Determination of NADH and NADPH Using Ion Chromatography and High Resolution Accurate Mass Spectroscopy

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Key Words
HR/AM, Metabolomics

Goal
Demonstrate High Resolution Accurate Mass determinations of NADPH and NADH using ion chromatography coupled to the Q Exactive Mass Spectrometer.

Introduction
Nicotinamide adenine dinucleotide (NAD+/NADH) and nicotinamide adenine dinucleotide phosphate (NADP+/NADPH) (Figure 1) are important biological compounds that donate and accept electrons in many anabolic and catabolic functions. NAD+ and NADH donate and accept electrons in photosynthesis through the Calvin Cycle to convert light energy and carbon dioxide into carbohydrates. They participate in the Citric Acid Cycle to convert acetyl-CoA metabolized from fats, carbohydrates, and proteins into energy in the form of adenosine triphosphate (ATP).1–3 NADP+ and NADPH provide the reducing power used for the synthesis of lipids, fatty acids, and cholesterol.2,3

In addition to its redox function, researchers have shown that NAD+/NADH is a substrate for other enzymes including transferases, synthases, and sirtuins.4–6 The NAD+/NADH ratio is important to signaling reactions inside and outside the cell, calcium mobilization, cell death, and aging.5–8 Disease states, such as hypertension, renal failure, and cancers are associated with an imbalance of these ratios.2–5 The therapeutic normalization of these ratios has been found to increase lifespan in rodents and is proposed to prevent neurodegenerative diseases and to inhibit the spread of cancer by metastasis.2,4,5,7

In addition to its redox function, researchers have shown that NADP+/NADPH are substrates for heme proteins, such as cytochrome P450.6,8 NADPH also reacts with Reactive Oxygen Species (ROS), and generates free radicals as part of the immune response.9,10 NADPH is a mediator that moderates calcium levels, gene expression, aging, and cell death.6,9,10 Analysis for both NAD+/NADH and NADP+/NADPH compounds are therefore of interest to pharmaceutical companies to control disease states and to researchers studying metabolomics, autoimmune diseases, extending longevity, and cancer.

Figure 1. Chemical structures.
A recent publication on the metabolome of head and neck cancer cells demonstrated the superior separations and increased sensitivity of polar metabolites using ion-exchange chromatography with High Resolution/Accurate Mass spectroscopy (HR/AM). Therefore ion-exchange separations with HR/AM spectroscopy was selected for this method.

The method described in this Technical Note, combines the ion chromatography (IC) separation and continuous online desalting of the IC suppressor with the high-resolution accurate-mass (HR/AM) spectroscopy (IC-HR/AM) advantages to analyze and identify specific mass/charge. NADH and NADPH are separated by IC using a high concentration (100 mM KOH) of electrolytically generated eluent on a metal-free Reagent-Free™ Ion Chromatography (RFIC™) system. The IC suppressor provides continuous inline desalting after the separation by replacing the cations (potassium) of the eluent and analytes with hydronium ions. This desalting converts the eluent to water bringing the baseline to nearly zero, while the analyte is converted to the acid of the anions. The desalting of potassium hydroxide eluent makes the eluent compatible with mass spectrometry. The eluent sample stream is then infused with solvent and detected in Full Scan and selected-ion monitoring (SIM) modes.

**Equipment**

**Chromatography**
- Thermo Scientific Dionex ICS-2100 RFIC system
- Thermo Scientific Dionex AS-AP Autosampler with temperature control

**Mass Spectrometry**
- Thermo Scientific™ Q Exactive™ High Resolution Accurate Mass™ (HR/AM™) spectrometer

**Reagents and Standards**
- 18 MΩ-cm resistivity degassed deionized water
- Fisher Scientific reagents
  - Ammonium hydroxide, 29%, ACS grade
  - Acetonitrile, HPLC grade
  - Methanol, HPLC grade
  - NADPH, ACS grade
  - NADH, ACS grade

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**Conditions**

**Chromatographic**
- **Columns:** Thermo Scientific™ Dionex™ IonPac™ AG20/AS20 guard and separation columns, 2 × 50 mm/2 × 250 mm
- **Eluent Source:** Thermo Scientific Dionex EGC III KOH Cartridge
- **Eluent:** 100 mM KOH
- **Flow Rate:** 0.25 mL/min
- **Temperature:** 30 °C
- **Injection Volume:** 10 µL

**IC System**
- **Backpressure:** 2200 psi
- **Background:** < 1 µS
- **Suppressor Mode:** External water regenerant at 1.0 mL/min
- **Suppressor/Desalter:** Thermo Scientific™ Dionex™ AERS™ 500 Anion Electrolytically Regenerated Suppressor in external water mode by Thermo Scientific Dionex AXP Auxiliary Pump

**Detection**
- **A:** Suppressed conductivity
- **B:** MS Full Scan, SIM, high resolution accurate mass

**IC-MS Interface**
- **Desalter:** Suppressor is providing desalting
- **Solvent:** 0.0017% Ammonium hydroxide in methanol
- **Solvent Flow:** 0.25 mL/min by Dionex AXP-MS pump

**Mass Spectrometry**
- **Probe:** Heated ESI probe v2
- **Mode:** Full Scan (m/z 70–1000), SIM
- **Resolution:** 140,000 FWHM, 1 ppm, external mass calibration

**Software**
- Thermo Scientific™ Excalibur™ version 2.2
- Thermo Scientific™ DCMSLink™ 2.13

The consumables for this application are shown in Table 1.
<table>
<thead>
<tr>
<th>Product name</th>
<th>Description</th>
<th>Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chromatography</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dionex EGC III KOH cartridge</td>
<td>Anion Eluent Generator cartridge</td>
<td>074532</td>
</tr>
<tr>
<td>Thermo Scientific Dionex CR-ATC Continuously</td>
<td>High-pressure electrolytic anion trap column</td>
<td>060477</td>
</tr>
<tr>
<td>Regenerated Anion Trap Column</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dionex IonPac AS20 Anion-Exchange Columns</td>
<td>Guard column, 2 × 50 mm</td>
<td>063066</td>
</tr>
<tr>
<td></td>
<td>Separation column, 2 × 250 mm</td>
<td>063065</td>
</tr>
<tr>
<td>Dionex AERS 500 Anion Electrolytically Regenerated Suppressor</td>
<td>Suppressor/desalter for 2 mm columns</td>
<td>082541</td>
</tr>
<tr>
<td>Thermo Scientific Dionex Suppresser External Regen Installation Kit</td>
<td>External water kit for suppressor/desalter regenerant</td>
<td>038018</td>
</tr>
<tr>
<td>Dionex AS-AP Autosampler Vial Kit</td>
<td>1.5 mL glass vials and caps, package of 100</td>
<td>055427</td>
</tr>
<tr>
<td><strong>Chromatography &amp; MS Interface</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black PEEK Tubing (0.010 in, 0.25 mm i.d.)</td>
<td>Used for IC-MS interface and Dionex AXP (solvent) pump to T-connector</td>
<td>052306</td>
</tr>
<tr>
<td>Dionex AXP Auxiliary Pump</td>
<td>Auxiliary pump supplying water to the suppressor in external water mode</td>
<td>063973</td>
</tr>
<tr>
<td>Dionex AXP-MS Pump</td>
<td>Auxiliary pump used to supply methanol to mix with the IC eluent stream prior to the MS</td>
<td>060684</td>
</tr>
<tr>
<td>T-connector</td>
<td>T-Connector for IC-MS interface</td>
<td>053593</td>
</tr>
<tr>
<td>Twisted pair of wires</td>
<td>Cable to connect the Dionex ICS-2100 system to the Q Exactive HR/AM mass spec</td>
<td>043598</td>
</tr>
<tr>
<td>12-Pin and 2-Pin TTL Connector Plugs</td>
<td>2-Position connector to Q Exactive</td>
<td>921370</td>
</tr>
<tr>
<td></td>
<td>12-Position connector to Dionex ICS-2100 RFIC system</td>
<td>923686</td>
</tr>
<tr>
<td>HESI II ESI Probe v2</td>
<td>Heated Electrospray Ionization Probe which provides increased sensitivity</td>
<td>OPTON-20037</td>
</tr>
</tbody>
</table>

Figure 1. Consumables list.
Standard Preparation
It is important to use 18 MΩ-cm resistivity deionized water for standards, eluent, and autosampler flush solution. It is recommended to degas the deionized water intended for eluent used for anion determinations. (An appropriate degassing method is vacuum filtration with ultrasonic agitation.) Using deionized water with resistivity less than 18 MΩ-cm can reduce sensitivity, introduce contamination, and affect calibration, thereby resulting in inaccurate quantification. Results can vary and contamination introduced from samples can affect the chromatography.

0.017% (v/v) Ammonium Hydroxide Diluent for Working Standards and Samples
The 0.017% (v/v) ammonium hydroxide diluent was prepared by diluting 12 µL of 29% ammonium hydroxide in 20 mL of deionized water.

10% (v/v) Acetonitrile Diluent for Stock Standards and Samples
A 100 mL of 10% acetonitrile was prepared by diluting 10 mL of HPLC grade acetonitrile with 90 mL deionized water.

0.0017% (v/v) Ammonium Hydroxide in Methanol Desolvation Solution
The desolvation solution was prepared by diluting 60 µL of 29% ammonium hydroxide in 1 L of methanol.

NADH and NADPH Standards
Separate stock standards of 1 mg/mL NADH and 5 mg/mL NADPH were prepared by dissolving 1 mg of NADH and 5 mg of NADPH, respectively in 1 mL each of 10% (v/v) Acetonitrile Diluent. A mixed stock standard of 0.1 mg/mL of NADH and NADPH was prepared from the stock standards and 10% Acetonitrile Diluent. All stock standards were stored at -20 °C. The 0.1 mg/mL mixed stock standard was thawed 1 h prior to use, and then diluted with 0.017% (v/v) Ammonium Hydroxide Diluent to prepare the 0.3, 1, 3, 5, 10, 30, 100, 300, and 1000 ng/mL working standards. The 0.3, 1, 3, 5 ng/mL standards were analyzed only when the MS was in Full Scan mode.

Sample Preparation
Only standards were evaluated with this method.

Instrument Setup
To set up this application:
1. Configure the IC system to the MS system. Connect a USB cable from the Dionex AS-AP autosampler to the Dionex ICS-2100 RFIC system. Connect the twisted pair cable from the Dionex ICS-2100 RFIC system to the Q Exactive. Set up and power-up the Dionex ICS-2100 system, and configure the system electronically with Excalibur DCMSLinks.
2. Install the degassed deionized water for eluent generation.
3. Install and condition the Dionex EGC III KOH cartridge, Dionex CR-ATC II trap column, guard column, separation column, and the Dionex AERS 500 suppressor according to the Dionex ICS-2100 IC system, Dionex AS-AP autosampler, and consumable product manuals and the flow diagram shown in Figure 2.12–17

Figure 2. Flow diagram.
IC-MS Interface
Prime the Dionex AXP pumps with deionized water for suppressor external water mode and with the desolvation solution for the IC-MS interface. Install the twisted pair wires into the 12-pin connector in the “TTL Input” positions according to the Dionex ICS-2100 RFIC system product manual. Install the other end into the “TTL Output” on the Q Exactive mass spectrometer.

Q Exactive MS Conditions
The Q Exactive conditions are shown in Tables 2 and 3.

Table 2. Q Exactive MS conditions.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheath Gas</td>
<td>60</td>
</tr>
<tr>
<td>Sweep Gas</td>
<td>1</td>
</tr>
<tr>
<td>Capillary Temperature</td>
<td>325 °C</td>
</tr>
<tr>
<td>Heater Temperature</td>
<td>475 °C</td>
</tr>
<tr>
<td>Resolution</td>
<td>140,000</td>
</tr>
<tr>
<td>Maximum IT</td>
<td>200 ms</td>
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<tr>
<td>Auxiliary Gas</td>
<td>20</td>
</tr>
<tr>
<td>Spray Voltage</td>
<td>3.25 kV</td>
</tr>
<tr>
<td>S-sens RF level</td>
<td>80</td>
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<tr>
<td>Mode</td>
<td>SIM with 3 Ion Multiplexing</td>
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<tr>
<td>AGC Target</td>
<td>1e5</td>
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<tr>
<td>Isolation Width</td>
<td>1.5 m/z</td>
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<tr>
<td>Positive/Negative Mode</td>
<td>Negative</td>
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Table 3. Ions used in SIM mode.
<table>
<thead>
<tr>
<th>mz</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>744.08382</td>
<td>NADPH [M-H]^-</td>
</tr>
<tr>
<td>371.53827</td>
<td>NADPH [M-2H]^-</td>
</tr>
<tr>
<td>664.11749</td>
<td>NADH [M-H]^-</td>
</tr>
</tbody>
</table>

Results and Discussion
Method
Ion-exchange chromatography is an ideal method to separate ionic compounds such as NADH and NADPH. High-resolution accurate-mass (HR/AM) spectroscopy provides specific molecular identification by providing high resolution to identify mass/charge within 1 ppm. Here, we show the advantages and the results of combining IC with HR/AM. NADH and NADPH are separated within 4 min by anion-exchange chromatography using electrolytically generated potassium hydroxide. The eluent is desalted with an electrolytic anion suppressor as it leaves the column. Then the eluent and sample stream are infused with solvent prior to HR/AM spectroscopy detection of the analytes.

The Dionex IonPac AS20 anion-exchange column was selected for this application because it is ideal for highly retained anions, such as NADH and NADPH. The method was optimized for fast elution of NADH and NADPH, 3.4 min and 3.7 min, respectively using 100 mM KOH electrolytically generated in line at 0.25 mL/min (data not shown).

To evaluate the sensitivity of this method, 10 µL of 100 µg/mL NADH and NADPH was injected using methanol with and without 0.0017% ammonium hydroxide for the desolvation solution (Figure 3). Figure 4 shows that both NADPH and NADH demonstrated a LOD of 1 ng/column at 10^5 counts. Slightly better peak symmetry was achieved using methanol with ammonium hydroxide than without it.

![Figure 3. Benefits of post-column solvent addition for increased sensitivity.](image-url)
To confirm the high resolution mass accuracy capabilities of this method and system, a 1000 ng/mL standard was evaluated resulting in 10,000 pg on column (Figure 4, Table 4). Full Scan mass spectra across the NADH and the NADPH peaks showed mass accuracy and precision within 1 ppm.

The single and double charge states of NADH and NADPH are shown in Figure 5.

In an IC-MS application, it is critical that the eluent is compatible. To verify the efficiency of the suppressor, the Total Ion Chromatogram (TIC) of an NADH injection was evaluated (Figure 6). Sodium adducts are very common in electrospray process due to sodium extractables leached from glass bottles. In this case sodium is introduced from the only glass bottle, the solvent bottle (ammonium hydroxide solution and methanol). This solution is introduced after the suppressor. In contrast, the potassium adduct is very small. The results confirm that the KOH has been neutralized to water by suppression due to the minor potassium presence in the TIC, whereas the sodium adduct is prominent.

During the experimental evaluation, Full Scan MS revealed NADPH degradation products occurring in standards stored longer than 24 h (Figure 7). This compound, m/z = 380.5439 was seen as a doublet to NADPH at RT 3.16 min. The m/z matches [M+H2O-2H]/2 (~0.85 ppm) and is surmised to be a water addition product which then loses water in the source to produce an interfering ion to NADPH.

### Table 4. Summary of high resolution/accurate mass.

<table>
<thead>
<tr>
<th></th>
<th>Accurate m/z</th>
<th>Measured m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH-1</td>
<td>744.08382</td>
<td>744.0839–744.0842</td>
</tr>
<tr>
<td>NADH-1</td>
<td>664.11749</td>
<td>664.1171–664.1181</td>
</tr>
</tbody>
</table>

Figure 4. Mass accuracy and scan speed.

Figure 5. NADPH and NADH charge states.

Figure 6. Small potassium adduct confirming efficient removal of eluent by the anion suppressor.

Figure 7. Full Scan reveals NADPH degradation product.
Detection Limits
The LODs were determined in SIM mode using 0.3 to 3 ng/mL standards and in Full Scan mode using 3 to 30 ng/mL standard (Figures 8–10). Detection of both analytes was sensitive in SIM mode, with LODs of 3 pg on column whereas the LODs were 30 pg on column in Full Scan mode. As expected, SIM mode results in at least 10x increased sensitivity than in Full Scan mode.

Conclusion
This application demonstrates the advantages of combining IC with the high resolution accurate mass and sensitivity capabilities of mass spectroscopy.

This application demonstrates a fast, accurate, and sensitive method to detect NADH and NADPH, which is needed for metabolomic research studies. Here, the two analytes are eluted within 4 min from a Dionex IonPac AS20 anion-exchange column on an integrated RFIC system. As the analytes elute, they are infused with methanol/ammonium hydroxide at the IC-MS interface and detected with HR/AM in Full Scan and SIM modes by the Q Exactive. The LODs are 2 pg/column in SIM mode and 50 pg/column in Full Scan mode. The measured m/z were within 1 ppm of theoretical accurate mass.

Acknowledgements
We would like to acknowledge Dr James Cox, Research Assistant Professor of Biochemistry, Director of the Metabolomics Core Facility from the University of Utah, in Salt Lake City, Utah for initiating this project and providing the standards.
References


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