

Simple Separation and Detection Techniques for the Analysis of Carbohydrates

Michael G. Hvizd,¹ Bruce Bailey,² Christopher Crafts,² Marc Plante,² and Ian Acworth²

¹Thermo Fisher Scientific, Bannockburn, IL, USA; ²Thermo Fisher Scientific, Chelmsford, MA, USA

Abstract

Carbohydrates are difficult to analyze because they are very polar compounds, exhibit similar structural characteristics, and do not have a suitable chromophore. Their analysis is often performed directly using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) with a high pH mobile phase. This technique has been approved for use in a variety of official methods for the analysis of carbohydrates in foods. Another common technique is to derivatize the sample with a suitable fluorescent tag and use HPLC with fluorescence detection for the analysis. This approach can assist with the separation of sugar isomers and provide sensitive detection, although derivatization can contribute to increased assay variability.

HPLC column technology for the separation of carbohydrates has advanced during the past several years. New hydrophilic interaction liquid chromatography (HILIC) mode separations are providing a unique way to separate polar carbohydrates using simple organic/water mobile phases. This chromatographic approach not only provides suitable separation, but also enables the use of sensitive nebulizer-based detectors (i.e., mass spectrometry [MS] or charged aerosol detection). The work presented here describes the use of the Thermo Scientific Dionex Corona™ Charged Aerosol Detector (CAD™) for the measurement of simple carbohydrates. The method has a limit of detection of <10 ng on column and a wide dynamic range that covers ng to µg levels with high reproducibility.

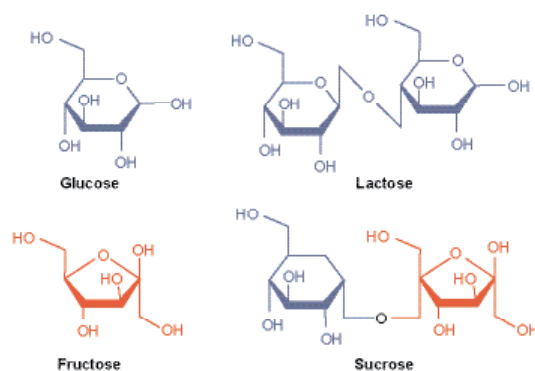
The determination of more complex carbohydrates—for example, glycans liberated from glycoproteins—can be achieved using a combination of MS and charged aerosol detection. Because the Dionex charged aerosol detector is a universal detector, there is no need to form fluorescent tagged derivatives, thus simplifying their analysis. The Dionex charged aerosol detector is used for quantitative analyses while the MS is used for structural verification. Data showing the utility of the LC-MS-CAD platform for analysis of glycoproteins is presented.

Introduction

Simple sugars are common components of beverages such as fruit juice and soft drinks. The similar structural characteristics of several simple sugars are shown in Figure 1. Carbohydrates possess weak chromophores, so UV detection cannot be practically employed for their analysis. These compounds can be directly oxidized using PAD techniques¹ or can be measured by fluorescence detection after preparation of a suitable derivative.² Recently, universal detection techniques such as evaporative light scattering detection (ELSD) have been employed, however, the lack of sensitivity and nonlinear nature limit their use. Although the PAD method can achieve low ppb levels of sensitivity, many food- and beverage-related applications do not require this level of sensitivity.

These compounds are highly soluble in water and are often poorly retained and separated by techniques such as reversed-phase chromatography on C18 columns. Carbohydrate separation can be performed using ion-exchange or with HILIC-mode chromatography. HPAE techniques provide excellent separation of complex samples containing many different carbohydrate species. However, the commonly used eluents contain nonvolatile species that are incompatible with most nebulizer-based detectors (e.g., MS, ELSD, CAD). HILIC separations typically involve high organic/ aqueous mobile phases with volatile additives, which are ideal for nebulizer-based detectors.

FIGURE 1. Structures of simple carbohydrates.



26146

The charged aerosol detector is a universal detector capable of measuring nonvolatile compounds, and is mass sensitive and can be added to HPLC or UHPLC platforms. This detector provides the most consistent response for all nonvolatile and some semivolatile analytes of all nebulizer-based HPLC detection techniques.³ The detector works by charging particles and is not dependent on light scattering, which has large variability and generally lower sensitivity.

Experiments were conducted to illustrate the separation of various carbohydrates from foods or fruit juices using HILIC. Direct and reproducible detection was performed using the Corona CAD. Glycoprotein analysis was conducted using a porous graphite column (PGC) with combined charged aerosol and MS detection.

Chromatography and Sample Preparation

Fruit Juice HPLC Method Conditions

Column: Shodex Asahipak® NH₂P-50 4E, 250 × 4.6 mm, 5 μm
Mobile Phase: 78% Acetonitrile, 22% water
Flow Rate: 1.4 mL/min
Column Temp.: 55 °C
Postcolumn Temp.: 30 °C
Injection Volume: 2 μL for sample, 5 μL for standards
Analysis Time: 15 min per sample
Detector: Thermo Scientific Dionex Corona *ultra*[™]
Charged Aerosol Detector,
nitrogen 35 psi; filter Corona
Stock Standard: 1 mg/mL each standard in 70% acetonitrile
Sample Prep.: Add 20 mL of 70% acetonitrile to 1 g juice sample,
mix, and then centrifuge for 2 min at 13,000 RPM

Food Analysis HPLC Parameters

Column: Shodex Asahipak NH₂P-50 4E, 250 × 4.6 mm, 5 μm
Mobile Phase: 70:30, Acetonitrile/water
Flow Rate: 1 mL/min
Column Temp.: 35 °C
Injection Volume: 10 μL
Detector: Corona CAD; nitrogen 35 psi; filter high
Sample Prep.: Honey and corn syrup samples were weighed
then were diluted in mobile phase

Glycoprotein Analysis

Column: PGC Hypercarb, 150 × 4.6 mm, 5 μm
Flow Rate: 1.0 mL/min
Mobile Phase: Acetonitrile/water/0.1% TFA: 4–18%,
Acetonitrile linear gradient >40 min
Sample Prep.: N-Linked glycans were released by standard procedures
using recombinant N-glycanase or endoglycosidase H.
O-Glycans were chemically released either
by traditional reductive β-elimination (RBE),⁴ or by
nonreductive ammonia/ammonium carbonate (NAC).⁵

UHPLC Method Conditions

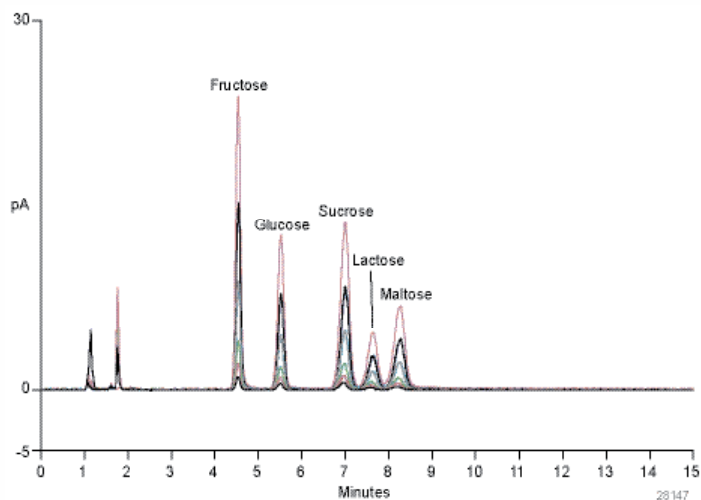
Column: BEH HILIC, 2.1 × 50 mm, 1.7 μm
Mobile Phase: Acetonitrile/5 mM ammonium formate, pH 3, (91:9)
Flow Rate: 1.8 mL/min
Column Temp.: 40 °C
Injection Volume: 2 μL
Detector: Corona *ultra*[™]; nitrogen 35 psi; filter medium

Results and Discussion

Analysis of Carbohydrates in Fruit Juices

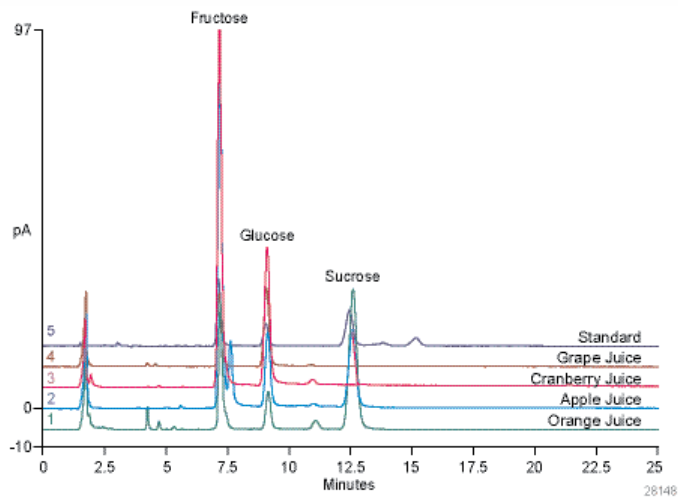
The analysis of fruit juice samples was performed using a polymeric amino column in HILIC mode. This column provided adequate separation while offering good stability and low column bleed characteristics compared to silica-based support materials, which was important for good sensitivity using charged aerosol detection. Improved selectivity and speed of separation of the various carbohydrate species present in these samples were achieved by decreasing water content and elevating the column temperature. Figure 2 illustrates the separation of fructose, glucose, sucrose, lactose, and maltose in less than 10 min. The limit of detection (LOD) for carbohydrate analysis using charged aerosol detection is approximately 5 ng on column (1 μg/mL using a 5 μL injection), thereby providing much better sensitivity than RI or ELSD detection.

FIGURE 2. Calibration standards for fruit juice carbohydrate analysis (25–1000 ng on column).



Sample preparation of various fruit juice samples for analysis using this technique was quite simple. An aliquot of juice sample was weighed and diluted in a solution that was compatible with the HILIC mobile phase conditions. After centrifugation, an aliquot of the sample was transferred to an autosampler vial for direct analysis of their sugar content as shown in Figure 3. Trace levels of several unknown peaks were observed.

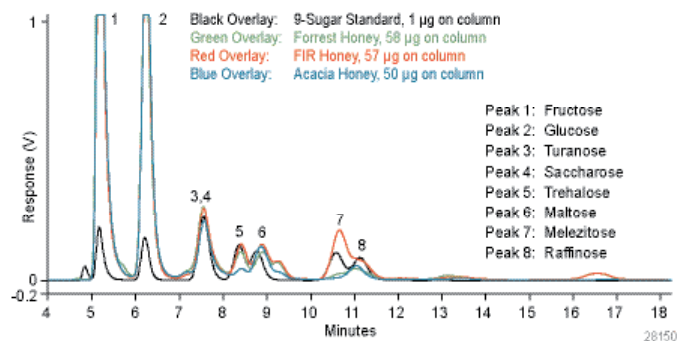
FIGURE 3. Direct analysis of carbohydrates in various fruit juice samples.



Analysis of Carbohydrates Found in Food Products

This work also describes use of the Corona CAD detector for measurement of a number of sugars typically found in honey. The method can be used to determine sugar abundance in different varieties of honey (Figure 4). This is an example of detection of analytes that only possess weak chromophores.

FIGURE 4. Comparison of carbohydrates found in different honey products.



HILIC chromatography with the Corona CAD was also used for the measurement of different corn syrups e.g., 43/43 carbon-refined syrup (Figure 5). These products are often used in the production of table syrups, jams, and jellies. The method has excellent sensitivity (components typically <10 ng on column), reproducibility (%RSD typically 0.6–1.8), and a dynamic range that covers ng to µg levels. This allows the extended characterization of higher dextrose equivalent (DE) chains as illustrated in the inset of Figure 5.

FIGURE 5. Reproducibility of 43/43 high DE corn syrup analysis (10 µg on column; six replicate injections). Inset shows peaks eluting between 8 and 19 min, magnified ~10 fold.

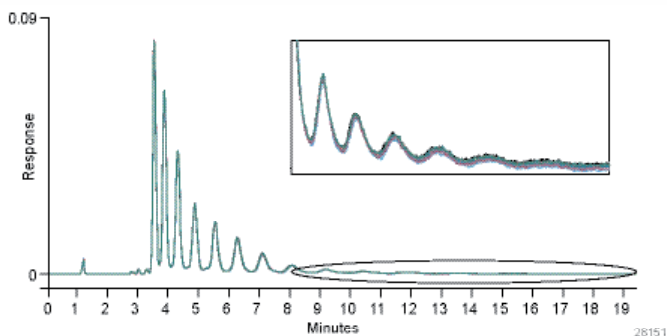
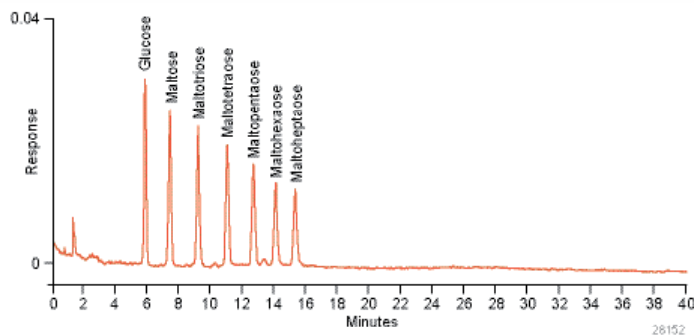


Figure 6 shows another example of the use of charged aerosol detection for measurement of oligosaccharides. The method has a LOD of <5 ng on column (Figure 6), and a dynamic range that covers ng to µg levels. Consistent response factors for the various oligosaccharides are observed using charged aerosol detection, because the slopes of these calibration curves are quite similar (data not shown).

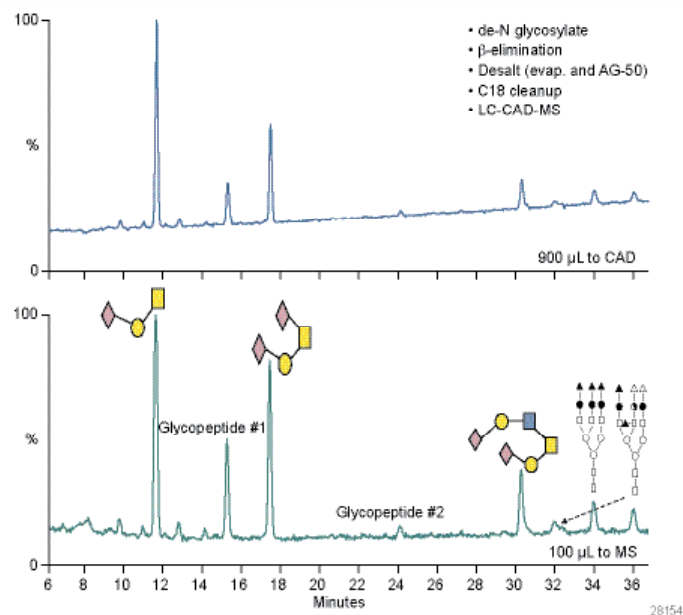
FIGURE 6. Analysis of oligosaccharide standards (200 ng each on column).



Profiling and Characterization of N- and O-Linked Glycans Released from Glycoproteins

The analysis of the carbohydrate structures on recombinant proteins is becoming increasingly important for many pharmaceutical companies as more protein- and peptide-based biological therapeutics are being developed. N- and O-linked glycans, released from bovine fetuin and other glycoproteins, were profiled by PGC RP-HPLC with charged aerosol detection, and characterized simultaneously by online MS (Figure 7). This approach provides an optimal workflow between glycan release, cleanup, routine profiling, and online LC-MS. Because the Corona CAD is a universal detector, the glycans do not require derivatization with a fluorescent dye as is currently practiced in the biopharmaceutical industry. This helps to simplify sample preparation and eliminate artifactual peaks resulting from fluorescence-labeling chemistry.

FIGURE 7. Fetuin: reductive β -elimination: LC-CAD-MS QToF (+ mode) using a PGC (Hypercarb) column.



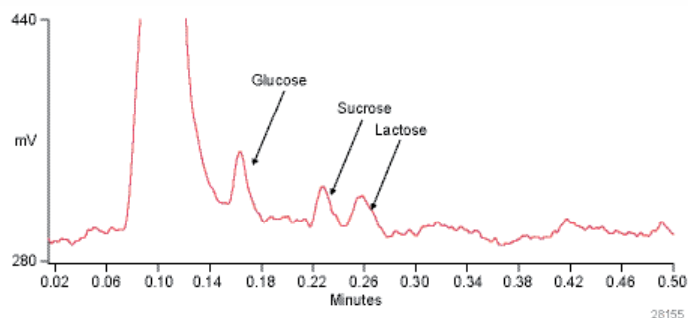
Both charged aerosol and MS detection require nonvolatile eluents and their use in parallel is thus easily achieved by a postcolumn flow split. This provides highly complementary data where charged aerosol detection can be used for quantitation and MS can be used for peak identification. The use of simple, volatile eluents also facilitates offline structural elucidation studies when fraction collection is employed.

The use of charged aerosol detection for unlabeled glycans (alditols) with flow splitting for simultaneous MS detection is simple and sensitive. Any collected fractions are generally clean (buffer-free), thus greatly simplifying further MS-based analysis. Reproducible profiles of unlabeled glycan alditols are achieved with charged aerosol detection and these provide quantitative data. This data together with HPLC retention times and online MS-derived mono-saccharide compositions, the combined technique provides an initial level of glycan identification.

UHPLC Analysis of Carbohydrates

New UHPLC-type HILIC columns allow rapid and sensitive analysis of simple carbohydrates as illustrated in Figure 8. In this example, the analysis of sugars is complete in <30 sec with the Corona *ultra* detector sensitivity extending into the sub-nanogram range. Future development with this type of column technology should allow significant improvements in both speed, separation, and sensitivity for carbohydrate analysis.

FIGURE 8. Injection of 0.5 ng of sugars, glucose, sucrose, and lactose.



Conclusions

The HPLC-CAD methods shown here demonstrate the capability to both accurately and precisely determine simple carbohydrates. The analysis of carbohydrates in fruit juices and foods was easily performed by weighing the sample and diluting the material in mobile phase compatible solutions. Direct injection of samples onto a HILIC column provided adequate separation of sugars with low ng detection levels achieved using the Corona CAD detector.

With the described conditions, the use of HILIC-CAD provided sufficient selectivity for a variety of sample matrices. However, there were some examples where some analytes were not completely resolved (e.g., honey). In these cases, the use of a more selective detector (e.g., MS), alternative separation conditions with charged aerosol detection or the use of HPAE-PAD may be required.

The N- and O-linked structures most relevant to recombinant glycoproteins can also be profiled and characterized using the proposed method of PGC RP-HPLC-CAD-MS. For glycoprotein analysis, charged aerosol detection avoids some of the pitfalls of PAD (high salt) and fluorescence (O-glycan peeling, fluorescent impurities, ion-pairing agents) that can hamper LC-MS and downstream offline MS/MS analysis of unknowns. The Corona CAD detector is a simple and robust instrument, making it amenable for use in routine development and manufacturing QC operations. It should prove useful when applied to the analysis of oligosaccharides by greatly simplifying the efforts and expertise needed for oligosaccharide profiling of N- and O-linked sugars.

Acknowledgements

The authors would like to thank Dr. Andrew J. S. Hanneman and Jason C. Rouse at Wyeth Pharmaceuticals for providing the data on LC-MS analysis of glycans.

References

1. Rocklin, R. D.; Clarke, A. P.; Weitzhandler, M. Improved Long-Term Reproducibility for Pulsed Amperometric Detection of Carbohydrates via a New Quadruple-Potential Waveform. *Anal. Chem.* **1998**, *70*, 1496–1501.
2. Anumula, K. R.; Dhume, S. T. High Resolution and High Sensitivity Methods for Oligosaccharide Mapping and Characterization by Normal Phase High Performance Liquid Chromatography Following Derivatization with Highly Fluorescent Anthranilic Acid. *Glycobiology* **1998**, *8*, 685–694.
3. ESA—A Dionex Company. *Dependence of Response on Chemical Structure*, Application Brief 70-8913 Rev B.
4. Carlson D. M. Structures and Immunochemical Properties of Oligosaccharides Isolated from Pig Submaxillary Mucins. *J. Biol. Chem.* **1968**, *243*, 616–626.
5. Huang, Y.; Mechref, Y.; Novotny, M. V. Microscale Nonreductive Release of O-Linked Glycans for Subsequent Analysis through MALDI Mass Spectrometry and Capillary Electrophoresis. *Anal. Chem.* **2001**, *73*, 6063–6069.

www.dionex.com/



Thermo Scientific Dionex products are designed, developed, and manufactured under an ISO 9001 Quality System.

©2011 Thermo Fisher Scientific Inc. All rights reserved. Ashipak is a registered trademark of Showa Denko K.K. All trademarks are the property of Thermo Fisher Scientific Inc. and its subsidiaries. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.

U.S./Canada	(847) 295 7500	Denmark	(45) 36 36 90 90	Sweden	(46) 8 473 3380	India	(91) 22 2764 2735
Brazil	(55) 11 3731 5140	France	(33) 1 39 30 01 10	Switzerland	(41) 62 205 9966	Japan	(81) 6 6885 1213
Austria	(43) 1 616 51 25	Germany	(49) 6126 991 0	United Kingdom	(44) 1276 691722	Korea	(82) 2 2653 2580
Benelux	(31) 20 683 9768	Ireland	(353) 1 644 0064	Australia	(61) 2 9420 5233	Singapore	(65) 6289 1190
	(32) 3 353 42 94	Italy	(39) 02 51 62 1267	China	(852) 2428 3282	Taiwan	(886) 2 8751 6655

Thermo
SCIENTIFIC
Part of Thermo Fisher Scientific