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

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Technical Note 149

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]

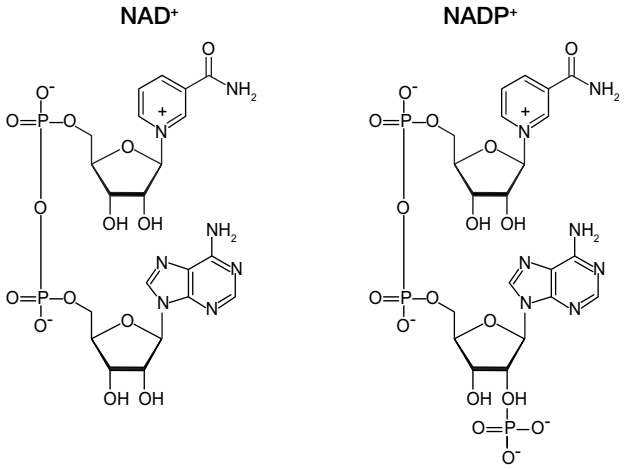
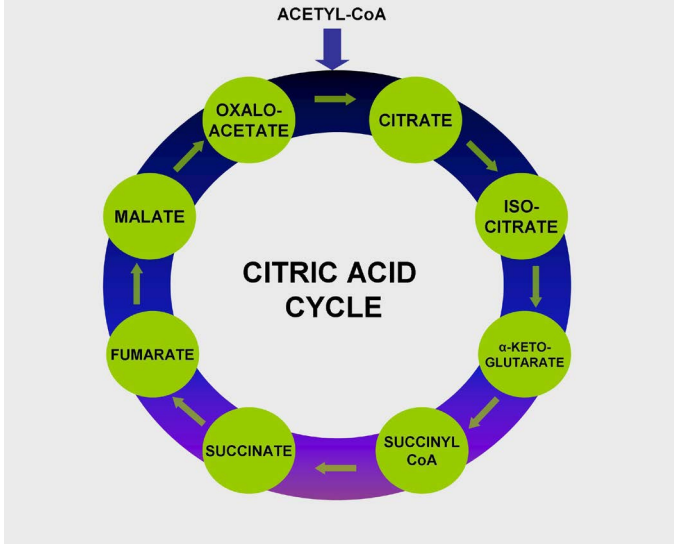


Figure 1. Chemical structures.



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.[2,3,6,7]

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.

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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 spectrometry (HR/AM).[11] Therefore ion-exchange separations with HR/AM spectrometry 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) spectrometry (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

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™ DCMS^{Link}™ 2.13

The consumables for this application are shown in Table 1.

Figure 1. Consumables list.

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

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 DCMS[Links].
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]

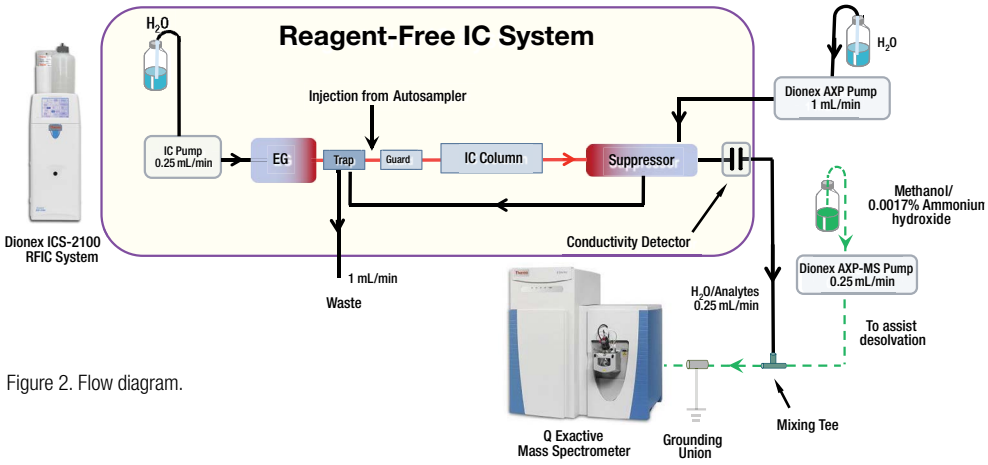

Figure 2. Flow diagram.

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.[11] 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.

Parameter	Condition
Sheath Gas	60
Sweep Gas	1
Capillary Temperature	325 °C
Heater Temperature	475 °C
Resolution	140,000
Maximum IT	200 ms
Auxiliary Gas	20
Spray Voltage	3.25 kV
S-sens RF level	80
Mode	SIM with 3 Ion Multiplexing
AGC Target	1e5
Isolation Width	1.5 m/z
Positive/Negative Mode	Negative

Table 3. Ions used in SIM mode.

mz	Condition
744.08382	NADPH $[M-H]^-$
371.53827	NADPH $[M-2H]^{2-}$
664.11749	NADH $[M-H]^-$

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) spectrometry 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 spectrometry 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.

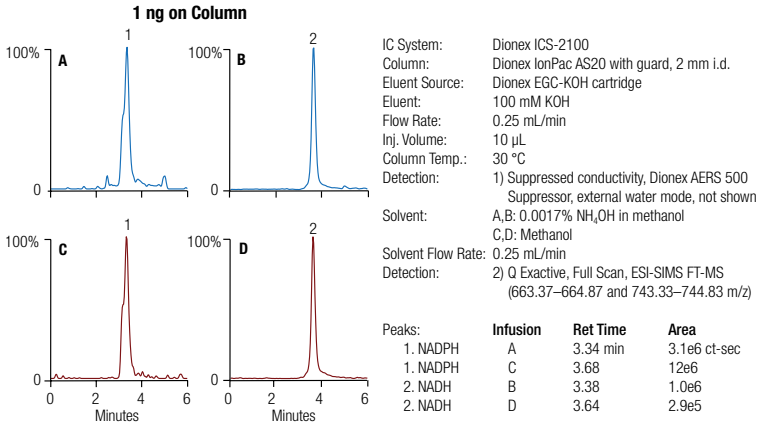

Figure 3. Benefits of post-column solvent addition for increased sensitivity.

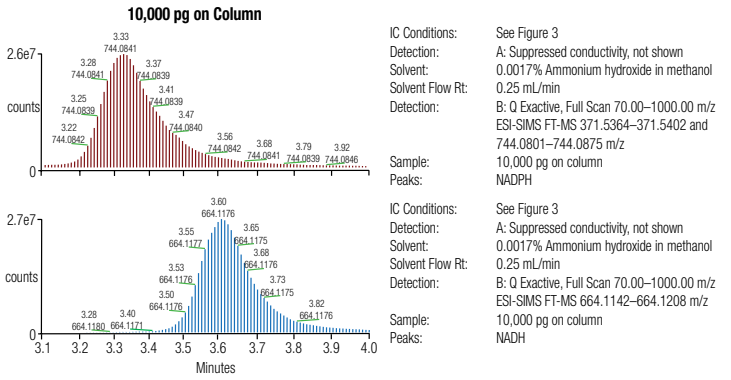

Figure 4. Mass accuracy and scan speed.

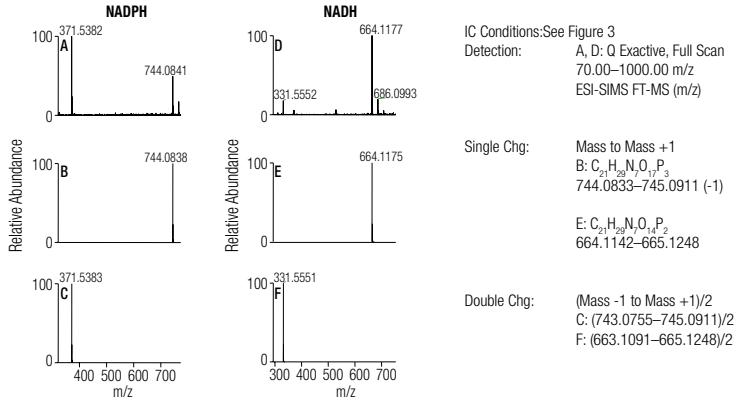

Figure 5. NADPH and NADH charge states.

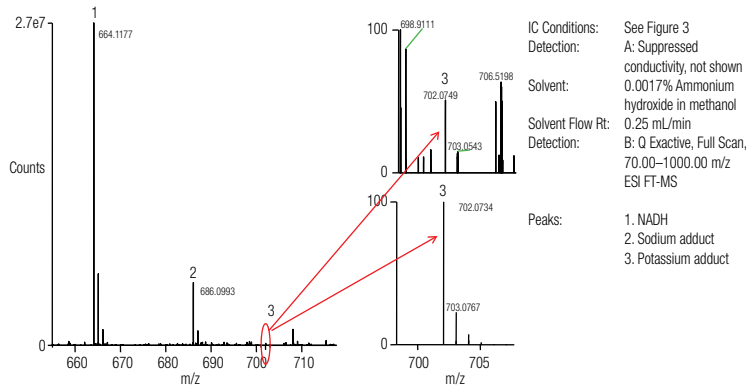

Figure 6. Small potassium adduct confirming efficient removal of eluent by the anion suppressor.

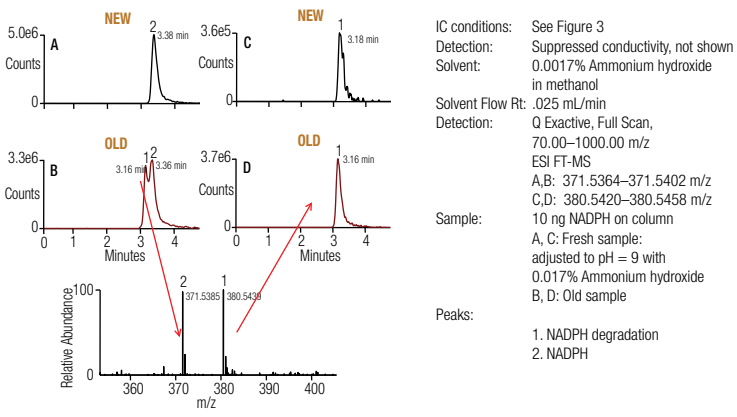

Figure 7. Full Scan reveals NADPH degradation product.

Table 4. Summary of high resolution/accurate mass.

	Accurate m/z	Measured m/z
NADPH-1	744.08382	744.0839–744.0842
NADH-1	664.11749	664.1171–664.1181

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 $[M^+H_2O^-2H]/2^-$ (~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.

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 spectrometry.

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.

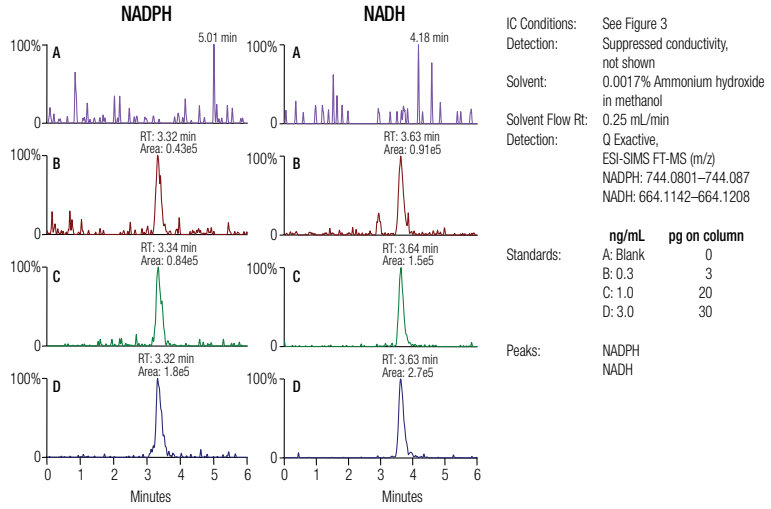

Figure 8. SIM detection limits.

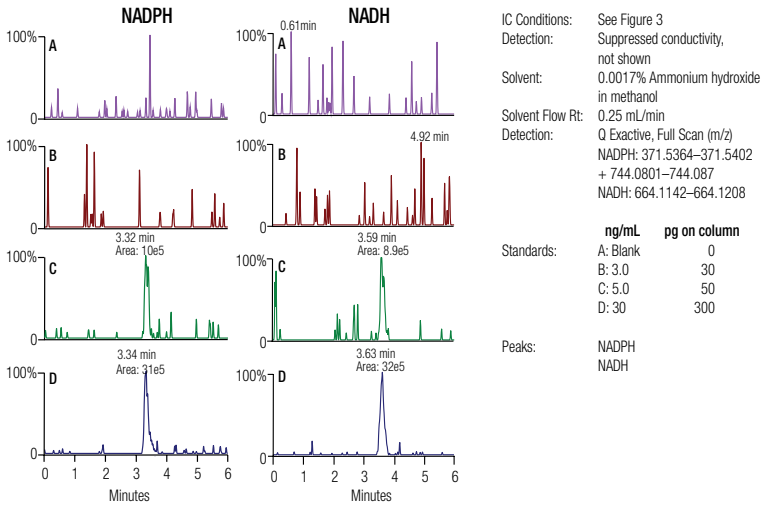

Figure 9. Full Scan detection limits.

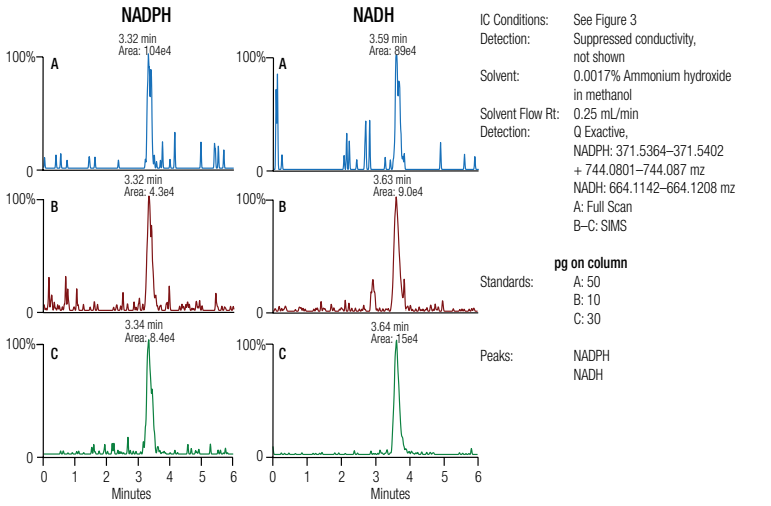

Figure 10. SIM vs Full Scan.

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