Technical Note 137

Determination of Carbohydrates in Urine by Capillary HPAE-PAD

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Key Words

HPIC, Capillary IC, ICS-4000, 3-*O*-methyl-D-glucose
L-rhamnose, D-xylose, D-mannitol, lactulose

Goal

Demonstrate the determination of monosaccharides and disaccharides in urine by HPAE-PAD on a Thermo Scientific™ Dionex™ ICS-4000 HPIC™ system.

Introduction

Researchers have studied the presence of certain carbohydrates such as mannitol, rhamnose, xylose, 3-*O*-methylglucose, and lactulose in both urine and serum samples to evaluate disease states in animals and humans.[1–12] Because carbohydrates are poor chromophores, they are difficult to detect by UV absorption without lengthy and costly derivitization. However, carbohydrates can be determined directly by High Performance Anion-Exchange chromatography with Pulsed Amperometric Detection (HPAE-PAD), a well-established technique that eliminates the need for derivitization, in turn saving time and money (i.e., reagent and disposal costs). In HPAE-PAD, neutral carbohydrates are ionized by the strong base eluent and then separated by anion-exchange chromatography. The carbohydrates are then detected by PAD with a gold working electrode using a four-potential waveform that is selective and sensitive for carbohydrates. Determination of these carbohydrates of interest have been previously demonstrated by Hurum and Rohrer in Thermo Scientific Application Note (AN) 1006 using HPAE-PAD on a 3 × 150 mm Thermo Scientific™ Dionex™ CarboPac™ PA20 column.[1]

Here we combine the advantages of a Reagent-Free™ IC (RFIC™) system and a capillary format ion chromatography system to determine monosaccharides and disaccharides in synthetic urine. In an RFIC system, the hydroxide eluent is electrolytically generated inline to deliver accurate and precise concentrations for isocratic or gradient separations by only adding deionized water. Eluent generation eliminates carbonate contamination and errors associated with manual preparation. A capillary scale system with µL/min flow rates can run 24/7, always on and always ready for samples. Eluent consumption and waste generation are reduced to 15 mL/day and eluent generator cartridges can last up to 18 months. In this work, the separation and detection of the same carbohydrates of interest discussed in AN 1006 are demonstrated using a capillary Dionex CarboPac PA20 anion-exchange column on the Thermo Scientific™ Dionex™ ICS-4000 Dedicated Capillary HPIC™ system. The carbohydrates are separated by HPAE using an electrolytically generated hydroxide eluent from 10 to 35 mM KOH at 0.008 mL/min. The analytes are detected by PAD, using a Gold on PTFE working electrode, and a four-potential waveform. The resulting method is direct, selective, and sensitive down to pmol concentrations.

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Equipment

- Dionex ICS-4000 HPIC system*
- Thermo Scientific™ Dionex™ IC Cube™
- Thermo Scientific™ Dionex™ Electrochemical Detector (ED)
- Thermo Scientific™ Dionex™ Electrochemical Cell, reference electrode with gasket, and working electrode with gasket
- Thermo Scientific™ Dionex™ AS-AP Autosampler
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.1 with SR2 MUa build or later

* A Thermo Scientific™ Dionex™ ICS-6000 IC system can be used for equivalent results

Reagents and Standards

- 18 MΩ-cm resistivity degassed deionized water
- pH Buffer solutions, pH 7 (Fisher Scientific, P/N SB108-500); pH 10 (Fisher Scientific, P/N SB115-500)

Table 1. ACS Grade Reagents

ACS Grade Reagents, Fisher Scientific	Part Number
Lactulose	AC22593-1000
ß-Lactose	
L-(+)-Rhamnose monohydrate	AC17408-0250
D-Mannitol	BP686-500
Sucrose	S6-500
D-Glucose	D15-500
Synthetic Urine Solution	83611
3-*O*-Methyl-D-glucose	
D-(+)-Galactose	AC15061-1000
D-(+)-Xylose	BP708-250
D-(+)-Cellobiose	AC10846-0250
D-(-)-Ribose	AC13236-0250

Samples

Described in Application Note AN 1006.[1]

Conditions	
Columns:	Dionex CarboPac PA20 column set (0.4 × 150 mm)
Eluent Source:	Thermo Scientific™ Dionex™ EGC-KOH Eluent Generator Cartridge (Capillary)
Eluent:	10 mM KOH (-7 to 1 min); 10–30 mM KOH (1 to 9 min); 30–35 mM KOH (9 to 16 min); 35 mM KOH (16 to 21 min); 10 mM KOH (21 to 37 min)
Flow Rate:	0.008 mL/min
Column Temp.:	30 °C
Compartment Temp.:	27 °C
Inj. Volume:	0.4 µL
Detection:	PAD, Gold on PTFE, 0.001" thick gasket, Four-Potential Carbohydrate waveform
Reference Electrode:	pH-Ag/AgCl
Background:	10–20 nC
Noise:	< 10 pC

The consumables and accessories for this application are listed in Table 2.

Table 2. Consumables list for the Dionex ICS-4000 system with ED detection

Product name	Description of High-Pressure Capillary Part	Part Number
Thermo Scientific™ Dionex™ EG Degas HP cartridge	High-pressure EG degas cartridge, up to 5000 psi	AAA-074459
Thermo Scientific™ Dionex™ CRD Bypass and Suppressor Bypass cartridges	Bypass cartridges (needed to complete flow path)	072056/072055
Thermo Scientific™ Dionex™ IonPac™ ASTC-500 Column/PEEK (Victrex plc) tubing	Trap column, 2 mm, installed between pump and Dionex EGC KOH cartridge. PEEK tubing	085359
Dionex high-pressure fittings (blue)	Bolts/Ferrules	074449/074373
Dionex AS-AP Autosampler vial kit, polypropylene	1.5 mL vials, packages of 100	079812
	0.3 mL vials, packages of 100	055428
Columns	Dionex CarboPac PA20 carbohydrate separation column, 0.4 × 150 mm	072072
	Dionex CarboPac PA20 carbohydrate guard column, 0.4 × 35 mm	072073
Dionex EGC-KOH cartridge	Anion Eluent Generator cartridge for capillary flow rates	072076
Thermo Scientific™ Dionex™ CR-ATC Continuously Regenerated Anion Trap Column	Anion Electrolytic trap column for capillary flow rates.	072078
Electrochemical Detector	ED Detector module for capillary or analytical flow rates.	072042
Electrochemical Cell	ED Cell body includes PEEK Yoke Block	072044
ED Cell Inlet Tubing kit	Kit Includes: 9" capillary tubing for cell inlet, long neck black PEEK connector, black PEEK split cone ferrule	074221
Reference Electrode/Gasket	pH-Ag/AgCl Gasket for capillary applications	061879 072162
Disposable Working Electrode/Gasket	Gold on PTFE, package of six[++]	066480
	0.001" thick PTFE gasket for capillary applications, Package of two	072117
	0.015" thick polypropylene gasket for mg/L concentrations	057364
ED Support Block	For use with 0.001", 0.002", 0.015" thick gaskets with disposable working electrodes	062158

[++] Kits include 0.002" gaskets intended for analytical flow rate. Do not use for this application.

Standard and Sample Preparation

Detailed instructions are described in AN 1006.[1]

Tip: It is important to use 18 MΩ-cm resistivity, deionized water for standards, eluent, and autosampler flush solution. It is recommended to routinely degas the deionized water intended for eluent in carbohydrate determinations. An appropriate degassing method is vacuum filtration. 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 may affect the chromatography. Consult Thermo Scientific Technical Note (TN) 71 for more information on water quality for HPAE-PAD.

Instrument Setup and Installation

This single-channel, Dionex ICS-4000 HPIC capillary IC system offers modular detection options while requiring little lab bench space. Although this technical note describes only the electrochemical detector, the system has the flexibility for analyte detection using either conductivity, electrochemical, or conductivity with charge detection for a variety of applications. The Dionex ICS-4000 HPIC system operates at system pressures up to 5000 psi, and therefore can use 4 µm particle-size columns for sample analysis.

Install and configure the Dionex AS-AP Autosampler to the Dionex ICS-4000 HPIC system according to the product manuals and Technical Note 136 Configuring a High-Pressure Integrated Capillary IC System for Electrochemical Detection.[13–15] Install the Dionex IC Cube and ED detector into the Dionex ICS-4000 system while the instrument is powered-off. To configure the system, follow the instructions in Thermo Scientific TN 136.[13] Install the Thermo Scientific™ Dionex™ IonPac™ ASTC-500 trap column between the pump and the Dionex EGC KOH cartridge using black PEEK tubing (P/N 078497). Temporarily leave the tubing to the Dionex EGC KOH capillary cartridge disconnected to flush the trap column of air and particles. First initiate the priming function on the pump (1 mL/min), point the Dionex IonPac ASTC-500 column upward, and flush for 30 min to allow air to escape. After 30 min, turn off the pump prime, and connect the tubing to the Dionex EGC-KOH capillary cartridge. Hydrate and condition the capillary Dionex EGC-KOH cartridge and Dionex CR-ATC device according to the product manuals and the Dionex ICS-4000 Operator's manual.[16,17] Plumb the consumable products and modules of the Dionex ICS-4000 HPIC system, according to Figures 1 and 2. Detailed installation instructions are described in the product manuals and TN 136.[13–17]

Tip: To achieve the best chromatography with HPIC, it is important to use the blue-colored high pressure connectors and ferrules (Table 2) for all connections prior to the ED cell. The Dionex ICS-4000 HPIC integrated RFIC system is designed to operate at pressures up to 5000 psi. Install the Dionex CRD Bypass and Dionex Suppressor Bypass cartridges in the Dionex IC Cube to complete the flow path for electrochemical detection applications (Figure 2). To achieve the best chromatography with capillary IC, minimize void volumes in all connections by using precision-cut tubing by the manufacturer, high-pressure connectors and fittings (colored blue), and seating the ferrule > 2 mm above the end of the tubing. These tips are thoroughly discussed in Thermo Scientific TN 113 "Practical Guidance for Capillary IC".[18] Extra care should be used to prevent introducing air in all consumables or tubing by observing a steady flow before installing the next device in line.

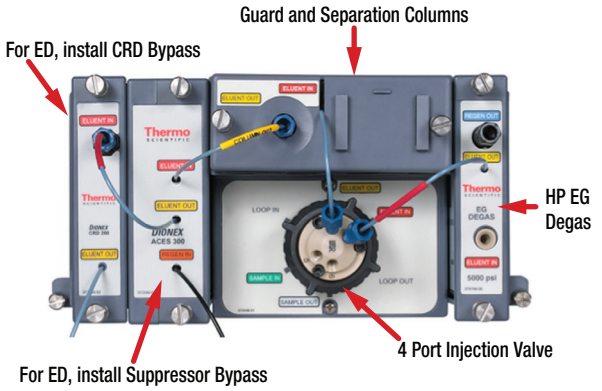

Figure 2. Dionex IC Cube

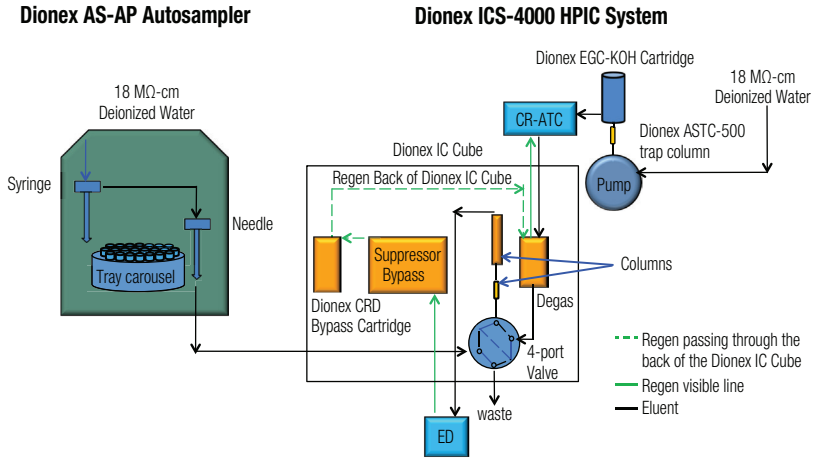

Figure 1. Flow diagram for the Dionex ICS-4000 HPIC System for HPAE-PAD

Installing the Electrochemical Cell with a pH-Ag/AgCl Reference Electrode

The installation procedures are thoroughly described in TN 136, the Dionex ICS-4000 Operator's manual, and the ED User's Compendium for Electrochemical Detection.[13,14,19]

Tips: Always wear powder-free gloves when handling the electrochemical cell. If this is a new ED cell, disassemble the cell and discard the shipping gasket. Caution: Do not touch the working electrode with any paper products including the lab wipes, as this can contaminate the working electrode. Remove all plugs on the cell inlet and cell outlet to prevent cell over pressure during the installation. First condition the pH-Ag/AgCl reference electrode in a solution of pH 7 buffer. The installation procedures are thoroughly discussed in TN 136.

To prepare the cell body for capillary applications, remove the titanium inlet tube and rinse the cell body, well of the reference electrode, and well of the inlet tube thoroughly with deionized water. Then rinse the working electrode gasket (0.001" thick PTFE gasket) and Support Block with deionized water and dry with a lab tissue. (The thinner 0.001" thick gaskets are used for capillary applications, whereas the 0.002" thick gaskets are used for applications on 2 to 4 mm i.d. columns.) Rinse the Gold on PTFE working electrode and shake-off the excess water (rather than drying it with a lab tissue). Assemble the gasket, disposable working electrode cell, Support Block, and Yoke Block assembly according to TN 136 and ED User's Compendium for Electrochemical Detection.[13,19] Avoid any wrinkles in the gasket, as this will cause a poor fit, subsequent leaks, and poor detection.

Calibrate the reference electrode using pH buffer 7 and pH buffer 10 and the instructions given by the "pH Calibration button" on the Chromeleon ED Panel. Remove the o-ring gasket from the reference electrode, and then install the gasket for the pH-Ag/AgCl reference electrode into the bottom of the reference electrode well and gently, but firmly, screw-in or rotate the reference electrode until it is finger-tight. Install the fully assembled ED cell into the Dionex ICS-4000 ED module. Immediately complete the final plumbing to the cell by installing the PEEK inlet tubing from the column outlet to the cell inlet well. Turn-on the Dionex EGC-KOH cartridge and Dionex CR-ATC capillary column and set eluent concentration to 10 mM KOH. Connect the Suppressor Bypass tubing to the cell outlet after observing the eluent flowing out of the cell outlet.

Creating an Instrument Method Using the Chromeleon Wizard

To create a new instrument method using the Chromeleon 7 CDS, select Create, Instrument Method, and specific Instrument. Table 3 describes the specific conditions for this application.

The compartment temperature is typically set for 15 °C for applications using a capillary suppressor. In ED the suppressor is not present, so the compartment temperature is set to within 3 °C of the separation temperature at 30 °C. Temperature control is important to maintain consistent separations; therefore it is important to conduct separations when the Dionex ICS-4000 door is closed and to set both temperatures as described in Table 3. The waveforms are thoroughly discussed in the ED User's Compendium.

Table 3. Additional conditions to create an instrument method using PAD

Page Title	Page	Mode	Action
Sampler Options	Injection	Injection Mode	PushCap
		Capillary Overfill+	50 (times)
		Accept Recommended Values+	Click on button
	General Settings	Temperature	Specify if needed
		Accept Recommended Values+	Click on button.
		Wait for Temperature	Click box if using the temperature option.
		Injection Wash Property	Enter AfterInj (After injection).
EDet Mode Options	Mode	DC or Integrated Amperometry	Click on box for Integrated Amperometry.
Integrated Amperometry	Cell Control	On	
	Reference Electrode	Type	Select Ag/AgCl
	Waveform	Type	Select "Gold Standard PAD" waveform with the Ag/AgCl reference electrode from pull down menu.
	Data Collection	Hz	Enter 2.0 for carbohydrates.
	pH	Lower and upper	Enter 11 and 13 for the lower and upper pH limit, respectively.
	Temperature	Column	Click on use box. Enter 30 (°C).
		Compartment	Click on use box. Enter 27 (°C).

+ At the 50x overfill setting, 20 µL of sample is withdrawn from the sample in full loop injection. For limited samples reduce the overfill setting to 20x which will withdraw 8 µL of sample. The Dionex AS-AP autosampler also has partial loop and limited sample modes which will reduce the total amount of sample withdrawn from the vial. These modes are thoroughly discussed in the product manual.[15]

Results and Discussion

The Gold on PTFE disposable working electrode was selected for this application because the electrode is more robust when using the 100 mM KOH wash and has a longer life than the standard gold disposable working electrode on polyester used for carbohydrate determinations.[20] This application followed the same conditions in AN 1006 adjusted to capillary flow rates. Additionally, a four-minute hold at 35 mM KOH was added and the equilibration time was extended 15 additional minutes to ensure the analytes were eluted from the column and the baseline was stable for the next injection. Figure 3 shows the separation of ten carbohydrates within 16 min. The critical pairs 3-O-methylglucose/rhamnose (Peaks 2 and 3) and lactose/lactulose Peaks 9 and 10 are partially resolved from each, similar to the previous results run on a 3 mm version of the same column (Figure 4).

Figure 5 compares the separations of four carbohydrates of interest (3-O-methylglucose/rhamnose, and lactose/lactulose) in deionized water (Chromatogram A, the bottom chromatogram colored blue) and in synthetic urine (Chromatogram B, top chromatogram colored brown). The overlaid chromatograms possess identical baseline profiles where the peaks elute at the same retention time in both the water and synthetic urine samples. The only differences are with the void peak (Peak 1). The void peak is a negative peak for the sample in the water matrix and a positive peak in the urine sample where the urine salts elute. These results nicely demonstrate that the high salt content of the synthetic urine sample did not overload the Dionex CarboPac PA20 capillary column. These results on the capillary format Dionex CarboPac PA20 column are similar to those previously achieved on the 3 mm version of the same column.

Instrument:	Thermo Scientific Dionex ICS-5000 IC System
Column:	Dionex CarboPac PA20, 3 × 150 mm
Eluent Source:	Thermo Scientific EGC-KOH III cartridge
Gradient:	10 mM KOH from -7 to 1 min, 10–30 mM KOH from 1–9 min, 30–35 mM KOH from 9–16 min
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL
Temperature:	30 °C
Detection:	PAD, Gold on 0.002" thick gasket PTFE (Disposable 4-Potential Carbohydrate waveform)
Samples:	Mixed carbohydrate standard in DI water, 1.67 µg/mL each with 0.32 µg/mL ribose

Peaks:

1. Mannitol	6. Xylose
2. 3-*O*-Methylglucose	7. Sucrose
3. Rhamnose	8. Ribose
4. Galactose	9. Lactose
5. Glucose	10. Lactulose

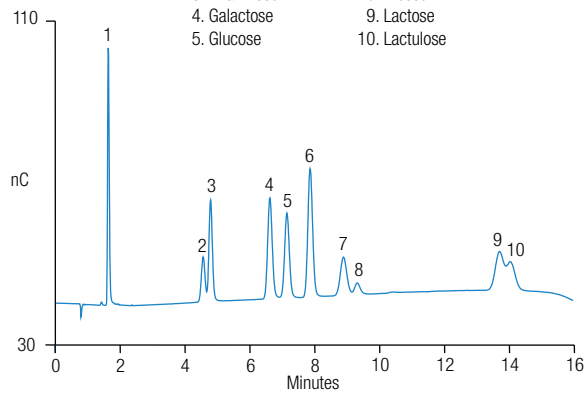

Figure 4. Separation of 10 Carbohydrates on a 3 × 150 mm Dionex CarboPac PA20 Column (AN 1006[1])

Instrument:	Dionex ICS-4000 Capillary IC system
Column:	Dionex CarboPac PA20, 0.4 mm
Eluent Source:	Dionex EGC-KOH Cartridge (Capillary) with Dionex CR-ATC Column (Capillary)
Gradient:	10 mM KOH (-7 to 1 min),10–30 mM KOH (1–9 min), 30–35 mM from (9–16 min)
Flow Rate:	0.008 mL/min
Inj. Volume:	0.4 µL
Column Temp.:	30 °C
Detection:	PAD, Au disposable, 4-Potential Carbohydrate waveform
Gasket:	0.001" PTFE
Ref. Electrode:	Ag/AgCl
Standard:	1.67 µg/mL each plus 0.32 µg/mL ribose in water

Peaks:

1. Mannitol	6. Xylose
2. 3-*O*-Methylglucose	7. Sucrose
3. Rhamnose	8. Ribose
4. Galactose	9. Laclose
5. Glucose	10. Lactulose

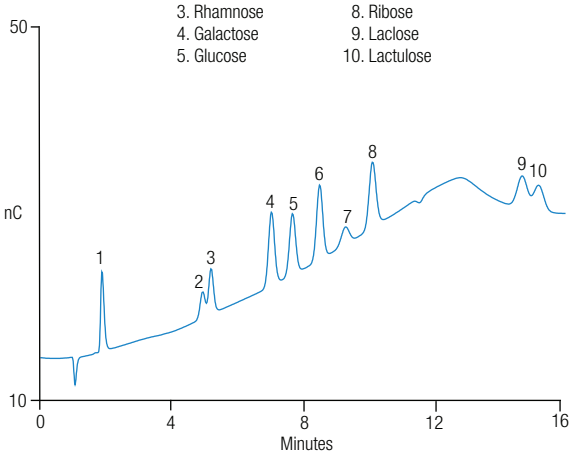

Figure 3. Carbohydrates of interest for urine analysis

Instrument:	Dionex ICS-4000 IC System
Column:	Dionex CarboPac PA20 set, 0.4 × 150 mm
Eluent Source:	Dionex EGC-KOH Cartridge (Capillary) with Dionex CR-ATC Column (Capillary)
Gradient:	10 mM KOH (-7 to 1 min),10–30 mM KOH (1–9 min), 30–35 mM KOH from (9–16 min)
Flow Rate:	0.008 mL/min
Inj. Volume:	0.4 µL
Column Temp.:	30 °C
Detection:	PAD, Au disposable, 4-Potential Carbohydrate waveform
Gasket:	0.001" PTFE
Ref. Electrode:	Ag/AgCl
Sample Prep.:	600-fold dilution in water
Samples:	1.67 µg/mL after dilution A) in water, B) in synthetic urine

Peaks:

1. Void Volume	4. Rhamnose
2. Mannitol	5. Xylose
3. 3-*O*-Methylglucose	6. Lactulose

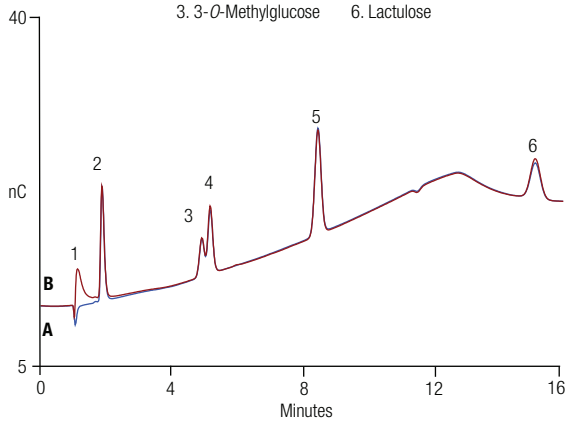

Figure 5. Carbohydrates in A) Water and B) Synthetic Urine

Conclusion

Determinations of monosaccharides and disaccharides are important to scientists researching disease states as discussed in the references. This application was developed and previously demonstrated in AN 1006 using a 3 mm Dionex CarboPac PA20 column set.[1] Here we demonstrated that the same application can be run in capillary format on the dedicated capillary IC, the Dionex ICS-4000 HPIC system. For more information on sialic acid applications, see references 21–23.

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