Delivering the Right Results

Characterization of Complex Biological Mixtures Using Multi-Reflection TOFMS in Different Mass Resolution Modes

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OBJECTIVE

Purpose: To demonstrate the analytical utility of low, intermediate, and high mass resolution modes of a multi-reflecting time-of-flight mass spectrometer (MR-TOFMS).

Methods: The MR-TOFMS used in this study was constructed with two parallel sets of planar and grid-free ion mirrors in combination with periodic lenses positioned in the field free region. This ion optics concept provides third order energy and first order spatial focusing in both directions, producing high mass resolution and low ion losses. By applying a pulser to the first lens assembly, ions can be diverted toward the detector after 2 reflections or returned to the flight path trajectory for a total of 64 reflections.

Results: Results demonstrate the analytical utility of this flexible flight tube design. Low resolution mode provides a resolving power of 3,000 which is suitable for high throughput screening and rapid sample comparison. Intermediate resolution mode with a resolving power of 50,000 yields greater spectral detail and mass accuracies of less than 1 ppm ideal for elemental formula determination. High resolution mode provides a resolving power of 100,000 allowing separation of isobars abundant in highly complex mixtures with mass accuracies below 1 ppm error.

INTRODUCTION

The utility of low, intermediate and high resolution modes was investigated for the analysis of polar lipids extracted from E. coli B. Low resolution mode was used to provide a rapid comparison between two samples to distinguish changes in the phosphatidylethanolamine (PE) profiles as a result of induced oxidation. Intermediate resolution mode was used in combination with liquid chromatography to separate components of this complex mixture and provide structural evidence of PE oxide formation using in-source collision induced dissociation (CID). High resolution mode was used to confirm presence of PE oxides free from spectral interference for direct infusion analysis.

ANALYTICAL METHODS

Samples: Polar extracts of E *coli* B were purchased from Avanti Polar Lipids, Inc. Extracts contain the following components:

Components	Weight Percent
Phosphatidylethanolamine (PE)	67.0
Phosphatidylglycerol (PG)	23.2
Cardiolipin (CL)	9.8

PE Oxidation: PEs are known to oxidize under relatively mild conditions. In this study, a small amount of the lipid extract was exposed to air at room temperature for 24 hours.

Analysis: Samples were analyzed by direct infusion and liquid chromatography.

- **Direct infusion:** Extracts were diluted ten fold prior to analysis. Running buffer consisted of a mixture of methanol with 10 mM ammonium acetate/isopropanol/water in ratios of 90/5/5. A small amount of Agilent tuning mix was added to samples prior to analysis for mass calibration and internal standardization.
- **Liquid chromatography:** Separation was carried out using the Eksigent expressLC™-100 at 10 ul/min under isocratic conditions using a mobile phase consisting of methanol with 10 mM ammonium acetate/isopropanol/water in ratios of 90/5/5. Extracts were diluted 10 fold in the mobile phase prior to injection of 100 nl. Analytes were separated on an Agilent Zorbax SB-C18 column with dimensions 150 x 0.5 mm x 5 um.
- **Ionization:** Positive mode electrospray ionization was used. For in-source CID, the skimmer voltage was elevated from 35 to 95V to induce fragmentation.

INSTRUMENTATION

An illustration of the MR-TOFMS used in this study is shown in Figure 1. The benefits of this design include:

- High ion transmission–the use of gridless mirrors and precision focusing minimize ion losses in the flight region.
- High mass resolution in a compact design–convolution of the flight path along with high-order focusing and spatial confinement of the ion beam allow for high mass resolution within a small flight tube package of approximately 30 inches.
- Large mass range–the 'open loop' design allows for the separation of ions across a large mass window.
- Selectable resolution mode–ions can be diverted toward the detector after 2 reflections for low resolution operation or returned to the flight path trajectory for a total of 64 reflections for high resolution analysis.

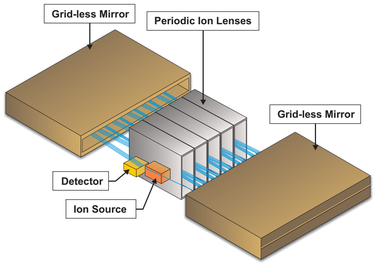
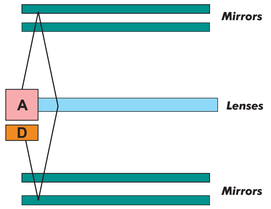


Figure 1. MR-TOFMS flight tube illustration

DIAMOND RESOLUTION MODE

Characteristics:

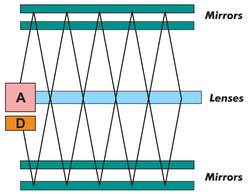
- Ions are diverted toward detector after 2 reflections
- Provides high speed, low resolution data at full mass range
- Suitable for rapid sample screening and targeted analysis
- Mass range: up to 2,500 m/z
- Resolving power: 3,000
- Mass accuracy: as low as 1 ppm (spectrum limited)
- Extraction frequency: 20 kHz



NORMAL RESOLUTION MODE

Characteristics:

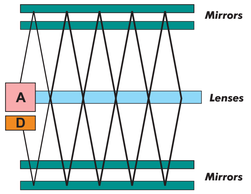
- Ions travel full flight path of 32 reflections
- Provides intermediate speed and resolving power data at full mass range
- Suitable for high resolution and mass accuracy work
- Mass range: up to 2,500 m/z
- Resolving power: 50,000
- Typical mass accuracy: <1ppm
- Extraction frequency: 1 kHz



ZOOM-IN RESOLUTION MODE

Characteristics:

- Ions are diverted back into flight tube after the first 32 reflections for a total of 64 reflections
- Provides lower speed but a high resolving power over a smaller mass range
- Suitable for high resolution and high mass accuracy work
- Mass window: up to 1,000 m/z
- Resolving power: 100,000
- Typical mass accuracy: <1 ppm
- Extraction frequency: 0.5 kHz



EXAMPLE WORKFLOW

The example given in the following sections outlines a workflow utilizing all the three resolution modes for characterizing lipid extracts.

Diamond Mode:
- **Fast Screening of Lipid Extracts**
- **Compare PE Profiles**

Normal Mode:
- **LC Identification of Oxidized PEs**
- **Structural Verification by CID**

Zoom-In Mode:
- **Confirmation of PE Oxides**
- **Elimination of Spectral Interferences**

DIAMOND MODE: SAMPLE SCREENING

Diamond Mode provides very rapid acquisition at low resolution suited for high throughput screening techniques such as flow injection analysis or high speed chromatography. This is well suited for profiling a large number of samples for the hypothesis generation phase of experimentation. In the example below, lipid extracts of before and after exposure to atmosphere for 24 hours were compared using direct infusion in Diamond Mode. Figure 2 shows an example section of spectra of an unexposed sample containing the main component peaks.

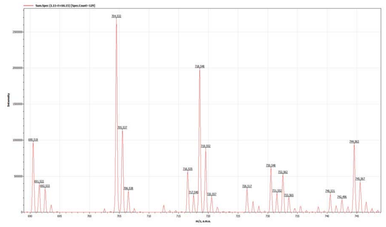


Figure 2. Direct infusion of lipid extract in Diamond Mode.

PE peaks could be tentatively identified based on accurate mass assignment (Table 1). In low resolution mode, mass errors are limited by the abundance of overlapping spectral isomer peaks. However, in the rapid screening phase it is only necessary to identify changes between samples.

Lipid	Measured (m/z)	Resolving Power	Mass Error (ppm)
PE C28:0	636.4726	2864	19
PE C30:1	662.4761	3292	1
PE C31:1	676.4960	3271	7
PE C32:1	690.5186	2500	17
PE C32:0	692.5329	2634	15
PE C18:1	704.5317	2425	13
PE C34:2	716.5347	2495	17
PE C34:1	7185481	2436	14
PE C35:1	732.5623	2475	12
PE C36:2	744.5628	2474	12

Table 1. Tentative Identification of PEs in Diamond Mode

Figure 3 shows a comparison of PEs in exposed and unexposed lipid samples. In this example workflow, a hypothesis that the exposed samples were reduced in PE due to oxidation of the unsaturated acyl moieties can be generated.

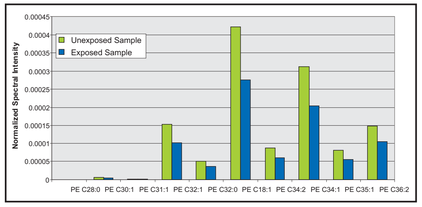


Figure 3. Comparison of PEs in unexposed and exposed lipid extracts. Data sets were normalized to the internal standard m/z 922 from the Agilent tuning mix and the endogenous phosphatidylglycerol (PG C30:0) m/z 712.

NORMAL MODE: IDENTIFICATION

In the next phase of testing, evidence for the formation of PE oxides based on the hypothesis generated in the first phase can be tested. Lipid extracts of exposed samples were analyzed by LC in Normal Resolution Mode to verify the presences of PE oxides. Figure 4 shows the resulting separation of PEs, PGs, and PE oxides. The greater resolving power of 50,000 yields mass accuracies below 1 ppm for all compounds measured.

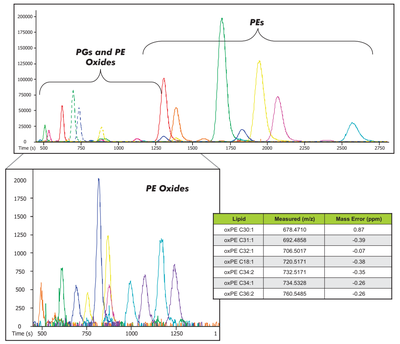


Figure 4. Presence of PE oxides determined using Normal Mode in combination with LC separation.

IN-SOURCE CID: STRUCTURAL IDENTIFICATION

Lipid extracts of exposed samples were analyzed a second time with a higher skimmer voltage to induce dissociation of the ions. Although ion intensities were low, the characteristic neutral loss of ethanolaminephosphate head groups (m/z 141) were observed at sufficient intensity for all but one of the PE oxides (Table 2). This additional piece of information increases confidence in the PE oxidation hypothesis by demonstrating that the oxide resides on the acyl moieties and not the PE head group.

Lipid Fragment	Measured (m/z)	Mass Error (ppm)
oxPE C30:1	537.4502	2.0
oxPE C31:1	551.4674	0.8
oxPE C32:1	565.4821	-0.9
oxPE C18:1	579.4995	2.0
oxPE C34:2	591.4994	1.8
oxPE C34:1	ND	---
oxPE C36:2	619.5299	0.5

Table 2. Neutral loss fragment ions observed for in-source CID of oxidized PEs.

ZOOM-IN MODE: CONFIRMATION

Zoom-In Mode can be used in situations where Normal Mode does not provide sufficient resolution for a desired analysis. In our example workflow, after establishing the mass range of interest, the exposed samples were analyzed by direct infusion in Zoom-In mode over a mass range 500 to 1,500 m/z. The high mass resolving power of Zoom-In mode allows separation of the PE oxides from spectral interferences observed when analyzing samples by direct infusion in Normal Mode. Figure 5 shows an example of separation of the PE oxide C32:1 from an M+2 isotope of PE C33:1. In Normal Mode, the PE oxide is observed as a shoulder on the PE C33:1 isotope peak. In Zoom-In Mode, the ion peaks are virtually baseline resolved.

Because separation of our desired components can be achieved in the mass spectrometer, further experiments can be carried out in Zoom-In Mode using direct infusion or flow injection without chromatography for higher throughput analysis. This may be used to generate further supportive data in future experiments involving time course or mass balance studies of oxide formation.

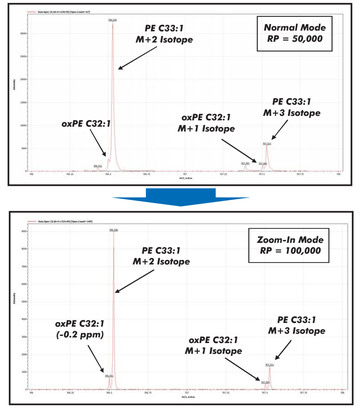


Figure 5. Zoom-In Mode can be used to separate oxidized PEs from interfering PE isotope peaks.

CONCLUSIONS

The analysis of lipid extracts demonstrates the flexibility of selective resolution modes in an anticipated workflow. Diamond Mode allows rapid sample screening for high throughput analysis. Normal Mode yields high mass resolution and high mass accuracy for compound identification. Zoom-In Mode enables separation of isobar with high resolution and mass accuracy over a smaller mass window.

ACKNOWLEDGMENTS

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