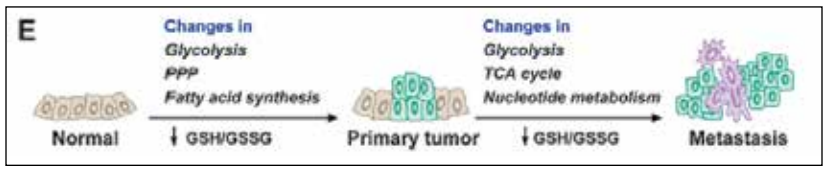



*Figure 1. A two-step metabolic progression hypothesis during mammary tumor progression.[1]*

Reactive oxygen species produced during tumor progression result in a decreased glutathione GSH (reduced)/GSSG (oxidized) redox pool, which impairs genome stability, tumor suppressor gene function, and control over cell proliferation. Continued GSH/GSSG depletion in the primary tumor may also contribute to general metastatic ability, and includes further changes in glycolysis and tricarboxylic acid cycle (TCA cycle) and increased nucleotide (PPP) flux for replication.

## EXPERIMENTAL

### Cell sample preparation

Two rodent breast cancer cell lines, MTln3 (highly metastatic) and MTC (poorly metastatic), were used and cultured in Eagle's minimal essential medium and supplemented with 5% fetal bovine serum (Invitrogen).

Cells were grown in 10-cm tissue culture dishes, and the media were replaced 24 h and 2 h prior to metabolite extraction. All samples were harvested at subconfluence.

Metabolism was quenched, and metabolites were extracted by aspiration of media and immediate addition of 4 mL of 80:20 methanol/water at 80 °C to simultaneously lyse cells and quench metabolism.

### LC conditions

| | |
|---|---|
| System: | Waters®<br>ACQUITY UPLC System |
| Column: | ACQUITY UPLC<br>HSS T3 Column<br>2.1 x 100 mm, 1.8 µm |
| Column temp.: | 40 °C |
| Flow rate: | 300 µL/min |
| Mobile phase A: | Water, 0.1% Formic acid |
| Mobile phase B: | Acetonitrile,<br>0.1% Formic acid |
| Injection vol.: | 10 µL |

Gradient:

| Time (min) | % A | % B | Curve |
|---|---|---|---|
| Initial | 99 | 1 | Initial |
| 8.0 | 50 | 50 | 6 |
| 8.1 | 1 | 99 | 6 |
| 11.0 | 1 | 99 | 6 |
| 11.1 | 99 | 1 | 6 |
| 15.0 | 99 | 1 | 6 |

Staging of the metabolic reprogramming using metabolomics could pinpoint the metabolic processes that are essential for breast cancer transformation and invasiveness, which may yield biomarkers and new directions for therapeutics. In this application note, we present a metabolomics study that combines targeted and untargeted approaches for breast cancer biomarkers analysis. Figure 2 illustrates the workflow for this study.

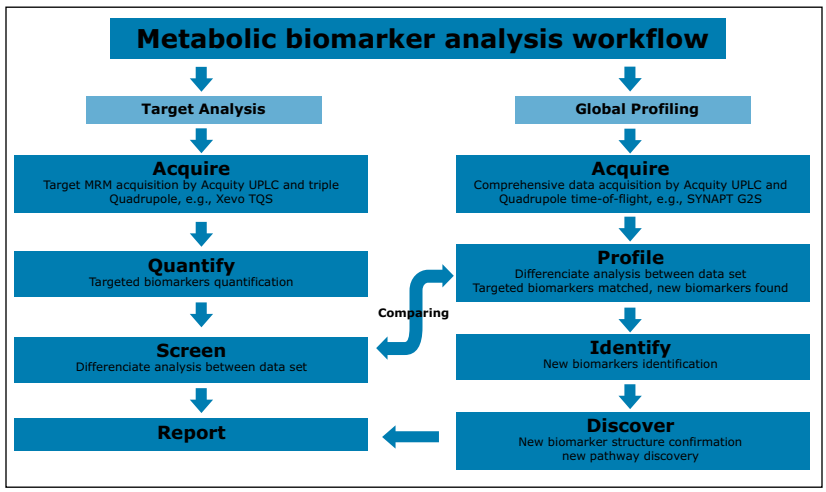



*Figure 2. Metabolomics biomarker analysis workflow.*

### Mass spectrometry

#### SYNAPT G2 HDMS for untargeted global analysis

The SYNAPT G2 HDMS was operated in both positive and negative $MS^E$ modes. The capillary voltage used was 2.0 kV with the source and desolvation temperatures set at 120 °C and 400 °C, respectively.

In the $MS^E$ acquisition mode, the instrument alternates between a low and high collision energy state on alternate scans. This allows for collection of precursor and fragment ion information of all species in an analysis without the sampling bias that is introduced with other common methods, such as DDA where a specific *m/z* must be isolated before fragmentation.

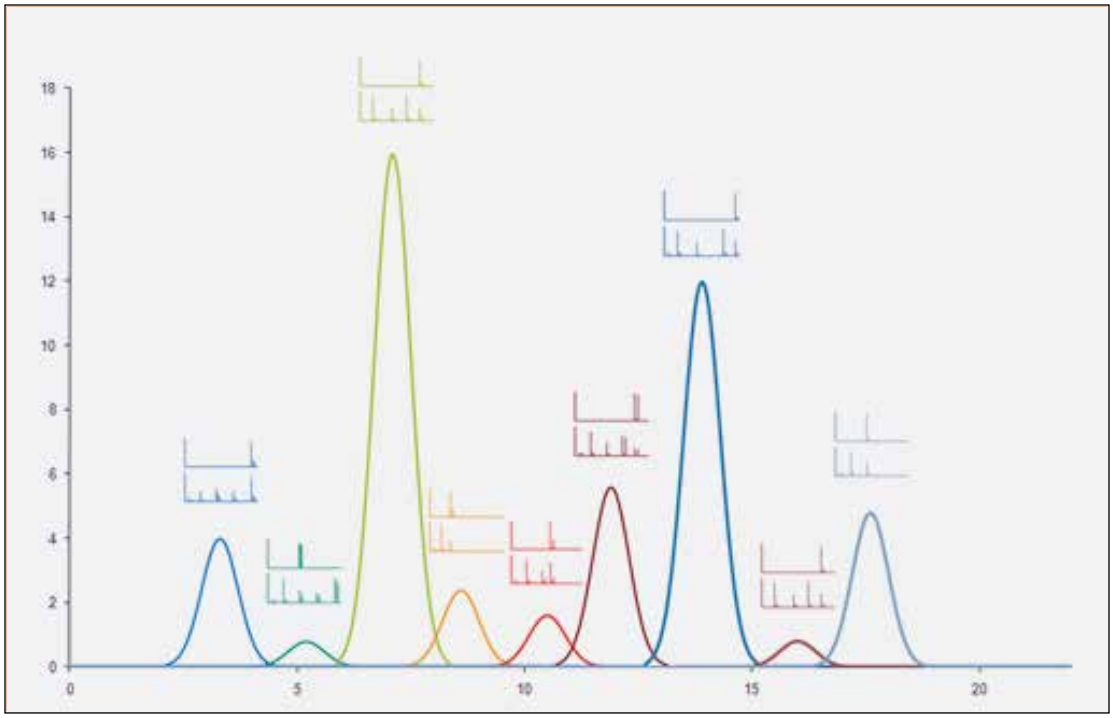



*Figure 3. MS$^E$ enables collection of comprehensive precursor and product ion information for virtually every component of a mixture.*

**Xevo TQ MS for targeted analysis**

The Xevo TQ MS was operated in both positive and negative MRM modes. The capillary voltage used was 2.0 kV with the source and desolvation temperatures set at 150 °C and 650 °C, respectively. The desolvation gas flow was set at 1200 L/hr and the collision gas (argon) flow 0.18 mL/min (4 x $10^{-3}$ mBar), with MS1/MS2 resolution at unit mass.

## RESULTS AND DISCUSSION

Targeted analysis was used to survey known metabolic pathways that are key to cancer aggressiveness, as outlined in Figure 1. Figure 4 shows many of the targeted metabolite markers that are elevated in the MTln3 cells from both the heat map (top) and relative change scale bar plot (bottom). Analysis of experimental data supports a Warburg effect cancer model.[2] For highly aggressive MTln3 cells, high cytosolic NADH is indicated by a glycolytic/TCA cycle signature of an increased malate/aspartate shuttle, shown in Figure 6. High AMP levels for MTln3 cells suggest the elevated malate/aspartate shuttle cannot keep up with cellular energy needs. High levels of amino acids seen in MTln3 cells are necessary for cell growth.

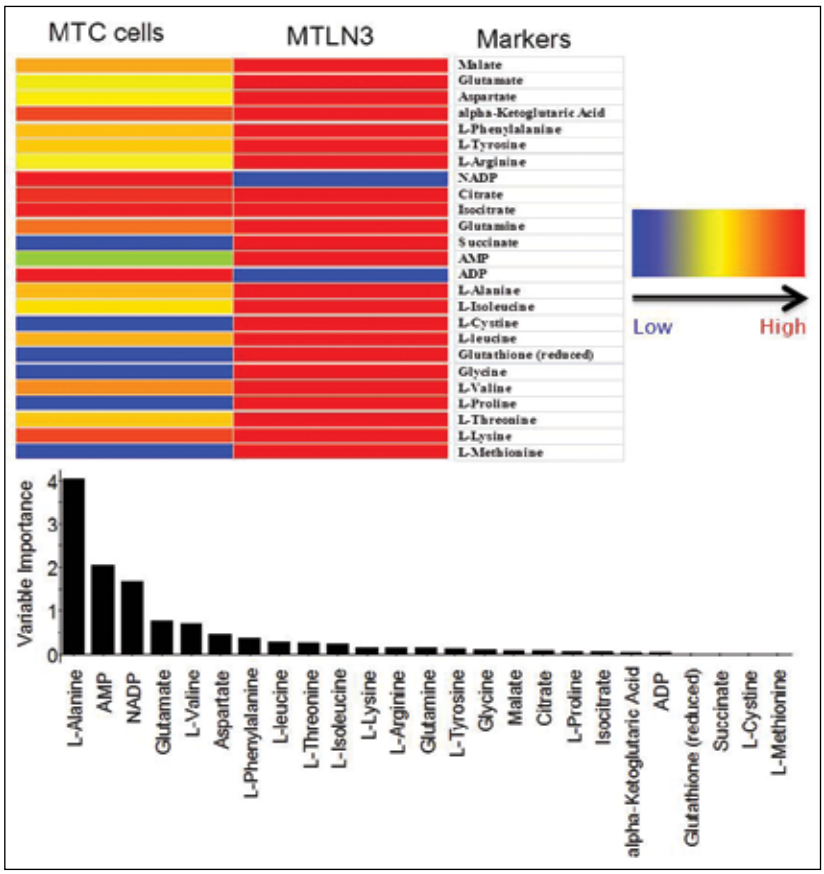



*Figure 4. Both the heat map (top) and the relative change scale bar plot (bottom) markers indicate the elevation of the metabolites in the MTLn3 cells.*

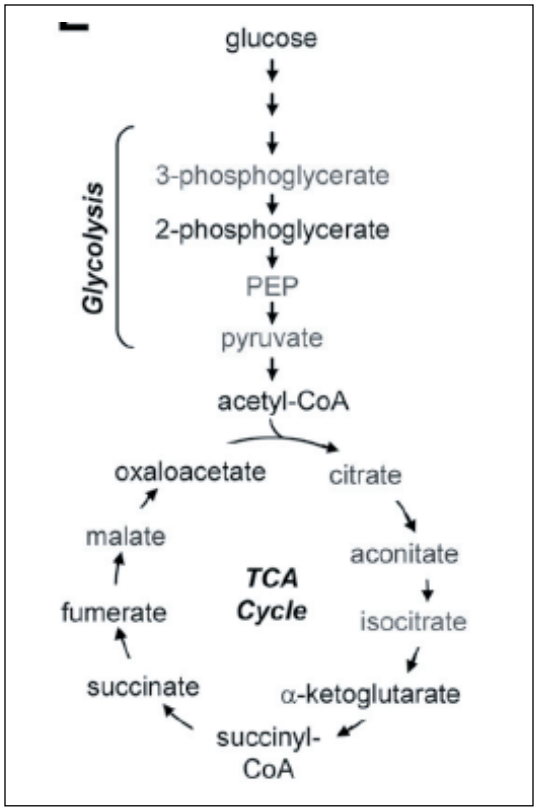



*Figure 5. Glycolysis and TCA Cycle.*

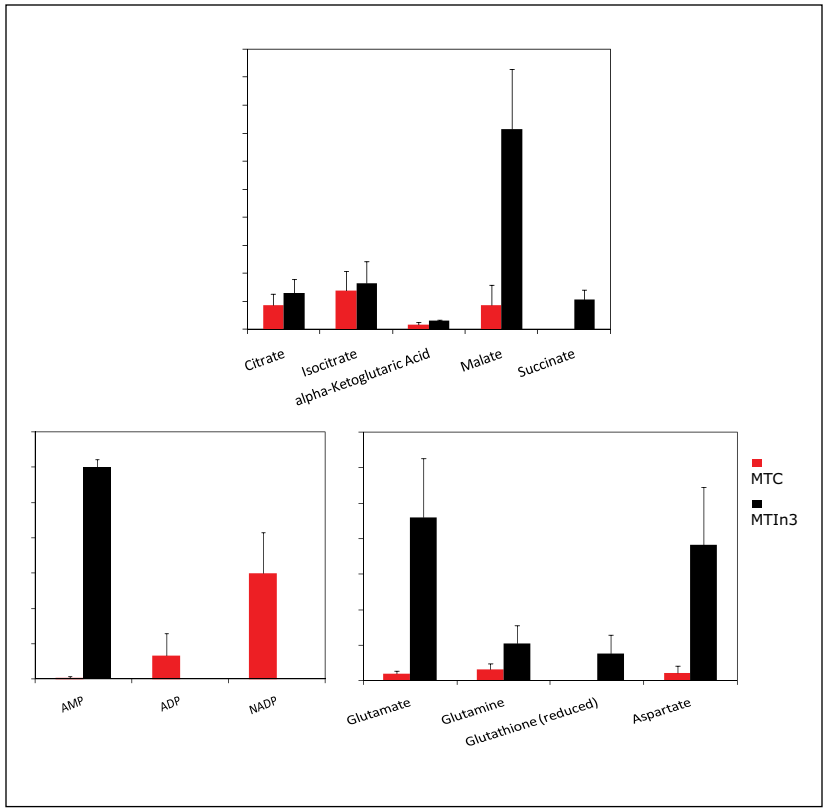



*Figure 6. Targeted analysis quantitative bar charts for selected Glycolysis and TCA Cycle metabolites.*

As shown in Figure 6, adenosine monophosphate (AMP) is extremely high in concentration. This indicates that the highly aggressive MTln3 cells require a large amount of energy. Even with a high malate/aspartate shuttle, adenosine triphosphate (ATP) production cannot keep pace.

AMP functions as an energy sensor and regulator of metabolism. When ATP production does not keep up with needs, a higher portion of a cell's adenine nucleotide pool is made available in the form of AMP. AMP then stimulates metabolic pathways that produce ATP in the MTln3 cells.

The "signature" of high levels of malate, glutamate, aspartate, and alpha ketoglutarate (as high as 10 fold) means high cytosolic NADH must use these carriers for transport into the mitochondria to turn into ATP. Highly aggressive cancer cells, such as MTln3, have high glycolysis and need the malate/aspartate/glutamate/ alpha-ketogutarate shuttle system to satisfy the ATP needs. This shuttle cannot work fast enough because AMP is still very high.

For untargeted global profiling analysis, all the samples were run in triplicate. A QC sample was made by mixing equal volumes of each sample. 10 QC samples were injected prior to the first sample in the experiment. A QC sample was also injected every 10 sample injections.

For the data analysis, MarkerLynx XS Application Manager[3] was used to integrate and align chemical and biological MS data points and convert them into Exact Mass Retention Time (EMRT) pairs. Those EMRT pairs can then be used for multivariate statistical analysis, such as principle component analysis (PCA-X), partial least-squares to latent structures data analysis (PLS-DA), and orthogonal PLS data analysis (OPLS-DA) to visualize and interpret the information-rich and complex MS data, as shown in both Figures 8 and 9.

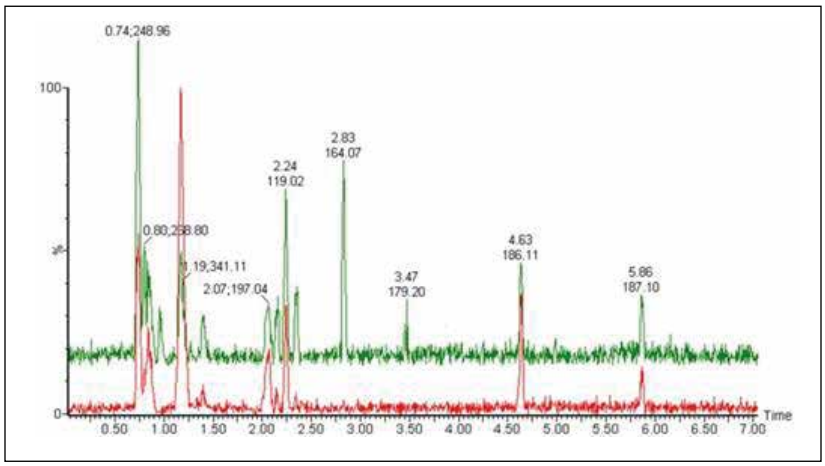



*Figure 7. A comparison of two typical chromatograms from the MTC and MTln3 cells. The figure clearly shows that there is difference between these two samples.*

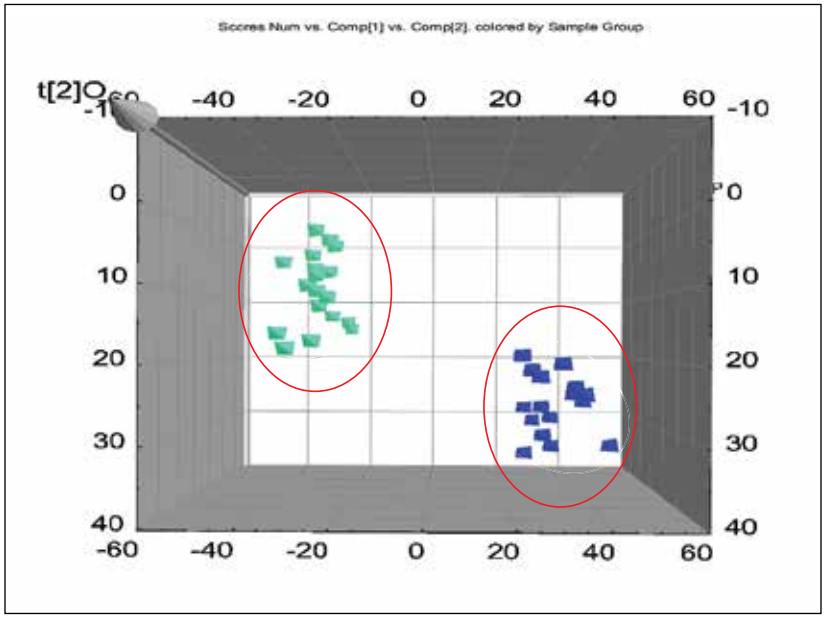
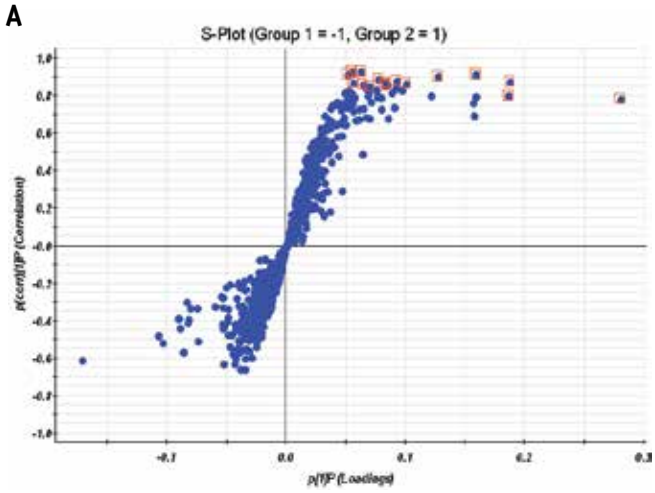



*Figure 8. The difference between the two sets of sample cells further demonstrated in the 3D score plot by multivariate statistical analysis using MarkerLynx XS Application Manager.*

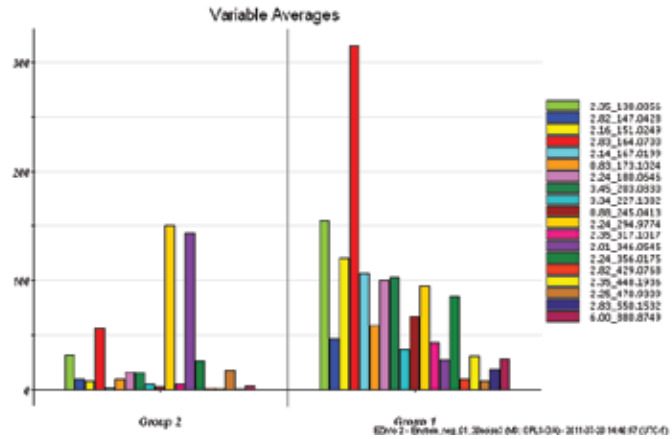
A




B

| Primary ID | Retention Time | Mass | p[1]P | p(corr)[1]P | Factor of Change | Group 2 | Group 1 |
|---|---|---|---|---|---|---|---|
| 2.35_130.0856 | 2.35 | 130.0856 | -0.243075 | -0.909009 | 4.8 | 31.7854 | 154.042 |
| 2.82_147.0428 | 2.82 | 147.0428 | -0.135959 | -0.936749 | 5.0 | 9.43344 | 47.0847 |
| 2.16_151.0249 | 2.16 | 151.0249 | -0.230581 | -0.896521 | 14.9 | 8.10109 | 120.593 |
| 2.83_164.0700 | 2.83 | 164.07 | -0.348204 | -0.876591 | 5.6 | 55.8486 | 314.817 |
| 2.14_167.0199 | 2.14 | 167.0199 | -0.222401 | -0.936049 | 42.9 | 2.47802 | 106.219 |
| 0.83_173.1024 | 0.83 | 173.1024 | -0.156022 | -0.965926 | 6.3 | 9.25443 | 58.1429 |
| 2.24_180.0646 | 2.24 | 180.0646 | -0.190133 | -0.856732 | 6.3 | 15.8021 | 99.4386 |
| 3.45_203.0830 | 3.45 | 203.083 | -0.184289 | -0.810462 | 6.7 | 15.3536 | 103.003 |
| 3.34_227.1382 | 3.34 | 227.1382 | -0.121099 | -0.929172 | 6.5 | 5.61931 | 36.7191 |
| 0.88_245.0413 | 0.88 | 245.0413 | -0.173638 | -0.906563 | 23.9 | 2.79646 | 66.908 |
| 2.24_294.9774 | 2.24 | 294.9774 | 0.131324 | 0.591855 | 1.6 | 149.841 | 94.4725 |
| 2.35_317.1017 | 2.35 | 317.1017 | -0.133452 | -0.933264 | 7.9 | 5.4566 | 43.0295 |
| 2.01_346.0545 | 2.01 | 346.0545 | 0.187943 | 0.595604 | 5.4 | 142.677 | 26.5841 |
| 2.24_356.0175 | 2.24 | 356.0175 | -0.163462 | -0.906209 | 3.3 | 26.0152 | 84.934 |
| 2.82_429.0768 | 2.82 | 429.0768 | -0.0618265 | -0.889218 | 9.2 | 1.05934 | 9.70496 |
| 2.35_448.1936 | 2.35 | 448.1936 | -0.112912 | -0.858168 | 20.1 | 1.56151 | 31.3196 |
| 2.25_470.9309 | 2.25 | 470.9309 | 0.0546198 | 0.634535 | 2.2 | 17.7269 | 8.16951 |
| 2.83_550.1532 | 2.83 | 550.1532 | -0.087934 | -0.865465 | 38.9 | 0.478954 | 18.6351 |
| 6.00_880.8749 | 6 | 880.8749 | -0.105049 | -0.87448 | 7.1 | 3.88319 | 27.4549 |

C




D

| | ID | Ret. Time | m/z |
|---|---|---|---|
| 1 | (1) 2-oxo-5-aminovalerate | 2.3509 | 130.0851 |
| 2 | (1) 2-dehydropantoate | 0.8430 | 145.0606 |
| 3 | | 2.1538 | 151.0253 |
| 4 | (1) 1-methylguanine | 2.8270 | 164.0701 |
| 5 | (1) 4-Hydroxymandelate | 2.1418 | 167.0200 |
| 6 | (1) cis-Aconitate | 0.8282 | 173.1028 |
| 7 | | 2.2371 | 180.0647 |
| 8 | (1) 1,4-dihydroxy-2-naphoate | 3.4520 | 203.0831 |
| 9 | | 0.8751 | 245.0414 |
| 10 | | 3.4522 | 261.0164 |
| 11 | | 2.3533 | 275.9776 |
| 12 | | 2.3510 | 317.1018 |
| 13 | Nicotinate-D-ribonucleotide | 2.8257 | 334.9577 |
| 14 | | 2.8297 | 335.9543 |
| 15 | Adenosine monophosphate (AMP) | 2.0107 | 346.0547 |
| 16 | | 2.2388 | 354.0155 |
| 17 | | 2.2408 | 356.0187 |
| 18 | (1) 11beta,21-Dihydroxy-3,20-oxo-5beta-pregna... | 2.3521 | 361.1035 |
| 19 | Xanthosine monophosphate (XMP) | 2.3507 | 363.1059 |
| 20 | (1) Carbobenzyloxy-L-phenylalanyl-L-serine | 2.8252 | 385.0694 |
| 21 | | 2.2397 | 537.0873 |
| 22 | | 2.8260 | 550.1470 |

*Figure 9. The selected markers from the S-Plot (A) can be transformed into table (B) and bar chart (C) to illustrate their contribution to the differences between the two sample cell lines. The markers can also be used for database searching for identification through in-house or online databases, such as ChemSpider. A couple of new markers, 2-dyhydropantoate and 4-hydroxymandelate were also found from the database. They are indications of increased methyl transferase activity, key to function of biosynthetic pathways. New markers (D) were found from database searching by untargeted analysis.*

The markers from MarkerLynx statistical analysis were validated, in part, by identifying hits in pathways complementary to those found in targeted analysis, and builds belief in new untargeted hits found. For example, untargeted analysis, shown in Figures 4 and 6, indicated high AMP and phosphoenelpyruvate (PEP) along with high cis-aconitate in aggressive cells. AMP was identified from targeted analysis; cis-aconitate supports targeted analysis finding for increased flux into the TCA cycle, and PEP for increased glycolysis. Markers/carriers (malate/aspartate shuttle) for high cytosolic NADH from targeted analysis are complementary to untargeted findings of high nicotinamide-D-ribonucleotide, a step in NAD synthesis degradation product of amino acids found to be elevated by targeted analysis. Targeted analysis found high levels of aspartate, isoleucine, tyrosine, arginine, and others. Untargeted analysis showed markers for amino acid degradation with high 2-oxo-5-aminovalerate, a breakdown product of arginine; 1,4 dihydrooxy-2-naphoate, a breakdown product of tyrosine; alpha-hydroxyisovalerate, a marker for branched chain amino acid (isoleucine) breakdown; and homoserine, a breakdown product of aspartate.

Among the new markers found from database searching by untargeted analysis are 2-dehydropantoate and 4-hydroxymandelate, as shown in Figure 9D. They are indications of increased methyl transferase activity, which is key to function of biosynthetic pathways. Our results show that any of these pathways appears to be upregulated in the MTln3 cells.

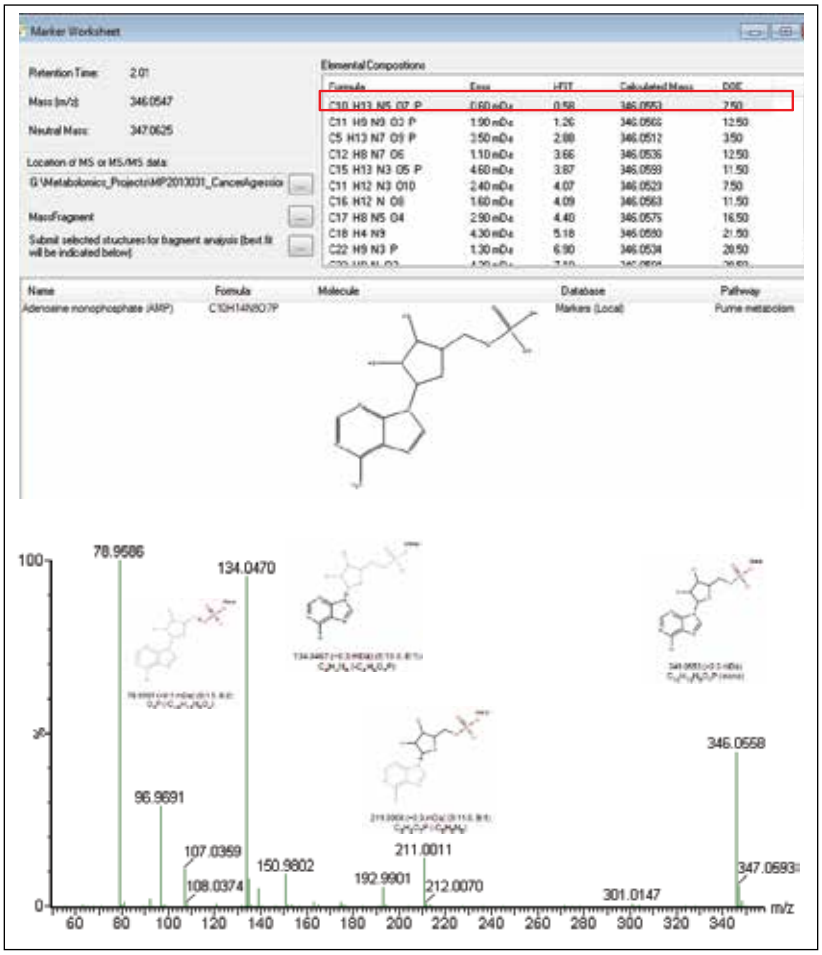



*Figure 10. The Chemspider database searching result for AMP indicates a positive identification of the compound (top). The MS/MS spectrum for AMP from the MS$^E$ data acquisition confirms the structure of AMP (bottom). Fragmentation structures were matched using the MassFragment Software.*

## CONCLUSIONS

We have successfully demonstrated a metabolomics study workflow that combines targeted and untargeted approaches for breast cancer biomarker analysis.

Aggressive cell MTLn3 and non-aggressive cell MTC show dramatically different concentrations of the biomarkers, such as malate and AMP in glycolysis and TCA cycle, which indicates glycolysis is higher in MTln3 cells.

Known markers of cancer aggressiveness can be analyzed by a targeted approach using Xevo TQ or Xevo TQ-S.

Hits are validated by identifying hits in pathways complementary to those found in targeted analysis using SYNAPT G2; this builds belief in new untargeted hits identified/discovered.

New markers and thereby new pathways can be discovered by untargeted SYNAPT G2 analysis. One example would be 2-dehydropantoate and 4-hydroxymandelate, which are markers for increased methyl transferase activity. Methyl transferase activity is key to the function of biosynthetic pathways.

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# A Definitive Lipidomics Workflow for Human Plasma Utilizing Off-line Enrichment and Class Specific Separation of Phospholipids

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[1]Waters Pacific, Singapore
[2]Centre for Life Sciences, National University of Singapore, Singapore

## APPLICATION BENEFITS

Large-scale quantitative and comparative lipidomic studies require the use of simple and high throughput workflows. To answer this need, a tandem quadrupole-based workflow has been developed. The benefits of this targeted lipidomics workflow include the following:

- Simplified extraction of lipids using Ostro™ sample preparation chemistry
- 96-well plate format allows for high throughput extraction with automation technologies
- BEH HILIC chemistry provides well-defined, predictable class-based separation of lipids
- ACQUITY UPLC® System allows shorter run times per sample
- Xevo® TQ-S MS offers highly sensitive detection and quantification of lipids across a large dynamic range
- TargetLynx™ processing method provides rapid, automated, and quantitative data analysis of all the lipids of interest in a large sample batch

## WATERS SOLUTIONS

Ostro Sample Preparation
Xevo TQ-S Mass Spectrometer
ACQUITY UPLC System
ACQUITY UPLC BEH HILIC Columns
TargetLynx Application Manager

## KEY WORDS

Plasma, phospholipid extraction, Ostro, HILIC, TQ-S, TargetLynx

## INTRODUCTION

Lipids play many important roles in maintaining homeostasis of living organisms. Lipidomics analyses could further our understanding of mechanisms of disease, including the identification of biomarkers and potential drug targets.

Biofluids such as plasma are typically complex, with large lipid diversity across many orders of concentration. These, together with the chemical complexity of lipids, present demanding analytical challenges ranging from the sample preparation stage to the analytical techniques used to identify and quantify key lipids. Today, many variations of the Bligh and Dyer method are used for total lipid extraction and purification, with equal amounts used for mass spectrometric analysis.

Recent advances in lipidomics have made use of developments in chemistries and instrumentation, most notably the use of off-line enrichment or solid-phase sample preparation products[1,2] and the coupling of UltraPerformance LC® with mass spectrometry. However, there is little standardization across platforms and workflows for a complete analysis.

Presented here is a tandem quadrupole-based phospholipid analysis workflow from extraction to separation, identification and quantification of the phospholipids from a single vendor. These commercially available products packaged as a complete solution are provided to ease the strains and increase the productivity of laboratories undertaking longitudinal studies spanning hundreds of lipids over thousands of samples.

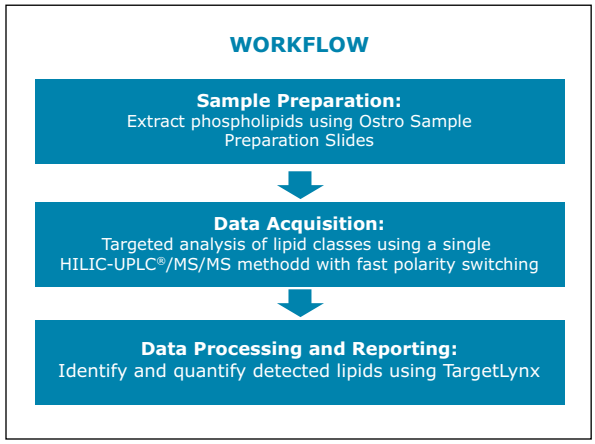


*Figure 1. Workflow for the targeted analysis of phospholipids in human plasma.*

## EXPERIMENTAL

### Method conditions

### LC conditions

| | |
|---|---|
| System: | ACQUITY UPLC |
| Column: | ACQUITY UPLC BEH HILIC 2.1 x 100 mm, 1.7 µm |
| Column temp.: | 30 °C |
| Mobile phase A: | 95:5 acetonitrile/water with 10 mM ammonium acetate, pH 8.0 |
| Mobile phase B: | 50:50 acetonitrile/water with 10 mM ammonium acetate, pH 8.0 |
| Gradient: | 0% to 20% B for 10 min |
| Flow rate: | 500 µL/min |
| Injection volume: | 3.0 µL, partial loop |

### MS conditions

| | |
|---|---|
| Mass spectrometer: | Xevo TQ-S |
| Ionization mode: | ESI, +/- switching |
| Capillary voltage: | 3.8 kV (+) / 1.9 kV (-) |
| Desolvation temp.: | 450 °C |
| Desolvation gas: | 1000 L/h |
| Source temp.: | 150 °C |
| Collision cell pressure: | 3.6 x $10^{-3}$ mBar |

### Sample description

Human plasma samples were obtained from the Centre for Life Sciences, National University of Singapore. The protocol described here follows a recently published application note.[3]

100 µL of human plasma was loaded into each well of a Waters® Ostro Sample Preparation Plate fitted onto a vacuum manifold. 800 µL of ethanol was added to each well and mixed thoroughly by aspirating the mixture 10x using a micropipette. A vacuum of approximately 15" Hg was applied to the plate until the solvent was completely drained. This step was repeated with another 800 µL of ethanol with the total fraction collected labelled as the "flow through."

800 µL of elution solvent (4.5:4.5:1.0 chloroform/methanol/triethylamine) was added to each well, and the fraction was collected under 15" Hg vacuum as the "eluate." This step was repeated, bringing the total fraction volume to approximately 1600 µL.

Both the eluate and flow through fractions were dried down under nitrogen, and reconstituted with 200 µL 1:1 (v/v) chloroform/methanol. 1 µL of the eluate fraction was combined with 99 µL of the flow through fraction to give a 1:100 dilution. This combined sample was then injected into the UPLC/MS system.

| Lipid class | Polarity | MRM time window (min) | No. of species detected | Cone voltage (V) | Collision energy (V) |
|---|---|---|---|---|---|
| Monohexylceramide (MonoHexCer) | + | 0 to 2 | 16 | 20 | 30 |
| Phosphatidylglycerol (PG) | - | 1 to 3 | 19 | 55 | 45 |
| Dihexylceramide (DiHexCer) | + | 2 to 4 | 16 | 20 | 30 |
| Phosphatidylinositol (PI) | - | 3 to 5 | 26 | 48 | 30 |
| Phosphatidylethanolamine (PE) | - | 4 to 6 | 33 | 48 | 40 |
| Phosphatidylcholine (PC) | + | 5 to 7 | 47 | 36 | 30 |
| Lyso-Phosphatidylinositol (LPI) | - | 5 to 7 | 11 | 48 | 30 |
| Lyso-Phosphatidylethanolamine (LPE) | - | 6 to 8 | 11 | 36 | 24 |
| Sphingomyelin (SM) | + | 7 to 9 | 18 | 36 | 24 |
| Lyso-Phosphatidylcholine (LPC) | + | 8 to 10 | 11 | 42 | 26 |

*Table 1. Xevo TQ-S MRM method.*

## Sample description

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| | Manual | Automation |
|---|---|---|
| **Lipid Class** | Overall %CV | Overall %CV |
| GluCeramides | 13.2 | 9.7 |
| Phosphatidylglycerol (PG) | 10.2 | 7.2 |
| Phosphatidylinositol (PI) | 10.2 | 5.8 |
| Phosphatidylethanolamine (PE) | 16.0 | 6.4 |
| Phosphatidylcholine (PC) | 9.9 | 7.5 |
| Lyso-Phosphatidylinositol (LPI) | 23.0 | 13.0 |
| Lyso-Phosphatidylethanolamine (LPE) | 16.0 | 6.4 |
| Sphingomyelin (SM) | 11.9 | 9.1 |
| Lyso-Phosphatidylcholine (LPC) | 14.4 | 8.4 |

*Table 2. Comparison of well-to-well reproducibility (%CV) of the Ostro plate for manual versus automated sample handling.*

Using an automated liquid handler to process the plasma samples, as shown in Figure 2, the well-to-well reproducibility (%CV) improved compared to manually performing the extraction. This was true for all classes of lipids analyzed, with improvements ranging from 25% (SM) to 60% (PE).

### MRM method setup on Xevo TQ-S

In reversed-phase chromatography of lipids, separation is governed by lipophilicity, alkyl chain length, and degree of saturation for each individual lipid. These result in broad MRM acquisition time windows[4] that, in turn, negatively affect the instrument's duty cycle, thus hindering accurate quantification.

In the HILIC-UPLC/MS method used in this application note, there was a clear and reproducible separation of the various classes of lipids. This was observed very clearly by the difference in retention times of PCs and SMs, which are normally difficult to identify and quantify using reversed-phase methods.[5, 6]

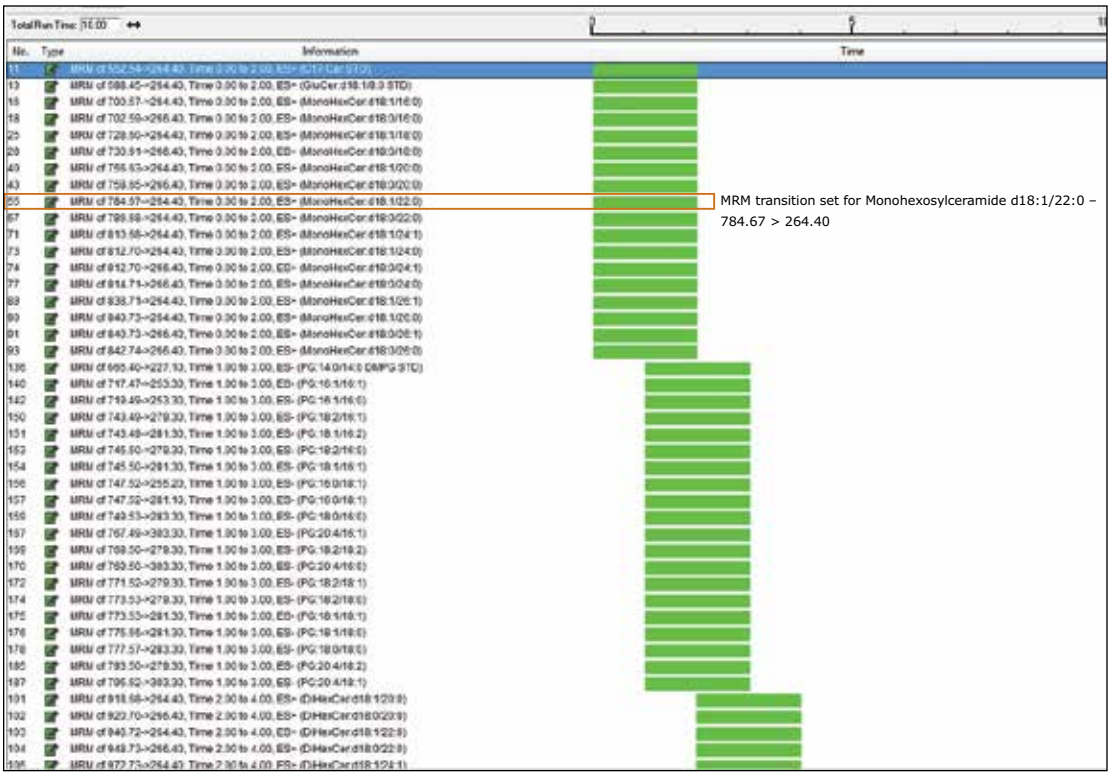


*Figure 2. Typical screen shot of the Xevo TQ-S MRM method editor.*

By leveraging the reproducible retention times of the lipid classes, MRM acquisition time windows were reduced to two minutes per class, as shown in Table 1. This allowed for the creation of a single MS method to analyze the combined "flow through" and "eluate" fractions in a single run. A total of 215 MRM transitions (+/- polarity) including internal standards were created in this method. Figure 3 shows each lipid transition set up as a single function, for example, monohexosylceramide d18:1/22:0, which will limit the addition or subtraction of lipids to only those of interest to the operator.

## Data processing and reporting

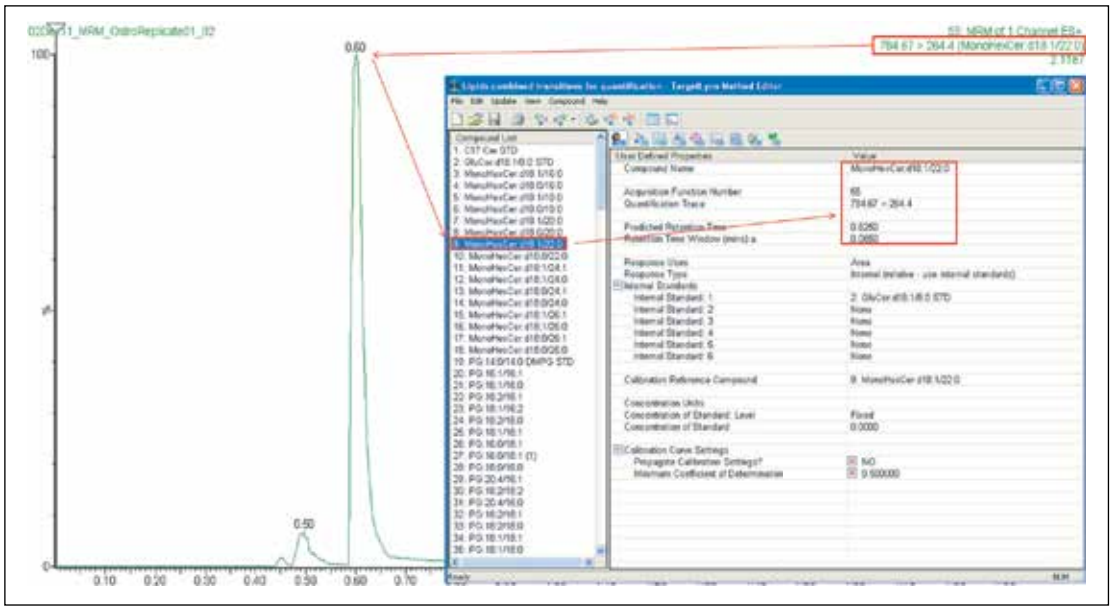

*Figure 3. Typical lipid MRM trace using monohexosylceramide d18:1/22:0 as an example.*

A complementary data processing method was created using the TargetLynx Application Manager, as shown in Figure 4. The insert shows how easily peak information can be "dragged and dropped" into the data processing method.

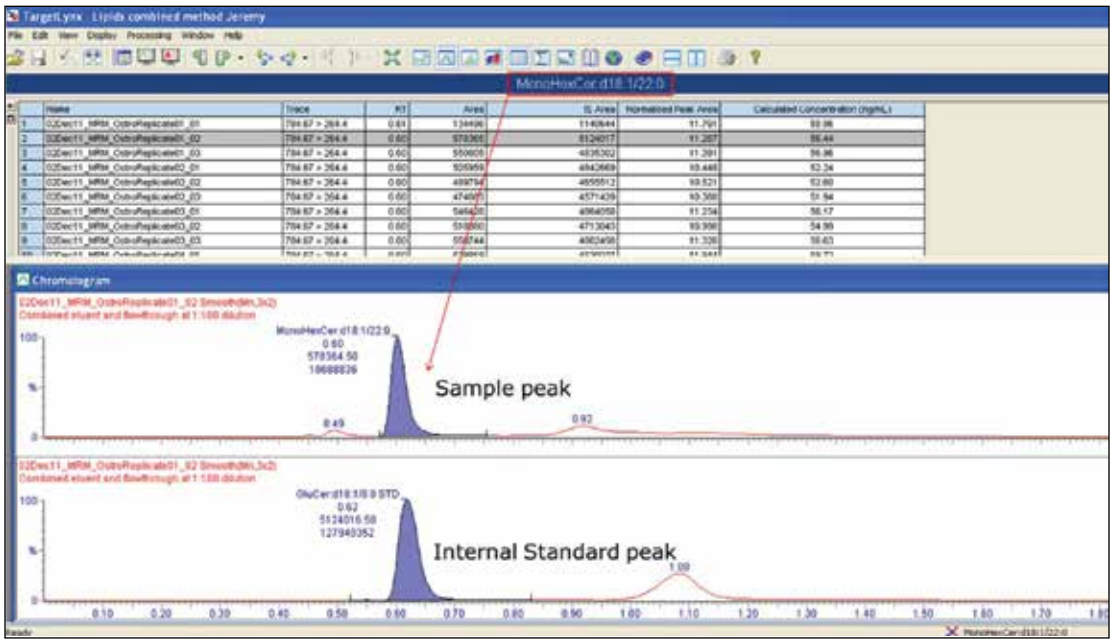


*Figure 4. Quantification of monohexosylceramide d18:1/22:0 MRM trace using TargetLynx. Chromatographic peaks are automatically detected and quantified against a standard for a series of samples.*

Once the processing method had been set with the appropriate transitions and retention times for each lipid, batch processing for any number of samples run under the same conditions described above can be performed. Figure 4 shows a typical TargetLynx results view. Using monohexosylceramide d18:1/22:0 as an example, the application manager automatically integrates both the sample peak and the pre-defined internal standard (IS) peak and calculates the concentration of the lipid in the sample against the known spiked concentration of the IS. A user-defined report can then be printed, or these results can be exported into a number of popular generic formats for further statistical analysis.

## CONCLUSIONS

Consistent, reliable, and rapid identification and quantification of hundreds of lipid species can now be performed in a single run by the application of this workflow. The high throughput nature of the workflow utilizing automation technologies and automated data processing and reporting means that large-scale comparative lipidomic studies can be routinely used by laboratories around the world. In addition, the consistency obtained from this standardized platform means that data can be shared and compared across various sites, thereby enabling a greater understanding of the global lipidome and its associations with diseases.

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# Rapid and Simultaneous Analysis of Plasma Catecholamines and Metanephrines Using Mixed-Mode SPE and Hydrophilic Interaction Chromatography (HILIC) for Clinical Research

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## APPLICATION BENEFITS

- Retention and baseline resolution of monoamine neurotransmitters and metanephrines without the need for ion-pairing reagents
- Rapid, simultaneous quantification of plasma metanephrines and catecholamines
- 5x analyte concentration without the need for evaporation and reconstitution
- Linear, accurate, and precise results down to 10 pg/mL

## WATERS SOLUTIONS

Oasis® WCX 96-well µElution Plate

96-well Sample Collection Plate

ACQUITY UPLC® BEH Amide Column

ACQUITY UPLC System

Xevo® TQ-S Mass Spectrometer

UNIFI® Scientific Information System

## KEY WORDS

Catecholamines, metanephrines, HILIC, SPE, LC-MS/MS, sample preparation

## INTRODUCTION

Clinical researchers are often interested in measuring elevated concentrations of plasma catecholamines and their O-methylated metabolites (metanephrines). However, these compounds (in particular, norepinephrine, epinephrine, and dopamine) can be a challenge to analyze via reversed-phase LC-MS/MS due to their polarity. As a result, many research laboratories still analyze this panel using ion-pairing reagents and electrochemical detection (ECD). While reversed-phase LC-MS/MS has been used successfully, challenges still exist due to ion-suppression from matrix components, insufficient retention, and inadequate separation of normetanephrine and epinephrine.

Hydrophilic interaction chromatography (HILIC) is increasingly becoming a method of choice for the analysis of polar compounds.[1-6] Expanding upon earlier published methods,[6-7] this application note describes the extraction and analysis of monoamine neurotransmitters and metanephrines from plasma. HILIC-based chromatographic separation is achieved using a Waters® ACQUITY UPLC BEH Amide Column. Waters Oasis WCX 96-well µElution Plates are used to extract these compounds from plasma. The use of mixed-mode weak cation exchange solid-phase extraction (SPE) plates, in combination with the amide column for HILIC chromatography and the Waters Xevo TQ-S mass spectrometer, result in a rapid, robust method with excellent linearity, accuracy and precision, as well as minimal matrix effects.

## EXPERIMENTAL

### LC conditions

| | |
|---|---|
| LC system: | ACQUITY UPLC |
| Column: | ACQUITY UPLC BEH Amide, 1.7 µm, 2.1 x 100 mm |
| Column temp.: | 30 °C |
| Sample temp.: | 10 °C |
| Mobile phase A (MPA): | 95:5 Water:ACN containing 30 mM $NH_4HCOO$, pH 3.0 |
| Mobile phase B (MPB): | 15:85 Water:ACN containing 30 mM $NH_4HCOO$, pH 3.0 |
| Needle washes: | Strong and weak needle washes were both placed in MPB |

The gradient ramp is shown in Table 1 and includes an initial hold, followed by a shallow ramp and an increase in flow rate to re-equilibrate the column. The entire cycle time is 4.0 min.

### MS conditions

| | |
|---|---|
| MS system: | Xevo TQ-S |
| Ionization mode: | ESI positive |
| Capillary voltage: | 0.5 kV |
| Cone voltage: | Compound specific (see Table 2) |
| Desolvation gas: | 900 L/hr |
| Cone gas: | 150 L/hr |
| Desolvation temp.: | 550 °C |
| Source temp.: | 150 °C |

Data were acquired and analyzed using UNIFI Software.

| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| 0 | 0.6 | 0.0 | 100.0 |
| 1.0 | 0.6 | 0.0 | 100.0 |
| 2.0 | 0.6 | 10.0 | 90.0 |
| 2.1 | 1.0 | 10.0 | 90.0 |
| 2.5 | 1.0 | 30.0 | 70.0 |
| 2.6 | 1.0 | 0.0 | 100.0 |
| 3.9 | 1.0 | 0.0 | 100.0 |
| 4.0 | 0.6 | 0.0 | 100.0 |

*Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the methods section.*

Combined stock standards containing 10-µg/mL of dopamine (DA), 3-methoxytyramine, (3-MT) metanephrine (MTN), and normetanephrine (NMT) and 50-µg/mL of norepenephrine (NE) and epinephrine (EP) were prepared in methanol containing 0.1% ascorbic acid to prevent oxidation. A combined internal standard stock solution composed of 10-µg/mL D3-metanephrine, D3-normetanephrine, D4-dopamine, D6-epinephrine, and D6-norepinephrine, was also prepared in methanol containing 0.1% ascorbic acid. Working internal standard solutions were prepared daily in 5% MeOH with 0.1% formic acid at a concentration of 2.5 ng/mL.

Human plasma (sodium heparin) was obtained from Biological Specialty Corporation (Colmar, PA). Pooled plasma (6 lots) was used to prepare calibration and quality control samples.

### Sample preparation

Pooled plasma samples (250 µL) were pre-treated with 250-µL of 50-mM $NH_4CH_3COO$ and 50-µL of an internal standard working solution (2.5 ng/mL). Pre-treated samples were loaded in individual wells of an Oasis WCX 96-well µElution Plate that had been conditioned with 200-µL of MeOH and 200-µL of $H_2O$. After loading the samples, wells were washed with 200-µL of 20-mM $NH_4CH_3COO$ followed by 200-µL of 50:50 ACN:IPA. The 96-well plate was then dried under vacuum for 30 s to remove as much solvent as possible from the sorbent bed. The target compounds were eluted from the plate with 2 x 25-µL aliquots of 85:15 ACN:$H_2O$ containing 2% formic acid into an 700-µL 96-well sample collection plate (p/n 186005837). 15-µL of the eluate was injected onto the UPLC®-MS/MS System.

## RESULTS AND DISCUSSION

The structures of all compounds are shown in Figure 1 along with their individual logP values, demonstrating the highly polar nature of many of these compounds. Table 2 shows the retention times and individualized MS parameters of each compound, including MRM transitions, cone voltage, and collision energy.

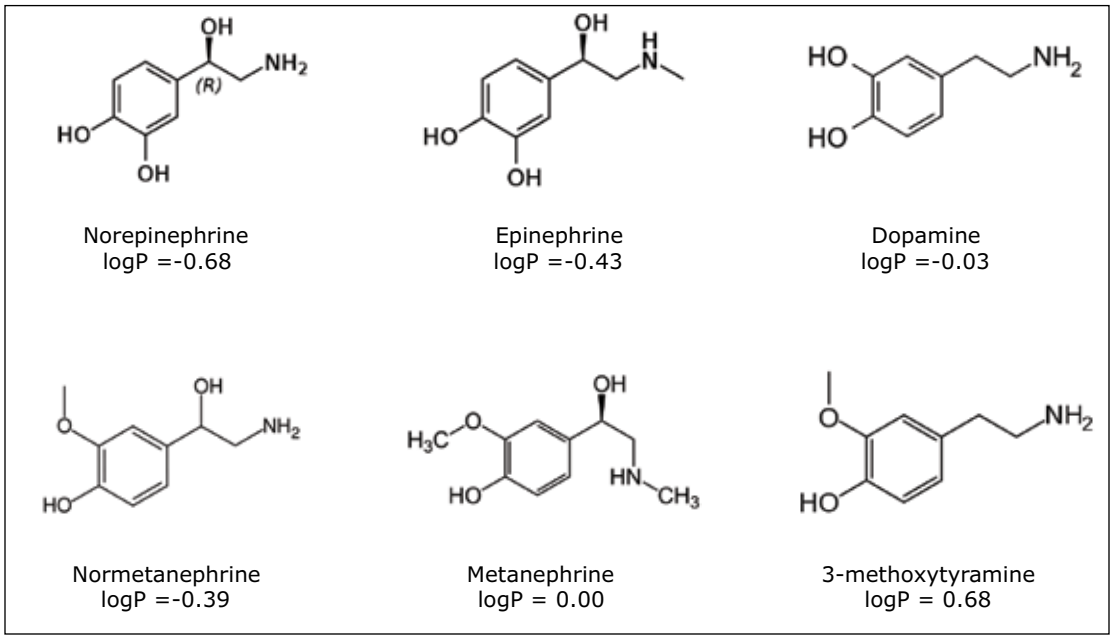


*Figure 1. Names, molecular structures, and logP values of catecholamines and metanephrines.*

| Analyte | RT (min) | MRM transitions *m/z* | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|
| **3-Methoxytyramine** | 0.84 | 151.1>91.2 | 17 | 22 |
| | | 151.1>119.2 | 17 | 18 |
| **Metanephrine** | 0.91 | 180>165.1 | 35 | 16 |
| | | 180>148.1 | 35 | 20 |
| **Normetanephrine** | 1.17 | 166.1>134.1 | 50 | 16 |
| | | 166.1>149.1 | 50 | 10 |
| **Dopamine** | 1.25 | 137.1>91.1 | 50 | 18 |
| | | 154.1>137.2 | 29 | 10 |
| **Epinephrine** | 1.40 | 184.1>166.1 | 15 | 8 |
| | | 166.1>107 | 15 | 18 |
| **Norepinephrine** | 1.98 | 152>135.2 | 46 | 14 |
| | | 152>79.2 | 20 | 20 |

*Table 2. Mass spectral parameters used for analysis of catecholamines and metanephrines.*

Figure 2A shows the chromatography of all compounds from a 20 pg/mL calibration standard using the ACQUITY UPLC BEH Amide Column. Previous work[6] had shown that 30 mM $NH_4HCOO$ and 15% water in MPB resulted in an ideal balance of ionic strength and solubility, enabling the resolution and peak shape seen in Figure 2A. One important feature of this separation is the resolution between NMT and EP. These two compounds have the same molecular formula and can interfere with each other if not adequately separated. Figure 2A demonstrates the baseline separation of these compounds in HILIC mode, enabling their unambiguous identification and quantification. Figure 2B shows the HILIC chromatography of an unspiked plasma sample, demonstrating the ability to determine endogenous concentrations of 3-MT, MTN, NMT, DA, EP, and NE (7.0, 31.7, 70.6, 0.0, 29.4, and 360.9 pg/mL, respectively).

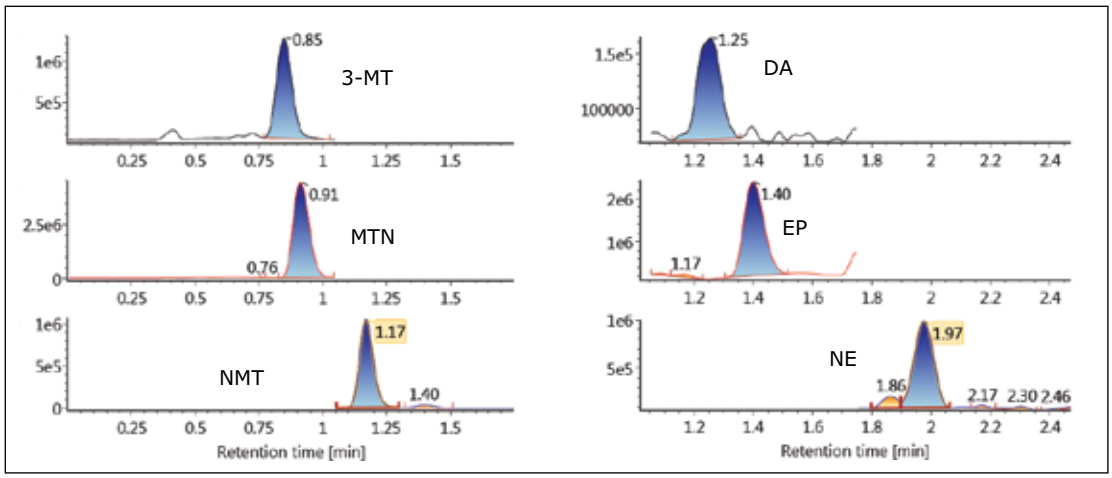


*Figure 2A. Chromatography of catecholamines and metanephrines on the ACQUITY UPLC BEH Amide Column, 1.7 µm, 2.1 x 100 mm. Representative calibration standard spiked at 20 pg/mL (100 pg/mL for EP and NE).*

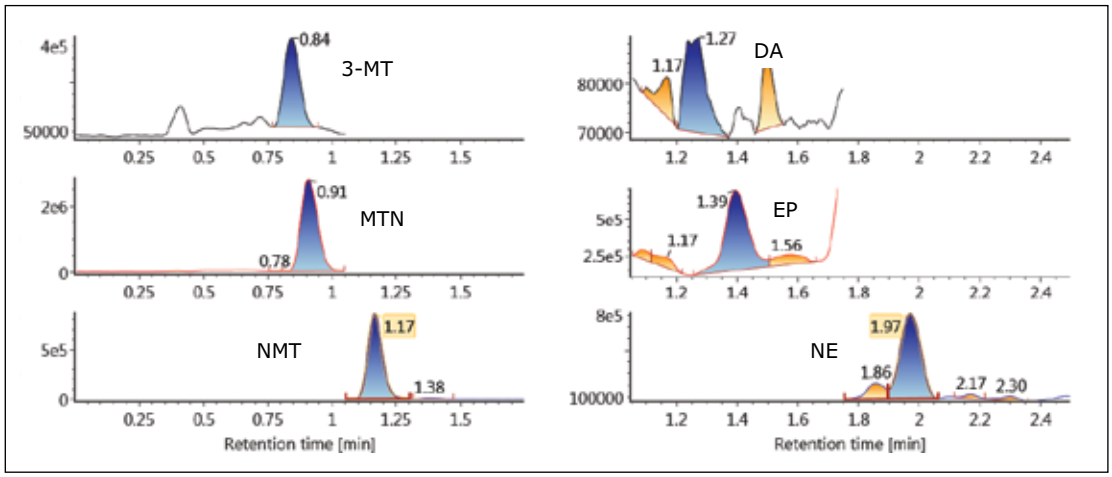


*Figure 2B. Chromatography of catecholamines and metanephrines on the ACQUITY UPLC BEH Amide Column, 1.7 µm, 2.1 x 100 mm. Representative method blank. Endogenous concentrations of all compounds are listed in Table 3.*

### Recovery and matrix effects

Extraction recoveries and matrix effects are shown in Figure 3. Recoveries ranged from 54% for NE to 90% for DA, with an average recovery of 76.4%. Matrix effects averaged -6.9%. The largest matrix effects were -23% and -22% for NE and DA, respectively, but were negligible for all other compounds. These results highlight another advantage of HIILIC chromatography, the ability to minimize matrix effects when analyzing polar compounds.

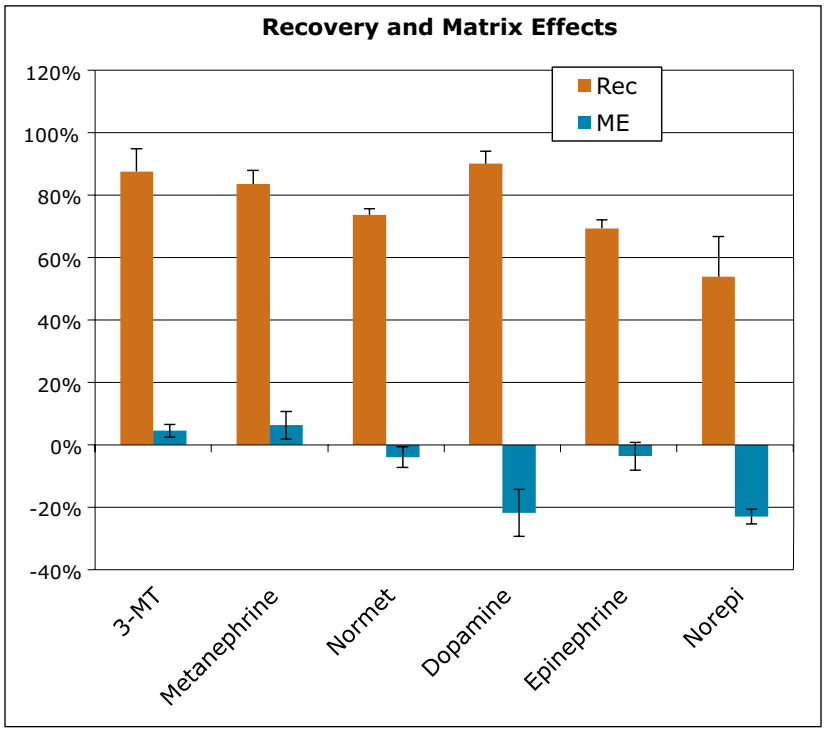


*Figure 3. Recovery and matrix effects for catecholamines and metanephrines extracted from urine using Oasis WCX 96-well µElution Plates (N=4). Error bars indicate standard deviations. All compounds were spiked at 100 pg/mL into pooled human plasma.*

### Quantitative results

Calibration curves and quality control samples were prepared via the standard addition method by spiking pooled plasma samples with known concentrations of analytes. Two ranges of calibration curves were used, reflecting different expected concentrations of various compounds in plasma. Calibration levels for 3-MT, metanephrine, normetanephrine, and dopamine ranged from 10–2,000 pg/mL. Calibration levels for epinephrine and norepinephrine ranged from 50–10,000 pg/mL. After data processing, the endogenous concentrations were extrapolated from the resulting calibration curves. These data were used to correct the actual calibration concentrations. For example, the plasma sample used for calibration was determined to contain 31.7 pg/mL of metanephrine, so the calibration concentrations were adjusted to 41.7–2031.7 pg/mL. The resulting calibration curves showed excellent linearity, with $R_2$ values of 0.999 or greater for all compounds. Figure 4 shows representative calibration curves for DA and MTN, both of which have $R_2$ values greater than 0.999. Table 3 summarizes the calibration data for all compounds. Mean % deviations from expected calibration values were less than 1% for all analytes. In addition, the maximum % deviations from calibration values are listed and show that with the exception of epinephrine, the maximum % deviation for all calibrators was less than 10%. The calculated endogenous concentration of compounds in the pooled plasma used for calibration is also listed, along with the corrected calibration ranges for each compound.

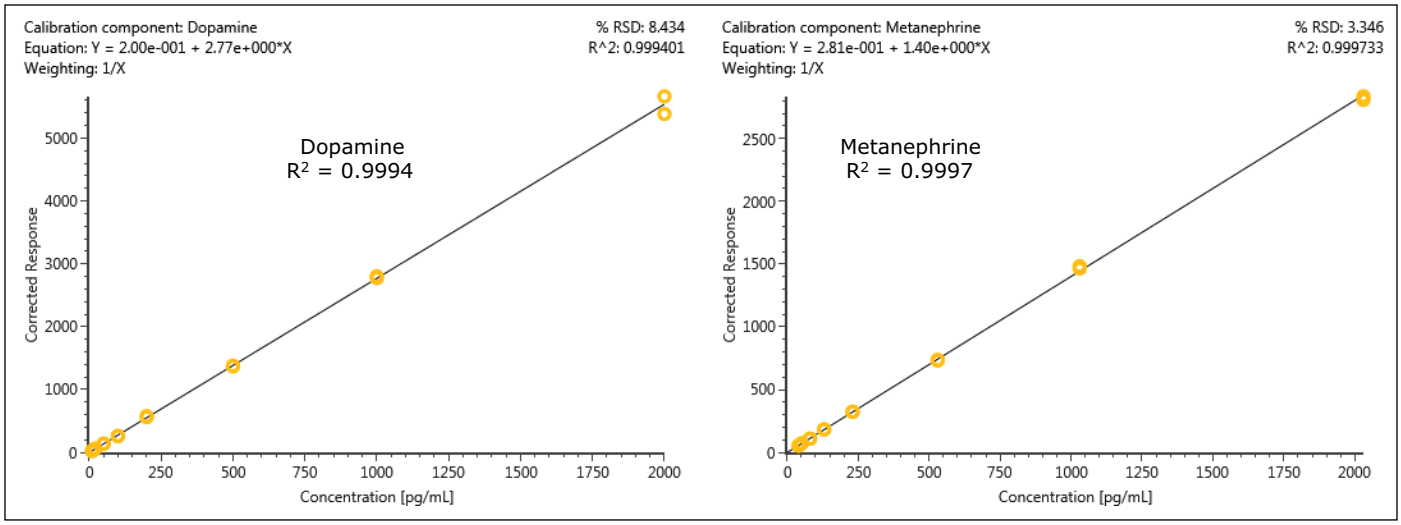


*Figure 4. Representative calibration curves for dopamine (DA) and metanephrine (MTN) extracted from plasma samples. The data were fitted with a 1/x weighted linear fit. Basal concentrations for DA and MTN were 0 and 30 pg/mL, respectively.*

| | $R^2$ | *Mean % Dev.* | Max % Dev. | Endogenous (pg/mL) | Corrected Calibration Range |
|---|---|---|---|---|---|
| **3-MT** | 0.9993 | 0.25% | 2.89% | 7 | 17-2007 |
| **Metanephrine** | 0.9997 | 0.00% | 2.50% | 32 | 42-2042 |
| **Normetanephrine** | 0.9998 | 0.00% | 1.72% | 71 | 81-2081 |
| **Dopamine** | 0.9994 | -0.33% | 4.57% | 0 | 10-2000 |
| **Epinephrine** | 0.9990 | 0.84% | 11.83% | 29 | 79-10079 |
| **Norepinephrine** | 0.9995 | 0.00% | 2.59% | 361 | 411-10411 |

*Table 3. Summary of calibration data for plasma metanephrines and catecholamines. Mean % deviation indicates the average % deviation of all calibration points from their theoretical concentrations. The max % deviation indicates the maximum deviation over the entire calibration range. The calculated endogenous concentrations are listed and used to correct the calibration range.*

Quality control samples (N=6) were overspiked at 200, 500, 2000, and 4000 pg/mL for EP and NE and at 40, 100, 400, and 800 pg/mL for the remaining compounds. QC results were accurate and precise (see Table 4). All QC values were within 10% of their target values, and most were within 5%. In addition, all coefficients of variation (%CV) were less than 10%. This demonstrates that the method is linear, accurate, and precise over a calibration range that includes the entire scope of expected values for normal and pathologically elevated samples.

| | QC spike concentration | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 40 pg/mL | | | 100 pg/mL | | | 400 pg/mL | | | 800 pg/mL | | |
| | Mean | S.D. | %CV | Mean | S.D. | %CV | Mean | S.D. | %CV | Mean | S.D. | %CV |
| 3-MT | 99.9% | 7.4% | 7.4% | 99.2% | 3.0% | 3.1% | 105.9% | 1.8% | 1.7% | 93.9% | 2.6% | 2.8% |
| Metanephrine | 99.9% | 2.0% | 2.0% | 97.6% | 0.8% | 0.8% | 107.3% | 1.2% | 1.1% | 94.6% | 1.7% | 1.7% |
| Normetanephrine | 99.8% | 1.6% | 1.6% | 96.8% | 1.7% | 1.8% | 104.6% | 0.4% | 0.4% | 93.4% | 1.0% | 1.1% |
| Dopamine | 97.0% | 7.2% | 7.4% | 91.2% | 3.4% | 3.7% | 103.7% | 3.1% | 3.0% | 95.6% | 2.7% | 2.8% |
| | 200 pg/mL | | | 500 pg/mL | | | 2000 pg/mL | | | 4000 pg/mL | | |
| | Mean | S.D. | %CV | Mean | S.D. | %CV | Mean | S.D. | %CV | Mean | S.D. | %CV |
| Epinephrine | 97.3% | 4.3% | 4.4% | 98.8% | 2.2% | 2.2% | 100.8% | 1.4% | 1.4% | 97.0% | 2.6% | 2.6% |
| Norepinephrine | 105.1% | 7.7% | 7.4% | 102.6% | 8.2% | 8.0% | 96.7% | 1.3% | 1.3% | 97.1% | 4.2% | 4.3% |

*Table 4. Quality control results for plasma catecholamines and metanephrines. Concentrations refer to the spiked concentration. Accuracies were calculated by comparing the result of the sum of the spiked concentration and endogenous calculated values in the plasma sample to the theoretical sum of these concentrations.*

## CONCLUSIONS

The extraction and analysis of plasma catecholamines and metanephrines using Oasis WCX µElution Plates and an ACQUITY UPLC BEH Amide Column in HILIC mode is detailed. Extraction using the Oasis WCX µElution Plate resulted in low matrix effects and consistent recoveries for all compounds that translated into excellent analytical accuracy and precision. In addition, the ability to elute the samples in an extremely low volume (50 µL) enabled 5x sample enrichment without the extra time or risk associated with evaporation and reconstitution. The ACQUITY UPLC BEH Amide Column used for HILIC separation resulted in rapid and efficient separation of all compounds, with baseline resolution between normetanephrine (NMT) and epinephrine (EP). It also enabled the analysis of the monoamines, dopamine, norepinephrine and epinephrine. Quantitative results were excellent, with highly linear responses across the entire calibration range and excellent accuracy and analytical precision.

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# The Application of UPLC/MS$^E$ for the Analysis of Bile Acids in Biological Fluids

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## APPLICATION BENEFITS

UPLC®/MS$^E$ can be used to measure bile acids reproducibly and reliably in biological fluids. This method analyzes a wide range of endogenous metabolites to provide both a targeted assay and a global metabolite profiling approach in the same analytical run. Use of this metabolic approach allows for a more comprehensible interpretation of metabolite changes, and it can be easily extended to other sample types and studies.

## WATERS SOLUTIONS

ACQUITY UPLC® Columns

ACQUITY UPLC System

QTof Premier™ Mass Spectrometer

MarkerLynx™ XS Application Manager

## KEY WORDS

Bile acids, endogenous metabolites, liver damage, hepatic and biliary tract disease

## INTRODUCTION

Individual bile acids are endogenous markers of liver cell function and studies of both qualitative and quantitative bile acid changes have been conducted as a result of liver and intestinal diseases. The measurement of serum bile acid concentrations can provide information pertaining to liver damage, as well as hepatic and biliary tract diseases.[1-4] However, traditional chromatographic methods have not typically provided sufficient separation in order to differentiate between structurally similar bile acids. Utilization of the Waters® UltraPerformance LC® (UPLC) Technology high resolution chromatographic system has greatly improved the abilility to separate metabolites from endogenous matrice. UPLC provides superior resolution, sensitivity, and throughput compared with conventional LC approaches. Using UPLC, previously co-eluting metabolites can be separated and matrix effects, such as ESI ion suppression, are minimized. By combining UPLC with oa-TOF mass spectrometry, both high-resolution and exact mass measurements can be achieved, aiding the identification of metabolites. This application note describes a new, sensitive UPLC-MS approach developed to measure bile acid reproducibly and reliably in biological fluids. Over 30 individual bile acids were separated and detected in a 5-minute window using an ACQUITY UPLC HSS T3 2.1 x 100 mm, 1.8 µm Column coupled to a Q-ToF Premier Mass Spectrometer. Bile acids were extracted from serum using methanol and a gradient elution of water and acetonitrile was employed, which also enabled the detection of a wide range of endogenous metabolites, such as lipids. MS$^E$ data were acquired using a patented acquisition method that collects precursor and product ion information for virtually every detectable species in a mixture. This allowed for characteristic metabolite fragmentation information to be obtained in a single analytical run, easily distinguishing glycine and taurine bile acid conjugates. This assay was applied to the study of the hepatotoxin galactosamine (galN) in rat. Serum bile acid changes were observed after galN treatment, including elevated taurine-conjugated bile acids, which correlated to liver damage severity. This UPLC-MS approach to bile acid analysis offers a sensitive and reproducible tool that will be of great value in exploring both markers and mechanisms of hepatotoxicity.

## EXPERIMENTAL

### UPLC conditions

| | |
|---|---|
| LC System: | ACQUITY UPLC |
| Column: | ACQUITY UPLC HSS T3, 2.1 x 100 mm, 1.8 µm |
| Column temp.: | 40 °C |
| Sample temp.: | 4 °C |
| Mobile phase A: | Water |
| Mobile phase B: | Acetonitrile |
| Flow rate: | 0.5 mL/min |
| Injection volume: | 5 µL |

Gradient:

| Time (min) | % A | Curve |
|---|---|---|
| 0.0 | 0 | 0 |
| 2 .0 | 0 | 6 |
| 12.0 | 5 | 6 |
| 17.0 | 5 | 6 |
| 18.0 | 100 | 6 |
| 22.0 | 100 | 6 |
| 22.5 | 5 | 6 |
| 23.0 | 0 | 6 |
| 26.0 | 0 | 6 |

| | |
|---|---|
| Data processing: | MarkerLynx XS Application Manager |

### MS conditions

| | |
|---|---|
| MS system: | Q-ToF Premier Negative electrospray mode |
| Scan range: | 50 to 1000 Da |
| Capillary voltage: | 2.4 Kv |
| Source temp.: | 120 °C |
| Desolvation temp.: | 350 °C |
| Cone voltage: | 35 V |
| Desolvation gas flow: | 900 L/hr |
| Collision energy (CE): | Low CE: 5 eV |
| High CE: | ramp of 10 to 70 eV |

## METHODS

### Animal dosing

Table 1 details the dosing of 40, six-week old male Sprague Dawley rats. The animals were euthanized 24 hrs after galactosamine (galN) or vehicle administration. Serum was isolated from blood samples collected at necropsy from the abdominal vena cava and stored at -40 °C pending analysis.

| Group | Sex | No. of Animals per group | Galactosamine (mg/kg) |
|---|---|---|---|
| 1 | M | 8 | 0* |
| 2 | M | 40 | 415 |

*0.9% saline

*Table 1. Animal and treatment details. Control = Group 1 and treated = Group 2.*

### Clinical chemistry and histopathology

*Clinical Chemistry Analysis*. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin levels were analyzed using a Vitros 950 analyzer (Ortho-Clinical Diagnostics, Rochester, NY).

*Histological Analysis*. Liver samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Liver sections were assigned the following histoscores: 0) absence of hepatocellular necrosis, 1) minimal necrosis, 2) mild necrosis, 3) moderate necrosis, and 4) marked necrosis.

### Sample preparation

Ice-cold methanol (150 µL) was added to 50 µL serum and vortexed for 30 seconds. The samples were then incubated at -20 °C for 20 mins, centrifuged at 13,000 rpm for 10 min, and the supernatant was removed to a clean tube. The supernatant was then dried down in a vacuum evaporator (Savant), reconstituted in 100 µL water, and transferred into 96-well 350 µL plates.

## RESULTS AND DISCUSSION

The UPLC-MS assay developed allowed for the reproducible separation and detection of 24 fully identified bile acids, plus 10 tentatively identified bile acids. All bile acids eluted within a 4 min window (6 to 10 mins) with good separation of individual bile acids, as shown in Figure 1. This method offers shorter analysis time than conventional HPLC methods (data not shown), and therefore allows significantly increased sample throughput.

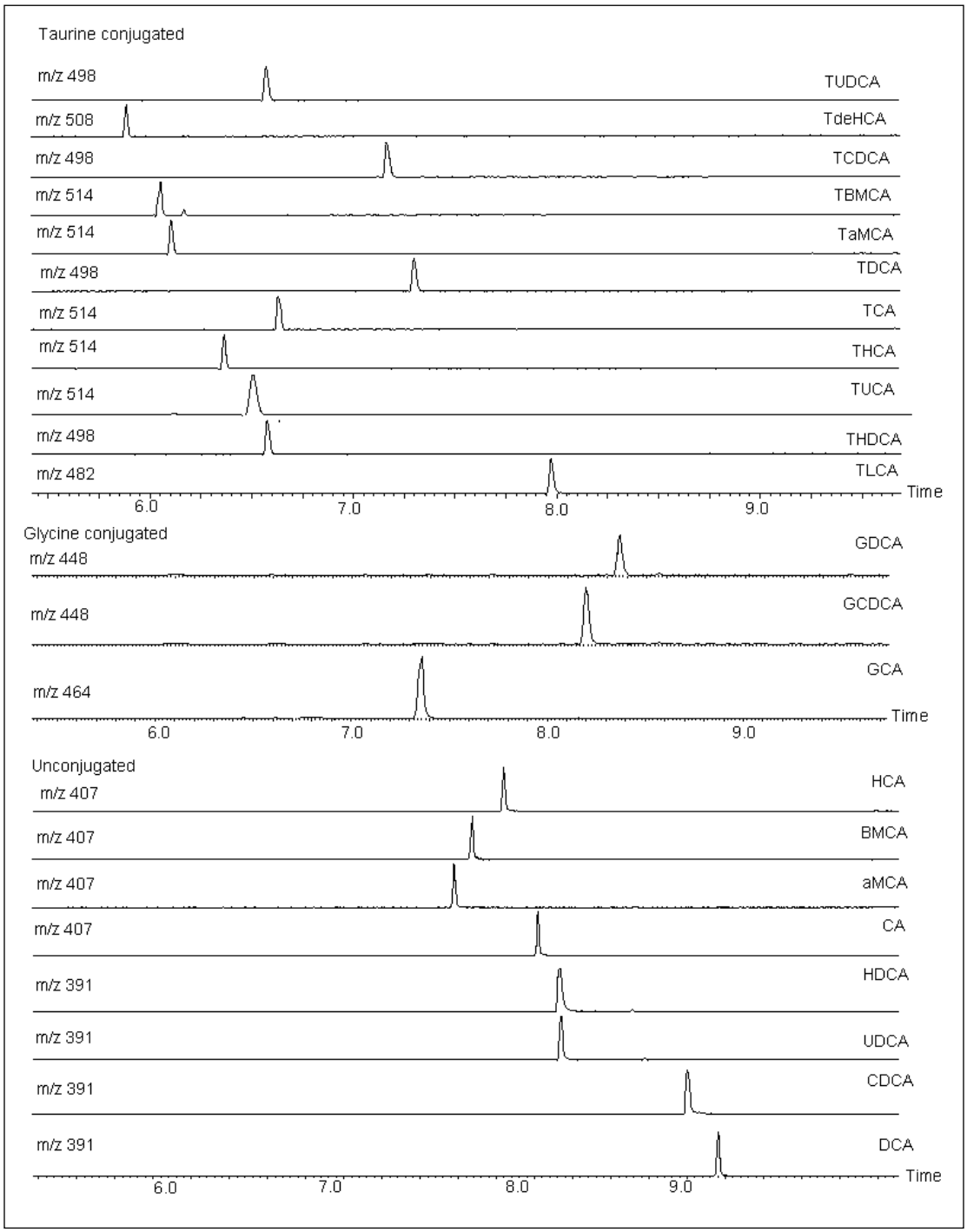


*Figure 1. Excellent separation of bile acids using UPLC-MS. 24 bile acids were fully identified, with 10 putative identifications, some of which are illustrated here.*

Bile acids ionize strongly using negative mode ESI, producing a prominent precursor $[M-H]^-$ ion in the low energy data and informative fragmentation data in the high energy data. This allows conjugates to be easily distinguished by fragment ion analysis. Glycine conjugates give rise to a diagnostic ion at 74 *m/z* and taurine conjugates at 79.9, 106.0, and 124.0 *m/z* respectively, as shown in Figure 2. This information was obtained using $MS^E$, facilitating the determination of the different conjugation classes of bile acids in a single run.

Importantly, in addition to bile acids, this assay allows for the detection of a wide range of endogenous metabolites, providing additional, complementary metabolite information. This is of great utility in metabonomics studies, as sample numbers may be large and it is desirable to obtain maximum information in a single analytical run. This provides reductions in sample volume, throughput time, and solvent consumption.

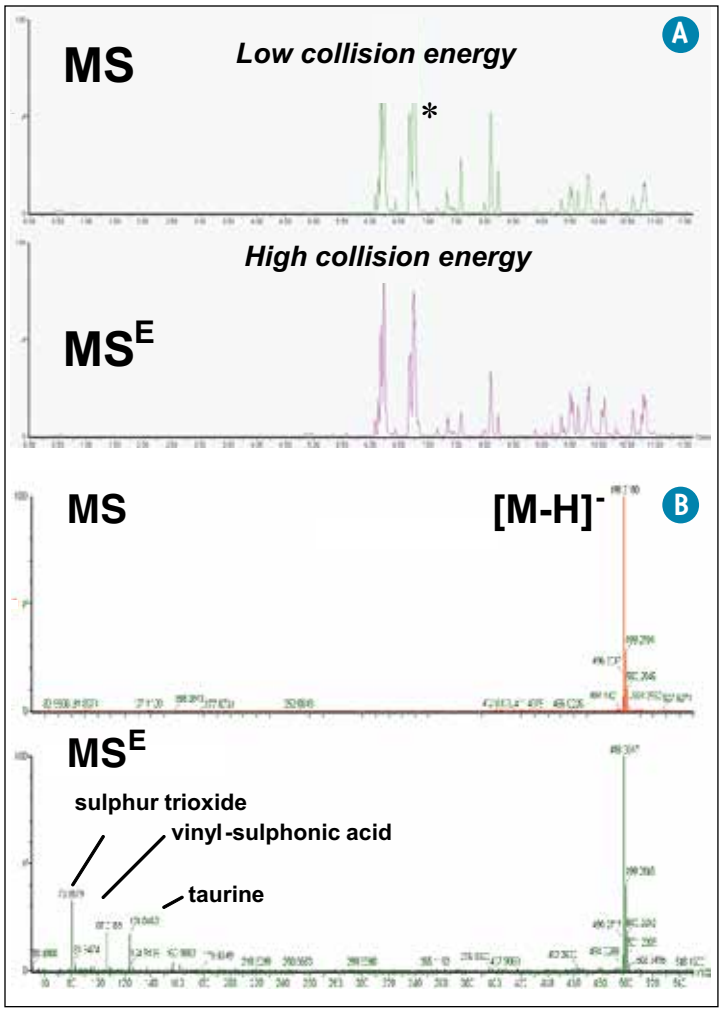


*Figure 2.*
*A) *Taurodeoxycholic acid (m/z 498) in (top) the low collision energy run and (below) the elevated collision energy total ion chromatograms. B) (top) The corresponding low collision energy and (below) high collision energy spectra, showing characteristic fragment ions. The application of this UPLC-$MS^E$ approach allows for the identification of over 30 bile acids in a single analytical run, reducing sample analysis time and the amount of sample required.*

Figure 3 shows representative base peak intensity (BPI) UPLC-MS chromatograms of serum from a control animal and those treated with galactosamine, demonstrating varying degrees of severity of liver damage. Dramatic serum BA changes were visible after galactosamine treatment, as shown in Figure 3, with obvious increases in the taurine-conjugated BAs. These increases correlated with the extent of liver damage (as determined by histoscore) and also the clinical chemistry (AST and ALT).

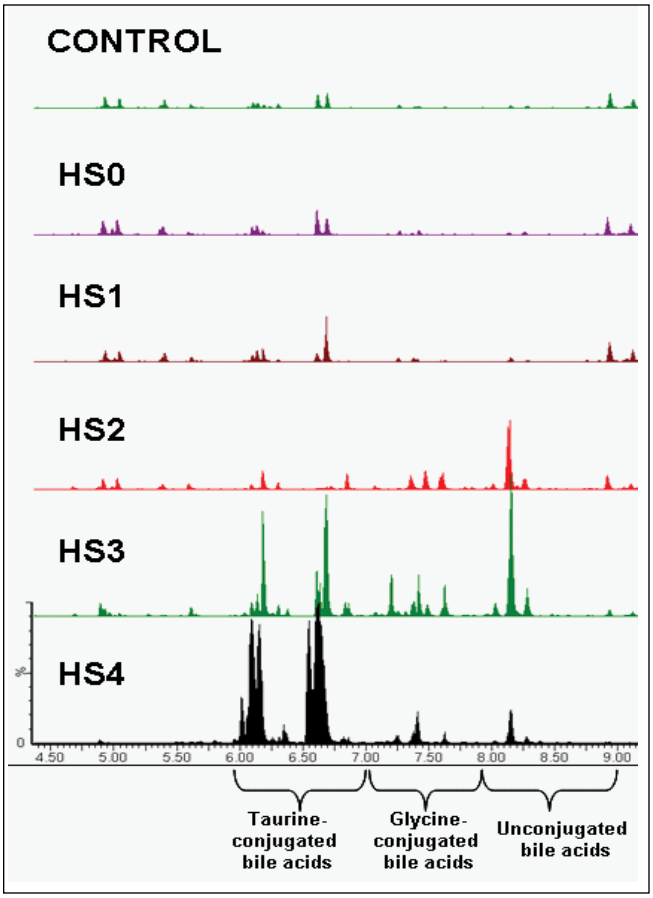


*Figure 3.*
*Selected region of the base peak ion (BPI) chromatograms of animals after galactosamine treatment, graded by histoscore. HS0 denotes no liver damage, whereas animals with a score of HS4 had marked liver damage. Taurine-conjugated bile acids were greatly elevated as liver damage increased; glycine conjugated bile acids showed some elevation whereas conjugated bile acids were unchanged.*

## CONCLUSIONS

UPLC-MS$^E$ offers a reliable and reproducible approach for the analysis of bile acids in biological fluids. This was demonstrated with serum samples applied to a toxicity study, and could easily be extended to other sample types and studies. The employment of a 26-min gradient allowed for the analysis of a wide range of endogenous metabolites, providing both a targeted assay and a global metabolite profiling approach in the same analytical run. This approach enhances the information obtained, leading to a more comprehensive interpretation of metabolite changes.

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# Targeted Lipidomics Using the ionKey/MS System

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## APPLICATION BENEFITS

The ionKey/MS™ System allows for fast and robust LC-MS lipidomics analyses with considerable reduction in solvent consumption and increase in sensitivity when compared to 2.1 mm I.D. chromatography. Potential applications include large-scale lipid profiling and low-abundance lipids analyses in biological materials.

## WATERS SOLUTIONS

ionKey/MS System

ACQUITY UPLC® M-Class

ionKey™ Source

Xevo® TQ-S

iKey Separation Device CSH™ C18

MassLynx™

## KEY WORDS

Lipidomics, lipid, microfluidics, metabolomics

## INTRODUCTION

Lipidomics is the comprehensive analysis of hundreds of lipid species in biological samples. Lipids play prominent roles in the physiological regulation of many key biological processes such as inflammation and neurotransmission. Alterations in lipid pathways have been associated with many diseases including cardiovascular diseases, obesity, and neurodegenerative disorders.

The ability to measure the wide array of lipid species in biological samples could help our understanding of their roles in health and disease. The need for a fast, comprehensive and sensitive analysis of the hundreds of lipid species challenges both the chromatographic separation and mass spectrometry.

Here we used the novel ionKey/MS System, which utilizes the iKey™ Separation Device packed with 1.7 µm particles for fast and robust chromatographic separation. By integrating microscale LC components into a single platform design, the device avoids problems associated with capillary connections, including manual variability, leaks, and excessive dead volume. This integrated microfluidic device is suitable for lipidomics analyses with considerable advantages when compared to analytical scale LC-MS analysis.

## EXPERIMENTAL

### LC conditions

| | |
|---|---|
| LC system: | ACQUITY UPLC M-Class |
| Sample loop: | 1 µL |
| Column: | iKey CSH™ $C_{18}$ 130 Å, 1.7 µm, 150 µm x 100 mm |
| Column temp.: | 55 °C |
| Flow rate: | 2 µL/min |
| Mobile phase A: | Acetonitrile/Water (60/40) with 10 mM ammonium formate + 0.1% formic acid |
| Mobile phase B: | Isopropanol/Acetonitrile (90/10) with 10 mM ammonium formate + 0.1% formic acid |
| Volume injected: | 0.2 - 0.5 µL |

Gradient:

| Time (min) | %A | %B | Curve |
|---|---|---|---|
| Initial | 55.0 | 45.0 | Initial |
| 1.00 | 40.0 | 60.0 | 6 |
| 10.00 | 1.0 | 99.0 | 6 |
| 16.00 | 1.0 | 99.0 | 6 |
| 16.01 | 55.0 | 45.0 | 6 |
| 18.00 | 55.0 | 45.0 | 6 |

### MS conditions

| | |
|---|---|
| Mass spectrometer: | Xevo TQ-S |
| Acquisition mode: | MRM |
| Ionization mode: | ESI positive |
| Capillary voltage: | 3.0 KV |
| Source temp.: | 120 °C |

### Materials

Lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL) and Nu-Chek Prep (Elysian, MN). Total lipid extract from bovine brain was purchased from Avanti Polar Lipids. Mouse plasma (10 µL) was extracted with isopropanol (490 µL). The solution was then allowed to stand for 30 min in ice, vortexed and then centrifuged (10,000 x g, at 4 °C for 10 min). The supernatant was collected in a new vial, evaporated to dryness under vacuum and kept at -80 °C until further analysis. Immediately prior to analysis, all lipid extracts were re-suspended in isopropanol/acetonitrile/water (50/25/25, 250 µL).

## RESULTS AND DISCUSSION

For the analysis of lipids, we used the ionKey/MS System, comprised of the Xevo TQ-S Mass Spectrometer, the ACQUITY UPLC M-Class, the ionKey source and the iKey Separation Device. The iKey contains the fluidic connections, electronics, ESI interface, heater, e-cord, and the chemistry, permitting operation at high pressure with sub 2 micron particles, leading to highly efficient LC separations of lipid molecules. By integrating microscale LC components into a single system design, we avoided problems associated with capillary connections, including manual variability, leaks and excessive dead volume. Lipidomics analyses were conducted using small volumes of lipid standards and lipid extracts from typical biological samples including plasma and brain tissues (0.2 µL). We separated lipids at flow rates of 2 µL/min using a ACQUITY UPLC M-Class engineered with 150 µm I.D. x 100 mm ceramic channel packed with CSH™ $C_{18}$ 130 Å 1.7 µm particles size (Fig. 1). The small column diameter (150 µm) of the iKey device allows low injection volumes (0.5 µL) and low flow rates (2 µl/min) increasing up to 10x the sensitivity compared to regular analytical columns (*e.g.*, 2.1 mm I.D.) (Fig.1). Mobile phase consumption was reduced compared to 2.1 mm I.D. chromatography albeit maintaining comparable chromatographic resolution and analysis times (Fig. 2)[1].

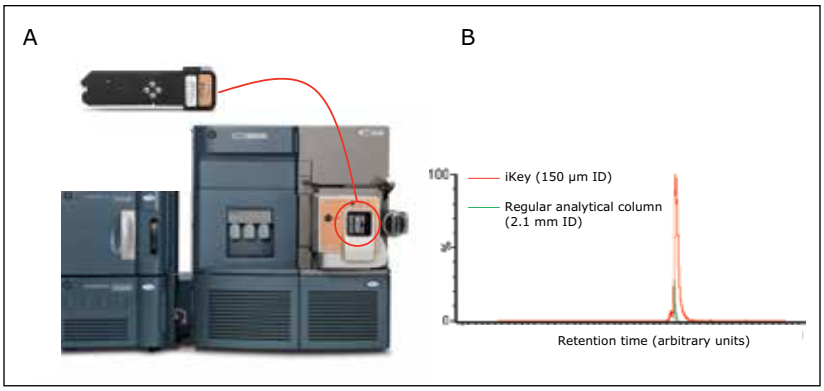


*Figure 1. (A) The ionKey/MS System: comprised of the Xevo TQ-S, the ACQUITY UPLC M-Class, the ionKey source and the iKey Separation Device. (B) Representative analysis of phosphatidylcholine (14:0/14:0) using ionKey/MS (red line) as compared to regular UPLC/MS[1] (green line).*

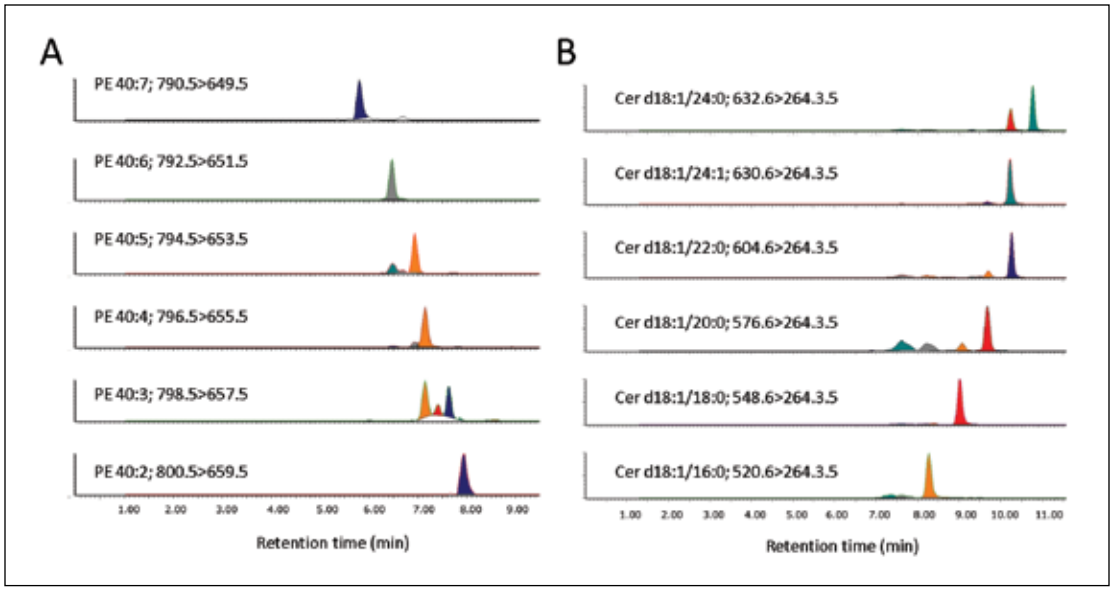


*Figure 2. Representative extracted ion chromatograms of A) glycerophospholipids (e.g., phosphatidyletahnolamines, PE) extracted from bovine brain and B) sphingolipids (e.g., ceramides, Cer) extracted from mouse plasma. Samples were analyzed using the ionKey/MS System.*

We conducted targeted lipidomic analyses using Xevo TQ-S in MRM mode and monitored 215 lipid species belonging to various lipid classes including phosphatidylethanolamines (PE), lyso PE, phosphatidylcholines (PC), lyso PC, ceramides (Cer), sphingomyelins, hexosylceramides, lactosylceramides and cholesterol esters (Table 1). Targeted lipids were measured over approximately five orders of dynamic range (Fig. 3 and 4). Lipids were separated according to acyl chain length and number of double bonds. Quantification was performed using TargetLynx™ Application Manager (Fig. 5). Initial reports in peer reviewed journals showed the advantages of using the ionKey/MS system in real world applications dealing with the analysis of low abundance lipids.[2,3]

| Lipid Class | No. MRMs | Cone voltage | Collision energy |
|---|---|---|---|
| **PE** | 45 | 26 | 18 |
| **Lyso PE** | 18 | 26 | 18 |
| **PC** | 44 | 42 | 26 |
| **Lyso PC** | 19 | 42 | 26 |
| **Ceramide** | 19 | 20 | 30 |
| **Sphingomyelin** | 20 | 36 | 24 |
| **HexosylCeramide** | 19 | 20 | 26 |
| **LactosylCeramide** | 16 | 20 | 30 |
| **Cholesteryl Ester** | 15 | 36 | 24 |

*Table 1. Overview of the MRM method used.*

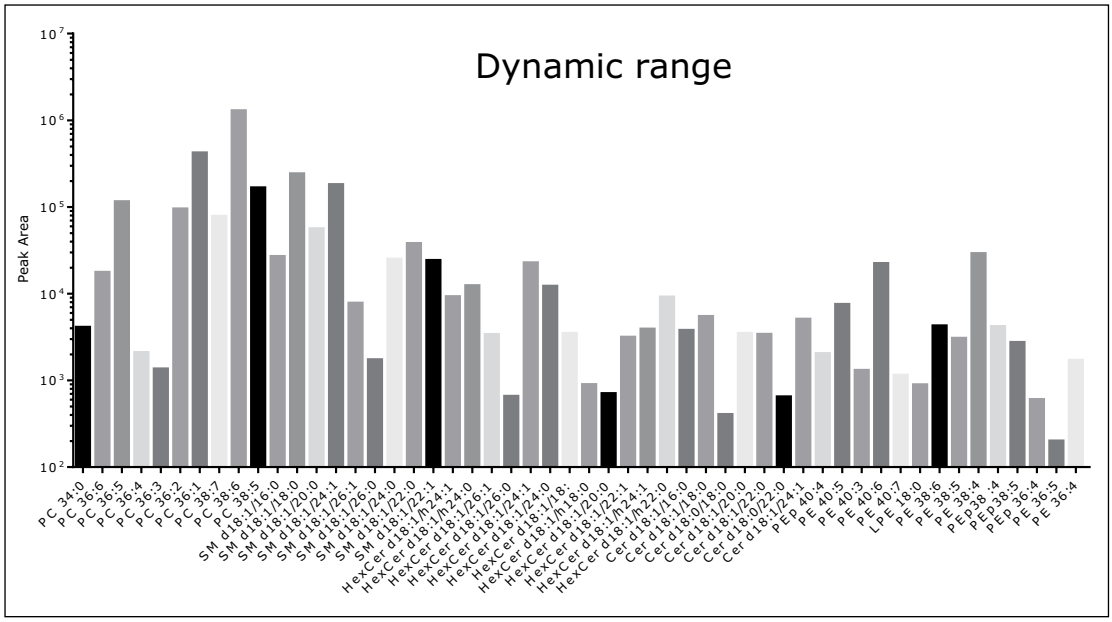


*Figure 3. Intensities of selected lipids extracted from bovine brain.*

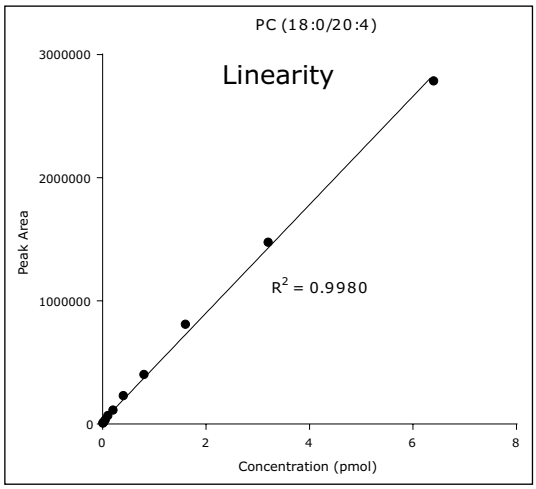


*Figure 4. Linearity of response for a selected phosphatidylcholine species (PC).*

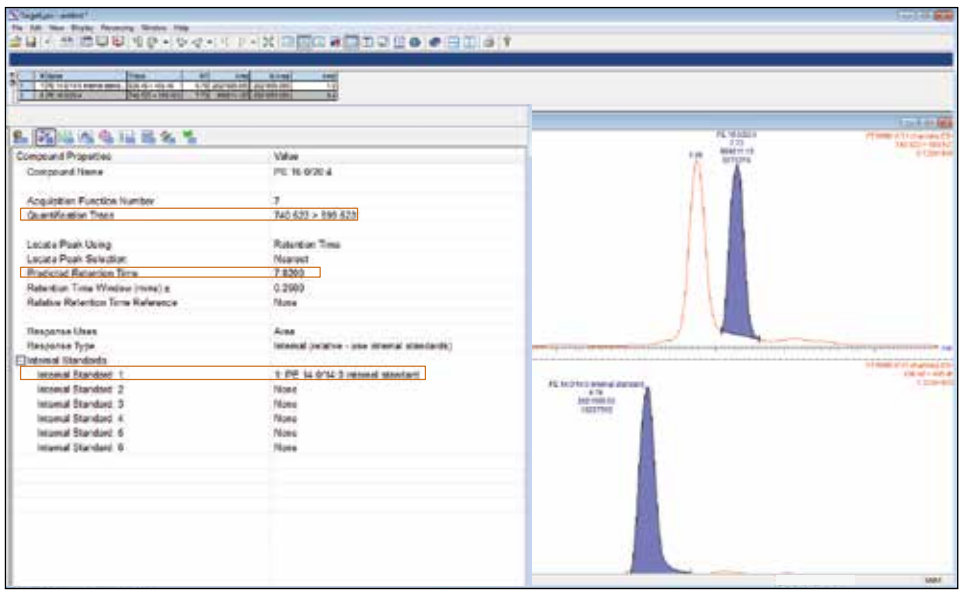


*Figure 5. Quantification can be performed using TargetLynx. MRM and retention times are automatically extracted and normalized by comparison to selected internal standard.*

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# Multiplexed Analysis of Steroid Hormones Using ionKey/MS

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## APPLICATION BENEFITS

A highly analytically sensitive multiplexed assay was developed for targeted quantitation of five steroids in human serum. The use of the ionKey™ source and the 150 µm iKey™ Separation Device yielded a 100–400 fold increase in on-column sensitivity while at the same time decreasing solvent usage by 150 fold as compared to standard flow methods. The increased on-column analytical sensitivity allowed for simplification of the steroid extraction procedure which in turn streamlined the sample preparation and reduced per/sample assay cost.

## WATERS SOLUTIONS

ionKey/MS™ System

nanoACQUITY® UPLC®

ionKey Source

Xevo® TQ-S Mass Spectrometer

iKey Separation Device BEH C18

MassLynx™

## KEY WORDS

Xevo, TQ-S, iKey, ionKey/MS, multiplexed

## INTRODUCTION

The measurement of steroids in human serum is an important clinical research tool. Traditionally, these assays are performed using a variety of biochemical techniques including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and chemiluminescent immunoassay (CLIA). However, immunoassays suffer from antibody cross-reactivity with structural isomers which has been shown to result in an overestimation of steroid levels. Recently, LC-MS/MS has emerged as a viable alternative for this important assay in the clinical research setting. While providing improved accuracy as compared to antibody based techniques, standard flow LC-MS/MS assays also consume high levels of solvent and often require time-consuming sample extraction procedures such as liquid-liquid or solid phase extraction to adequate analytical sensitivity.

In this application, we report the use of the newly developed 150 µm ionKey/MS System for the multiplexed quantitation of five important steroid compounds in human serum: testosterone, dihydrotestosterone, progesterone, cortisone, and cortisol. The reduced flow method results in a 150 fold decrease in solvent consumption and a 100–400 fold increase in on-column analytical sensitivity.[1]

## EXPERIMENTAL

### Method conditions

### LC conditions

| | |
|---|---|
| LC system: | nanoACQUITY UPLC |
| Sample loop: | 5 uL |
| Column: | iKey BEH $C_{18}$ 130, 1.7 µm, 150µm x 50mm |
| Column temp: | 45 °C |
| Flow rate: | 3.06 µL/min |
| Mobile phase A: | Water + 0.1% formic acid |
| Mobile phase B: | Methanol + 0.1% formic acid |
| Volume injected: | 0.5 µL using partial loop mode |

Gradient:

| Time (min) | %A | %B | Curve |
|---|---|---|---|
| Initial | 90.0 | 10.0 | Initial |
| 0.25 | 90.0 | 10.0 | 6 |
| 1.00 | 45.0 | 55.0 | 6 |
| 7.50 | 5.0 | 95.0 | 6 |
| 8.00 | 55.0 | 10.0 | 6 |
| 12.00 | 55.0 | 10.0 | 6 |

### MS conditions

| | |
|---|---|
| Mass spectrometer: | Xevo TQ-S |
| Acquisition mode: | MRM |
| Ionization mode: | ESI positive |
| Capillary voltage: | 3.2 kV |
| Source temperature: | 100 °C |
| Source offset: | 50V |
| Collision gas: | argon |
| Dwell times for all compounds: | 0.011 s |

### Sample preparation

Serum samples were precipitated with 3.7 volumes of ice cold methanol containing stable isotope-labeled internal standards for each steroid at a level of 10 ng/mL. Samples were incubated at -80 °C for 30 minutes, centrifuged at 3270 x g for 10 min and supernatant was collected. All sample preparation and injections were conducted in 96-well plates. 0.5 µL of extracted serum was injected and separation was performed using a nanoACQUITY UPLC connected to an ionKey source using a 150 µm iKey packed with BEH $C_{18}$ (1.7 µm particles). The column effluent was monitored using a Xevo TQ-S Mass Spectrometer operated in multiple reaction monitoring (MRM) positive ion electrospray mode.

| Compound | Time window (Min) | Transition | Cone (V) | Collision energy (V) |
|---|---|---|---|---|
| testosterone | 4.1-5.3 | 289.24>97.03 | 50 | 20 |
| dihydrotestosterone | 4.5-5.5 | 291>255 | 46 | 14 |
| d3 testosterone | 4.1-5.3 | 292.2>97.03 | 50 | 20 |
| d3 dihydrotestosterone | 4.5-5.5 | 294.1>258.2 | 46 | 14 |
| progesterone | 4.9-6 | 315>109 | 20 | 26 |
| $^{13}C_3$ progesterone | 4.9-6 | 318.2>112.2 | 20 | 26 |
| cortisone | 3.5-4.6 | 361>163.05 | 25 | 30 |
| cortisol | 3.5-5 | 363>327.14 | 25 | 16 |
| d4 cortisol | 3.5-5 | 367.2>331 | 25 | 22 |
| d7 cortisone | 3.5-4.6 | 368.2>169 | 25 | 22 |

*Table 1. MRM transitions and instrument settings for each compound. (Broccardo 2013)*

### Data analysis

Quantification was performed using linear regression against a standard curve in MassLynx Software. Peak areas for each compound were normalized to the corresponding internal standard in each sample.

## RESULTS AND DISCUSSION

Chromatographic separation of the five compounds is illustrated in Figure 1. An average peak width of 6s was achieved.

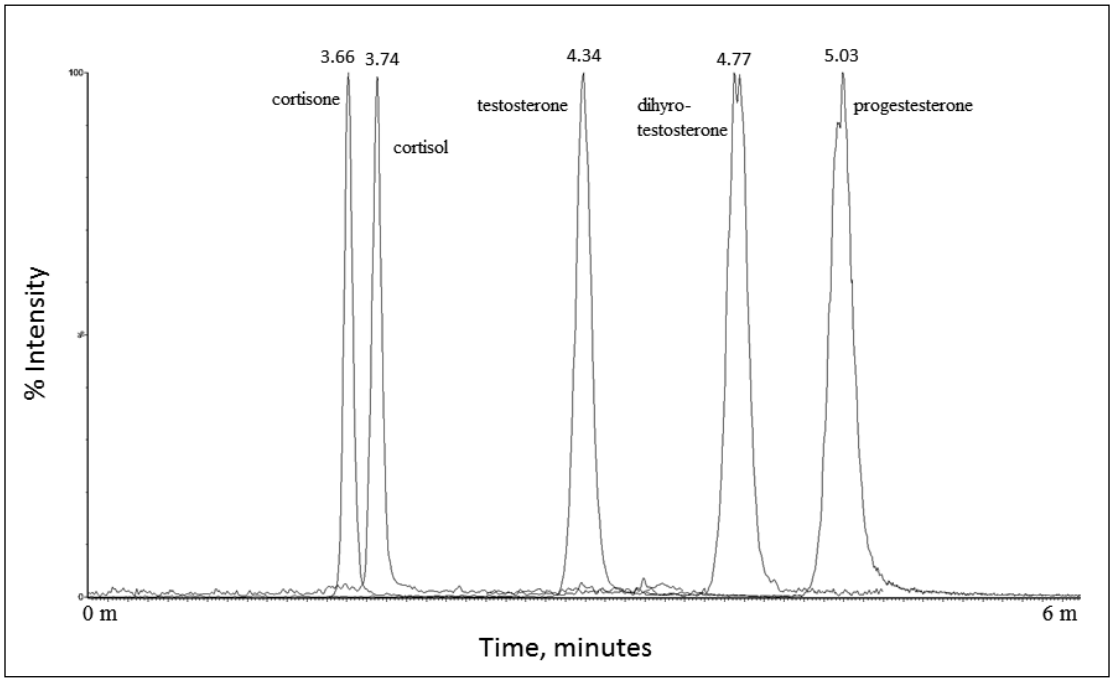


*Figure 1. Chromatographic separation of the 5 steroid compounds in human serum. (Broccardo 2013)*

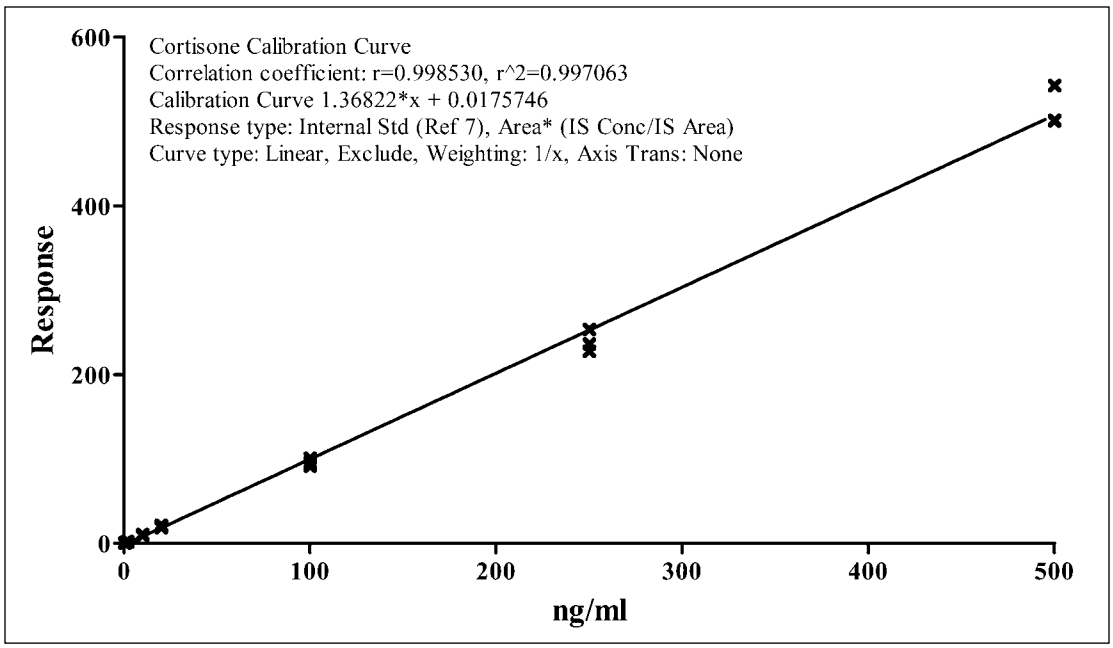


*Figure 2. Calibration curve for Cortisone measured in human serum. (Broccardo 2013)*

A typical calibration curve is shown in Figure 2. The correlation coefficients for all compounds were 0.99 or greater.

The lower limit of quantification (LOQ) and lower limit of detection (LOD) for all five compounds is listed in Table 2. These values represent a 100-400 fold increase in on column sensitivity as compared to published standard flow assays which typically require injection volumes of 50-200 µL of extracted sample;[2,4] only 0.5 µL of extracted sample is used in the assay presented here. Importantly, the LOQs for all compounds are within the range of clinical relevance reported in the literature.[3]

| Analyte | LOD (ng/mL) | LOQ (ng/mL) |
|---|---|---|
| Testosterone | 0.12 | 0.41 |
| Dihydrotestosterone | 0.42 | 1.40 |
| Progesterone | 0.03 | 0.40 |
| Cortisone | 0.09 | 0.29 |
| Cortisol | 0.57 | 1.90 |

*Table 2. LOD and LOQ values for the 5 steroids in human serum. (Broccardo 2013)*

## CONCLUSIONS

- The ionKey/MS System with the Xevo TQ-S and 150 µm iKey enabled the development of a low flow MRM assay of 5 steroids in human serum.
- The low flow regime resulted in an increase in on-column sensitivity and a 150 fold decrease in solvent consumption, as compared to standard flow methods in the literature.

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# Fast and Simple Free Fatty Acids Analysis Using UPC²/MS

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## APPLICATION BENEFITS

- Demonstrates the separation of free fatty acid (FFA) species based on chain length and number of double bonds
- No derivatization is required, which results in easier and fast sample preparation and eliminates artifact formation
- Organic phase lipid extract can be directly injected onto the system, saving time and reducing cost per analysis
- Less than three-minute chromatographic separation is up to 10X faster compared to GC/MS
- Unlike GC/MS, low volatile and very long chain fatty acids (>24 carbon atoms) can be easily analyzed with UPC²®

## WATERS SOLUTIONS

ACQUITY UPC²® System

TransOmics™ Informatics

Xevo® G2 QTof Mass Spectrometer

ACQUITY UPC² HSS Column

## KEY WORDS

Free fatty acids, UltraPerformance Convergence Chromatography™, UPC², TransOmics, time-of-flight mass spectrometry, UPC²/MS/MS

## INTRODUCTION

Fatty acids, both free and as part of complex lipids, play a number of key roles in metabolism – as major metabolic fuel (storage and transport of energy), as essential components of all membranes, and as gene regulators. In addition, dietary lipids provide polyunsaturated fatty acids that are precursors of powerful locally acting metabolites, e.g., eicosanoids.

The common fatty acids of animal and plant origin have even-numbered chains of 16 to 24 carbon atoms with 0 to 6 double bonds. Nature provides countless exceptions, however, and odd- and even-numbered fatty acids with up to nearly 100 carbon atoms exist. In addition, double bonds can be of the *cis* (Z) and *trans* (E) configuration and there can be innumerable other structural features, including branch points, rings, oxygenated functions, and many more.

Fatty acid chains may contain one or more double bonds at specific positions (unsaturated and poly unsaturated with *cis* (Z) or *trans* (E) configuration) or they may be fully saturated. The LIPIDMAPS systematic nomenclature for fatty acids indicates the location of double bonds with reference to the carboxyl group with "Δ".[1] Fatty acid structures also contain a methyl group at one end of the molecule (designated omega, ω) and a carboxyl group at the other end. The carbon atom next to the carboxyl group is called α carbon and the subsequent one the β carbon. The letter "n" is also often used instead of ω to indicate the position of the double bond closest to the methyl end.[2] Figure 1 outlines the structures of different straight chain fatty acids.

The isolation of free fatty acids (FFA) from biological materials is a complex task and precautions should be taken at all times to prevent or minimize the effects of hydrolyzing enzymes. After isolation, the typical chromatographic methods for analyzing fatty acids include gas chromatography/mass spectroscopy (GC/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS). However, there are shortcomings associated with each of these methods.

For example, GC methods require derivatization of the fatty acids to hydrolyze and convert to methyl esters, which is time-consuming and risks re-arrangement of the fatty acids during derivatization, leaving doubt as to whether the esters formed are from FFA or intact complex lipids. Moreover, the GC/MS analysis of low volatile, very-long-chain fatty acids with high molecular weight (>C24) is a problem even after fatty acid methyl ester (FAME) derivatization.

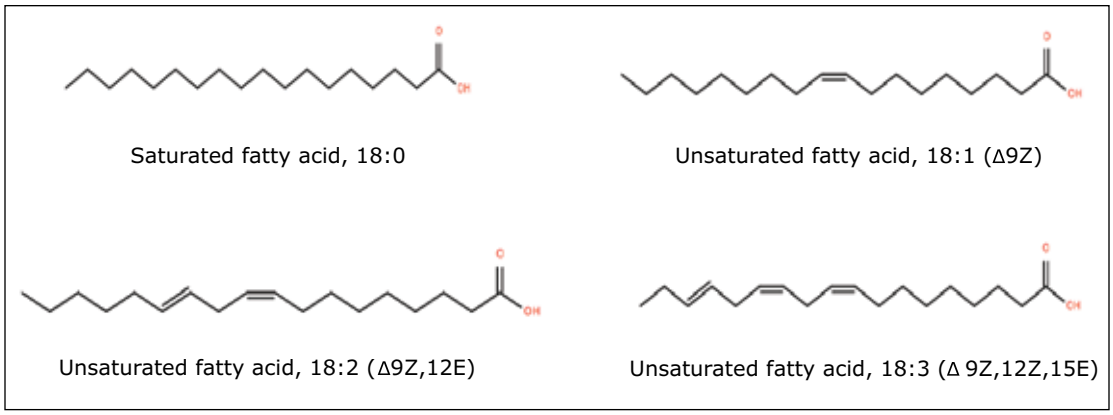


*Figure 1. Structure and nomenclature of different straight chain fatty acids with a methyl and a carboxyl (acidic) end. Fatty acids may be named according to systematic or trivial nomenclature. One systematic way to describe the position of double bonds is in relation to the acidic end of the fatty acids; symbolized as Δ (Greek delta) followed with numbers. All unsaturated fatty acids are shown with cis (Z) or trans (E) configuration of the double bonds.*

In LC/MS methods, although no sample derivatization is required, the runs typically involve labor-intensive and time-consuming sample preparation, and utilize toxic organic solvents, which are expensive to purchase and dispose. In a typical reversed phase (RP) LC/MS analysis, the organic extracts containing all the lipids have to be evaporated and re-constituted in a more compatible injection solvent.

Thus, it would be beneficial to have streamlined methods for the separation and determination of fatty acids. Here, we present a rapid, high-throughput and efficient method for the separation and analysis of FFA using UltraPerformance Convergence Chromatography (UPCC, or UPC$^2$) with mass spectrometry.

UPC$^2$ is a complementary, orthogonal separation technology that is taking its place alongside LC and GC. While all three use a stationary phase to interact with compounds of interest and a mobile phase to move compounds through the stationary phase and achieve separation, the techniques differ mainly by the mobile phases used.

GC is defined by using a gas as its mobile phase, LC is defined by using liquids as its mobile phase, and CC is defined by using both gas and liquids. It is this convergence of mobile phases in combination with a far greater choice of stationary phases that makes CC a powerful additional choice for laboratory scientists. Because UPC$^2$ can receive samples in organic solvents such as hexane and chloroform, it significantly simplifies the requirements for sample preparation, while maintaining all the advantages of RPLC.

Here, the analysis of fatty acids in the free form instead of FAME derivatives results in easier and faster sample preparation. The organic phase extract containing all the FFA can be injected directly into the system, which results in significant savings in sample preparation and analysis time, solvent costs, and solvent waste disposal. Additionally, artifact formation that can result from a derivatization procedure is eliminated.

## EXPERIMENTAL

### Method conditions

#### UPC² conditions

| | |
|---|---|
| System: | ACQUITY UPC² |
| Columns: | ACQUITY UPC² HSS $C_{18}$ SB 1.8 µm, 2.1 x 150 mm |
| Column temp.: | 50 °C |
| Sample vial: | Total Recovery Vial (p/n 186000385C) |
| Sample temp.: | 10 °C |
| Injection volume: | 0.5 µL |
| Flow rate: | 0.6 mL/min |
| Mobile phase A: | $CO_2$ |
| Mobile phase B: | Methanol in 0.1% formic acid |
| Make up: | Methanol in 0.1% $NH_4OH$ (0.2 mL/min) |
| Splitter: | Upchurch cross 1/16 PEEK |

#### Gradient

| Time (min) | %A ($CO_2$) | %B | Curve |
|---|---|---|---|
| 0.0 | 95 | 5 | Initial |
| 5.0 | 75 | 25 | 6 |
| 5.1 | 50 | 50 | 1 |
| 6.0 | 50 | 50 | 11 |
| 8.0 | 95 | 5 | 1 |

#### MS conditions

| | |
|---|---|
| Mass spectrometer: | Xevo G2 QTof |
| Ionization mode: | ESI negative |
| Capillary voltage: | 1.0 kV |
| Cone voltage: | 30 V |
| Source temp.: | 100 °C |
| Desolvation temp.: | 500 °C |
| Cone gas flow: | 10 L/h |
| Desolvation gas flow: | 600 L/h |
| Acquisition range: | 50 to 600 *m/z* |

### Sample preparation

#### FFA standard mixtures

Individual saturated FFA standards containing even carbon number $C_8$ to $C_{24}$ were purchased from Sigma. A complex model mixture of different FFA standards (GLC-85 in FFA form) was purchased from Nu-Chek Prep (Elysian, MN, USA). The list of FFA standards analyzed and other detailed information is provided in Table 1. A 1 mg/mL stock solution was prepared in chloroform, and 0.1 mg/mL working lipid mixtures were prepared in chloroform, then injected onto the UPC²/MS system.

#### Algae and algaenan produced oils

Oil produced from hydrous pyrolysis of algae and algaenan at low and high pyrolysis temperature were provided from Old Dominion University (Norfolk, VA, USA). Algae 1 and algaenan 1 were treated at a pyrolysis temperature of (310 °C); and Algae 2 and algaenan 2 were treated at a pyrolysis temperature of (360 °C).

Extraction of algaenan was performed by a modified extraction procedure. Briefly, lipids were removed from the algae by Soxhlet extraction with 1:1 (v/v) benzene/methanol solvent mixture for 24 hours. The residue was treated with 2N sodium hydroxide at 60 °C for two hours. The remaining residue was then washed excessively with deionized water, followed by treatment with Dowex 50W-x8 cation exchange resin to exchange any residual sodium. Finally, the solid was rinsed with deionized water. The oil samples were diluted 10 times in dichloromethane, and 1 µL was injected onto the UPC²/MS system.

#### Data acquisition and processing

When using multivariate data analysis for sample comparison, it is crucial that each sample is randomized and injected a minimum of three times to ensure that the data analysis is statistically valid. For this study, five replicates of each algae and algaenan oil extracts were acquired in $MS^E$ mode, an unbiased Tof acquisition method in which the mass spectrometer switches between low and elevated collision energy on alternate scans. Data analysis and FFA identification were performed using TransOmics Informatics for Metabolomics and Lipidomics (TOIML).

| Compound | Formula | Neutral mass | $[M-H]^-$ | Retention time (min) | Common name | Description |
|---|---|---|---|---|---|---|
| 1 | $C_4H_8O_2$ | 88.052429 | 87.045153 | 0.89 | Butyric acid | C4:0 |
| 2 | $C_6H_{12}O_2$ | 116.083730 | 115.076453 | 0.96 | Caproic acid | C6:0 |
| 3 | $C_8H_{16}O_2$ | 144.115030 | 143.107753 | 1.06 | Caprylic acid | C8:0 |
| 4 | $C_{10}H_2OO_2$ | 172.146330 | 171.139053 | 1.17 | Capric acid | C10:0 |
| 5 | $C_{11}H_{22}O_2$ | 186.161980 | 185.154704 | 1.23 | Undecylic acid | C11:0 |
| 6 | $C_{12}H_{24}O_2$ | 200.177630 | 199.170354 | 1.31 | Lauric acid | C12:0 |
| 7 | $C_{13}H_{26}O_2$ | 214.193280 | 213.186004 | 1.41 | Tridecylic acid | C13:0 |
| 8 | $C_{14}H_{28}O_2$ | 228.208930 | 227.201654 | 1.54 | Myrislc acid | C14:0 |
| 9 | $C_{15}H_{30}O_2$ | 242.224580 | 241.217304 | 1.67 | Pentadecylic acid | C15:0 |
| 10 | $C_{16}H_{32}O_2$ | 256.240230 | 255.232954 | 1.80 | Palmilc acid | C16:0 |
| 11 | $C_{17}H_{34}O_2$ | 270.255880 | 269.248604 | 1.97 | Margaric acid | C17:0 |
| 12 | $C_{18}H_{36}O_2$ | 284.271530 | 283.264254 | 2.11 | Stearic acid | C18:0 |
| 13 | $C_{20}H_{40}O_2$ | 312.302831 | 311.295554 | 2.41 | Arachidic acid | C20:0 |
| 14 | $C_{22}H_{44}O_2$ | 340.334131 | 339.326854 | 2.70 | Behenic acid | C22:0 |
| 15 | $C_{14}H_{26}O_2$ | 226.193280 | 225.186004 | 1.45 | Physeteric acid | C14:1 |
| 16 | $C_{15}H_{28}O_2$ | 240.208930 | 239.201654 | 1.57 |  | C15:1 |
| 17 | $C_{16}H_{30}O_2$ | 254.224580 | 253.217304 | 1.67 | Palmitoleic acid | 16:1 |
| 18 | $C_{17}H_{32}O_2$ | 268.240230 | 267.232954 | 1.81 | 10-HEPTADECENOIC Acid | C17:1 (Δ10) |
| 19 | $C_{18}H_{30}O_2$ | 278.224580 | 277.217304 | 1.76 | Gamma Linolenic Acid | C18:3 (Δ6,9,12) |
| 20 | $C_{18}H_{30}O_2$ | 278.224580 | 277.217304 | 1.86 | Linolenic Acid | C18:3(Δ9,12,15) |
| 21 | $C_{18}H_{30}O_2$ | 280.240230 | 279.232954 | 1.88 | Linoleic Acid | C18:2 |
| 22 | $C_{18}H_{34}O_2$ | 282.255880 | 281.248604 | 1.98 | Oleic Acid | C18:1 |
| 23 | $C_{18}H_{34}O_2$ | 282.255880 | 281.248604 | 1.98 | Elaidic Acid | C18:1T |
| 24 | $C_{20}H_{32}O_2$ | 304.240230 | 303.232954 | 1.93 | Arachidonic acid | C20:4 |
| 25 | $C_{20}H_{34}O_2$ | 306.255880 | 305.248604 | 2.04 | HOMOGAMMA LINOLENIC Acid | C20:3 (Δ8,11,14) |
| 26 | $C_{20}H_{34}O_2$ | 306.255880 | 305.248604 | 2.14 | 11-14-17-EICOSATRIENOIC Acid | C20:3 (Δ11,14,17) |
| 27 | $C_{20}H_{36}O_2$ | 308.271530 | 307.264254 | 2.17 | 11-14-EICOSADIENOIC Acid | C20:2 (Δ11, 14) |
| 28 | $C_{20}H_{38}O_2$ | 310.287180 | 309.279904 | 2.24 | 11-EICOSENOIC Acid | C20:1 (Δ11) |
| 29 | $C_{22}H_{32}O_2$ | 328.240230 | 327.232954 | 2.09 | Docosahexaenoic Acid | C22:6 |
| 30 | $C_{22}H_{40}O_2$ | 336.302831 | 335.295554 | 2.46 | Docosadienoic Acid | C22:2 |
| 31 | $C_{22}H_{38}O_2$ | 338.318481 | 337.311204 | 2.54 | Erucic Acid | C22:1 |
| 32 | $C_{24}H_{46}O_2$ | 366.349781 | 365.342504 | 2.83 | Nervonic acid | C24:1 |

*Table 1. A list of analyzed saturated and unsaturated standard FFA mixtures with corresponding retention time determined from Figure 3A.*

## RESULTS AND DISCUSSION

### Analysis of saturated FFA standards

Figure 2 shows the separation of saturated FFA with carbon chain length $C_8$ to $C_{24}$. The ACQUITY UPC$^2$ High Strength Silica (HSS) $C_{18}$ SB 1.8 µm, 2.1 x 150 mm Column provides an RP-like separation that results in effective separation of the different FFA species. The gradient is run under acidic conditions using a small percentage of formic acid (0.1% v/v in methanol) to improve the peak shape and decrease peak tailing.

The ACQUITY UPC$^2$ method is 10X faster (only a three-minute run) than GC/MS and RPLC methods, and uses less toxic and cheaper $CO_2$ as a solvent. A typical lipidomics study involves the analysis of thousands of biological samples, and the additional speed allows for large sample sets to be analyzed efficiently, improving the overall power of the experiment.

The FFA lipid molecular species separation mechanism is mainly based on hydrophobic interaction of the FFA carbon numbers and number of double bonds with the HSS $C_{18}$ SB material. Therefore, the elution order of the FFA species depends on the length and the number of double bonds on the fatty acid chain. Thus, the longer and the more saturated the acyl chain length the longer the retention time.

The co-solvent mobile phase B (methanol in 0.1% formic acid) can be optimized to increase the chromatographic resolution and peak capacity. The higher the percentage of the co-solvent, the shorter the retention time and the narrower the peaks. However, when analyzing a complex biological sample containing saturated and unsaturated FFA species with different carbon chain length, peak capacity is important in order to reduce coeluting lipid species. The co-solvent gradient 5% to 25% methanol in 0.1% formic acid was used for further analysis.

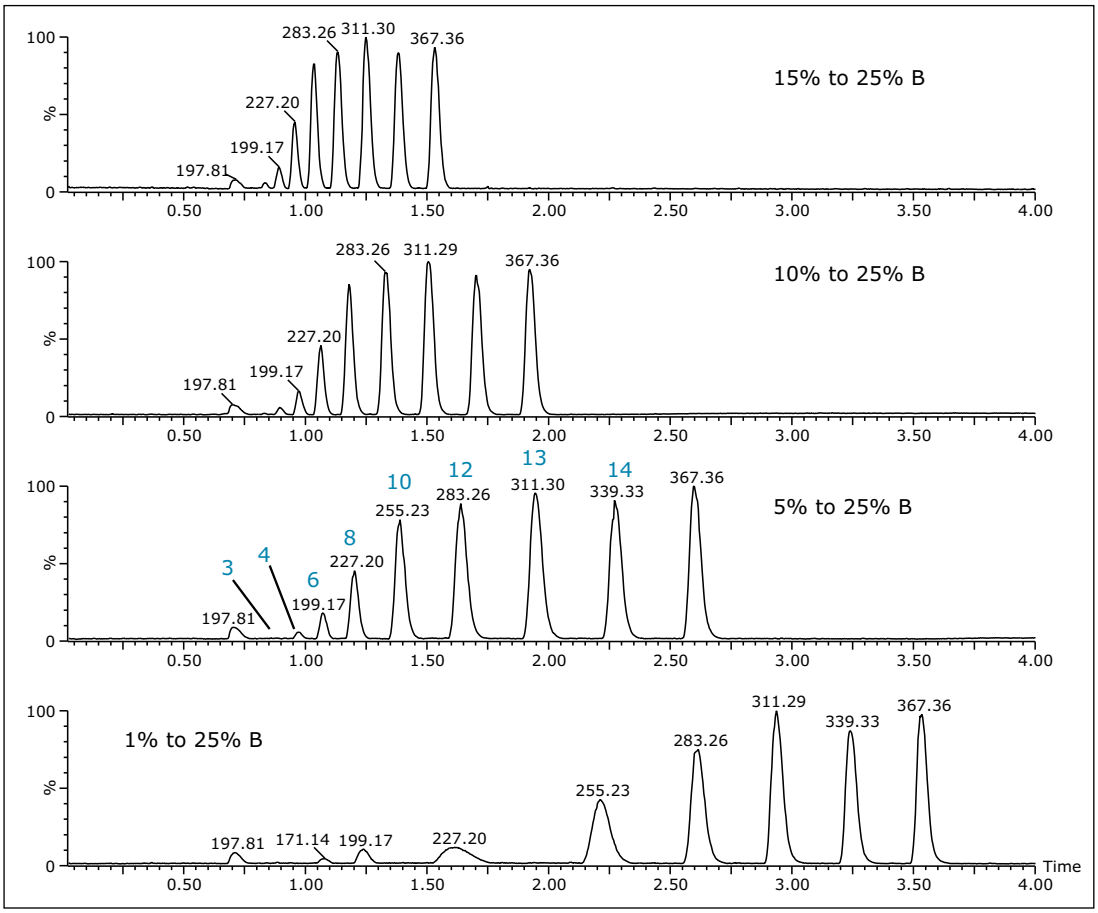


*Figure 2. The separation of saturated FFA with carbon chain length C8-C24 with various co-solvent gradient. For the lipid ID, see Table 1.*

## Analysis of complex saturated and unsaturated FFA standards GLC-85

Reversed-phase chromatography separates lipids according to both chain-length and degree of unsaturation. The problem lies in the fact that the dual nature of the reversed-phase separation process (a double bond in the fatty acyl chain reduces the retention time and the fatty acyl chain length increases the retention time) can hamper the analysis of real samples; the number of components is often so great that identification becomes difficult due to coelution (Figures 3A and B).

On the other hand, by using the precursor exact mass, corresponding product ion information and ion mobility (separation of lipid ions in the gas phase according to their size and molecular shape), each coeluting peak can be extracted and identified.

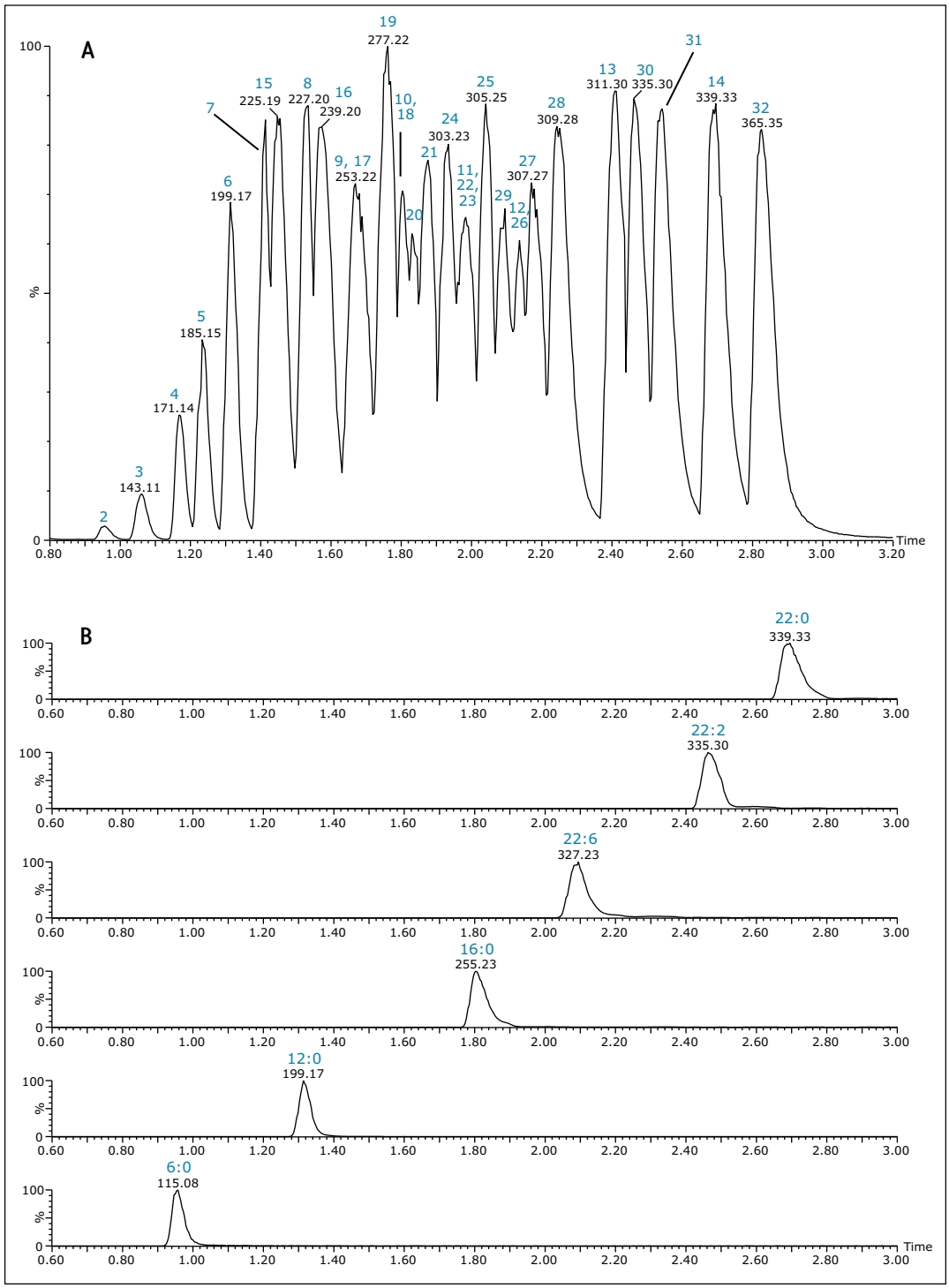


*Figure 3. A) The separation of complex standard mixture that contains saturated, unsaturated, short and long chain 32 different FFA species. B) The separation depends on both chain length and degree of unsaturation. In an RP separation, the fatty acyl chain length increases the retention time and the number of double bonds in the fatty acyl chain decreases the retention time. For the lipid ID, see Table 1.*

Another benefit of the method is the ability to separate between lipid isomers. FFA can have different biological functions based on the double bond position (e.g., omega-3 and omega-6). Figure 4 shows the separation of FFA isomers based on the position of the double bond. The separation of 18:3 (Δ6,9,12) and 18:3 (Δ9,12,15); and 20:3 (Δ8,11,14) and 20:3 (Δ11,14,17) isomers have been observed.

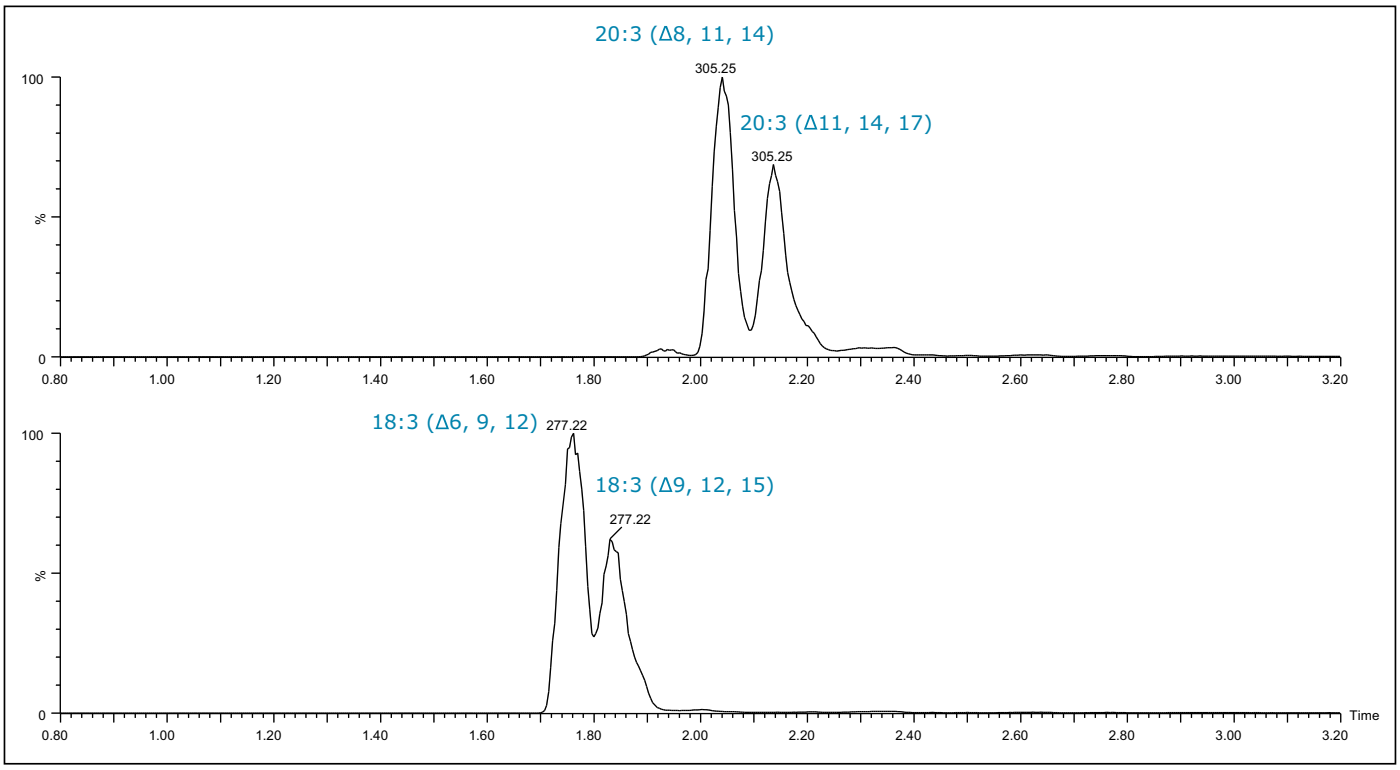


*Figure 4. Extracted ion chromatogram (from figure 3) showing the separation of isobaric lipid species based on the position of the double bond.*

## Biological application and data analysis using TransOmics

The developed UPC$^2$/Xevo G2 QTof MS method was applied with minor modifications for the profile of FFA in algae and algaenan extracts treated at low (310 °C) and high (360 °C) pyrolysis temperatures.

Algaenan is a non-hydrolyzable, insoluble biopolymer in the cell walls of several green freshwater and marine microalgae.[3] Figure 5 shows a representative chromatogram from algaenan 1 with the UPC$^2$ conditions used for the analysis. For complete analysis of the data, set the gradient 1% to 10% co-solvent mobile phase B (methanol in 0.1% formic acid) in 10 minutes was used.

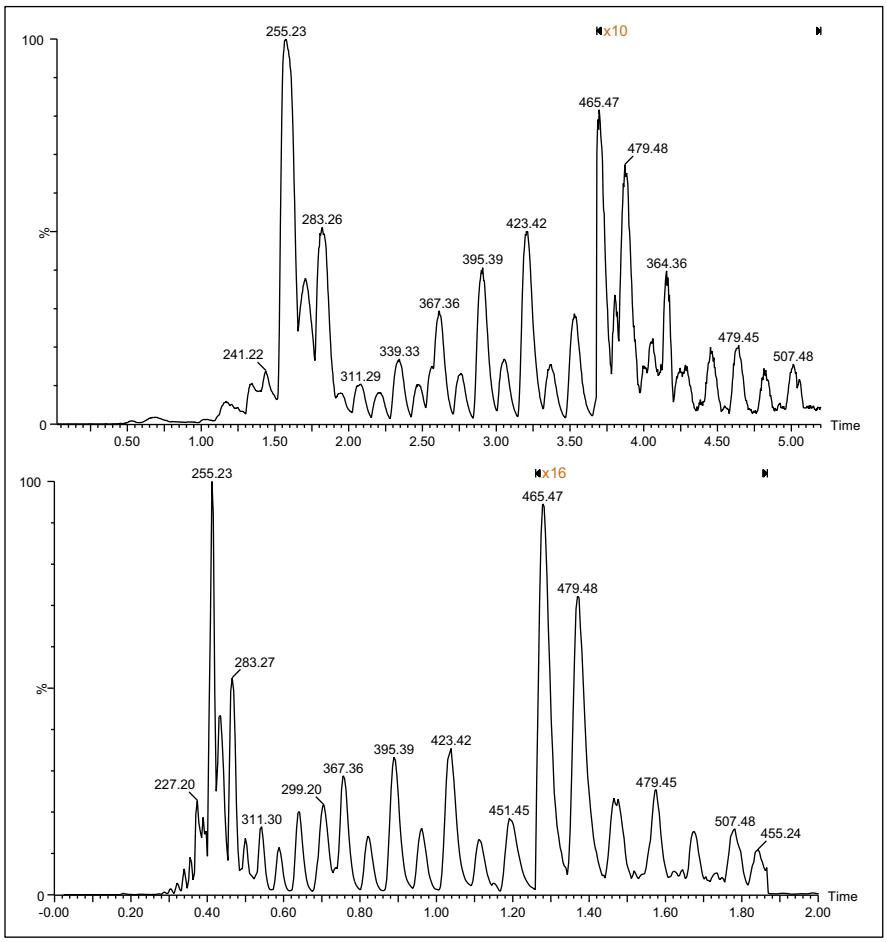


*Figure 5. Representative chromatogram from algaenan 1 with various co-solvent gradients (top 1% to 10% methanol in 10 minutes, lower 5% to 20% methanol in 10 minutes). (UPC$^2$ conditions: HSS $C_{18}$ SB (2.1 x 100 mm), flow rate= 1.5 mL/min. The other UPC$^2$ conditions are described in the method conditions).*

The lipid profiles of the algae and algaenan oil were investigated using TransOmics (TOIML) Software to determine the pattern and composition of FFA at two different pyrolysis temperatures. Differential analysis of results across different treatments can quickly be performed, thereby facilitating identification and quantitation of potential biomarkers. The software adopts an intuitive workflow approach to performing comparative UPC$^2$/Xevo G2 QTof MS metabolomics and lipidomics data analysis.

The workflow starts with UPC$^2$/MS raw data file loading, then retention time alignment and deconvolution, followed by analysis that creates a list of features. The features are then identified with compound searches and explored using multivariate statistical methods.

Principal component analysis (PCA) was used in the first instance to identify the combination of the FFA species that best describe the maximum variance between algae 1, algae 2, algaenan 1, and algaenan 2 oils (Figure 6). The PCA plot showed excellent technical UPC$^2$/MS measurements. The PCA plot effectively displays the inter-sample relationships in multi-dimensional hyperspace, with more similar samples clustering together and dissimilar samples separated.[4]

The clustering in Figure 6 indicates that algae 1 and algaenan 1 are different, but algae 2 and algaenan 2 have more similarity in their FFA compositions after high pyrolysis temperature treatment. Orthogonal projections latent structure discriminant analysis (OPLS-DA) binary comparison can be performed between the different sample groups (algae 1 vs. algae 2, algaenan 1 vs. algaenan 2, algae 1 vs. algaenan 1, and algae 2 vs. algaenan 2) to find out the features that change between the two groups.

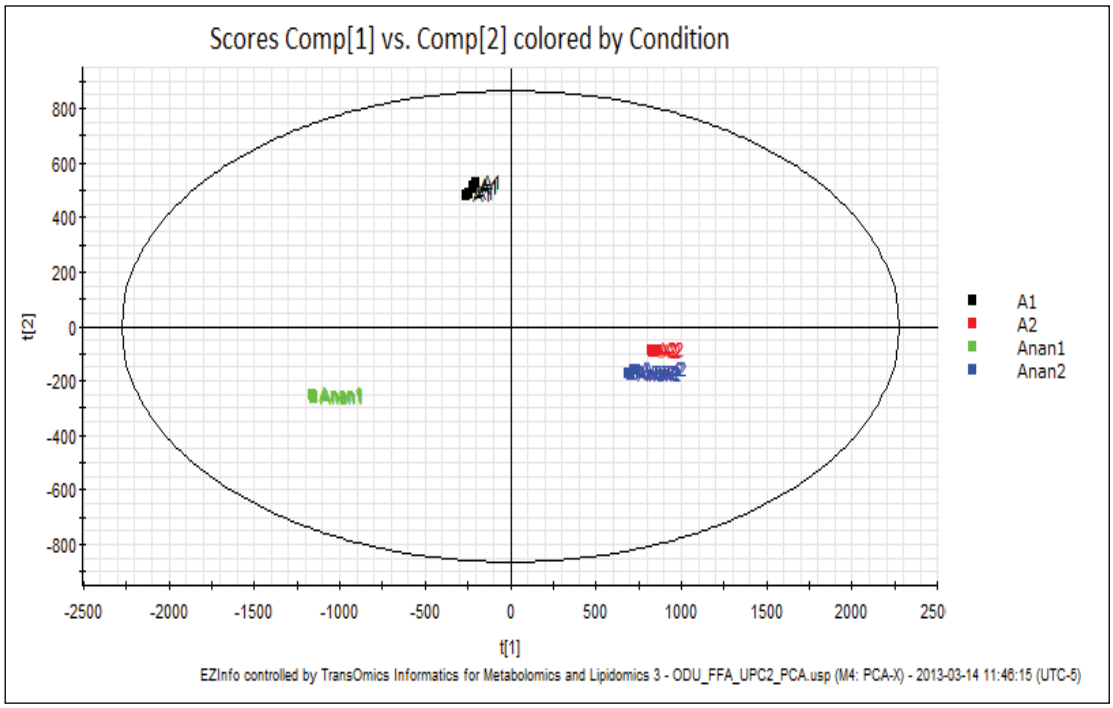


*Figure 6. Principal component analysis of algae and algaenan oil extracts treated at low and high pyrolysis temperature. (A1= algae at low pyrolysis temperature A2= algae at high pyrolysis temperature Anan1= algaenan at low pyrolysis temperature Anan2= algaenan at high pyrolysis temperature).*

As an example, the OPLS-DA binary comparison between algae 1 vs. algae 2 is shown in Figure 7A. As shown in the S-plot, the features that contribute most to the variance between the two groups are those farthest from the origin of the plot, highlighted in red (Figure 7B). These selected features can be exported to TransOmics for further identification. This helps the researcher focus on the features/compounds that change between samples instead of spending time on the whole data set.

Figures 7C and 7D show representative trend plots that change most between algae 1 and algae 2. Figure 8A shows the ion map, mass spectrum, and chromatogram across all the runs for FFA 29:0. This view allows to review compound measurements such as peak picking and alignment to ensure they are valid across all the runs. Figure 8B shows the normalized abundance of FFA 29:0 across all the conditions. FFA 29:0 is elevated in algeanan 1 compared to algae 1, algae 2, and algeanan 2; however, there is no significant difference between algae 2 and algeanan 2. Detailed investigation and comparison between algae 1 and algae 2 showed that algae 1 contains elevated levels of short (C9:0 to C13:0) and long (C31:0 to C37:0) chain FFA, whereas algae 2 contains elevated levels of medium (C14:0-C29:0) chain FFA. Similarly, the comparison between algaenan 1 and algaenan 2 showed that algaenan 1 contains elevated levels of long (C28:0 to C37:0) chain FFA, whereas algaenan 2 contains elevated levels of short and medium (C9:0 to C27:0) chain FFA.

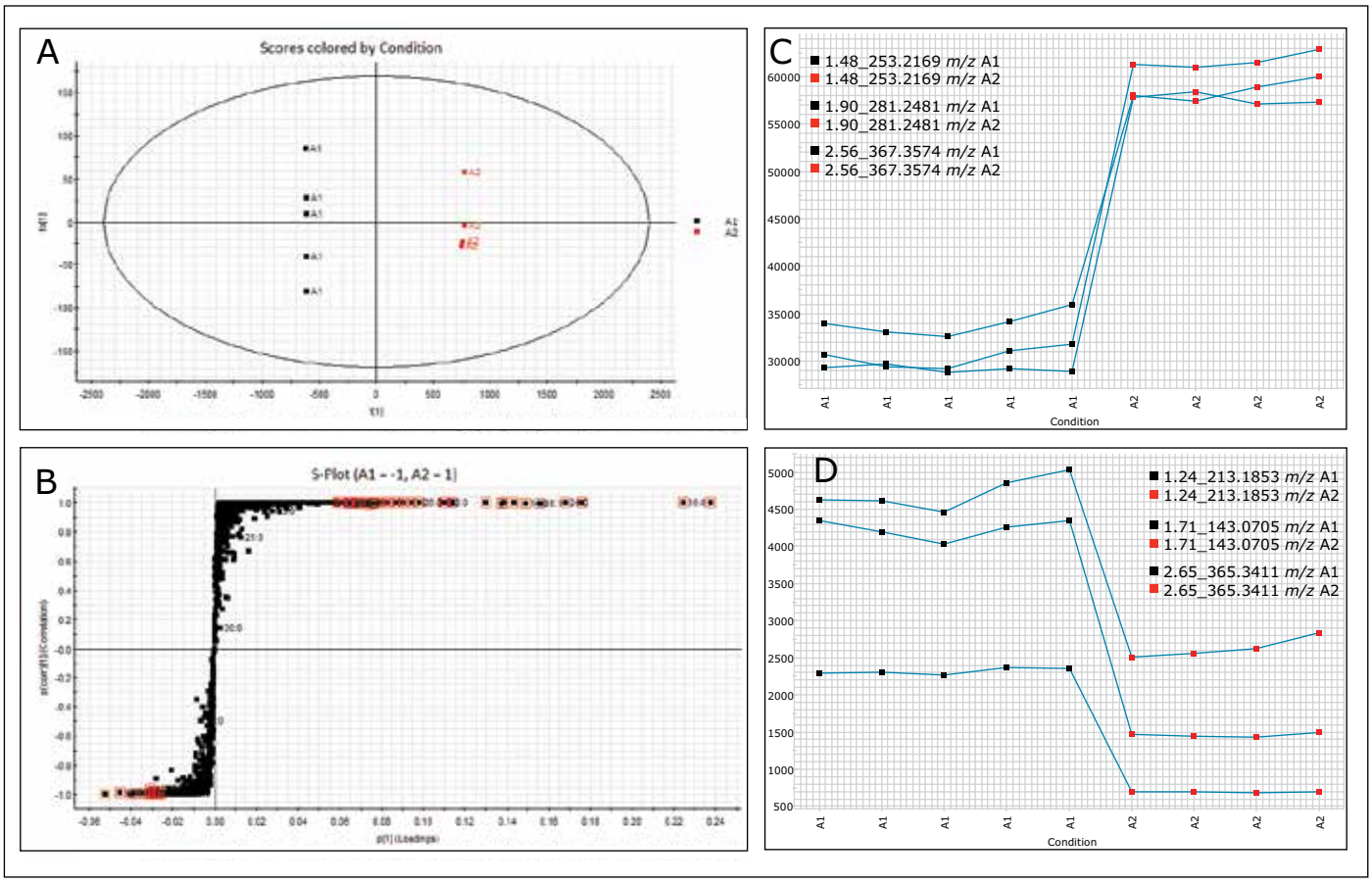


*Figure 7. (A) OPLS DA plot between algae 1 and algae 2 group difference. (B) S-plot indicating the major features (highlighted in red) that contribute to the group difference between algae 1 and algae 2. (C) Representative trend plot showing the major up-regulated 16:1, 18:1, and 24:0 FFA in A1 (D) Representative trend plot showing the major up-regulated 8:0, 13:0, and 24:1 FFA in A2. (A1= algae at low pyrolysis temperature A2= algae at high pyrolysis temperature).*

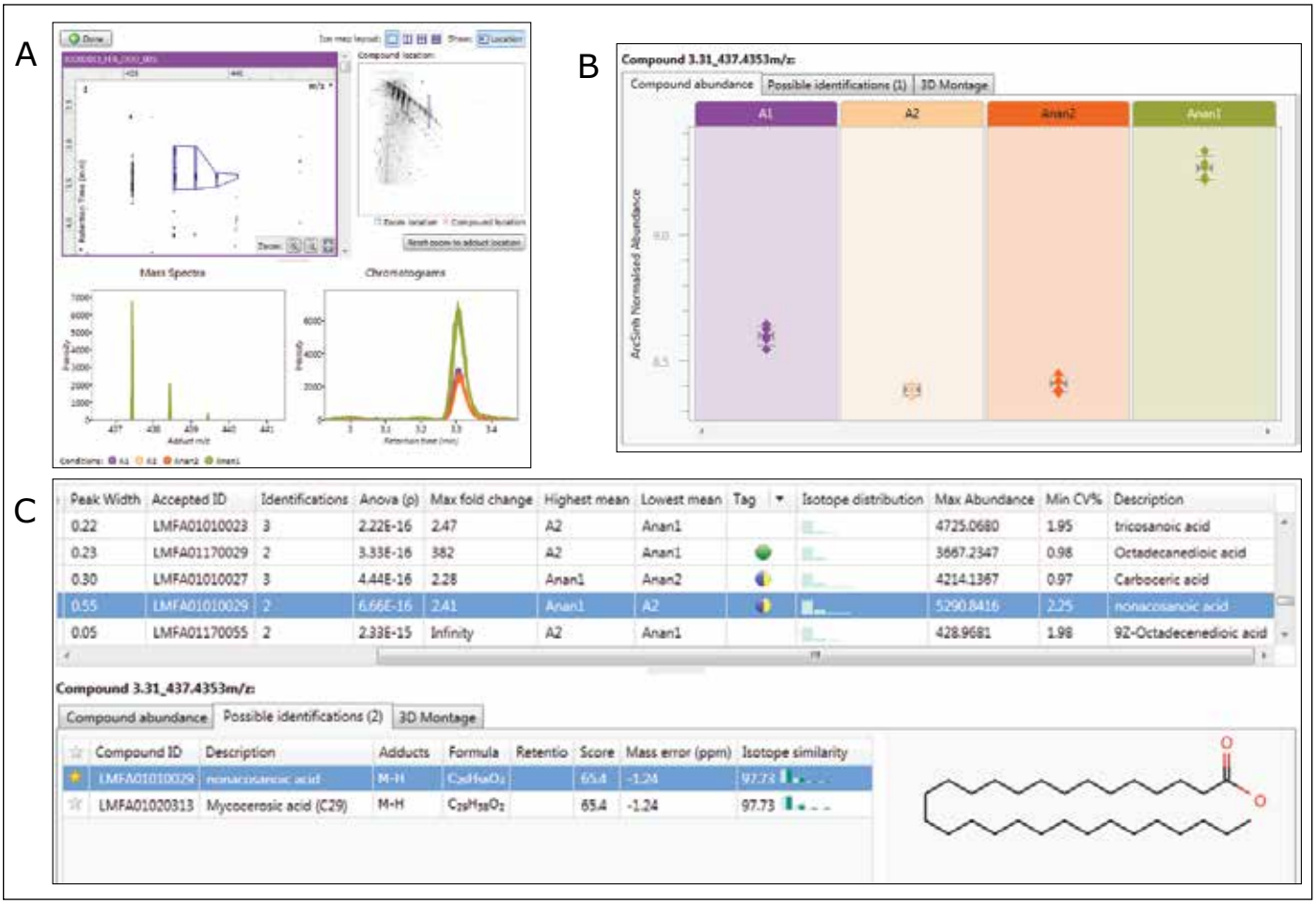


*Figure 8. (A) Selected FFA 29:0 showing its ion map, mass spectrum, and chromatogram across all the runs. (B) Normalized abundance of FFA 29:0 across all the conditions. (C) Identification can be performed by means of local or web-based database search. In this example, the feature with retention time and exact mass pair 3.31_437.4353 is identified as nonacosanoic acid (29:0 FFA). (A1= algae at low pyrolysis temperature, A2= algae at high pyrolysis temperature; Anan1= algaenan at low pyrolysis temperature Anan2= algaenan at high pyrolysis temperature).*

Identification can be performed by means of local or web-based (such as LIPID MAPS, HMDB, and METLIN) compound searches based on retention time, low energy exact mass, high energy fragment ion, theoretical isotope pattern distribution, and collision cross section area (CCS) (Figure 8C). In this example, the feature with retention time and exact mass 3.31_437.4353 is identified as nonacosanoic acid (29:0 FFA) based on retention time, low energy exact mass, and theoretical isotope pattern distribution. Figure 9 shows the expression and abundance profile of selected features according to their relative similarity between the different groups.

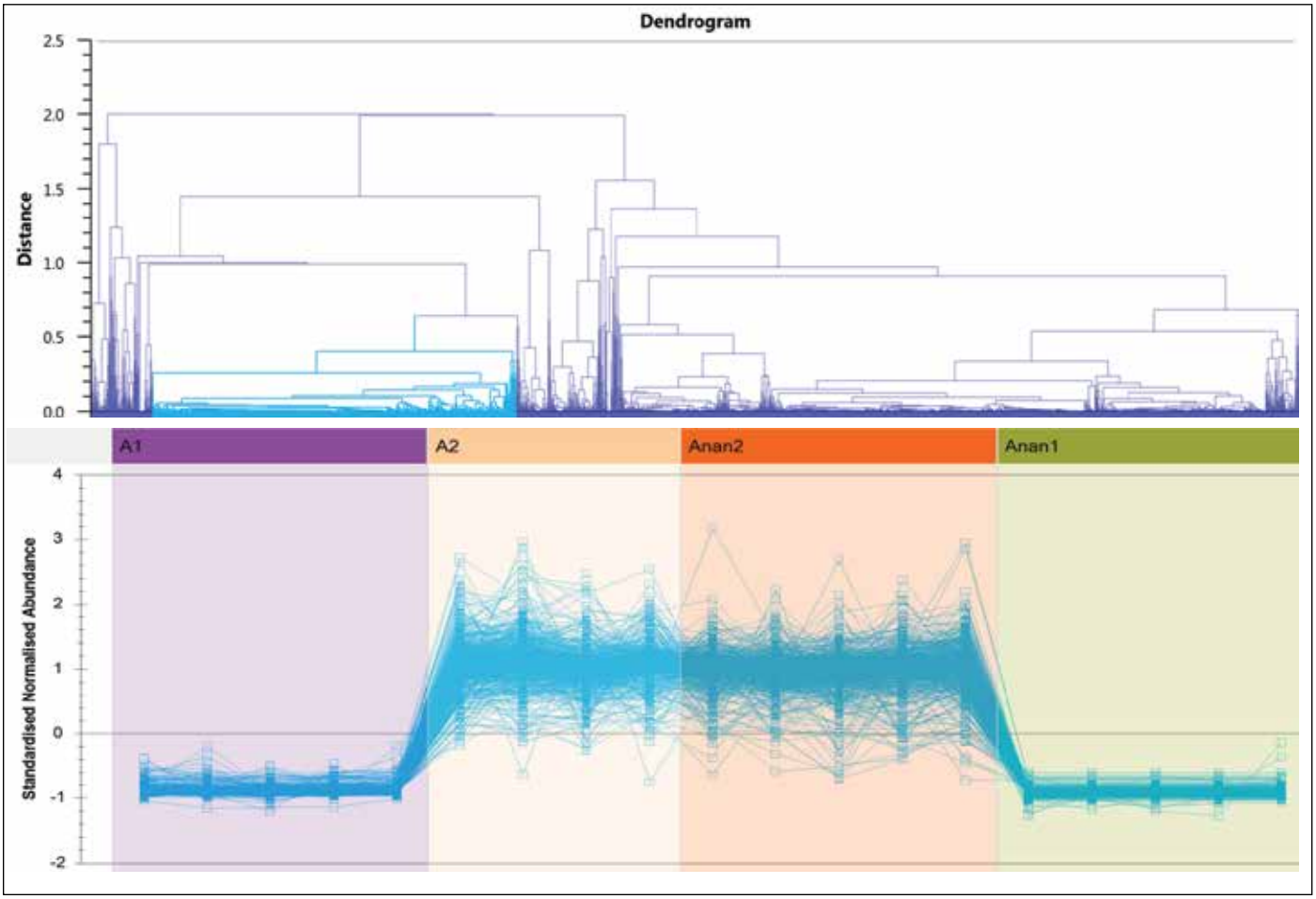


*Figure 9. Expression and abundance profile of selected features according to their relative similarity between the different groups.*

## CONCLUSIONS

The UPC$^2$/MS FFA analysis described provides a simple and fast method with a significant reduction in analysis time compared to alternative techniques such as GC/MS, which requires FAME derivatization. In addition, the organic layer extract containing the lipids can be injected directly into the system, omitting the need for solvent exchange for compatibility with reversed-phase LC methods.

Saturated and unsaturated FFA containing $C_8$ to $C_{36}$ carbons were separated and determined, including low volatile very long chain fatty acids (>24 carbon atoms) that have challenged GC/MS even after FAME derivatization. Data analysis and FFA identification was facilitated using TransOmics for Metabolomics and Lipidomics Software that adopts an intuitive workflow approach to performing comparative ACQUITY UPC$^2$/Xevo G2 QTof MS metabolomics and lipidomics data analysis.

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[ TECHNOLOGY BRIEF ]

# Bile Acid Profiling Using UltraPerformance Convergence Chromatography (UPC²) Coupled to ESI-MS/MS


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## GOAL

Develop a novel method for rapid profiling and quantification of bile acids using UltraPerformance Convergence Chromatography™ (UPC²™).

## BACKGROUND

Bile acids play an important role as the signaling molecules that regulate triglyceride, cholesterol, and glucose metabolism.[1] These signaling pathways have become the source of attractive drug targets for metabolic diseases. Also bile acids are used as biomarkers in serum to interpret liver diseases and the mechanism of bile acid-regulated metabolism.[2–5] Therefore, an analytical method that determines the bile acid profile in the body is beneficial.

The separation of bile acids is complex due to the presence of structural analogues including isomeric forms, and the polarity diversity between unconjugated and conjugated forms. In the past, gas chromatography (GC) and liquid chromatography (LC) have been used to analyze these compounds; however, several limitations have been observed. GC always requires exhaustive derivatization steps which lead to a loss in bile acids at each step. In addition, aliquots of the samples have to be extracted separately to determine the composition or the concentration of conjugated bile acids in GC analysis.

**UltraPerformance Convergence Chromatography combined with ESI-MS/MS enables the simultaneous profiling of 25 bile acids within 13 minutes.**

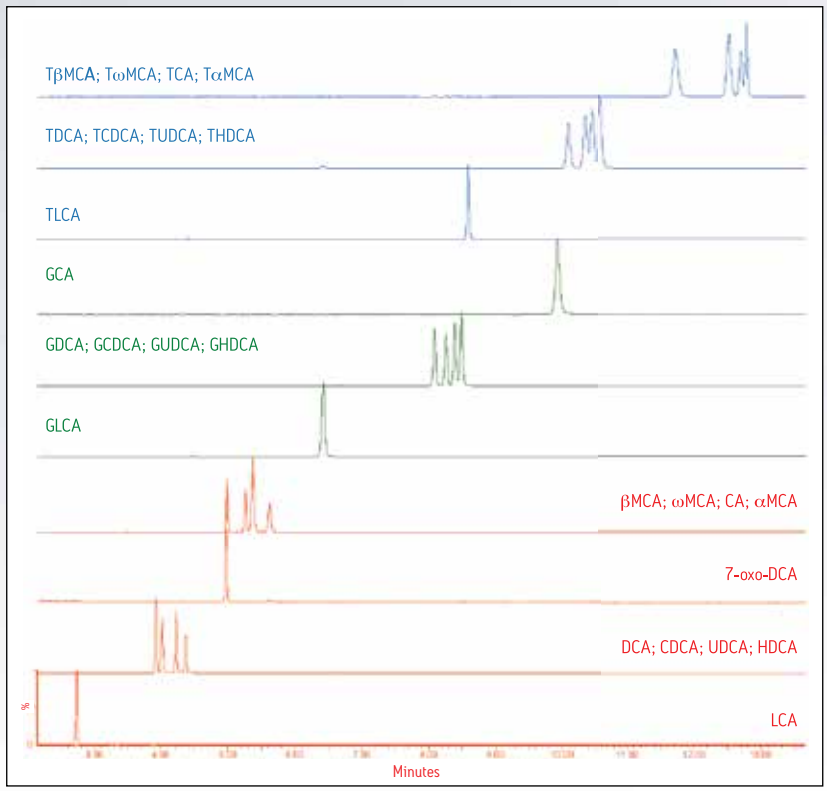


*Figure 1. Simultaneous analysis of a 25-bile acids standard mixture including glycine and taurine conjugates was achieved within 13 minutes. The compound name is shown in order of retention time on each MRM chromatogram.*

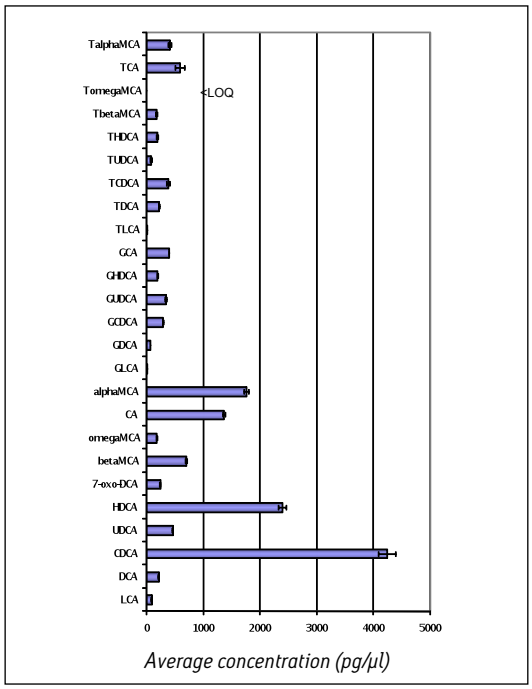


*Figure 2. The quantification of bile acids in rat serum by UPC²/MS (n=6).*

While LC can detect conjugated and unconjugated forms simultaneously, the separation can take up to 30 minutes for a sample. This application note describes a faster UPC²/MS method for bile acid profiling and quantification.

## THE SOLUTION

A method using supercritical carbon dioxide ($SCCO_2$)-based mobile phases was developed on the Waters® ACQUITY UPC²™ System to simultaneously separate 25 different bile acids including their conjugates. The use of $SCCO_2$ as the primary mobile phase allows for faster analyte diffusion and lower back pressure, resulting in faster analysis times. The ACQUITY UPC² System was connected to a Xevo® TQ-S Mass Spectrometer in order to enhance the sensitivity and specificity of the analysis. Separation results for 25 different bile acids (including glycine and taurine conjugates) are shown in Figure 1. All bile acids were separated within 13 minutes, which is approximately two-fold faster than previous methods of analysis in bile acid profiling. This method was optimized through systematic investigation of different stationary phases, column temperature settings, additives, and pH in the modifier. The detection mode using the Xevo TQ-S was ESI negative. No make-up flow resulted in maximum sensitivity.

The developed method was applied to the quantitation of bile acids and their conjugates in a real biological sample (rat serum). Commercially purchased rat serum was deproteinized using methanol in a ratio of 3:1 methanol/serum, vortexed, and centrifuged. The supernatant was then injected onto UPC²/MS/MS in order to determine the amount of each bile acid and their conjugates present in the serum sample. Figure 2 shows the results, indicating that this method can reproducibly determine the levels of individual bile acids and their conjugates in biological fluids.

## SUMMARY

The use of UltraPerformance Convergence Chromatography (UPC²) in conjunction with ESI-MS/MS enables the simultaneous profiling of 25 bile acids including conjugated forms in biological fluids. The profiling is achieved within 13 minutes by a sub-2-µm particle column on the system with acceptable resolution of the analytes. This application demonstrates that this novel separation method is highly suitable for determining the effects of bile acid levels in triglyceride, cholesterol, and glucose metabolism and has a potential to speed up the development of novel drug targets for metabolic diseases.

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# Method Development for the Analysis of Endogenous Steroids Using Convergence Chromatography with Mass Spectrometric Detection

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Waters Corporation, Milford, MA, USA

## APPLICATION BENEFITS

- Analysis of endogenous steroids in approximately two minutes
- Minimal sample prep without requiring sample derivitization
- Ability to separate compounds that are structurally very similar
- 50% to 95% reduction in analysis time relative to GC and LC methods enables high-throughput analysis
- Compatible with UV and mass spectrometric detection

## WATERS SOLUTIONS

ACQUITY UPC²™ System

ACQUITY UPC² BEH Column

Xevo® TQ Mass Spectrometer

Empower® 3 Software

MassLynx® Software

LCMS Certified Maximum Recovery Vials

## KEY WORDS

Steroid, hormone, metabolic, clinical research, UPC², method development, convergence chromatography

## INTRODUCTION

Steroid biosynthesis is a complex metabolic pathway utilizing simple precursors to synthesize multiple steroidal forms. This biosynthetic pathway is unique to animals and provides a common target for antibiotics and other anti-infective drugs. Precise and accurate steroid analysis is critical for the development of steroid-based therapeutics. For mass spectrometric analysis of steroids, due to their structural similarity, chromatographic separation of the steroids is essential prior to analysis. Typical research analyses utilize either gas (GC/MS) or liquid (LC/MS) chromatographic methods. GC/MS methods require sample derivitization prior to analysis resulting in analysis times of approximately 40 minutes.[1] For LC/MS methods, typical analysis times are approximately 12 minutes for HPLC, or four to five minutes with the use of more recent UHPLC methods.[2,3]

This study focuses on the application of convergence chromatography (CC), utilizing liquid $CO_2$ as the primary mobile phase, for the rapid chromatographic analysis of endogenous steroids (structures shown in Figure 1).

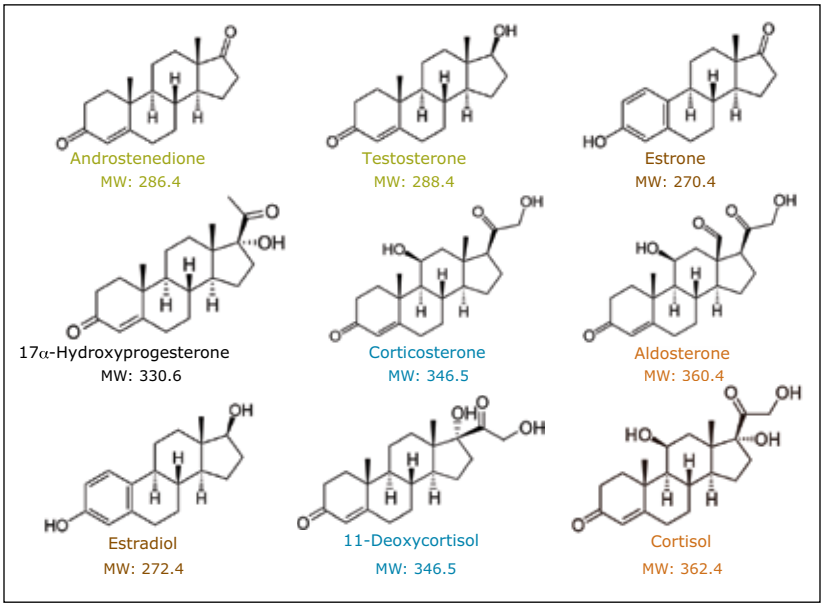


*Figure 1. Structures of steroids evaluated. Compounds with the same color font indicate compounds with similar molecular weights that generate similar MS fragments.*

## EXPERIMENTAL

### Sample description

Column screening (UV detection): A mixture of nine steroid standards was prepared at 0.2 mg/mL each, using a diluent of 88:12 methanol/ethanol.

Mass spectrometer evaluations: Often times, matrix interferences can limit the applicability of a technique. For this reason, standards were evaluated in a human plasma matrix. However, to insure these evaluations were indicative of technique sensitivity and not affected by recovery issues during sample preparation, the plasma samples were post-spiked after a 3:1 acetonitrile protein crash of the plasma. After centrifugation, the supernatant was collected and spiked with a mixture of the nine steroid standards. Spiking of steroids to various levels was achieved by serial dilution of the sample with additional crashed plasma.

### Method conditions

Screening columns:

ACQUITY UPC$^2$ BEH
1.7 µm, 3.0 x 50 mm
(p/n 186006562)

ACQUITY UPC$^2$ BEH 2-Ethylpyridine
1.7 µm, 3.0 x 50 mm
(p/n 186006580)

ACQUITY UPC$^2$ CSH Fluoro-Phenyl
1.7 µm, 3.0 x 50 mm
(p/n 186006571)

ACQUITY UPC$^2$ HSS $C_{18}$ SB
1.8 µm, 3.0 x 50 mm
(p/n 186006621)

### UPC$^2$ conditions

| | |
|---|---|
| System: | ACQUITY UPC$^2$ with PDA detector |
| Mobile phase A: | $CO_2$ (tank, medical grade) |
| Modifier B: | Methanol (Fisher Optima grade) |
| Column temp.: | 40 °C |
| ABPR: | 1800 psi |
| Gradient: | 2% to 17% modifier B in two minutes, re-equilibration at 2% modifier B for one minute |
| Flow rate: | 3.65 mL/min |
| UV detection: | 220 nm (compensated 380 to 480 nm) [40 pts/s] |
| Injection volume: | 1 µL |
| Needle wash: | 50:50 methanol/2-propanol |
| Seal wash: | Methanol |

### Data management

Empower 3 Software

### Make-up flow pump conditions

| | |
|---|---|
| Solvent: | Methanol with 2.5% water and 0.1% ammonium hydroxide |
| Flow rate: | 0.4 mL/min |

### MS conditions

| | |
|---|---|
| Mass spectrometer: | Xevo TQ |
| Capillary voltage: | 1 kV |
| Desolvation temp.: | 500 °C |
| Desolvation gas flow: | 750 L/h |
| Data management: | MassLynx Software |

Here, we present data collected with the ACQUITY® UltraPerformance Convergence Chromatography™ (UPC²®) System. In combination with stationary phases designed specifically for UPC², based on the bridged ethylene hybrid, BEH Technology,™ this technique results in the analysis of steroids in approximately two minutes. After initial method development using UV detection, the system was coupled to a tandem quadrupole mass spectrometer for analysis of steroid-spiked plasma samples. In addition to the significant reduction in analysis time relative to other techniques, convergence chromatography minimizes the consumption of mobile-phase solvents (e.g., methanol), thereby generating less waste for disposal and significantly reducing the cost of analysis per sample.

## RESULTS AND DISCUSSION

A generic two-minute screening gradient was used to evaluate the separation of the nine-steroid mixture on four different stationary phases to determine which would provide the best separation. The chromatograms in Figure 2 demonstrate the selectivity differences of the ACQUITY UPC² stationary phases, as well as the inherent speed of this chromatographic technique. Based on these results, the ACQUITY UPC² BEH stationary phase was chosen for additional application development with mass spectrometric detection.

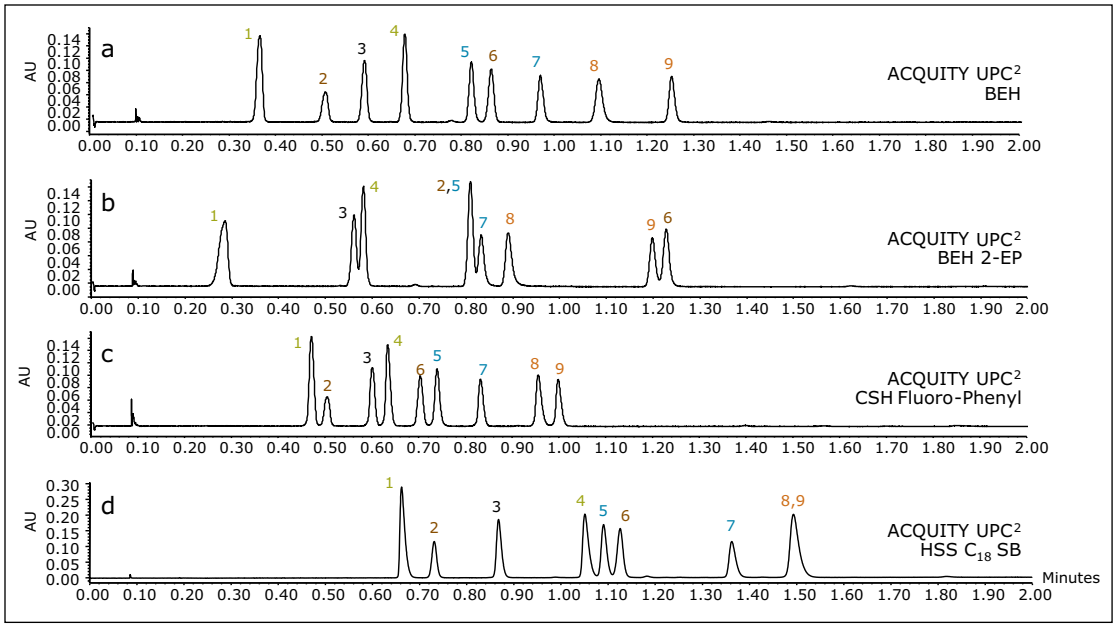


*Figure 2. UPC² separations (UV) of steroid standards on ACQUITY UPC² columns including: (a) BEH, (b) BEH 2-EP, (c) CSH™ Fluoro-Phenyl, and (d) HSS $C_{18}$ SB. All columns were 3.0 x 50 mm, 1.7-µm configurations except for the HSS $C_{18}$ SB which is a 1.8-µm particle size. Steroid compounds are the following: (1) androstenedione, (2) estrone, (3) 17a-OHP [17α-hydroxyprogesterone, (4) testosterone, (5) 11-deoxycortisol, (6) estradiol, (7) corticosterone, (8) aldosterone, and (9) cortisol. Colored peak assignments denote compounds with similar molecular weights and m/z fragments.*

Individual mass spectrometer (MRM) transitions were optimized by direct infusion of standards into the Xevo TQ MS using on-board fluidics, without the connectivity of the ACQUITY UPC$^2$ System (Table 1). After optimization of transitions, the mass spectrometer was coupled to the UPC$^2$ system using a mass spectrometer splitter, incorporating the addition of a make-up flow pump, to facilitate sample flow into the MS and subsequent ionization (Figure 3).

| Compound | Precursor | Product | Collision energy | Dwell | Cone voltage | Mode |
|---|---|---|---|---|---|---|
| Estrone | **271.05** | 153.1 | 30 | 0.005 | 25 | **ESI +** |
| | | **253.2** | **15** | | | |
| Androstenedione | **287.05** | 97.15 | 21 | 0.005 | 25 | **ESI +** |
| | | **109.2** | **26** | | | |
| Testosterone | **289.10** | 97.15 | 21 | 0.005 | 25 | **ESI +** |
| | | **109.15** | **26** | | | |
| 17α-Hydroxy progesterone | **331.10** | 97.15 | 21 | 0.005 | 25 | **ESI +** |
| | | **109.1** | **26** | | | |
| | | 313.3 | 16 | | | |
| 11-Deoxycortisol | **347.05** | **97.11** | **24** | 0.005 | 26 | **ESI +** |
| | | 109.14 | 26 | | | |
| Corticosterone | **347.05** | 105.1 | 42 | 0.005 | 24 | **ESI +** |
| | | **121.1** | **28** | | | |
| Aldosterone | **361.05** | 97.15 | 35 | 0.005 | 25 | **ESI +** |
| | | 315.2 | 20 | | | |
| | | **343.2** | **16** | | | |
| Cortisol | **363.05** | **121.2** | **25** | 0.005 | 25 | **ESI +** |
| | | 309.2 | 20 | | | |
| | | 327.2 | 15 | | | |
| Estradiol | **271.00** | **145.1** | **38** | 0.005 | 55 | **ESI -** |
| | | 183.1 | 38 | | | |

*Table 1. Multiple reaction monitoring (MRM) transitions used for the analysis of nine structurally related steroids. MS conditions for the MRM transitions were optimized using IntelliStart™ in infusion mode only (without the UPC$^2$ instrument). MRM transitions in bold are transitions chosen for monitoring.*

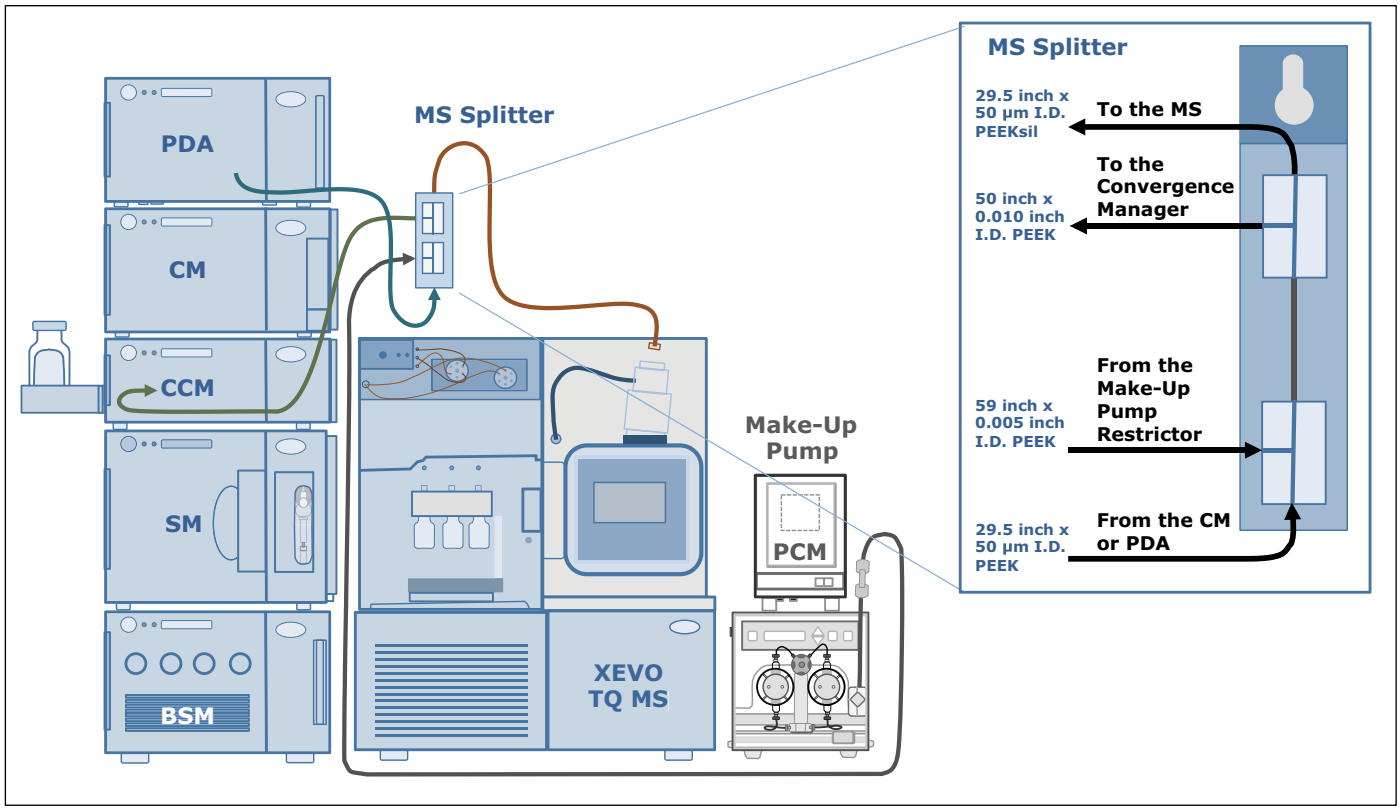


*Figure 3. ACQUITY UPC² System coupled to a Xevo TQ MS, includes a Binary Solvent Manager (BSM), Sample Manager (SM), Convergence Chromatography Manager (CCM), Column Manager (CM), Photodiode Array (PDA) Detector, Make-up Flow Pump with additional Pump Control Module (PCM). The mass spectrometer splitter is used to connect all components together.*

## Optimization of make-up flow

The make-up flow introduced through the mass spectrometer splitter has a dual purpose. It facilitates the post-mixer transfer of the sample through the tubing, as the $CO_2$ in the mobile phase starts to decompress as it reaches the mass spectrometer. This is especially important at low concentrations of the organic modifier in the mobile phase, as seen in the early stages of the current gradient profile. In addition, the use of additives in the make-up flow (such as water, ammonium hydroxide ($NH_4OH$), or formic acid (FA)) can assist in ionization of the analytes within the mass spectrometer source, thereby improving sensitivity. To optimize the make-up flow and additional MS conditions, a plasma sample spiked with the nine steroids (at 50 ng/mL) was used to evaluate various conditions including: additive used in make-up flow solvent, capillary voltage, desolvation temperature, and gas flow. The results of those evaluations are shown in Figure 4.

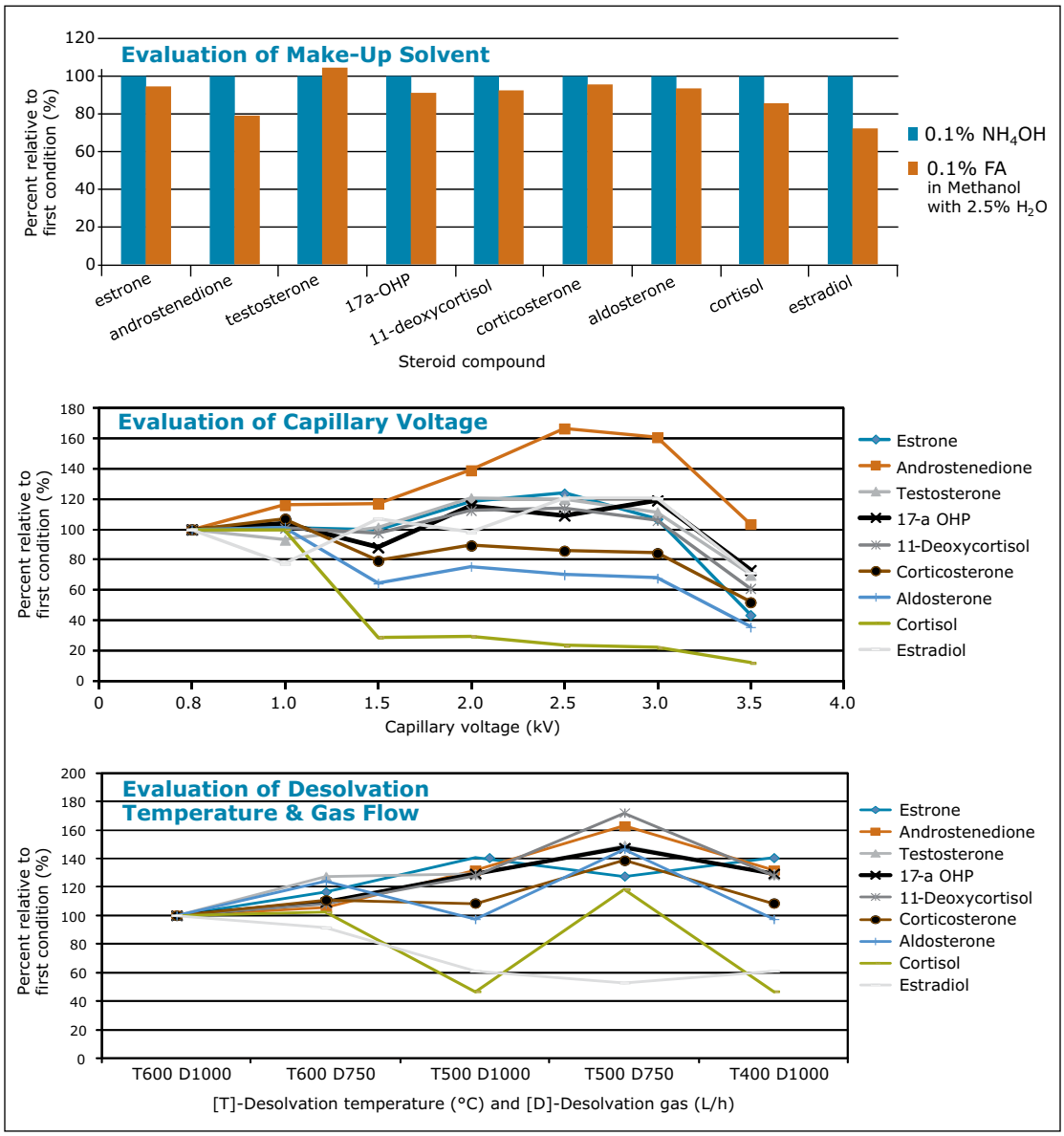


*Figure 4. Optimization of make-up flow solvent, capillary voltage (kV), desolvation temperature (°C), and gas flow (L/h). Values reported are in percentages relative to the first conditions evaluated.*

The top panel of Figure 4 shows eight of the nine steroids yielded higher MS signals (better ionization) when using ammonium hydroxide as an additive in the make-up flow. In addition, most of the steroid signals were ambivalent to capillary voltages between 0.8 and 3.0 kV, as shown in the middle panel of Figure 4. However, at voltages higher than 1.0 kV, the signal for cortisol diminished dramatically. Based on these evaluations, the optimum conditions were determined, with the best overall signal obtained for all steroids using a make-up solvent composed of methanol with 2.5% water and 0.1% ammonium hydroxide, and a flow rate of 0.4 mL/min. The optimum results were obtained by MS when using a capillary voltage of 1.0 kV, with a desolvation temperature of 500 °C, and a gas flow of 750 L/h (bottom panel of Figure 4). The resulting chromatography for the nine steroids post-spiked into the human plasma after protein crash is shown in Figure 5.

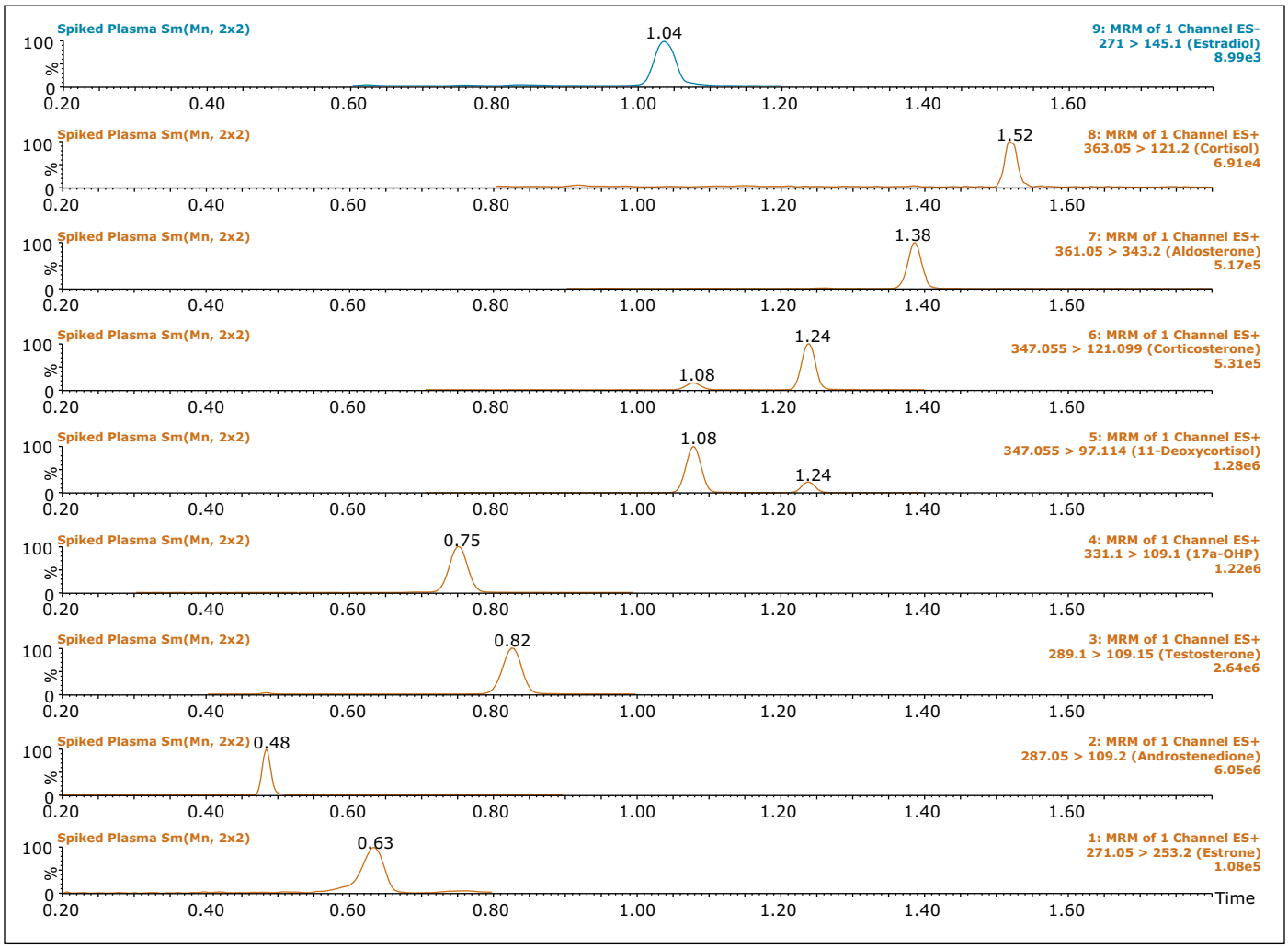


*Figure 5. Total ion chromatograms (TIC) of the nine steroids. Standards were post-spiked at a concentration of 50 ng/mL into a 3:1 acetonitrile protein crash of human plasma.*

## Reproducibility

As in any method development, the accuracy and reproducibility of the method is critical for success. To evaluate reproducibility of the method, the peak areas for the individual steroids were monitored over the course of 100 injections (using 1-µL injection volumes of 50-ng/mL steroid spiked in plasma). The RSD values for the peak areas ranged from 5.6% to approximately 13.7%. A representative example of the reproducibility results are shown in Figure 6. Similar results were obtained for the other steroids evaluated.

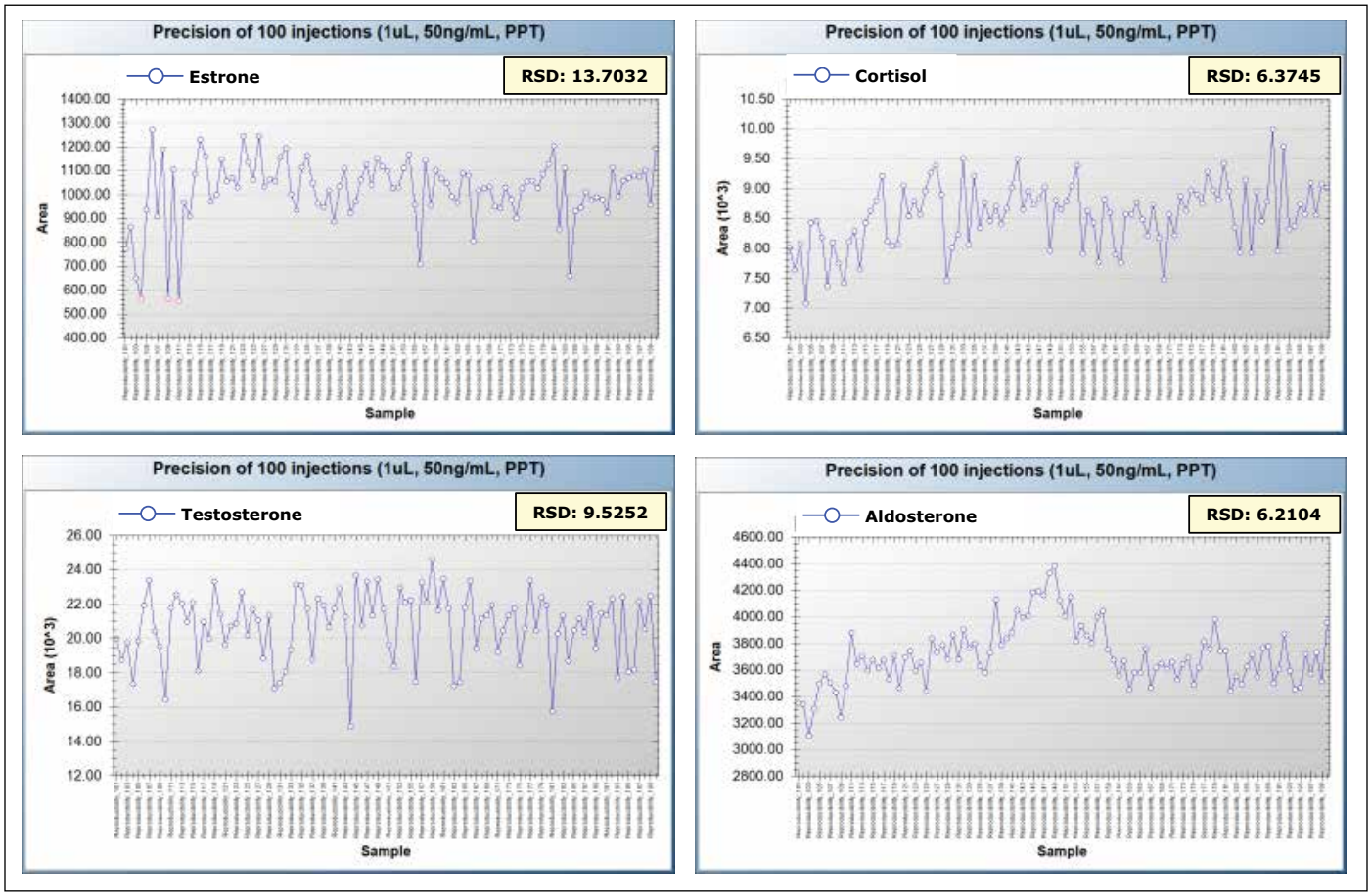


*Figure 6. Examples of injection-to-injection reproducibility over 100 injections (1 µL) of steroid-spiked (50 ng/mL) human plasma for estrone (top left), cortisol (top right), testosterone (bottom left), and aldosterone (bottom right). Peak area RSDs range from approximately 5.6% to 13.7%.*

To evaluate the linearity of response, calibration curves were generated using 5-µL injections of the spiked steroid plasma samples (after 3:1 acetonitrile protein crash). Concentrations of the steroids ranged from 0.98 to 500 ng/mL. Representative calibration curves are shown in Figure 7, with more complete data for each of the steroids shown in Table 2.

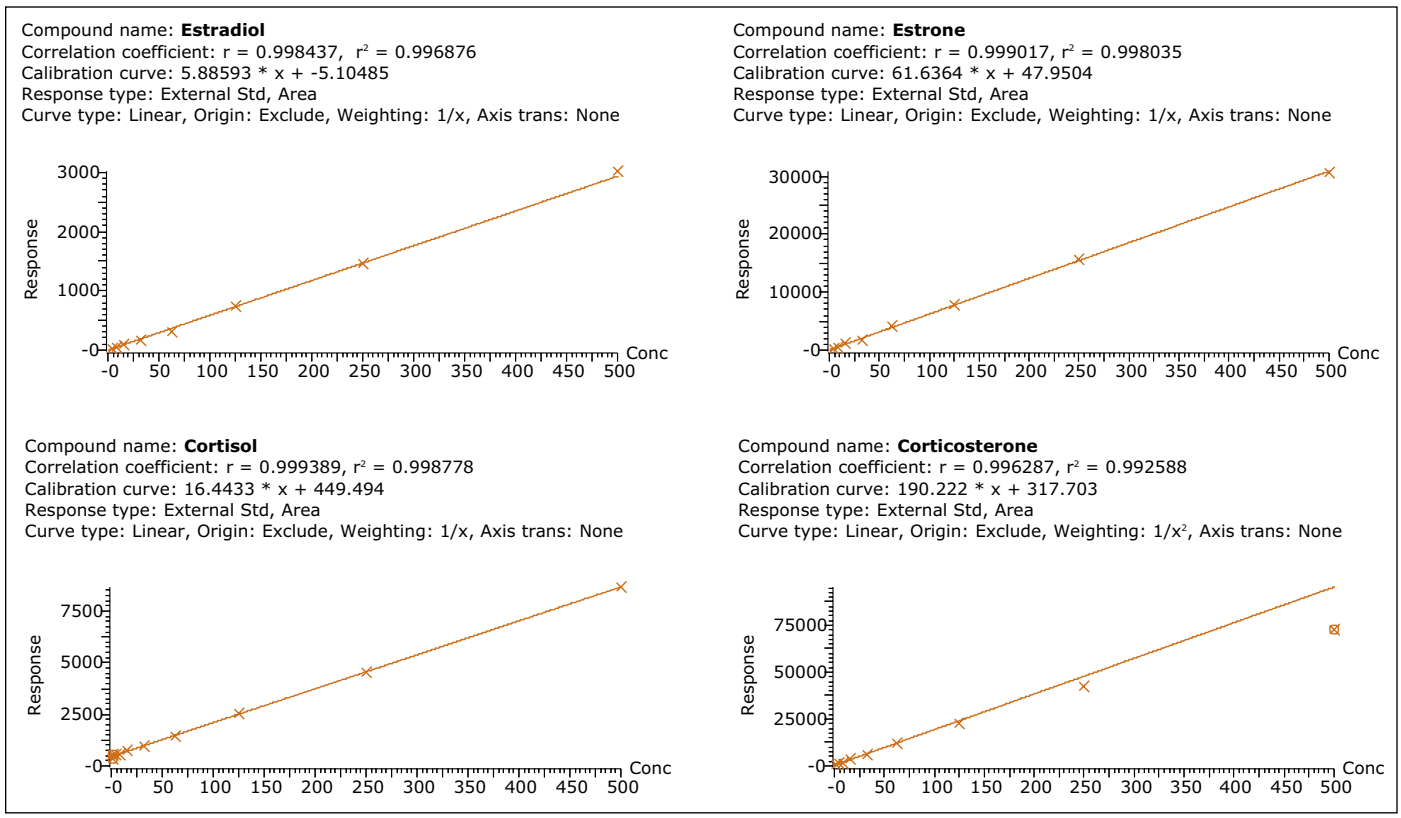


*Figure 7. Four representative calibration curves demonstrating the response linearity. All are 5-µL injections of 3:1 acetonitrile crashed human plasma spiked from 0.98 to 500 ng/mL with steroid standards.*

| Conc (ng/mL) | | Compound (% Dev) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Estradiol | Cortisol | Aldosterone | Corticosterone | 11-deoxy cortisol | 17a-OHP | Testosterone | Androstene-dione | Estrone |
| Std 0.5 | 1.0 | BLQ | E | 10.8 | -4.2 | 1.7 | 18.7 | E | E | BLQ |
| Std 1 | 2.0 | BLQ | E | (44.5) | 0.8 | -8.8 | -10.2 | E | -15.2 | BLQ |
| Std 2 | 3.9 | BLQ | 16.6 | -15.3 | 11.4 | 3.4 | 8.5 | E | -20.9 | -14.9 |
| Std 3 | 7.8 | 23.1 | -26.2 | -9.2 | 4.7 | 12.6 | -2.6 | -5.3 | 28.3 | 4.4 |
| Std 4 | 15.6 | -2.6 | 9.5 | -6.0 | 9.4 | 6.7 | -3.5 | -12.1 | 11.0 | 18.8 |
| Std 5 | 32.3 | -8.5 | 1.3 | 0.9 | -1.3 | -4.4 | -12.2 | 6.1 | (26.4) | -14.9 |
| Std 6 | 62.5 | -16.0 | -3.7 | 12.7 | -2.7 | -0.2 | -5.6 | -1.8 | -1.9 | 6.4 |
| Std 7 | 125.0 | -0.3 | 3.6 | 4.0 | -6.0 | 8.1 | 5.9 | 15.6 | -1.3 | 0.0 |
| Std 8 | 250.0 | -1.2 | -1.0 | 8.8 | -12.1 | -9.0 | 2.0 | 2.4 | (-40.1) | 1.2 |
| Std 9 | 500.0 | 2.9 | 0.0 | -6.6 | (-24.1) | -10.0 | -1.0 | -4.8 | (-20.6) | -1.0 |

%Dev <20%
% Dev 20 - 25%
%DEV >25%

BLQ – Below limit of quantification
E – Rejected standard due to endogenous levels in the blanks
() – Rejected Standard (large deviation)

*Table 2. Percent deviation from the calibration curve for each steroid at each level of spiking.*

## CONCLUSIONS

Convergence chromatography enables fast, accurate analysis of steroids with reduced analysis times relative to current LC and GC methodologies. UPC$^2$ offers scientists a unique workflow, application, and environmental impact benefits, compared to LC and GC platforms, with simplified sample preparation. Samples extracted in organic solvents can be injected without additional steps to exchange solvents for RP-compatible diluents. In addition, with $CO_2$ as the primary mobile phase, the cost of analysis per sample is reduced, using a more environmentally friendly solvent relative to conventional RP methods. This method demonstrates the separation power of convergence chromatography utilizing either UV or MS detection. While the limits of detection and quantification presented here are not compatible with the low levels of steroid concentrations typically found in biological samples (e.g. plasma), additional optimization of MS parameters, with the possibility of additional sample derivitization to improve ionization, may help to reach higher sensitivity levels. This could further enable the application of this method to the analysis of steroids for clinical research.

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# Enantiomeric and Diastereomeric Separations of Fragrance and Essential Oil Components using the ACQUITY UPC$^2$ System with ACQUITY UPC$^2$ Trefoil Columns

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Waters Corporation, Milford, MA, USA

## APPLICATION BENEFITS

- Shorter analysis times compared to chiral GC.
- The 2.5-µm particle chiral stationary phases provide high efficiency enantiomeric separations for fragrance compounds.
- The low system volume and extra-column volume of the ACQUITY UPC$^2$ System enables superior, faster, and more efficient enantiomeric separations of fragrance compounds compared to traditional SFC.
- UPC$^2$ solvents are more compatible with mass spectrometry, compared to those used in normal-phase chiral HPLC, enabling superior real time peak identification.

## WATERS SOLUTIONS

ACQUITY UPC2® Trefoil™

ACQUITY UPC2 System with ACQUITY UPC2 PDA Detector and ACQUITY® TQ Detector

MassLynx® Software

## KEY WORDS

Enantiomers, chiral stationary phase, fragrance, essential oils, UltraPerformance Convergence Chromatography (UPC$^2$), convergence chromatography (CC), Trefoil

## INTRODUCTION

Perception of aroma occurs at the olfactory membrane. This membrane is comprised in part of proteins and carbohydrates, which are chiral in nature. This makes it possible for the olfactory receptors to distinguish between enantiomers. Many enantiomers of fragrance molecules are perceived differently by our sense of smell.[1] For example, carvone is a chiral terpenoid where the R enantiomer smells like spearmint while the S enantiomer has the distinct odor of caraway seed.[2]

Chiral composition of fragrance molecules is important for many industries, including food, cosmetics, and consumer products, in controlling the olfactory perception of products.[1] In addition, chiral analyses are routinely performed to authenticate the natural sources of essential oils. Since naturally chiral sources of essential oils are generally more costly and have a greater perceived health benefit than their synthetic counterparts, rapid chiral analysis allows manufacturers to quickly exclude adulterated products containing inexpensive racemic synthetic materials at the time of purchase.[3]

Historically, chiral separations of fragrance compounds have primarily been carried out using chiral stationary phases (CSPs) in capillary gas chromatography (GC).[2,3,4] The analysis time often ranges from 15 to 50 minutes.[3] More recently, supercritical fluid chromatography (SFC) with CSPs has been applied to these separations, often resulting in comparable resolution and reduced run time.[5,6] Despite the great success in chiral separation by SFC, the associated instrumentation and CSPs have been slow to tap into the technology advancements that have taken place in the HPLC field. For example, one of most significant breakthroughs in HPLC in the past decade is the advent of Waters® UPLC® Technology, which utilizes sub-2-µm particles. ACQUITY UPLC® Systems retain the practicality and principles of HPLC while increasing the overall interlaced attributes of speed, sensitivity, and resolution. Until very recently, the standard particle size for commercially available CSPs has remained 5 µm.

Convergence chromatography is the next evolution in SFC. The Waters ACQUITY UPC$^2$ System is a holistically designed system that has similar selectivity to normal-phase chromatography and is built upon proven UPLC technology.

## EXPERIMENTAL

### Instrumentation

All experiments were performed on an ACQUITY UPC$^2$ System equipped with an ACQUITY UPC$^2$ PDA Detector and an ACQUITY TQ Detector. The system is controlled by MassLynx Software.

### Samples

The standard samples used in this study were purchased from TCI Americas, with their structures shown in Figure 1. Essential oils were purchased from various commercial sources. All samples were dissolved in tert-butyl methyl ether (TBME) for the analyses.

### UPC$^2$ conditions

| | |
|---|---|
| Column: | ACQUITY UPC$^2$ Trefoil AMY1 or CEL1 (2.5 µm, 3.0 x 150 mm) |
| Backpressure: | 1740 psi |
| Temperature: | 40 °C |
| Mobile phase A: | $CO_2$ |
| Mobile phase B: | Isopropanol |
| MS: | APCI positive mode |

Other key parameters are listed in their respective figure captions.

UltraPerformance Convergence Chromatography™ (UPC$^{2®}$) offers minimized system and dwell volume, enabling users to leverage the superior separation power inherent to smaller particle sizes.

We present herein the enantiomeric and diastereomeric separations of four fragrance compounds using Waters ACQUITY UPC$^2$ Trefoil AMY1 and CEL1 Columns on an ACQUITY UPC$^2$ System. Compared to the traditional method of analysis by GC, UPC$^2$ offered similarly high resolution with significantly shorter run times, resulting in improved productivity.

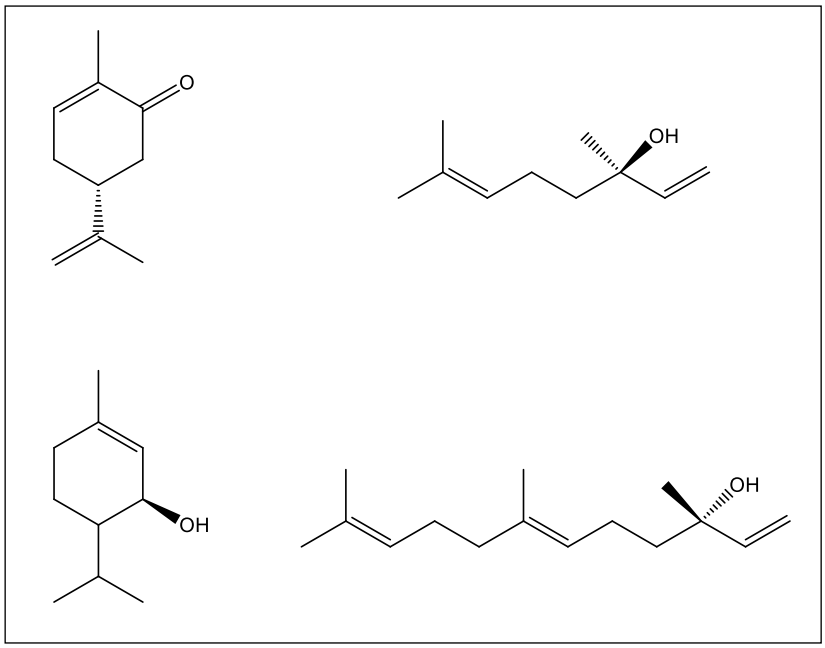


*Figure 1. Structures of the four fragrance compounds presented in this study.*

UltraPerformance Convergence Chromatography™ (UPC$^{2®}$) offers minimized system and dwell volume, enabling users to leverage the superior separation power inherent to smaller particle sizes.

We present herein the enantiomeric and diastereomeric separations of four fragrance compounds using Waters ACQUITY UPC$^2$ Trefoil AMY1 and CEL1 Columns on an ACQUITY UPC$^2$ System. Compared to the traditional method of analysis by GC, UPC$^2$ offered similarly high resolution with significantly shorter run times, resulting in improved productivity.

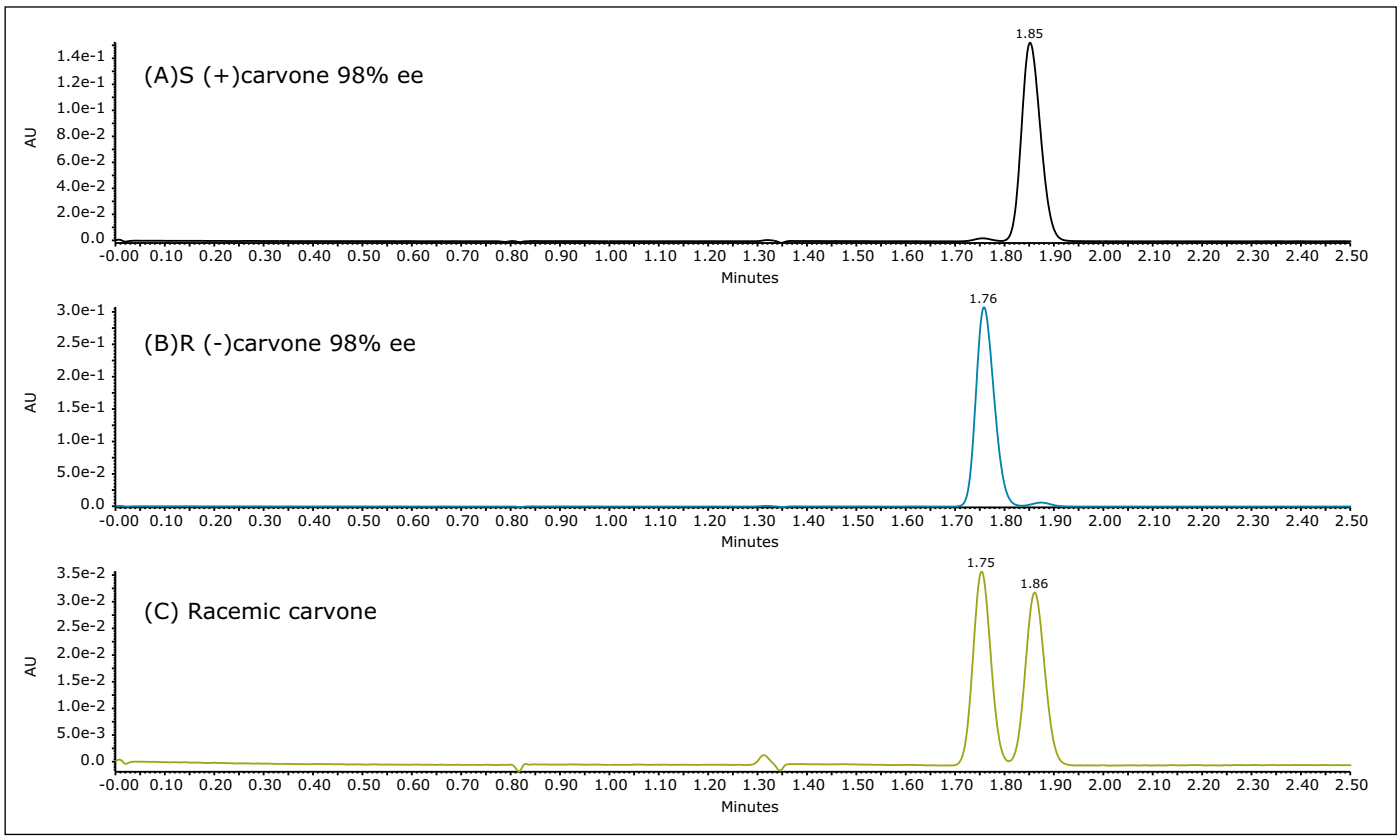


*Figure 2. UPC$^2$-UV chromatograms of the enantiomeric separation of carvone on an ACQUITY UPC$^2$ Trefoil CEL1 Column: (A) S (+) carvone; (B) R (-) carvone; and (C) racemic carvone. An isocratic method with 4% isopropanol was used. The flow rate was 0.9 mL/min.*

Linalool is a terpene alcohol with a soft floral odor, and can be found in different plant extracts. Figure 3A shows the enantiomeric resolution of the linalool standard on an ACQUITY UPC$^2$ Trefoil AMY1 Column. It is noted that the linalool standard was non-racemic (Figure 3A), suggesting the standard was derived from a natural source. The e. e. was estimated to be 40% in favor of the late eluting isomer. Figure 3B is the UPC$^2$-UV chromatogram of a commercially available lavender essential oil obtained under the same condition. The two linalool enantiomers were identified by both retention time and corresponding mass spectra (results not shown). It is noted that MS plays a critical role for the positive identification of the target analytes in a complex matrix. While bearing a similar selectivity to normal-phase LC, UPC$^2$ is inherently advantageous in incorporating MS detection due to its MS-friendly mobile phase. The linalool in this lavender essential oil exhibited a 92% e. e. in favor of the later eluting peak at 2.07 min.

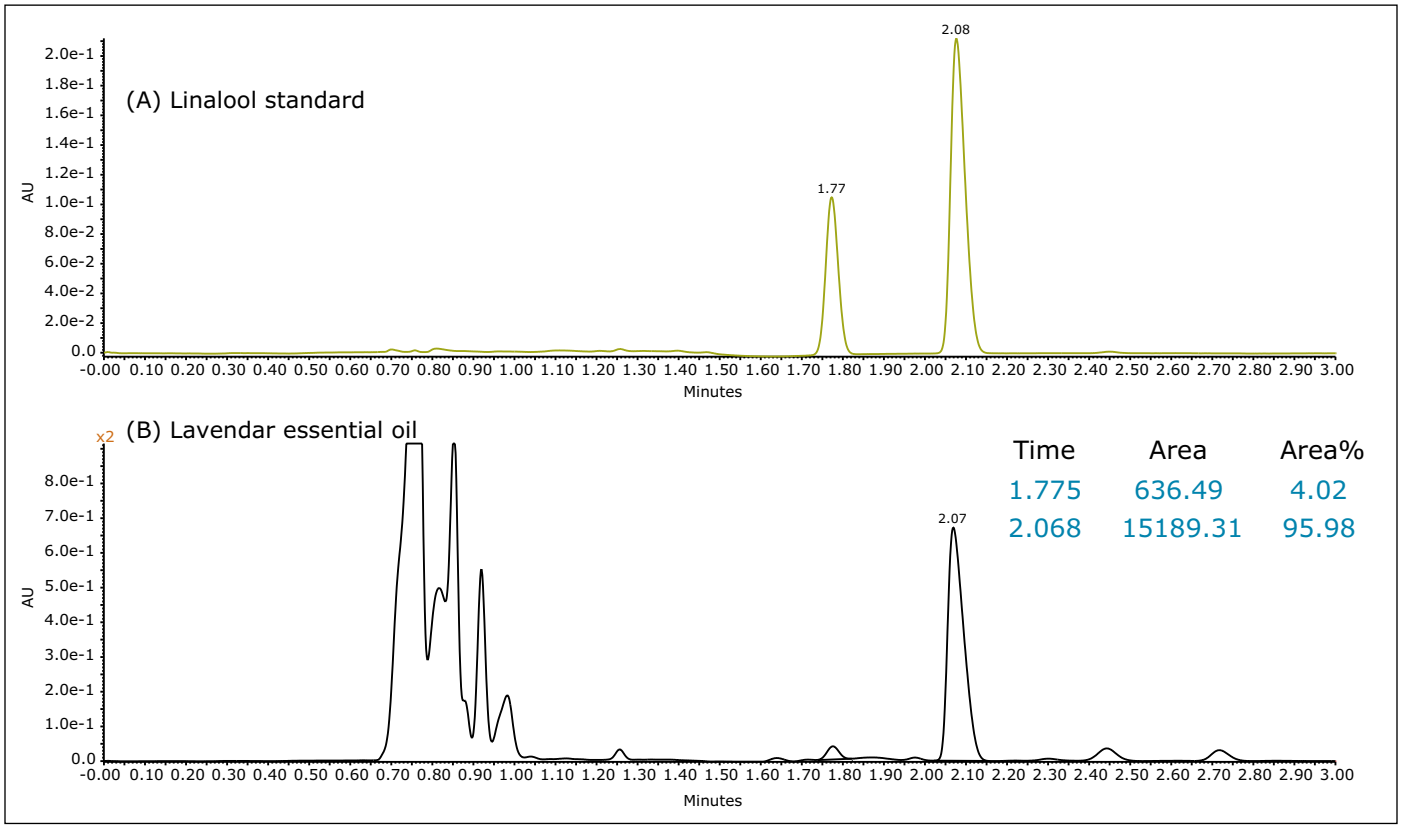


*Figure 3. UPC$^2$-UV chromatograms of (A) linalool standard (B) lavender essential oil on an ACQUITY UPC$^2$ Trefoil AMY1 Column. An isocratic method with 3% isopropanol was used for linalool. The flow rate was 1.0 mL/min.*

Similarly, terpinen-4-ol is a terpene with a pleasant conifer odor, and is a major constituent of tea tree oil. Figure 4A shows the enantiomeric resolution of the two isomers of a terpinen-4-ol standard on an ACQUITY UPC$^2$ Trefoil™ AMY1 Column. The terpinen-4-ol standard was nearly racemic (Figure 4A), suggesting its synthetic origin. Examination of a tea tree essential oil (Figure 4B) revealed that the terpinen-4-ol exhibited a 37% e. e. in favor of the early eluting isomer at 1.95 min.

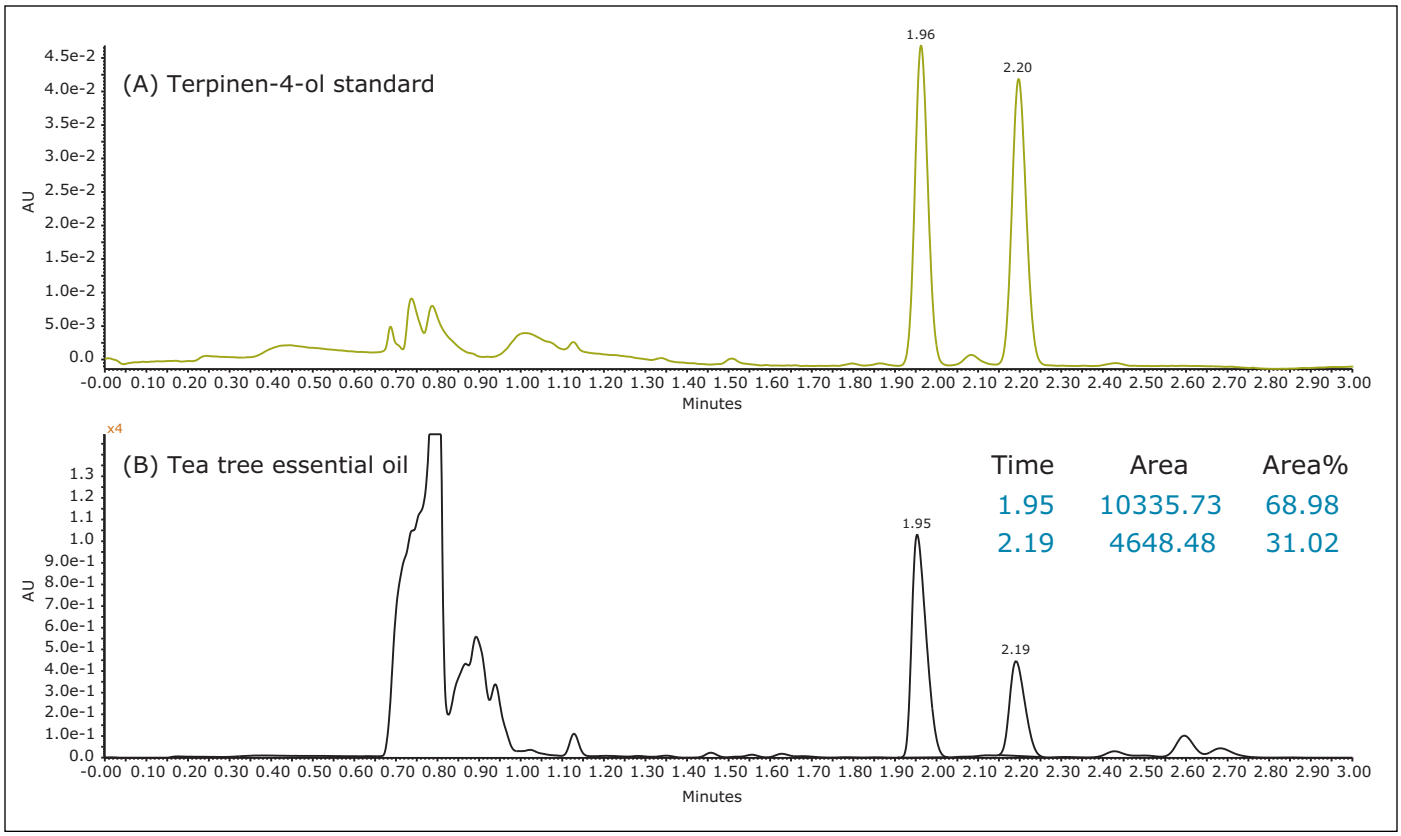


*Figure 4. UPC$^2$-UV chromatograms of (A) Terpinen-4-ol standard and (B) Tea Tree essential oil on an ACQUITY UPC$^2$ Trefoil AMY1 column. An isocratic method with 5% isopropanol was used. The flow rate was 1.0 mL/min.*

Nerolidol, which can be found in the neroli essential oil derived from the bitter orange plant, is a sesquiterpene with a pleasant woody odor reminiscent of fresh bark. The nerolidol molecule (Figure 1) contains a chiral center and a double bond generating cis/trans isomerism, resulting in four possible stereoisomers in a mixture. Figure 5 shows the simultaneous separation of all four nerolidol isomers on an ACQUITY UPC$^2$ Trefoil AMY1 column in less than 3 min. Figure 5B is the selected ion recording (SIR) for the isomeric mixture at *m/z* 205.2, corresponding to the $[(M+H)-H_2O]^+$ of nerolidol. The observation of the base peak of nerolidol resulting from the loss of water is consistent with other reports.[7]

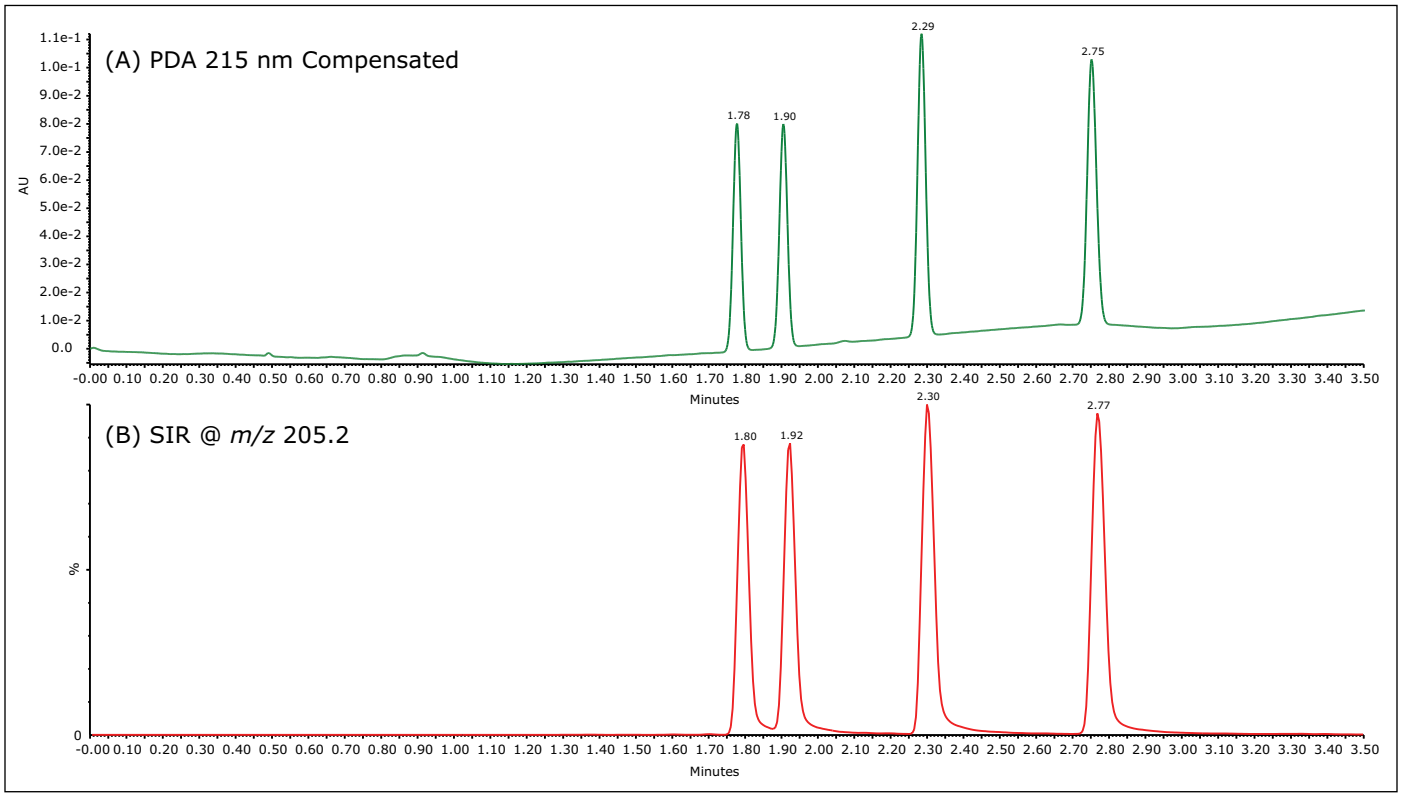


*Figure 5. UPC$^2$ chromatograms of a nerolidol standard separated on an ACQUITY UPC$^2$ Trefoil AMY1 Column: (A) UV at 215 nm with a compensation wavelength of 260-310 nm; and (B) SIR at m/z 205.2. The flow rate was 1.5 mL/min. A gradient of 2-7% isopropanol in 3.5 min was used.*

## CONCLUSIONS

In this application note, we have demonstrated the successful chiral separations of fragrance compounds on ACQUITY UPC$^2$ Trefoil AMY1 and CEL1 Columns using an ACQUITY UPC$^2$ System. The low system volume and extra-column volume of the UPC$^2$, combined with the reduced particle size of the ACQUITY UPC$^2$ Trefoil AMY1 and CEL1 Columns, enable superior, faster, and more efficient separations compared with traditional SFC and GC. The demonstrated analysis times range from 2 to 3 minutes, significantly shorter than the 15-50 minute analysis time typical for chiral GC,[3] allows for a fast authentication of the natural sources of essential oils. In all cases, the closely eluting isomers were baseline resolved. For the essential oil analysis, the oil samples were diluted and directly injected onto an ACQUITY UPC$^2$ System without tedious sample preparation. Furthermore, the inherent compatibility between UPC$^2$ and MS offered an unambiguous identification of the target analytes in a complex sample matrix. The high efficiency, short analysis time, and simple sample workup demonstrated in this study should be welcomed by industries where chiral analyses of fragrance compounds are routinely performed.

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