

Carbohydrate Analysis using HPLC with PAD, FLD, Charged Aerosol Detection, and MS Detectors

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Overview

Purpose: Sensitive applications for the analysis of simple carbohydrates, either as mono- or disaccharides, or as fairly small glycans liberated from glycoproteins typically containing 6–11 residues, are desired to enhance sampling rates. Simple, rapid and accurate methods have been developed for the analysis of carbohydrates using various chromatography and detection solutions.

Methods: A sensitive pulsed amperometric detection method for the analysis of simple carbohydrates was developed and examples are shown for impurity analysis of sugars used in positron emission tomography (PET). A second chemistry for the analysis of fluorescent derivatives of glycans from glycoproteins is described. The direct analysis of simple sugars using HILIC-mode chromatography with charged aerosol detection is described. Examples showing the use of this technique for their direct determination in fruit juices are shown. Finally, orthogonal use of charged aerosol detection and MS detectors for the analysis of glycans from glycoproteins is described.

Results: These methods enable the rapid separation of carbohydrate compounds at low levels and with minimal matrix interference.

Introduction

Carbohydrates are difficult to analyze because they have similar physical and chemical characteristics, and do not have a suitable chromophore for UV detection. Several different HPLC methods using various detector strategies (pulsed amperometric electrochemical, fluorescence following derivatization, charged aerosol detection and mass spectrometry) were developed to help study carbohydrates and examples for each approach will be presented. Although fluorescent tags improve the chromatographic resolution and detector sensitivity, they can lead to increased assay variability. Different HPLC modes can be used for carbohydrate separations, with ion exchange, hydrophilic interaction liquid chromatography (HILIC) and reversed phase (RP) on porous graphite column (PGC) being the most common. HPLC enables the development of simpler chromatographic methods with direct detection using PAD or mass detectors such as ELSD, MS, and charged aerosol detection. The Thermo Scientific Dionex Corona Charged Aerosol Detector (CAD™) is an ideal detector when combined with HILIC or RP/PGC for measuring different carbohydrates. It is a mass-sensitive detector that can measure any non-volatile, and many semi-volatile compounds, typically with low nanogram sensitivity. Unlike ELSD, it shows high sensitivity, wide dynamic range, high precision, and more consistent inter-analyte response, independent of chemical structure. For the analysis of glycans liberated from glycoproteins, the utility of the LC-MS with charged aerosol detection platform is illustrated where the Corona CAD is used for quantitative analyses while the MS provides structural verification. The advantage of this approach over methods using fluorescent tags is discussed.

Methods

General considerations for Pulsed Amperometric Detection

The Thermo Scientific Dionex UltiMate 3000 with PAD platform consisted of a base-compatible HPLC system, a Thermo Scientific Dionex Coulochem III electrochemical detector and a gold working electrode. Carbohydrates were separated using ion exchange and determined under basic conditions using a four-pulse waveform. It is essential to make sure no titanium is in the flow path as its degradation under basic conditions can lead to deterioration of column and electrode performance.

Carbohydrate Analysis by HPLC-EC PAD:

| | | | |
|-------------------|--------------------------------------------------------------------------------------|----------------|-----------|
| Pump: | Thermo Scientific Dionex ISO-3100 SD | | |
| Autosampler: | Thermo Scientific Dionex WPS-3000TSL Analytical Autosampler | | |
| Flow: | Isocratic at 0.50 mL/min. with constant He purge | | |
| Column: | Thermo Scientific CarboPak: PA20, 3 x 150 mm, 6.5 µm | | |
| Temperature: | 32 °C | | |
| Injection volume: | 50 µL partial loop | | |
| Mobile Phase: | 50 mM sodium hydroxide (NaOH), prepared from pellets, 99.99%, semiconductor grade | | |
| EC detector: | Coulochem™ III, Thermo Scientific Dionex model 5040 cell with Au Target: 25 µm Mylar | | |
| EC Parameters: | E1 | +200 mV 500 ms | AD 300 ms |
| | E2 | -2000 mV 10 ms | |
| | E3 | +600 mV 10 ms | |
| | E4 | -100 mV 10 ms | |
| Range: | 200 nC | | |

Analysis of Glycan Derivatives with Fluorescence Detector

Flow: 1.0 mL/min.
Column: TSKgel® Amide-80, 4.6 x 150 mm, 3 µm
Temperature: 35 °C
Injection volume: 5 µL partial loop
Mobile Phase A: 50 mM ammonium formate in water pH=4.4
Mobile Phase B: Acetonitrile
Gradient: 65%B to 53%B in 24 min, 0%B from 24.5 min to 26.5 min, re-equilibration from 27 min to 35 min.
FI Detector: Ex 320 nm; Em 420 nm
Glycan derivative formation: N-Glycanase (2 µL) was added to 100 µg protein in 50 µL buffer then placed overnight at 37 °C. Following release from protein, carbohydrates were cleaned up using Thermo Scientific Hypercarb SPE. Glycans were eluted from SPE using 40% acetonitrile/60% water/0.1% TFA. Samples were dried in a refrigerated speedvac at 4 °C. To dried samples, 20 µL 2-AA solution was added, mixed and heated at 65 °C for 3 hours. After reaching room temperature, excess labeling reagent was removed with 1 mL acetone wash (repeated 3 times) and samples were dried with a speedvac. Samples were then reconstituted with 200 µL MP A and then transferred to an autosampler vial for injection.

Direct Carbohydrate Analysis in Fruit Juice with Charged Aerosol Detection

Flow: Isocratic at 1.4 mL/min.
Column: Shodex™ Asahipak® NH2P-50 4E 4.6 x 250 mm, 5 µm
Temperature: 55 °C
Post column temperature: 30 °C
Inj. volume: 2 µL for sample, 5 µL for standards
Mobile Phase: 78% Acetonitrile, 22% Water
Detector: Corona™ ultra RS™
Nitrogen: 35 psi Corona filter: Corona
Sample Preparation: To 1 gram juice sample, add 20 mL of 70% acetonitrile, mix, and then centrifuge for 2 min @ 13,000 RPM

Glycoprotein Analysis by parallel Charged Aerosol Detection and MS

Flow: 1.0 mL/min.
Column: Thermo Scientific PGC Hypercarb 4.6 x 150 mm, 5 µm
Temperature: 55 °C
Post column temperature: 30 °C
Inj. volume: 5 µL partial loop
Mobile Phase: MeCN/H₂O/0.1% TFA: 4%–18% Acetonitrile gradient in 40 min
Detector: Corona ultra RS
Nitrogen: 35 psi Corona filter: Corona
Sample Preparation: N-linked glycans were released by standard procedures using recombinant N-glycanase or endo-H. O-glycans were chemically released either by traditional reductive β-elimination (RBE),¹ or by nonreductive ammonia/ammonium carbonate (NAC).² Cleanup of samples was performed by cation-exchange SPE (solid-phase extraction; collecting flow-through) and borate evaporation. Secondary cleanup of polypeptides using a C18 SPE (collect flow-through) was performed.

Results and Discussion

Direct Carbohydrate Analysis with PAD

Carbohydrates are commonly measured using high performance anion exchange chromatography in combination with pulsed amperometric detection (PAD). A simple PAD method was developed using the Coulochem III electrochemical detector with pulse mode. The chromatogram shown in Figure 1 illustrates replicate injections of carbohydrate standards (1 ng on-column). Good retention stability was observed (data not shown) and the assay reproducibility over 14 hours is shown in Table 1. The sensitivity of this method can reach a limit-of-detection (LOD) of less than 100 pg on-column (data not shown). This method was used for impurity testing of 2-deoxy-2-[¹⁸F]fluoro-D-glucose (FDG), which is the most widely used radiopharmaceutical for positron emission tomography. The synthesis of FDG involves nucleophilic displacement and hydrolysis. A contaminant resulting from both processes is 2-Deoxy-2-chloro-D-glucose (CIDG). Limits for CIDG have been set by USP, CMC, and EP and soon by the FDA. Due to the sensitivity and selectivity of PAD, sample preparation procedures are not required. HPAC-PAD is a well established, sensitive, and selective method for determining carbohydrates (Figure 2). The separation uses a CarboPak™ PA20 anion exchange column using an alkaline MP (50 mM NaOH) and meets the resolution requirements set by the USP method.

Table 1. Reproducibility statistics for 14 hour run (50 ng standard)

| | Glucose | | Fructose | | Sucrose | | Lactose | |
|------|---------|------|----------|------|---------|------|---------|------|
| | Height | Area | Height | Area | Height | Area | Height | Area |
| Mean | 178.3 | 15.9 | 85.9 | 8.7 | 44.6 | 5.1 | 95.2 | 12.9 |
| SD | 1.61 | 0.21 | 0.93 | 0.19 | 0.51 | 0.11 | 0.90 | 0.21 |
| RSD | 0.90 | 1.33 | 1.08 | 2.15 | 1.15 | 2.23 | 0.95 | 1.59 |

FIGURE 1. Triplicate injections of carbohydrate standards (1 ng on-column) using anion exchange chromatography and PAD detection.

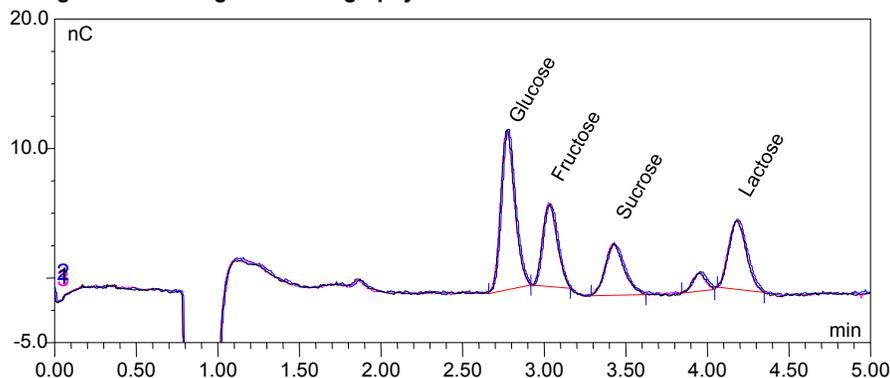
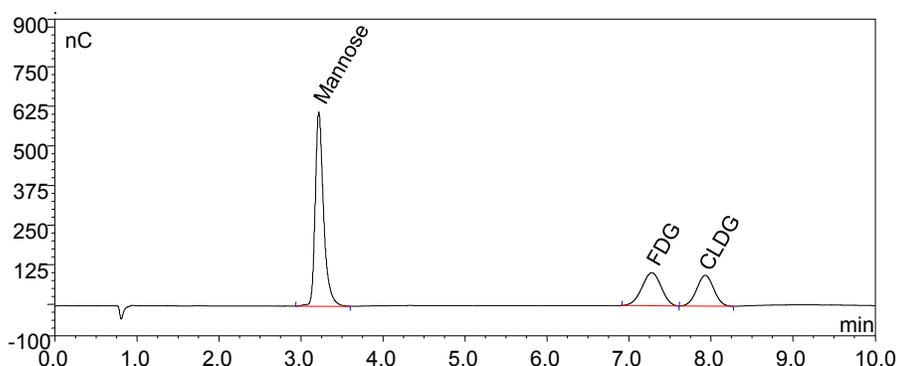


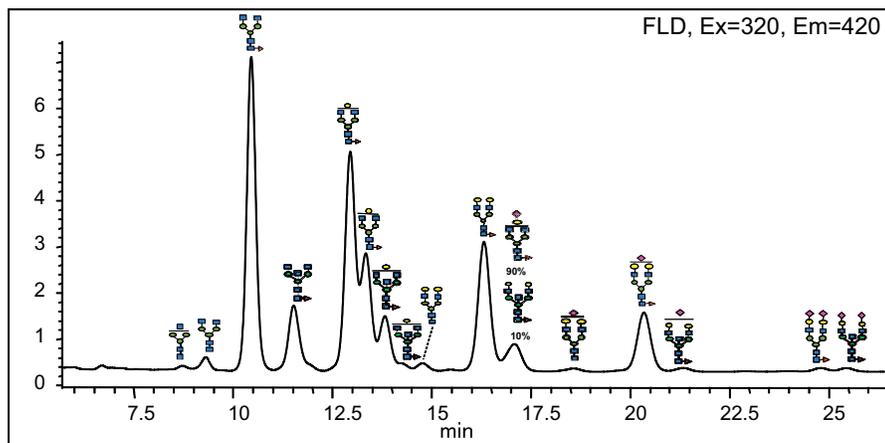
FIGURE 2. Chromatogram illustrating the separation and detection of 10 µg/mL FDG/CLDG / mannose using anion exchange chromatography and PAD detection.



Glycan Analysis with Fluorescence Detection_

The analysis of glycans using fluorescence detection requires the formation of a suitable derivative. In this case the highly fluorescent 2-anthranilic acid (2-AA) derivative was prepared according to the technique described by Anumula.³ A technical note illustrating a similar fluorescence method for glycan profiling using

FIGURE 3. Carbohydrate separation of 2-AA derivatives using HILIC-mode HPLC with fluorescence detection.



2-aminobenzamide (AB) has been published by Thermo Fisher Scientific.⁴ The proposed structure assignments shown in Figure 3 were based on accurate mass analysis.

Analysis of Carbohydrates in Fruit Juices using Charged Aerosol Detection

The analysis of simple carbohydrates was performed using a polymeric amino column in HILIC mode (Figure 4). This column, unlike silica-based columns, provides adequate separation while offering good stability and low column bleed. Improved selectivity and speed of separation of the various carbohydrate species was achieved by decreasing the water content and elevating the column temperature. Simple carbohydrates were eluted in less than 16 minutes and the LOD was ~5 ng on-column (1 µg/mL using a 5 µL injection), which is much better than those achieved by RI or ELSD. This method was applied to the measurement of carbohydrates in fruit juice samples (Figure 5). Other food sugars in honey and different corn syrups (data not shown) have also been successfully analyzed. The method can be used to easily determine sugar abundance in a variety of food products. This technique is a good example of detecting analytes that only possess weak chromophores with very simple analytical conditions and sample preparation

FIGURE 4. Carbohydrate measurement using HILIC-mode HPLC with charged aerosol detection.

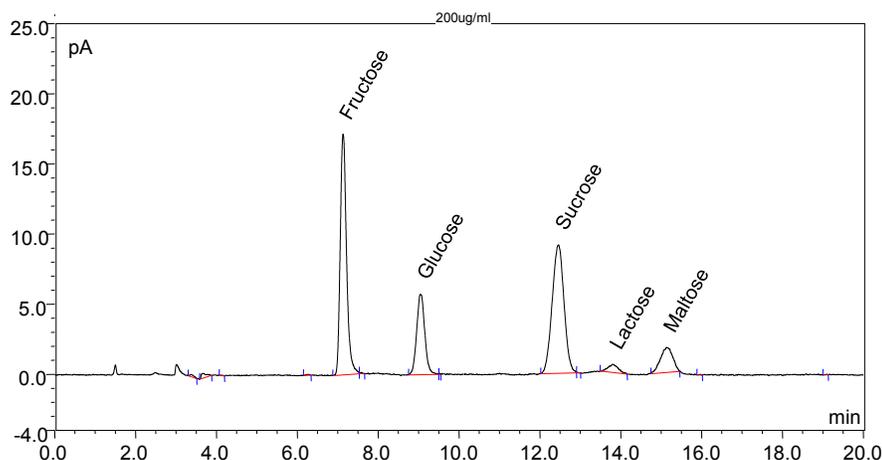
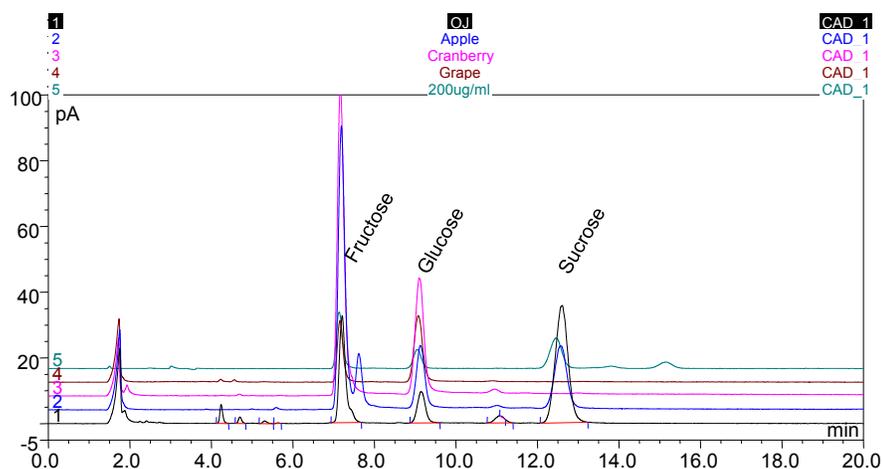


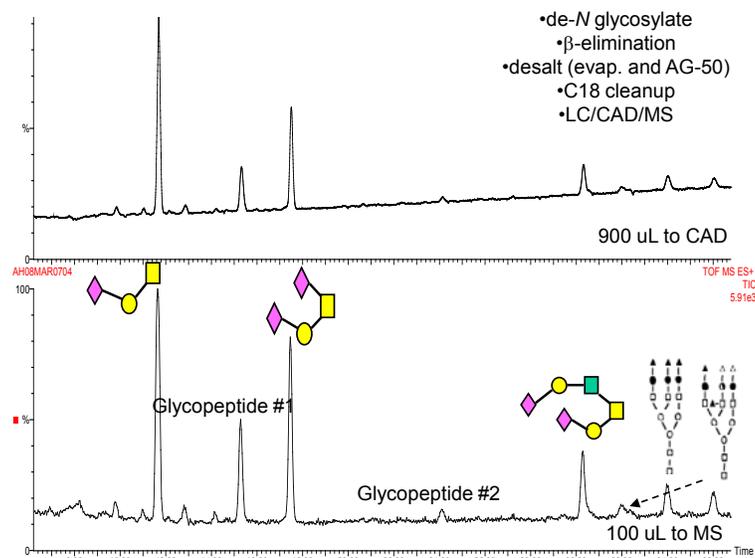
FIGURE 5. Direct analysis of carbohydrates in various fruit juice samples.



Glycoprotein Analysis with Charged Aerosol and MS detection

The Corona CAD and MS use the same volatile mobile phase. The two analytical techniques can also be used orthogonally with the Corona CAD providing the profile of the glycans and quantitative data, while MS is able to provide structural information. This is shown in Figure 6 for the analysis of glycans from fetuin. The combined technique is a powerful approach to glycan identification and quantification.

FIGURE 6. Fetuin: reductive β -elimination: LC/Charged Aerosol Detection/ MS QToF (+ mode) using a Hypercarb PGC column with parallel charged aerosol detection and MS.



Conclusions

- The UltiMate™ 3000 with PAD system offers a routine and robust approach for the direct determination of simple carbohydrates. The reproducibility of this method shows an RSD of less than 2% (height) over a 14 hour period. The mass sensitivity of this method allows for detection of 100 pg simple carbohydrates.
- The use of the derivatizing agent 2AA and fluorescence detection offers excellent sensitivity and selectivity for the measurement of glycans from glycoproteins.
- The direct HILIC-charged aerosol detection approach offers a simple way to both accurately and precisely determine simple carbohydrates. Fruit juices and food samples were easily processed by weighing the sample and diluting the material in mobile phase compatible solutions. Direct injection of samples using HILIC mode separation provided adequate separation of sugars with low ng detection levels achieved using the Corona CAD. Using this platform, useful product characterizations can be performed using mobile phases which are compatible with nebulizer based detectors. This helps to simplify analysis and the use of MS as an orthogonal detector enables confirmation of structures.
- The *N*- and *O*-linked glycans derived from recombinant proteins and other glycoproteins can be profiled and characterized using PGC RP-HPLC-Charged aerosol detection-MS. For glycoprotein analysis, detection by the Corona CAD avoids some of the pitfalls of PAD (high salt), and fluorescence (*O*-glycan peeling, fluorescent impurities, ion-pairing agents). These issues can hamper LC-MS and downstream offline-MS/MS analysis of unknowns. The Corona CAD is a simple and robust instrument and thus amenable for use in routine development and even manufacturing QC operations.

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