Protein therapeutics applications notebook

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Introduction

PROTEIN BIOPHARMACEUTICALS–CHALLENGES IN CHARACTERIZATION

Protein and monoclonal antibody (MAb) biopharmaceuticals form the largest part of the growing biologics drug market, and have transformed the biotechnology and biopharmaceutical industries in the last decade. Protein biopharmaceuticals include blood factors, thrombolytic agents, hormones, hematopoietic growth factors, interferons, interleukin-based products, tumor necrosis factor, and therapeutic enzymes.

MAb treatment is one of the most effective methods for diagnosis and treatment of a broad range of diseases, including autoimmune disorders, cardiovascular diseases, infectious diseases, cancer, and inflammation. This approach offers key advantages, such as reduced toxicity and side effects, and provides high targeted specificity.

There is an extensive development pipeline for protein and MAb therapeutics, further emphasizing the need for innovative analytic tools. During development and production of these products, it is essential to detect, characterize, and quantify impurities as well as structural variants and modifications, and to monitor product stability. This is key to demonstrating safety and efficacy and is required by the U.S. FDA and other regulatory agencies.

Common analytical tasks include:

- Purity check
- Aggregation analysis
- Fragmentation analysis
- Clips and truncations
- Post-translational modification analyses
  - Deamidation
  - *N*-acylation
  - Oxidation
  - Amino acid substitutions/truncation
  - Phosphorylation
- PEGylation
- Peptide mapping
- Glycan and glycosylation changes

BIOSEPARATION SOLUTIONS FOR PROTEIN AND MAb BIOPHARMACEUTICALS

Thermo Scientific offers next-generation analytical high-performance liquid chromatography (HPLC) systems for biomolecules, combined with an outstanding array of column innovations that address every facet of protein analysis and characterization.

The Thermo Scientific Dionex UltiMate 3000 Titanium HPLC system is fully biocompatible and supports robust and reliable operation, even with high-salt and pH buffers typically required for protein separation. The dual-gradient pump design, combined with an autosampler that also collects fractions, allows unprecedented throughput and automation.

The Thermo Scientific ProPac ion-exchange (IEX), hydrophobic interaction chromatography (HIC), and immobilized metal affinity chromatography (IMAC) columns provide high-resolution purity analysis and are the industry standards for charge and microheterogeneity characterization.

Monoliths have emerged as a key purification methodology. The Thermo Scientific ProSwift monolithic IEX and reversed-phase (RP) columns are useful for fast, high-resolution, and high-capacity protein separation and purification. They are excellent for rapid quantification and monitoring of biopharmaceuticals in research and development (R&D) and quality assurance/control (QA/QC).

Peptide mapping and LC/mass spectrometry (MS) characterization of structural modifications provide detailed structural information on protein and monoclonal antibody biopharmaceuticals. The UltiMate™ 3000 rapid separation LC (RSLC) and RSLCnano systems provide ultrafast, maximum resolution as well as higher sensitivity for in-depth characterization of protein sequence and modifications, thus meeting the key challenges in chromatography. The UltiMate 3000 RSLC platform is the perfect partner for MS to reveal protein modifications.

THERMO SCIENTIFIC AND DIONEX INTEGRATED SYSTEMS

Dionex Products are now a part of the Thermo Scientific brand, creating exciting new possibilities for scientific analysis. Now, leading capabilities in LC, ion chromatography (IC), and sample preparation are together in one portfolio with those in MS. Combining Dionex's innovation in chromatography with Thermo Scientific's leadership position in MS, a new range of powerful and simplified workflow solutions is now possible.

These Thermo Scientific integrated solutions can expand your capabilities and provide tools for new possibilities:

- IC and MS
- LC and MS
- Sample preparation and MS

High throughput and automation

Protein therapeutics applications notebook

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Application Update 174

On-Line High-Throughput Desalting to Prepare Samples for Mass Spectrometry

INTRODUCTION

Mass spectrometry (MS) or liquid chromatography-mass spectrometry (LC-MS) has become a powerful tool for the analysis of complex biochemical samples. These samples often contain high concentrations of salts that interfere with the ionization process and can result in lower peak intensities, compromised peptide/protein detection, and frequent contamination of the ion source. Therefore, it is essential to remove salts from samples before subjecting them to MS detection. Although the most common desalting method is off-line solid-phase extraction (SPE), it has the disadvantage of being time-consuming.[1] To save time, scientists have used on-line desalting based on the valve-switching technique.[2–4]

The work shown here describes a simple, fast, and effective on-line method to facilitate high-throughput sample desalting before MS detection. The entire process, including sample desalting and flushing, was completed within 2 min for an oligonucleotide sample and a bovine serum albumin (BSA) sample. This was accomplished with an UltiMate® 3000 HPLC system equipped with a dual gradient pump, an autosampler, and a column oven equipped with a 2p–10p valve.

EQUIPMENT

Dionex UltiMate 3000 HPLC system including:

- DGP-3600A Pump with SRD-3600 solvent rack with degasser
- WPS-3000TSL Autosampler
- TCC-3200 Thermostatted Column Compartment
- VWD-3400RS UV-vis Detector
- DAD-3000RS (for UV spectrum)

Chromeleon® Chromatography Data System 6.80 SR7

REAGENTS

Deionized water, Milli-Q® gradient A10

Acetonitrile (CH_3CN), HPLC grade, Fisher

Ammonium hydrogen carbonate (NH_4HCO_3),
Formic acid (HCOOH), Potassium bromide (KBr), analytical grade, SCRC, China

SAMPLES

An oligonucleotide sample containing large amounts of salts that remained from the production process was provided by a customer. Purified and desalted BSA was purchased from Shanghai Amin Biotech Co., Ltd., China.

RESULTS AND DISCUSSION

Method Development

In the production process of biochemical products (e.g., oligonucleotides and proteins), some manufacturers apply, or would like to apply, MS as part of their QA/QC analysis. Typically, SPE is used to remove high concentrations of salts from the products. The product is bound to the SPE resin, whereas the salts go to waste. The retained product is then eluted from the SPE resin and analyzed by MS. For best efficiency, desalting and elution must be as fast as possible. On-line SPE using HPLC instrumentation can meet this requirement. In fact, the process can be designed as on-line SPE-MS. Figure 1 shows the schematics of a configuration to perform method development for on-line SPE-UV. In this configuration, an HPLC column is added to create an on-line SPE-LC-MS system. Because most of the salts used in the production process do not have UV absorbance, a salt with UV absorbance (KBr) was added to the sample solution to monitor the success of desalting during method development. The UV detector monitors whether or not the salts are flushed out completely and also is used to decide the valve-switching time for sample elution. After valve switching, this UV detector also is used to confirm if the analytes are eluted. In Figure 1, an HPLC column is added to separate the analytes prior to UV detection. After method development, this system can be configured so that salts go directly to waste, and only the eluted analytes are sent to the column and detector. If desired, the MS detector can replace the UV detector; alternately, it can be added after the UV detector, either with or without the HPLC column. Figure 2 shows the chromatograms of desalting when KBr solutions with concentrations from 100 to 4000 µg/mL were injected onto the SPE column (Acclaim® Polar Advantage II [PA2] Guard, 4.3 × 10 mm, 5 µm) at a desalting flow rate of 1 mL/min. KBr is eluted completely within 1.5 min, even at 4000 µg/mL.

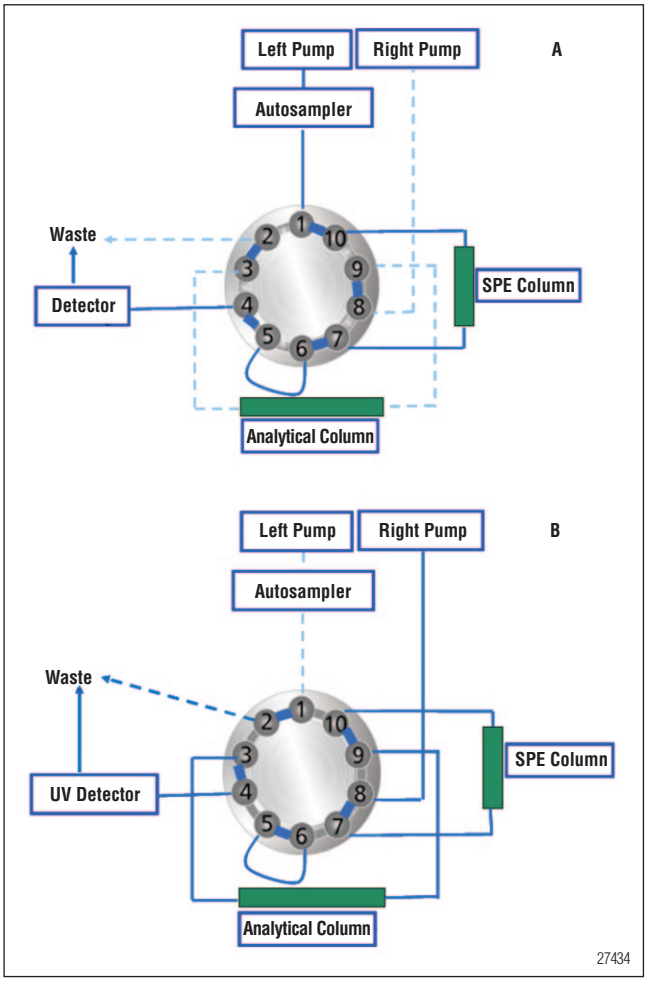

Figure 1. Schematics of valve-switching for sample desalting and elution. A) Loading and desalting (left pump): after injection, the analytes are retained on the SPE column, and the salts in the sample solution are flushed out of the SPE column. If KBr is added to the sample solution prior to injection, a huge peak can be observed at 227 or 215 nm when the SPE column is flushed. B) Sample elution (right pump): after desalting the sample is eluted. The retained analytes are eluted from the SPE column and sent through the analytical column, then to the UV detector.

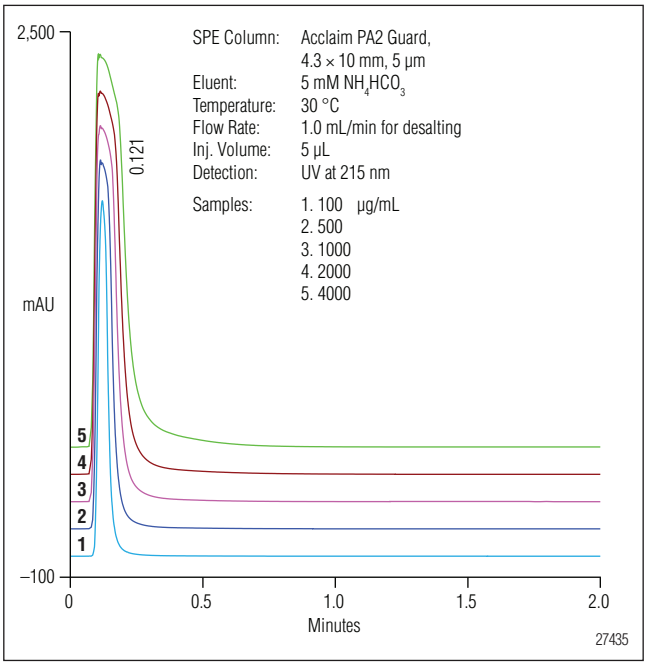

Figure 2. Chromatography of desalting of KBr solutions with different concentrations using an SPE column.

To achieve best peak intensities, the most commonly used mobile phases for LC-MS of oligonucleotides and proteins are basic or acidic solutions in an organic solvent. These mobile phases must be volatile. Solvent volatility is also important if the sample is injected into the MS without an analytical separation. Therefore, NH_4HCO_3 and HCOOH solutions were selected for desalting and SPE. The Acclaim PA2 column was chosen for desalting and SPE was selected because of its wide allowable pH range (pH 1.5–10) and its past success in binding oligonucleotides.[5]

If an analytical column is required for LC/MS, the Acclaim PA2 column is a good choice for the basic conditions of oligonucleotide separations because of its wide allowable pH range. For protein samples, the Acclaim 300 C18 column is a good choice.

In this document, an oligonucleotide sample and a protein (BSA) sample were used to develop the desalting method. The oligonucleotide sample was spiked with 2000 µg/mL KBr and then desalted with the Acclaim PA2 column, followed by elution from and subsequent separation on the PA2 column. Figure 3A shows the results of this experiment at a desalting flow rate of 1.0 mL/min.

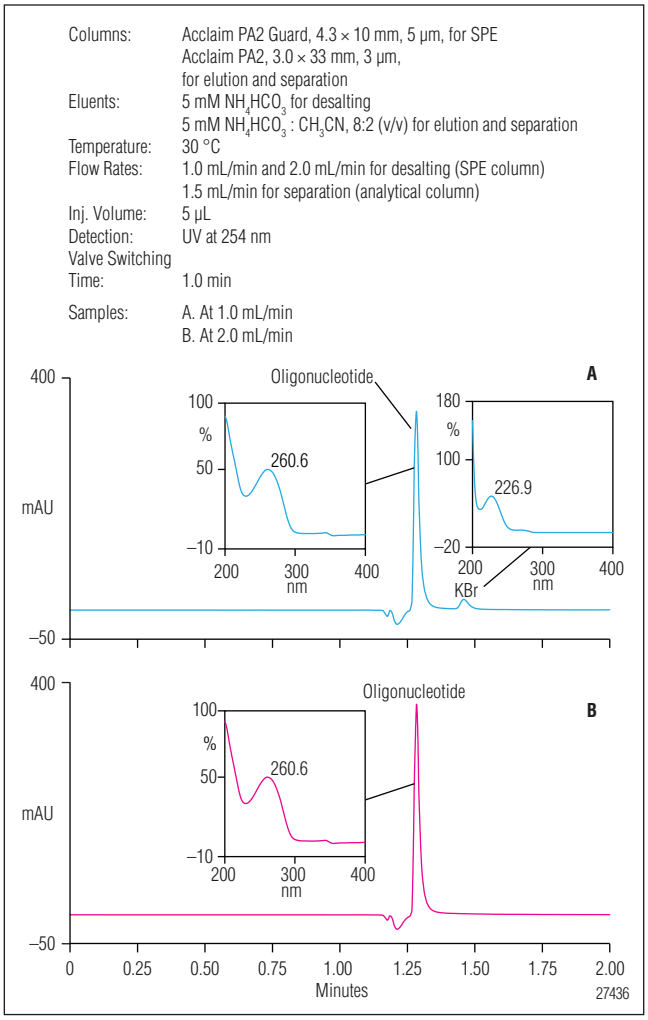

Figure 3. Chromatograms of an oligonucleotide sample spiked with 2000 µg/mL KBr and desalted at different flow rates. A) 1.0 mL/min and B) 2.0 mL/min.

A small peak was found following oligonucleotide (characteristic absorbance at 260 nm for oligonucleotide). Upon comparing the UV spectrum of the small peak to that of bromide, the authors concluded that the small peak was bromide and desalting was incomplete. There are two possible solutions for this problem. One possibility is to lengthen the desalting time. For example, KBr can be removed completely when the valve-switching time increases from 1.0 to 1.5 min. Another possible solution is to increase the flow rate from 1.0 to 2.0 mL/min, as shown in Figure 3B. The increased flow rate completely removes KBr. For the BSA sample, the same results are obtained. The higher flow rate also allows higher throughput desalting.

On-Line High-Throughput Desalting Using a Tandem Configuration

The desalting and elution can be accelerated using the tandem configuration shown in Figure 4 in conjunction with the program shown in Figure 5. The reproducibility of this tandem configuration was investigated using 10 consecutive injections of BSA spiked with 4000 µg/mL KBr (5 injections each on the two SPE columns). An overlay of the chromatograms from this experiment shows that this method is reproducible (Figure 6). The retention time RSD was 0.10%.

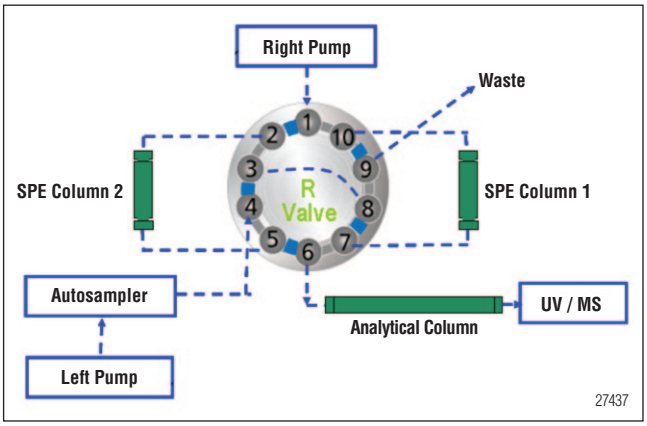

Figure 4. Schematics of valve-switching for fast on-line desalting using a tandem configuration. Cycle 1: 1) Valve switching to 1-2. Loading and desalting (left pump): after injection, the analytes are retained on SPE Column 1, and the salts in the sample solution are flushed out of SPE Column 1 to waste. 2) When the desalting completes, the valve switches to 1-10. Sample elution and analysis (right pump). Meanwhile, SPE Column 2 is now in line with the left pump. Cycle 2: same as Cycle 1, but the position of valve switching is reversed for injection to SPE Column 2. Two programs are required for each cycle and run in turn.

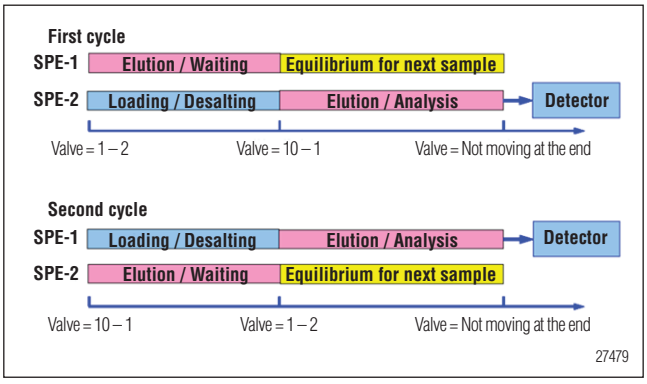

Figure 5. Program of the on-line tandem configuration for fast desalting.

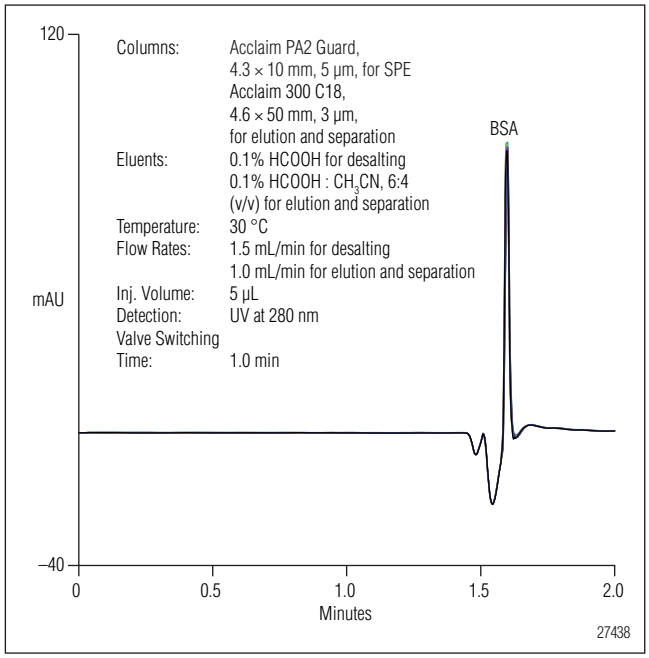

Figure 6. Overlay of ten chromatograms of a BSA sample spiked with 4000 µg/mL KBr.

When the BSA sample was desalted and separated using this tandem configuration, a small peak also was found with absorbance at 214, 254, and 280 nm, even when the desalting flow rate was 2.0 mL/min. Figure 7 compares chromatograms of BSA, BSA spiked with KBr, and UV spectra of KBr. Detection at three wavelengths revealed that the peak is an impurity from the BSA sample, and that sample desalting was complete.

When the sample salt concentration is not high, faster on-line high-throughput desalting and separation can be achieved by shortening the valve-switching time in the tandem configuration. Figure 8 overlays chromatograms of four consecutive injections of an oligonucleotide that was injected onto two SPE columns (two injections on each SPE column). The desalting, elution, and separation of the oligonucleotide sample (salt concentrations less than 1000 µg/mL) may be completed within 1.1 min when the valve-switching time is 0.4 min. The 0.0% RSD for retention time and 1.5% for peak area show that the faster on-line high-throughput desalting and separation tandem configuration is reproducible.

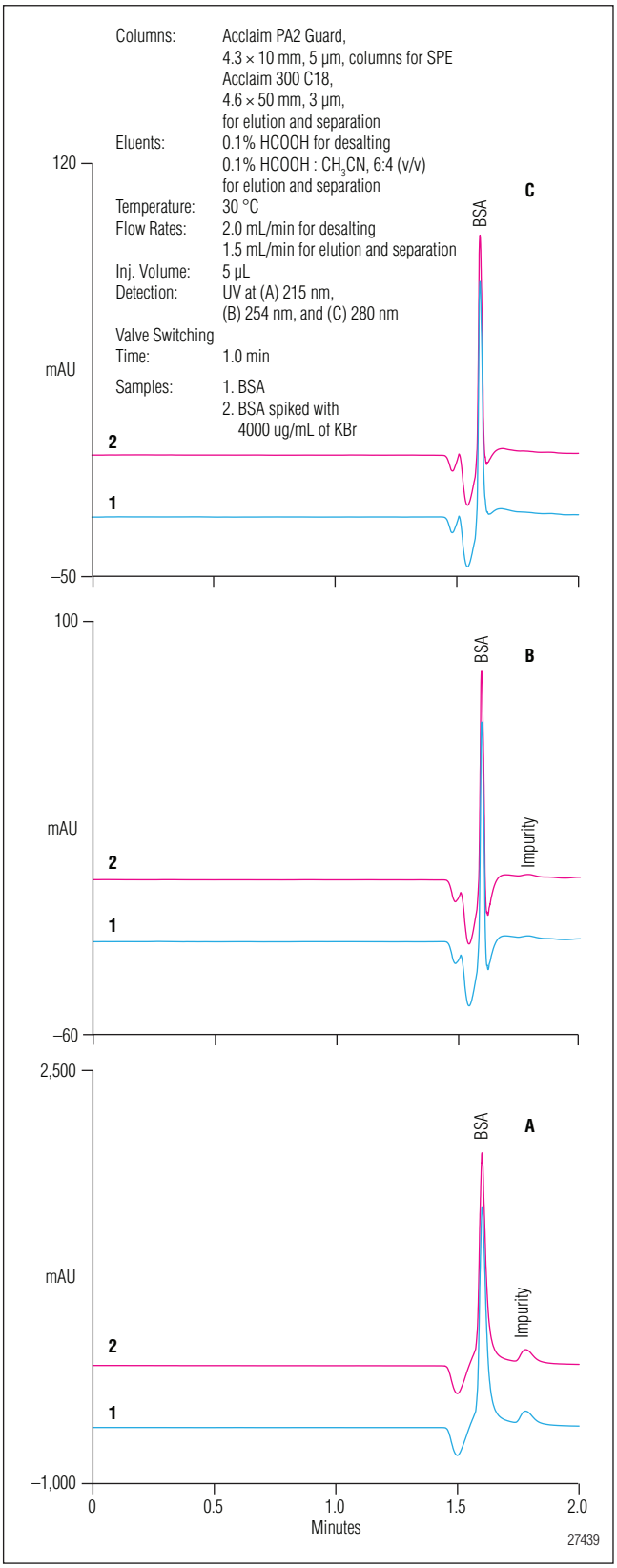

Figure 7. Overlays of chromatograms of BSA and BSA spiked with KBr at A) 215 nm, B) 254 nm, and C) 280 nm.

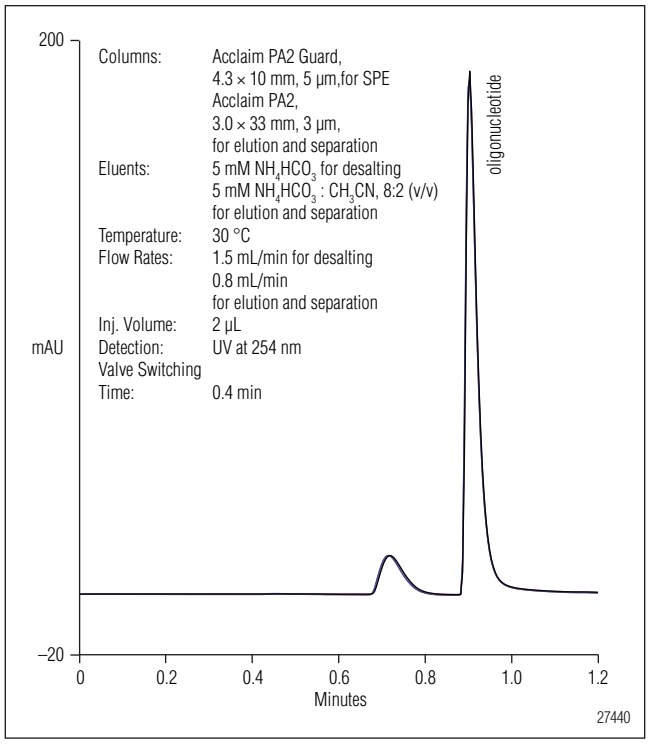

Figure 8. Overlay of chromatograms from four consecutive injections of an oligonucleotide onto two SPE columns (two injections on each SPE column).

CONCLUSION

This application update demonstrates a fast method to desalt oligonucleotide or protein samples for either on-line analysis by HPLC or LC/MS.

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Poster Note

Development of an Automated Method for Antibody Purification and Analysis

ANTIBODY PURIFICATION

One of the major problems in biotherapeutics is aggregation, which can produce variants of the active pharmaceutical ingredient. These product-related substances can have different efficacies than the main product and may cause serious side effects, such as anti-drug-antibody formation.

Protein aggregates are mostly the consequence of suboptimal production, purification, or handling conditions (e.g., temperature, pH). In the purification of antibodies, a protein-affinity separation is generally the first step. Affinity chromatography on protein A or G columns typically yields a purity of more than 95% in a single step. To verify the purification efficiency (or sample purity or antibody quality), a technique such as ion-exchange or size-exclusion chromatography (SEC) is needed.

The SEC technique provides the necessary selectivity to identify agglomerates and size-based variations of the main component. Ion-exchange stationary phases provide good selectivity for separation of charge variants of the protein biopharmaceutical. The variations may be very subtle or small, and finding the optimal chromatographic conditions requires optimization.

This work discusses the development of an automated solution for purification and separation of antibodies using a single UltiMate® 3000 HPLC system. In this process, the autosampler performs the injection, high-volume fraction collection, and reinjection of the collected fractions for analysis.

INSTRUMENTATION AND 2-D LC WORKFLOW

HPLC experiments were carried out using an UltiMate 3000 Titanium system (Dionex, Germany) equipped with a:

- Membrane degasser
- ×2 dual-gradient pump system
- Thermostatted column compartment
- Well-plate autosampler for both injection and automated fraction collection (Figure 1)
- UV detector equipped with an 11 µL flow cell

Figure 1. The UltiMate 3000 Titanium system and detail of the WPS-3000(B)TFC bioinert autosampler/fractionation solution.

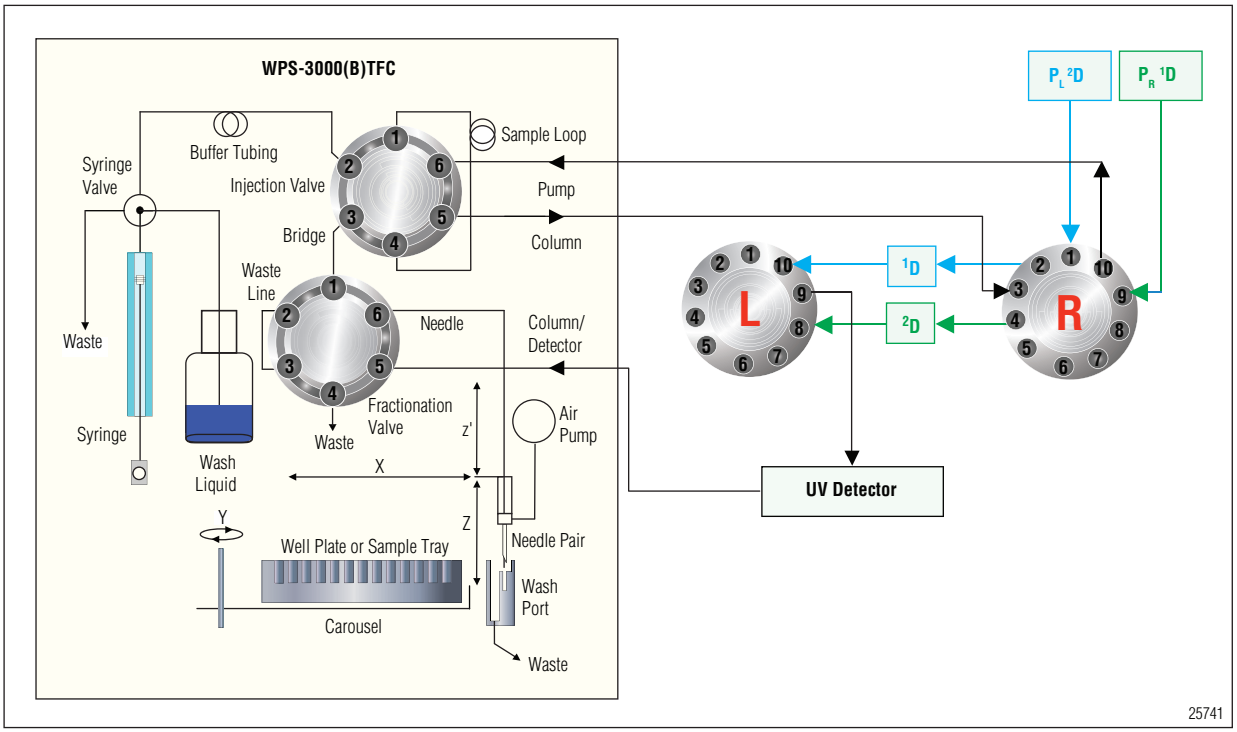

Figure 2. Fluidic setup of the automated off-line 2-D LC system.

The workflow and LC conditions for automated off-line 2-D LC included the following:

- 50 to 250 µL injection of an unpurified monoclonal/polyclonal antibody
- A first-dimension (^{1}D) affinity chromatography separation, at a flow rate of 1.5 mL/min using the following steps:
  – A column wash/equilibration step of 2 min
  – An elution step of 5 min
  – Automated peak detection followed by fraction collection into a well plate in the autosampler
- A second-dimension (^{2}D) separation, using either a:
  – Weak cation exchange separation at pH 5.5, applying a linear NaCl salt gradient,

  or

  – Size-exclusion chromatography separation (200 mM sodium phosphate, 250 mM NaCl, pH 6.3)

(Prior to the ^{2}D separation an optional neutralization buffer can be added, using the derivatization capabilities of the autosampler, to raise the pH of the fraction.)

LC CONDITIONS

A protein A column (Poros® 20 µm, 4.6 × 50 mm, 0.8 mL, Applied Biosystems) was used for ^{1}D affinity-based separations with UV detection at 214 and 280 nm. Affinity LC conditions: mobile phase A: 10 mM sodium phosphate, 150 mM NaCl, pH 7; mobile phase B: 50 mM Glycine-HCl, 150 mM NaCl, pH 2.5. 1D wash and equilibration step for 2 min at 100% A, followed by a 5 min wash step at 100% B. Flow rate: 1.5 mL/min, column temperature: 25 °C.

A ProPac® WCX-10 column (4.6 × 250 mm, Dionex) was used for the ^{2}D weak cation-exchange (WCX) LC separation applying a linear salt gradient of MES buffer/sodium-chloride solution, detection at 214 and 280 nm. Mobile phase A: 20 mM MES buffer pH 5.5 + 60 mM NaCl; mobile phase B: 20 mM MES buffer, pH 5.5 + 180 mM NaCl. The ^{2}D gradient on this column was from 45 to 85 % B (50 min), 6 min wash step at 100% B, and 12 min equilibration time at 45% B. Flow rate: 250 µL/min, column temperature: 25 °C.

An SEC column was used for the aggregation analysis (4.6 × 300 mm, BioLC, Japan), the mobile phase used was 200 mM sodium phosphate, 250 mM NaCl, pH 6.3.

RESULTS

Typically, the antibody is manually injected onto the affinity column. Here the autosampler was used to introduce the sample to this column. After an equilibration step with mobile phase A, the antibody was washed off the Protein A column with mobile phase B and fractionated into a 96 deep-well plate using peak-based triggers. These triggers can be easily optimized to either collect or discard the breakthrough peak and to collect the antibody peak with a trigger based on retention time and signal properties. There is also some room for accelerating this process, as the pump module can deliver up to 6 mL/min. However, a minimum fractionation/decision delay time (5 s) should be considered. If desired or required, a neutralizing buffer can be added to the fractions after fractionation to increase the pH, using the derivatization capabilities of the autosampler. For the antibody peak (Figure 3), a peak area RSD of 0.037% and a retention time RSD of 0.115% (t_R=3.31) was found.

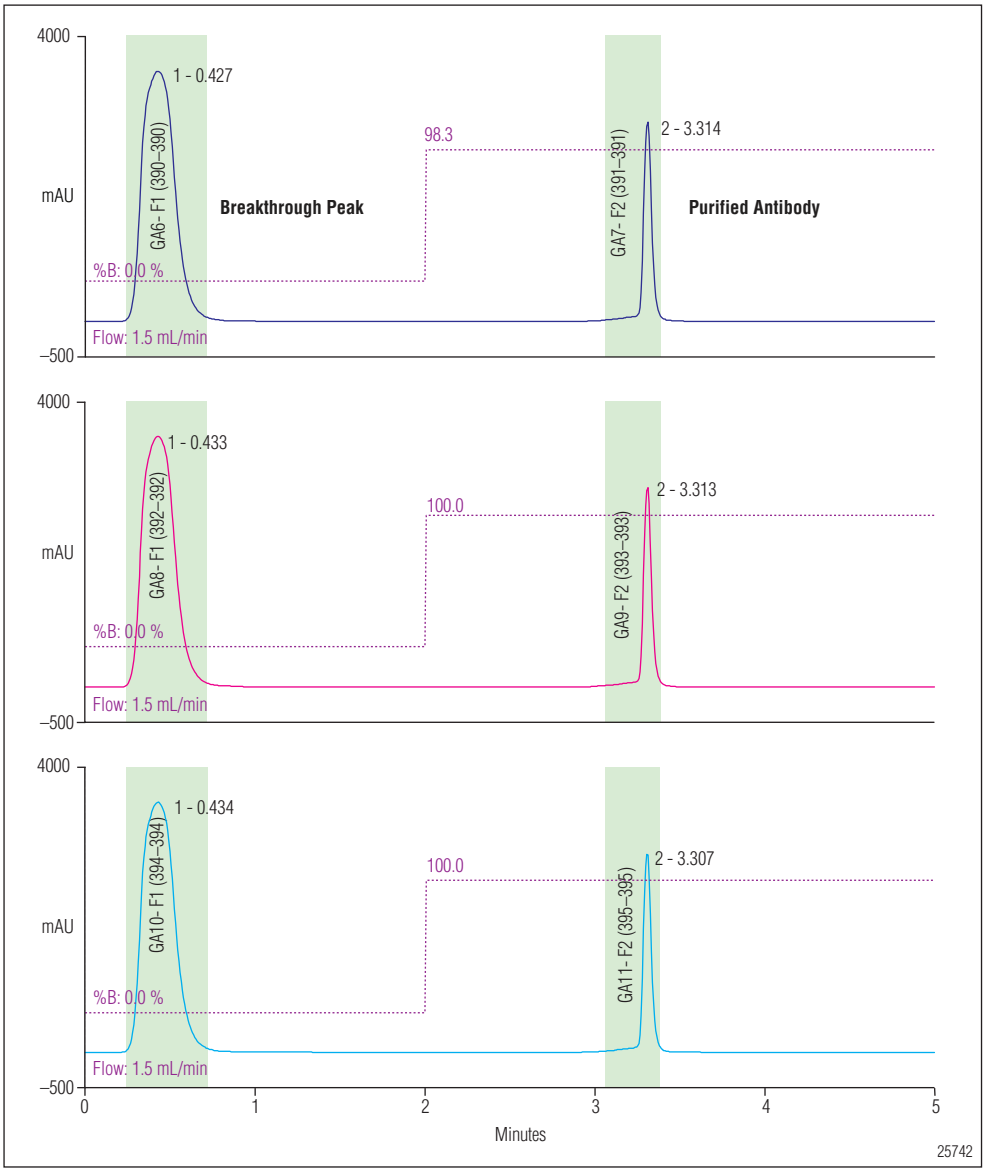

Figure 3. First-dimension affinity-chromatography separation of a polyclonal antibody; 0–2 min: wash step; 2–5 min: elution step. This set of chromatograms also illustrates the repeatability of the injection and automatic peak detection functionalities.

When a fraction is found that meets the requirements, a post-acquisition step can initiate the second dimension separation to start. This can be a WCX-based separation, yielding selectivity towards small charge differences in the antibody mixture (Figure 4, IEX of a monoclonal IgG_1 fraction), or SEC to separate the antibody from its dimers, aggregates, etc. (Figure 5).

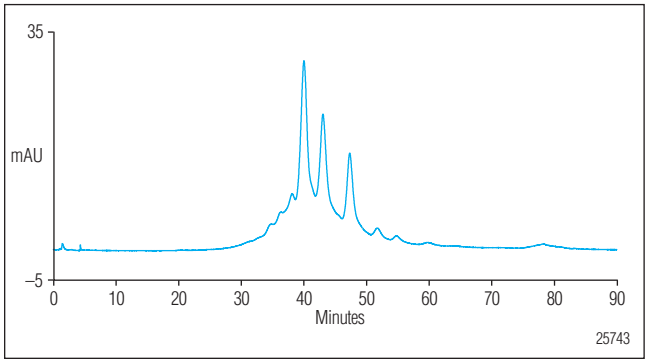

Figure 4. Example of a ^{2}D IEX separation (ProPac WCX-10, 250 × 4.6 mm I.D.) of a purified monoclonal antibody fraction.

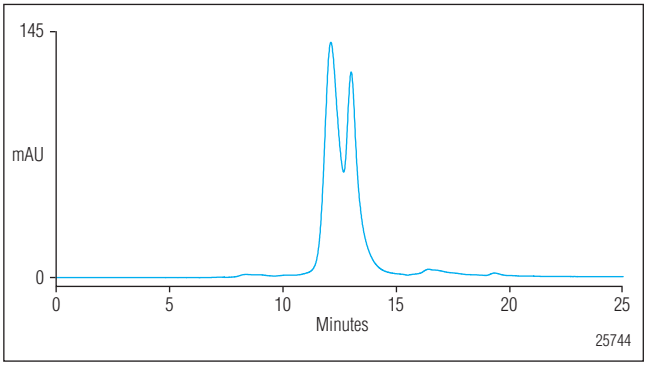

Figure 5. Example of a ^{2}D SEC separation of a purified polyclonal antibody fraction.

SUMMARY

The WPS-3000(B)TFC key features include:

- Dual-valve autosampler, enabling high-flow fraction collection and reinjection of fractions used in 2-D workflows
- Heating and cooling of sample compartment
- Easy-to-adapt fluidics to suit your application, full-loop injection volume ranges from 1 to 500 µL
- Accommodates multiple well plate types simultaneously
- Excellent repeatability for injection and fractionation

This solution enables automation of multistep analysis (including off-line 2-D LC) in combination with Chromeleon® Chromatography Management Software (Dionex). Sample handling and derivatization, purification, and HPLC analysis can be programmed both easily and in depth and gives the instrument unique capabilities in the fields of sample purification, standard HPLC, and QC.

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Poster Note

Should Your Autosampler Just Sit and Wait? Intelligent Instrument Control Allows Sharing HPLC Modules Across Systems

INTRODUCTION

In traditional HPLC configurations, some of the modules are used inefficiently:

- The autosampler is idle for most of the time.
- The thermostatted column compartment (oven) could hold more than one column.

This inefficiency can be improved if these modules could be utilized in more than one system.

With a Dionex UltiMate™ 3000 Dual LC system, which consists of a dual-gradient pump (two independent pumps in one enclosure), an autosampler, a thermostatted column compartment, and two detectors, the capabilities of two traditional LC systems can be achieved—we call this "parallel chromatography" (Parallel LC). UltiMate 3000 Dual LC systems allow sharing of the autosampler and the column compartment. This sharing facilitates better utilization of the potential productivity of these modules.

Parallel LC with an UltiMate 3000 Dual LC system has several advantages:

- It nearly doubles the throughput at a cost that is substantially lower than two complete HPLC systems.
- This gain in productivity can be reached without modifying and revalidating the existing analytical method.
- It can be used for isocratic as well as gradient methods.
- A Dual LC system can analyze a sample set with two different (orthogonal) methods at the same time.

However, control of such a complex system requires powerful software.

In this presentation we show:

- Analytical scenarios that are especially suitable for Parallel LC.
- Intelligent software solutions for trouble-free control of the two HPLC channels incorporated in a single UltiMate 3000 Dual LC system.

DEFINITIONS

Parallel LC system: A Dionex UltiMate 3000 Dual LC system consisting of a dual-gradient pump (two independent pumps in one enclosure), two detectors, and a shared autosampler and column compartment, configured in a way that it behaves like two independent HPLC systems.

Sub-system: One of the two separation channels of a Parallel LC system, which corresponds to a complete HPLC system.

Module: A single instrument in the HPLC system, e.g. a pump or a detector.

Sub-module: A Dionex UltiMate DGP-3600 ×2 Dual-Gradient pump encloses two ternary low-pressure gradient pumps in a single housing. In this case each independent pump is called a "sub-module."

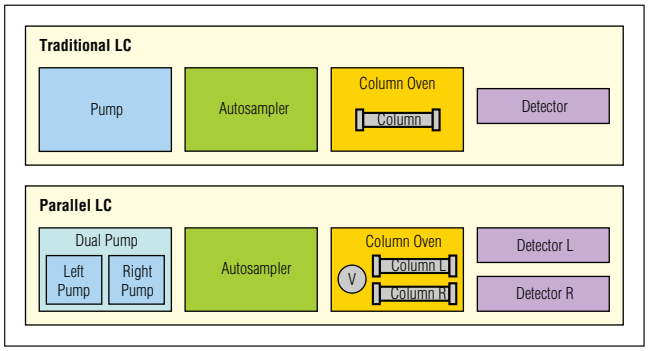


Figure 1. Schematic instrument setup for traditional and Parallel LC. Compared to a traditional setup, Parallel LC nearly doubles throughput by only adding a pump, a detector, and a switching valve (V). The autosampler and the column oven are shared between the two separation channels (sub-systems).

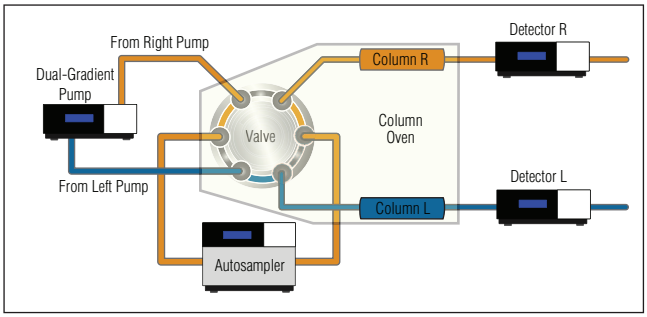


Figure 2. Flow scheme for UltiMate 3000 Parallel LC mode. The autosampler is currently in the flow path of the right pump. (A Dionex UltiMate DGP-3600 ×2 Dual-Gradient Pump encloses two ternary low-pressure gradient pumps in a single housing. The sub-devices are called left pump and right pump.)

INSTRUMENTATION

Figure 1 shows the schematics of a traditional and a Parallel LC.

A traditional LC system consists of the following modules:

- One pump
- One injection device, in most cases an autosampler
- One thermostatted column compartment (oven) with:
  – one column
- One detector

A Parallel LC setup contains:

- Two pumps*
- One shareable autosampler
- One shareable thermostatted column compartment with:
  – Two columns
  – One motorized switching valve (two-position six-port)
- Two detectors

* Optimally the two pumps can be built into the same housing, as in the Dionex UltiMate DGP-3600 ×2 Dual-Gradient Pump, which encloses two ternary low-pressure gradient pumps in a single housing. In this solution the relative cost of a single system is further reduced.

In Parallel LC, two columns and detectors are used in parallel in two separate flow paths ("sub-systems"). The autosampler is utilized in both sub-systems; a two-position six-port valve switches between the sub-systems (Figure 2). The two independent pump modules (left pump and right pump) in the dual-gradient pump deliver the flow to their dedicated sub-systems. Figure 3 shows a photo of an UltiMate 3000 Dual LC system configured for Parallel LC and lists the comprising modules.

APPLICATION EXAMPLES

For successful implementation of Parallel LC, there are a few requirements on the analytical methods that will be run on the two sub-systems:

Temperature Requirements: Both analytical methods must operate at the same temperature, because the two columns are placed in the same column compartment and the samples in the same autosampler.

Eluent Requirements: Because switching the autosampler between the two flow paths can transfer eluent of different composition between the sub-systems, the eluents must be miscible and the initial eluent conditions for both methods should support fast flow path switching.

Considering the above requirements there are a few application areas that are especially suitable for Parallel LC.

Figure 3. Dionex UltiMate 3000 Dual LC system for Parallel LC. The modules are arranged in a way that ensures optimal plumbing. Modules from top left: SRD-3600: six-channel solvent rack with degasser, DGP-3600A: analytical dual-gradient pump, WPS-3000TSL Analytical: thermostatted in-line split-loop well plate sampler, VWD-3400: four-channel variable wavelength detector, TCC-3100 Thermostatted Column Compartment with an integrated two-position six-port switching valve, and a second VWD-3400 detector.

Doubling Sample Throughput for a Single Method

In this scenario the same analytical method is run on both sub-systems. Samples are injected alternately to the two flow paths. By running an UltiMate 3000 Dual LC System in Parallel LC mode, the throughput can be increased by close to 100% compared to a traditional HPLC. This gain in productivity can be achieved both for isocratic and gradient methods.

Isocratic Methods

For isocratic runs the implementation is relatively simple, as all the above mentioned method requirements are fulfilled during the entire separation time.

Gradient Methods

For gradient methods the Eluent Requirements must be considered: switching of the autosampler between the sub-systems must be timed properly during the equilibration phase. Figure 4 shows how the gradients, valve switches, and injections are synchronized. As discussed later, the software provides the necessary tools for avoiding eluent mixing at undesired times.

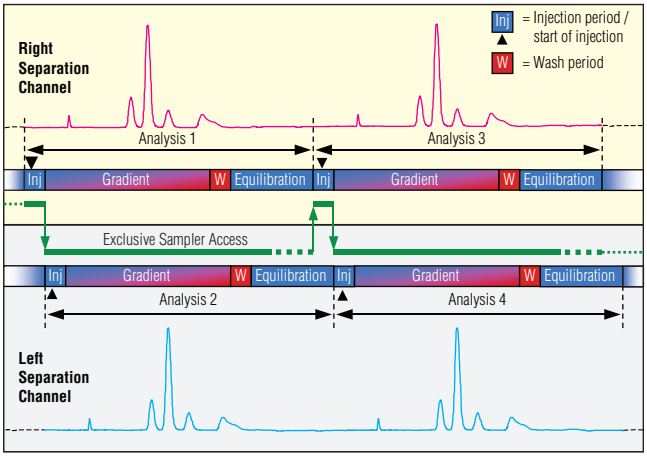


Figure 4. Running the same gradient method with Parallel LC. Proper timing of the valve switches ensures that a portion of the gradient does not interfere with the other separation channel during valve switching. The throughput is nearly doubled compared to a traditional LC system.

Analyses with Different Methods

Doubling throughput for a single method (see above) is perhaps the most obvious application of Parallel LC. However, Parallel LC is not limited to using the same method on both sub-systems. The methods can be different as long as the eluents are miscible at the time of the autosampler switching.

The samples that are injected on the two sub-systems can be different, or the same. The latter example is discussed in this presentation.

In many cases a single analytical run cannot resolve all the components in a complex sample and an "orthogonal separation" using a different column and/or eluent is necessary. Parallel LC provides great benefits for this situation:

- The injections on the two sub-systems can take place from the same vial. This decreases the sample preparation efforts.
- Chromeleon's reporting tools facilitate concise reporting of the combined results from the two methods. For example, presence of a compound can be confirmed based on presence (and spectrum) in both chromatograms; average amount can be calculated, etc.
- The complete result (i.e. combined results from the two methods) becomes available much faster for the single samples (compared to the scenario when the two analyses are performed sequentially, i.e. Method 2 is run on the same system after all samples have been analyzed with Method 1).

Isocratic Methods Using the Same Eluent

If both methods are isocratic and they use the same eluent (i.e. on two different columns) the implementation becomes relatively simple as eluent transfer between the flow paths is never an issue. Intelligent control and monitoring features ensure that the autosampler handles requests from both sub-systems to perform an injection at the same time. In such a case the software puts the second sub-system on hold until the first sub-system has completed the injection. This is shown in Figure 5.

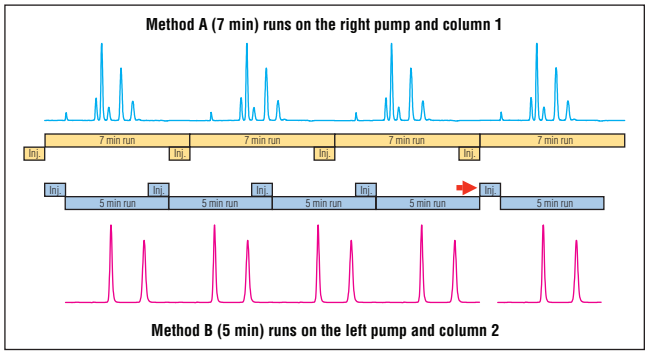


Figure 5. Running isocratic methods with the same eluents: The user does not need to worry about switching the autosampler between the sub-systems. In case of conflict (marked with red arrow) the software slightly delays the following injection and continues as soon as the autosampler becomes available.

Gradient Methods and Isocratic Methods Using Different Eluents

If the eluents are different or the methods use gradient elution the Eluent Requirements (see above) must be considered. As discussed later, the Chromeleon® Chromatography Management Software provides the tools for avoiding eluent mixing issues.

For a complete application example, see our presentation A Total Solution for Explosives Analysis by Reversed-Phase HPLC with a Parallel HPLC System.[1]

SOFTWARE SOLUTIONS

Control of a powerful solution such as Parallel LC requires powerful software.

In the presented solution, the instruments behave (from the user's point of view) as if two independent HPLC systems were present, despite the fact that some modules are shared.

The software has to ensure seamless cooperation of the two sub-systems and facilitate easy user interaction with them. Below we discuss the solutions Chromeleon provides for this.

System Configuration

First of all, the system must be configured with clear indication of which sub-system each module belongs to. Figure 6 shows the dialog boxes for configuring the shared modules.

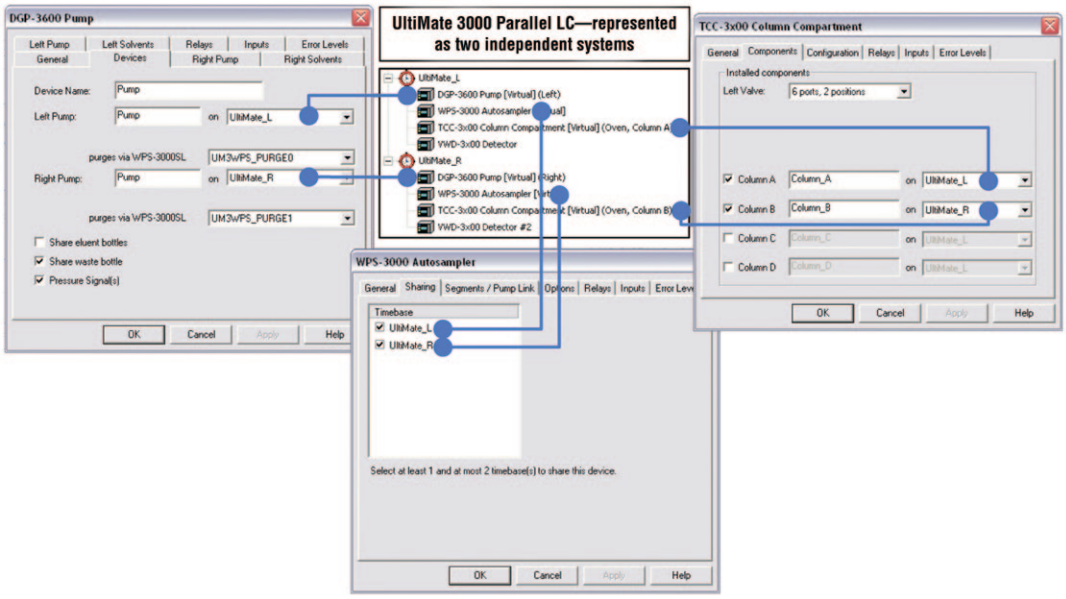


Figure 6. Configuring an UltiMate 3000 Parallel LC. In the shared dual-pump each of the independent sub-modules is assigned to its corresponding sub-system. For sharing the autosampler it is enough to indicate which sub-systems can access it. The column compartment is shared by assigning the two columns to the two different sub-systems.

Common and Specific Instrument Commands

A dual-gradient pump contains two independent pumps. There are however some instrument control commands that are valid for both sub-devices; for example, commands related to leak detection. The Chromeleon software ensures that common and specific commands are properly addressed during the runs.

Checking Method Parameters

Because the autosampler and the column compartment are shared, the two methods must use matching temperatures. If the user attempts to start methods that require different column oven or autosampler temperatures, the system will not start the second sample sequence and the user will be notified as shown in Figure 7.

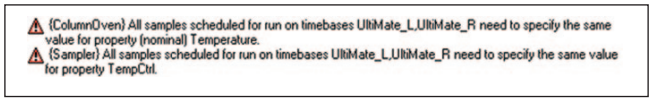


Figure 7. Chromeleon's Ready Check function ensures that sequences, including the control programs, are syntactically and semantically correct. For Parallel LC systems it also confirms that there are no conflicts regarding the shared parameters. If this is not the case, the user gets a clear message.

Exclusive Access to the Autosampler

Correctly timed switching of the autosampler between the two sub-systems is an important consideration with Parallel LC. Two complications must be avoided:

- Sample mixing: Remainder of a sample enters a foreign flow path.
- Eluent mixing: Eluent with improper composition enters a foreign flow path.

Sample Mixing

The software automatically prevents sample mixing, as each sub-system takes exclusive access of the autosampler while it performs an injection. The Exclusive Access Time is automatically calculated during generation of the instrument method (Program) based on instrument and method parameters (such as Capillary Void Volume, Inject Volume, and Flow) and considering a user-defined Flush Out Factor (Figure 8). If an injection request arrives from one sub-system while the other one is already injecting, the software will simply put this sub-system on hold until the other one releases its exclusive access.

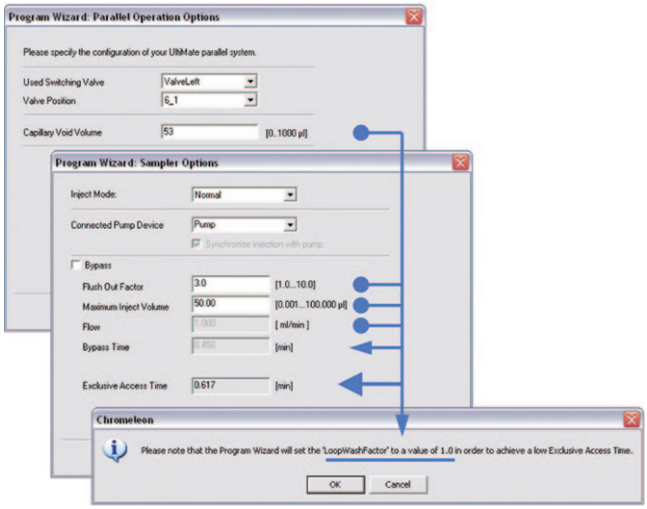


Figure 8. To prevent sample mixing (i.e. one sub-system taking control of the autosampler before the previous injection on the other sub-system is finished) each sub-system takes exclusive access of the autosampler throughout an injection. During generation of the instrument method (Program) the software automatically calculates the Exclusive Access Time. The calculation is based on instrument parameters (e.g. Capillary Void Volume) and method parameters (e.g. Flow, Inject Volume), and a user defined Flush Out Factor. Related parameters, like the Loop Wash Factor, are also optimized.

Eluent Mixing

Prevention of eluent mixing cannot be completely automated as the software is not aware of the chemical properties of the actual eluents in the two flow paths. However, the user can easily extend the period while one sub-system has exclusive access to the autosampler. This way the user can ensure not only that the injection has taken place, but also that the elution gradient has passed the autosampler before it is switched to the other flow path. This is illustrated in Figure 4.

Error Handling

The software ensures that a problem that only affects one sub-system does not (immediately) interfere with the analysis on the other one.

If there is a problem that affects a non-shared module, the other sub-system will continue to work without interruption. For example:

- If the pressure limit is exceeded on the left sub-system and it stops, the right sub-system can finish running its sequence of samples.

If the problem affects a shared module, the problem is handled in a way that ensures secure operation but influences the sub-systems as little as possible. For example:

- If there is a fatal problem with the shared column oven, like a leakage, this will stop both sub-systems. This is necessary for safe operation.
- If there is a problem with the shared autosampler while the left sub-system has exclusive access to it, the currently running sample can be finished on the right sub-system without interruption. The analyst can then either correct the problem, or deactivate the left sub-system and continue running exclusively on the right sub-system.

System Monitoring and Control

The software provides easy means of monitoring and controlling both sub-systems. Chromeleon's "Panel Tabsets" allow fast switching between user interface tabs. These allow immediate access to the control screens for any module (Figure 9).

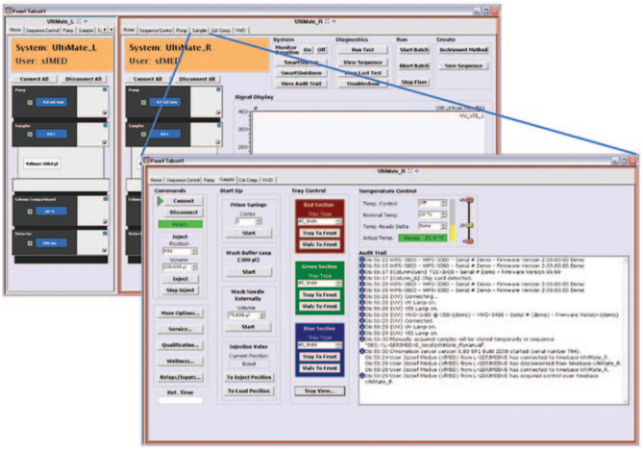
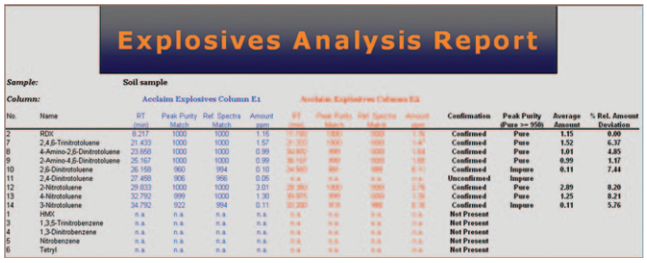


Figure 9. Panel Tabsets provide well structured overview and easy control possibilities for the two sub-systems. The screen can be shared between the sub-systems (as in the background) or it can be filled with the controls of a single module (as in the foreground).

Reporting

One application area where Parallel LC is especially advantageous is analyzing the same sample with orthogonal methods (see Application Examples above). In this case Chromeleon can create reports where data from the two methods are consolidated. For example, presence of a compound can be confirmed if present in both chromatograms or the average amount can be calculated.


Explosives Analysis Report

Sample: Soil sample

Column: Acclaim Explosives Column E1

No.	Name	RT (min)	Peak Purity Match	Ref. Spectra Match	Amount ppm	Confirmation	Peak Purity (Pure >= 950)	Average Amount	% Rel. Amount Deviation
2	RDX	8.217	1000	1000	1.15	Confirmed	Pure	1.15	0.00
7	2,4,6-Trinitrotoluene	21.433	1000	1000	1.57	Confirmed	Pure	1.52	6.37
8	4-Amino-2,6-Dinitrotoluene	23.658	1000	1000	0.99	Confirmed	Pure	1.01	4.85
9	2-Amino-4,6-Dinitrotoluene	25.167	1000	1000	0.99	Confirmed	Pure	0.99	1.57
10	2,6-Dinitrotoluene	26.158	960	994	0.10	Confirmed	Impure	0.11	7.44
11	2,4-Dinitrotoluene	27.458	906	966	0.05	Unconfirmed	Impure		
12	2-Nitrotoluene	29.833	1000	1000	3.01	Confirmed	Pure	2.89	8.20
13	4-Nitrotoluene	32.792	999	1000	1.30	Confirmed	Pure	1.25	8.21
14	3-Nitrotoluene	34.792	922	994	0.11	Confirmed	Impure	0.11	5.76
1	HMX	n.a.	n.a.	n.a.	n.a.	Not Present			
3	1,3,5-Trinitrobenzene	n.a.	n.a.	n.a.	n.a.	Not Present			
4	1,3-Dinitrobenzene	n.a.	n.a.	n.a.	n.a.	Not Present			
5	Nitrobenzene	n.a.	n.a.	n.a.	n.a.	Not Present			
6	Tetryl	n.a.	n.a.	n.a.	n.a.	Not Present			

Figure 10. Example of a consolidated Chromeleon report. The same soil sample was analyzed on two different columns (E1 and E2) in order to determine its contamination with explosives. Presence of a compound is confirmed if it is present in both methods. In this case the average amount is also calculated from the results given by the two methods.

CONCLUSION

Intelligent module sharing between HPLC systems can almost double the productivity of a traditional HPLC system, without the need for modifying and revalidating existing analytical methods.

Parallel LC is especially suitable in the following analytical scenarios:

- Doubling throughput: Analyzing different samples with the same isocratic or gradient* method on both sub-systems
- Orthogonal analysis: Analyzing the same samples with two different methods on the two sub-systems

Powerful Chromeleon software helps users while working with a Parallel LC system:

- Easy system configuration via dialog boxes
- Intelligent handling of exclusive access to the shared autosampler
- Prevention of typical user errors (syntax and logical errors as well)
- Intelligent error handling
- Easy system monitoring and control
- Powerful reporting, including consolidated data from the two sub-systems

*NOTE: For short gradient runs, Tandem LC can be an even more economical alternative.[2] Tandem LC is designed to operate with two identical columns running the same application. Compared to a traditional HPLC setup it needs an additional pump, but not an additional detector, as in Parallel LC.

REFERENCES

1. Maio, G.; Steiner, F.; Franz, H.; Tracy, M.; Liu, X.; Arnold F. A Total Solution for Explosives Analysis by Reversed-Phase HPLC with a Parallel HPLC System, Poster, Abstract #780-11P, Pittcon, Chicago, IL, February 25–March 1, 2007.
2. M. Karsten, B. Dolman, G. Maio, F. Steiner, H. Franz, F. Arnold and R. Swart: Increasing Throughput in LC and LC-MS with a Parallel HPLC System, Poster, Pittcon, Orlando, FL, March 12–17, 2006. (Available at www.dionex.com).

Poster Note

Automated MAb Workflow: from Harvest Cell Culture to Intact Mass Analysis of Variants

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Thermo SCIENTIFIC

Overview

Purpose: Demonstrate an automated monoclonal antibody (MAb) analysis two-dimensional (2D) workflow and intact mass detection.

Methods: Automated analysis is achieved with the Thermo Scientific Dionex UltiMate 3000 x2 Dual Titanium Biocompatible Analytical LC System using Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) software. The intact mass information is acquired on the Thermo Scientific Q Exactive mass spectrometer.

Results: This workflow enables the completion of affinity purification, size-exclusion analysis, and charge variant analysis in less than one hour. The intact mass analysis characterizes the structural difference of the MAb variants.

Introduction

During development of recombinant MAbs, a large of number of harvest cell culture (HCC) samples must be screened for IgG titer, aggregations, and charge variants. Affinity chromatography is often used first to purify MAbs, with typical yields of more than 95%. Size-exclusion chromatography (SEC) is used to identify and quantify MAb aggregations. Finally, ion-exchange chromatography (IEC) characterizes charge variants. For the final biopharmaceutical product approval and subsequent manufacturing processes, a comprehensive characterization of MAb purity, aggregate forms, and charge variants is required by the regulatory agencies.

In the present study, we automate a 2D high-performance liquid chromatography (HPLC) workflow using an integrated HPLC system. This system consists of a dual-gradient pump, a UV/VIS detector, a column oven, and an autosampler capable of both sample injection and fraction collection. First, the HCC is injected onto the POROS® A Protein A Affinity column and IgG fractions are collected by the autosampler. Subsequently, the IgG fractions are injected separately onto Thermo Scientific MAbPac SEC-1 and MAbPac™ SCX-10 columns for further analysis. The MAbPac SCX-10, 3 µm column was recently introduced in 4 × 50 mm format for high-throughput MAb variant analysis. This column delivers high resolution separation with a shorter run time using either salt or pH gradients. Incorporating this column into the workflow, we completed affinity purification, SEC and charge variant analyses in less than one hour. Furthermore, the fractions collected off the MAbPac SCX-10 column were analyzed by mass spectrometry (MS), and intact mass information of the MAbs demonstrated the presence of lysine variants.

Methods

Harvest Cell Culture

MAb HCC was a gift from a local biotech company. The HCC was filtered through a 0.22 µm membrane prior to sample injection.

Columns

- MAbPac SCX-10, 3 µm, 4 × 50 mm (P/N 077907)
- MAbPac SCX-10, 10 µm, 4 × 250 mm (P/N 074625)
- MAbPac SEC-1, 4 × 300 mm (P/N 074696)
- POROS A Protein A Affinity 20 µm Column, PEEK™, 4.6 mm x 50 mm, 0.8 ml (P/N 1-5022-24)

Liquid Chromatography System

HPLC experiments were carried out using an UltiMate™ 3000 x2 Dual Titanium System equipped with SRD-3600 Integrated Solvent and Degasser Rack, DGP-3600BM x 2 Dual-Gradient Micro Pump, TCC-3000SD Thermostated Column Compartment with two biocompatible 10-port valves, WPS-3000T(B)FC Analytical Dual-Valve Wellplate Sampler, VWD-3400RS Four Channel Variable Wavelength Detector equipped with a Micro Flow Cell, and PCM-3000 pH and Conductivity Monitor.

pH-Based Ion-Exchange Chromatography

In a scale-up purification, 1 mL of IgG was purified from the 3.8 mL HCC using Thermo Scientific Pierce Protein A Plus Agarose beads (P/N 22810). The protein concentration was determined at ~ 0.5 mg/mL. Approximately 100 µL of the purified IgG was injected onto a MAbPac SCX-10, 10 µm, 4 × 250 mm column and separated via pH gradient from pH 7.8 to pH 10.8. Mobile phase buffers contained 9.6 mM Tris, 11 mM imidazole, and 6 mM piperazine with pH values of either 6.8 (Buffer A) or 10.8 (Buffer B). The column was equilibrated at 40% B. Three min after sample injection, a linear gradient was run from 40% to 100% B in 30 min. Fractions were collected onto a 96-wellplate at a rate of 0.2 min per fraction from 17 to 27 min.

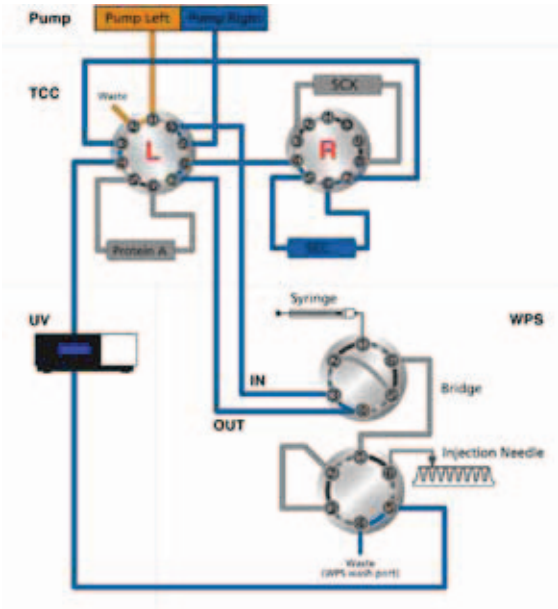
2D-LC Workflow

The workflow and LC conditions for automated off-line 2D-LC include the following:

- Injection of 50 µL of an unpurified HCC sample
- A first-dimension (^{1}D) affinity chromatography separation at a flow rate of 2.0 mL/min using the following steps:
  - A column wash/equilibration step of 0.75 min
  - An elution step of 1 min
  - Automated time-based fraction collection into a wellplate in the autosampler
  - Protein A column is regenerated by a 20% acetonitrile wash and reconditioned for the next analysis

  Total analysis time is approximately 3 min.
- A second-dimension (^{2}D) separation of the collected fraction includes one of the following:
  - SEC separation at a flow rate of 0.3 mL/min using an isocratic mobile phase
  - Strong cation-exchange separation at a flow rate of 0.6 mL/min using a salt gradient

FIGURE 1. Fluidic configuration of the automated off-line 2D-LC system using the wellplate bio-inert autosampler



LC-MS

HPLC: Thermo Scientific ProSwift RP-10R Monolithic Capillary Column (1.0 mm i.d. × 5 cm) was used for desalting. LC solvents were 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 50 ºC during analysis. Flow rate was 100 µL/min. After injection of MAb, a 5 min gradient from 10% B to 95% B was used to elute MAbs from the column.

MS: Using Q Exactive™ instruments, intact MAb was analyzed by ESI-MS for intact molecular mass. The spray voltage was 4 kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275 ºC . S-lens level was set at 55. In-source CID was set at 45 eV. Resolution was 17,500. The AGC target was set at 3E6 for full scan. Maximum IT was set at 200 ms.

Data Processing: Full MS spectra of intact MAbs were analyzed using Thermo Scientific Protein Deconvolution software 1.0 that utilizes the ReSpect algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the MAb. The averaged spectra were subsequently deconvoluted using an input *m/z* range of 2000 to 4000 *m/z*, an output mass range of 140000 to 160000 Da, a target mass of 150000 Da, and minimum of at least 8 consecutive charge states from the input *m/z* spectrum to produce a deconvoluted peak.

Results

In the first step of the chromatographic separation, HCC was injected onto the Protein A Affinity column. In order to collect sufficient amounts of IgG material for the ^{2}D analysis, 50 µL of HCC was injected. The IgG fraction was collected into a 96-wellplate using time-based triggers (Figure 2). The total collection time was 0.1 min. At 2 mL/min flow rate, the total volume collected was 200 µL. Chromeleon CDS software is capable of fraction collection using UV-based peak triggers, or both time and peak triggers together. In the configuration presented here, there was a 0.1 min delay time in fraction collection.

A transition sequence was used to switch the valves and direct the flow path to each ^{2}D analysis column. The ^{2}D analyses can be either SEC (Figure 3) or IEC (Figure 4). Collected fractions can be directly injected onto the ^{2}D column without further modifications. The injection volume for each ^{2}D was 25 µL.

The IEC analysis of the Protein-A purified fractions which used a linear salt gradient revealed many variants in the purified IgG fractions. A one-hour carboxypeptidase digestion (data not shown) eliminated several peaks and enhanced others, suggesting the presence of lysine variants. Use of the MAbPac SCX-10 3 µm column reduced the analysis time from ~60 to 20 min. The total analysis time for all three chromatographic steps was <60 min, which included the transition programs between different analyses. All these steps are automated, and therefore multiple HCC samples can be cycled through without user intervention.

Over the last few years, researchers have demonstrated that pH-gradient-based IEC is an effective method to separate acidic and basic proteins. In this study, we applied pH gradient to the separate MAb variants on a MAbPac SCX-10 column. As shown in Figure 5, separation of at least three variants was achieved. Major peaks 1, 2, and 3 eluted at 19.8, 20.8, and 22.1 min, respectively. Use of the PCM-3000 allowed real-time monitoring of the pH and conductivity of the eluent during all the analyses. The pH values for fractions containing Peaks 1, 2, and 3 were 8.5, 8.6, and 8.7, respectively. These fractions were analyzed on a Q Exactive mass spectrometer (Figure 6). On-line desalting using a reversed phase monolithic column was carried out prior to MS detection. The deconvoluted spectra (Figure 7) showed that the major component in Peak 1 has a 147992.703 *m/z*. Adjacent peaks at 148155.503 and 148315.903 *m/z* correspond to different glycoforms with 1 and 2 additional hexoses. The major component in Peak 2 has a 148210.650 *m/z*. The delta mass between Peak 1 and Peak 2 is 128 amu, corresponding to one lysine. Similarly, the delta mass between Peak 2 and Peak 3 (at *m/z* 148248.641) is also 128 amu. These data suggest that Peak 1 and Peak 2 correspond to lysine truncation variants of Peak 3.

FIGURE 2. Example of a ^{1}D affinity purification of IgG from HCC: the vertical yellow stripe indicates fractionation time.

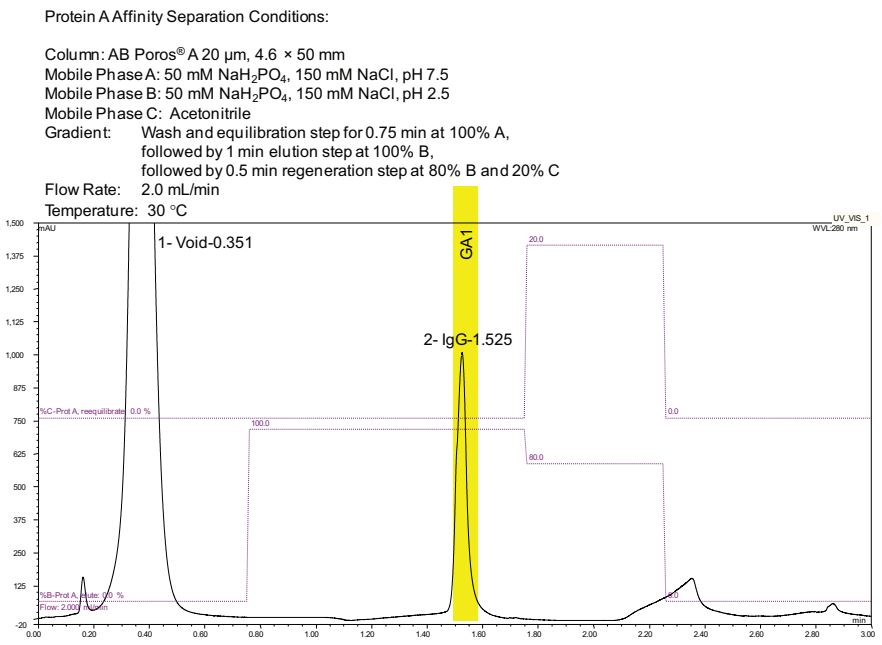


FIGURE 3. Example of an isocratic ²D SEC separation of a purified IgG fraction collected from the MAbPac SEC-1, 4 × 300 mm column

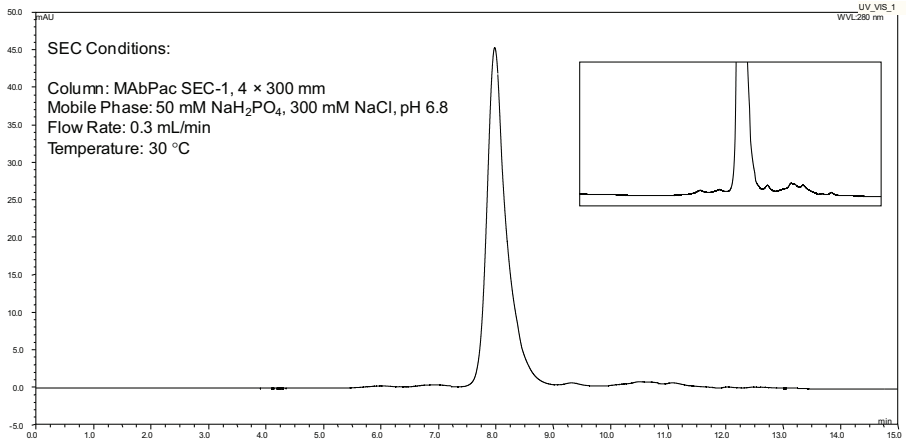


FIGURE 4. Example of a ²D SCX separation of a purified IgG fraction collected from the MAbPac SCX-10, 3 µm, 4 × 50 mm column

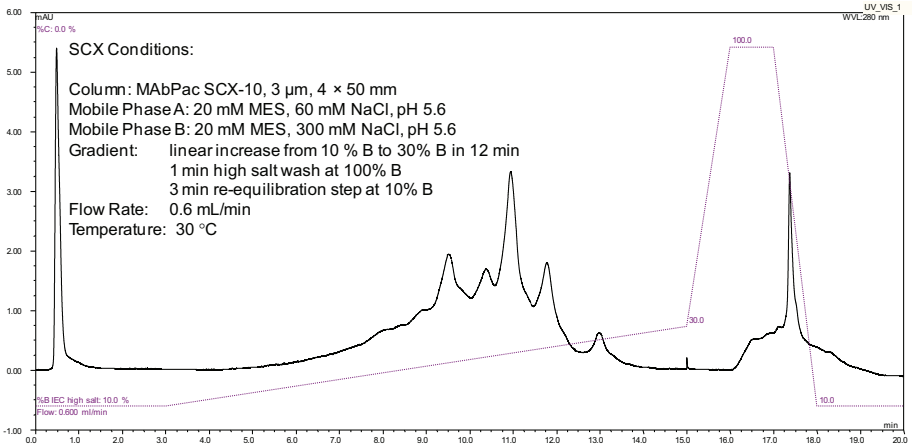


FIGURE 5. pH gradient separation of purifed IgG on a MAbPac SCX-10 column

pH-Gradient Separation Conditions:

Column: MAbPac SCX-10, 10 µm, 4 × 250 mm
Mobile Phase A: 9.6 mM Tris, 11 mM imidazole, and 6 mM piperazine, pH value 6.8
Mobile Phase B: 9.6 mM Tris, 11 mM imidazole, and 6 mM piperazine, pH value 10.8
Gradient: 3 min pre-equilibration at 40% B
followed by linear increase from 40 % B to 100% B in 30 min
followed by 7 min high pH wash at 100% B
followed by 15 min re-equilibration step at 40% B
Flow Rate: 1.0 mL/min
Temperature: 30 °C
Fraction Collection Rate: 0.2 min/well

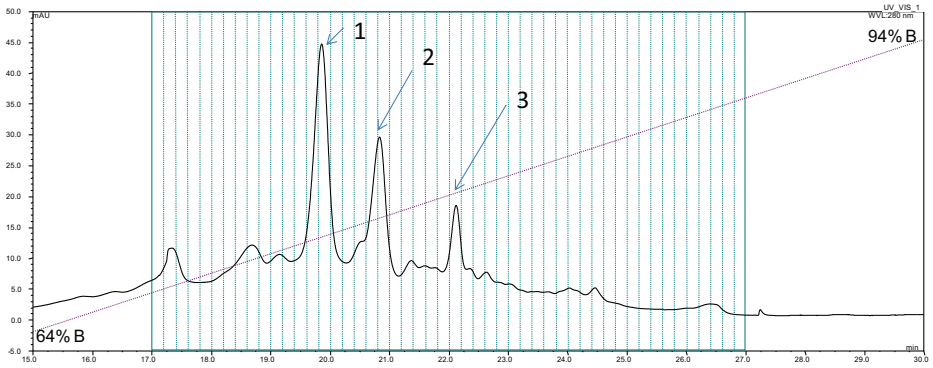


FIGURE 6. Full scan MS spectra

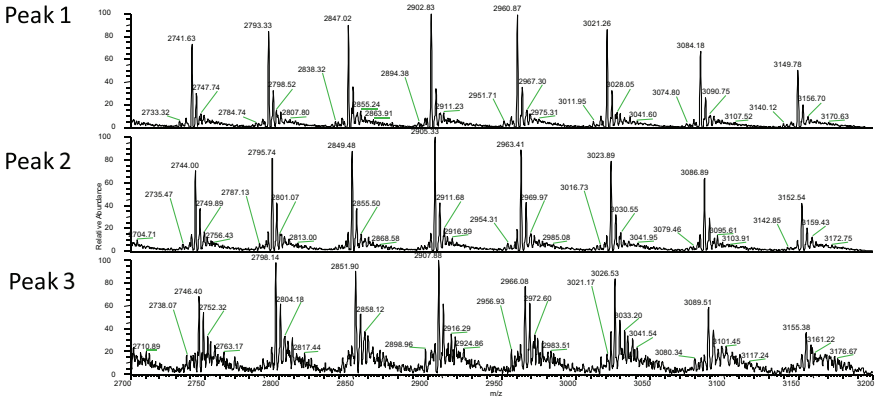
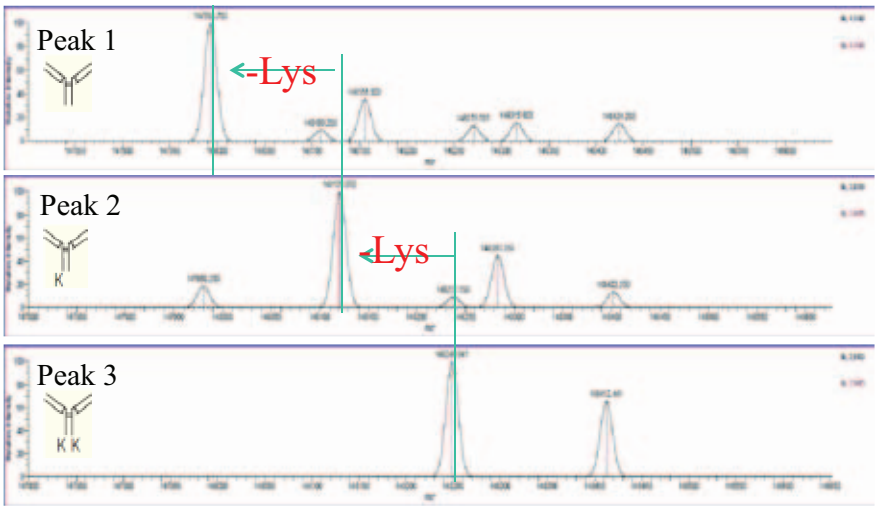


FIGURE 7. Deconvoluted MS spectra



Conclusion

- Using Protein-A Affinity, MAbPac SEC, and MAbPac SCX-10 columns, HCC was characterized by affinity purification, followed by SEC and charge variant analysis in less than one hour.
- The separation of the lysine variants demonstrated that the pH-based gradient method is an effective approach, orthogonal to salt gradient separation.
- The combination of off-line IEC separation and on-line LC MS detection provides an efficient way to obtain structural information of MAb variants.

References

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Acknowledgements

We would like to thank Terry Zhang at Thermo Fisher Scientific for assistance in acquiring the MS data.

Routine analysis:

Charge variant methods

Protein therapeutics applications notebook

High-Resolution Cation-Exchange Chromatography for Analysis of Protein Purity and Microheterogeneity

Proteins produced for medicinal purposes must be shown to be consistently pure from lot to lot. A protein's polypeptide backbone is subject to a wide variety of post-translational modifications that are either enzymatically or chemically produced (phosphorylation, glycosylation, oxidation, deamidation, etc.) and lead to the creation of protein isoforms. Purity analysis and isoform analysis require a high-resolution separation technique. The reference below and Dionex Application Notes 125, 128, 129, and 213 show that ion-exchange chromatography (IEC) provides high-resolution separations of protein isoforms.

To demonstrate the high-resolving power of IEC, we separated a ribonuclease A (RA) standard that was certified as 75% pure by UV absorbance. RA has an isoelectric point of 9.3 suggesting it would best be separated by a cation-exchange column. Figures 1 and 2 display that both the ProPac® WCX and SCX columns (weak and strong cation-exchangers) show the impurity of the RA standard, but with distinct profiles and the WCX column producing more discrete peaks. Depending on the analysis goals, either column could be used.

Reference

Weitzhandler, M.; Farnan, D.; Horvath, J.; Rohrer, J. S.; Slingsby, R. W.; Avdalovic, N.; and Pohl, C. Protein Variant Separations by Cation-Exchange Chromatography on Tentacle-Type Polymeric Stationary Phases. *J. Chromatogr. A* **1998,** *828,* 365–372.

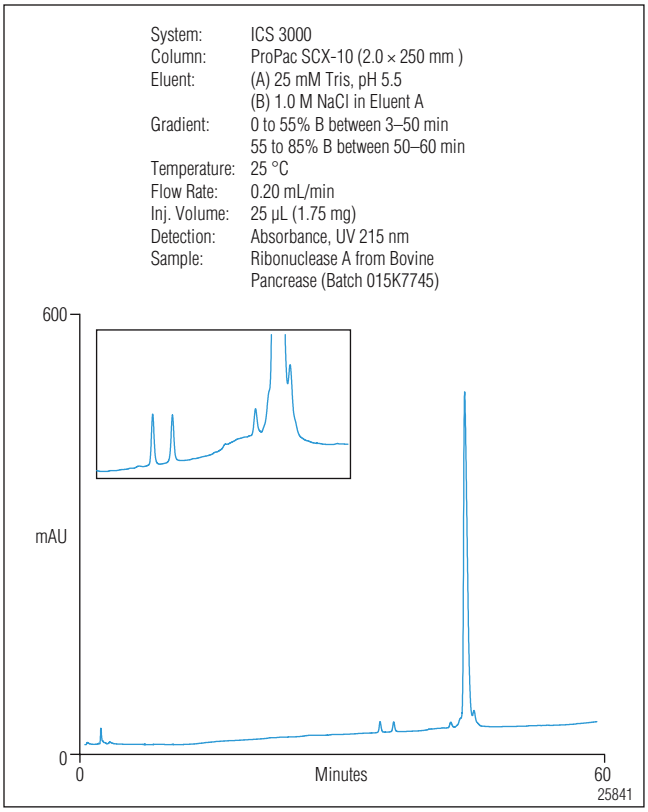

Figure 1. Separation of ribonuclease A from bovine pancrease using the ProPac SCX-10 column.

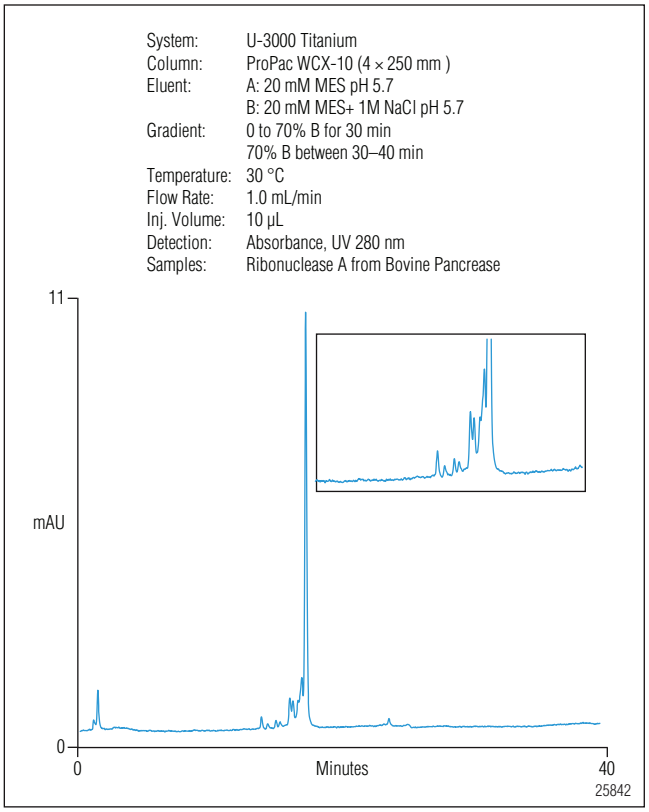

Figure 2. Separations of ribonuclease A from bovine pancrease using the ProPac WCX-10 column.

Thermo SCIENTIFIC

Application Note 126

Determination of Hemoglobin Variants by Cation-Exchange Chromatography

INTRODUCTION

Clinical laboratories frequently separate and quantify the levels of hemoglobin variants in blood samples. Two types of hemoglobin most commonly measured are glycosylated hemoglobins and hemoglobin sequence variants.[1,2] For the physician, the assay of glycated (nonenzymatically glycosylated) hemoglobin levels in the blood of a diabetic patient serves as an excellent indication of the average level of blood glucose during the preceding one to two months. In addition, the determination of hemoglobin variants helps to identify a variety of hemoglobinopathies, including sickle cell, hemoglobin C, and Bart's Disease. Moreover, these assays are extremely important in the diagnosis, treatment, and counseling of afflicted children.[3]

Typically, isoelectric focusing (IEF) gel electrophoresis is used for the analysis of hemoglobin variants, including HbS, HbC, HbF, HbA, and HbA_2. However, two IEF procedures are necessary: cellulose acetate electrophoresis at alkaline pH, followed by citrate agar electrophoresis at acidic pH for confirmation. In comparsion, the ProPac™ SCX-10 column successfully resolves these hemoglobin species in a single run within 20 min. This column uses a unique, hydrophilic-coated, polymeric resin with sulfonate functional groups on grafted linker arms. The physicochemical characteristics of this strong cation exchange support afford minimal band spreading and very high selectivity.[4]

This Application Note describes two examples that illustrate the use of the Dionex ProPac SCX-10 strong cation exchange column for the rapid, high-resolution separation of hemoglobin variants.

EQUIPMENT

DX-500 BioLC® Liquid Chromatograph
- GP50 Gradient Pump
- AD20 Variable Wavelength Absorbance Detector
- LC25 Chromatography Enclosure
- AS50 Autosampler (with a 50-µL sample loop)

PeakNet Chromatography Workstation

REAGENTS AND STANDARDS

High-purity deionized water prepared with a Milli-Q system (Millipore, Bedford, MA, USA)

Sodium phosphate, monobasic and dibasic, analytical-reagent grade (J. T. Baker, Phillipsburg, NJ, USA)

Sodium chloride, analytical-reagent grade (Fluka, Ronkonkoma, NY, USA)

Potassium cyanide, analytical-reagent grade (Sigma, St. Louis, MO, USA)

Hemoglobin variants (Helena Labs, Beaumont, TX, USA)

PREPARATION OF SOLUTIONS AND REAGENTS

Two eluents are used for this chromatography: 50 mM sodium phosphate with 2 mM potassium cyanide (KCN) (pH 6.0) and 50 mM sodium phosphate (pH 6.0) with 500 mM sodium chloride (NaCl) and 2 mM potassium cyanide (KCN). The sodium phosphate buffer system was prepared by diluting appropriate quantities of the monobasic and dibasic sodium phosphate concentrate solutions with water to attain the desired pH 6.0. The following procedure is a recommended starting point for obtaining

the desired eluents, but some deviation from this formula may be necessary after checking the pH when using reagents in other labs. If the pH is not 6.0, then adjust the proportions of monobasic and dibasic solutions added. The combined total volume of monobasic and dibasic solutions should remain at 500 mL to produce 50 mM sodium phosphate for 2 L of eluent.

1M Sodium Chloride

Dissolve 116.90 g sodium chloride in water, and fill to a final volume of 2.0 L. Filter through a 0.45-µm filter.

200 mM Potassium Cyanide

Dissolve 13.02 g anhydrous potassium cyanide in 1000 mL of water. Filter through a 0.45-µm filter. *Caution: exercise necessary care and precautions when handling concentrated potassium cyanide.*

200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate (Na_2HPO_4) in 1000 mL of water Filter through a 0.45-µm filter. Store frozen until needed.

200 mM Sodium Phosphate, Monobasic

Dissolve 27.60 g monohydrate monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$) in 1000 mL of water. Filter through a 0.45-µm filter. Store frozen until needed.

50 mM Sodium Phosphate, 2 mM Potassium Cyanide, pH 6.0

Combine 70 mL of 200 mM dibasic sodium phosphate, 430 mL of 200 mM monobasic sodium phosphate, 20.0 mL of 200 mM potassium cyanide, and 1480 mL water.

50 mM Sodium Phosphate with 0.5 M Sodium Chloride, 2 mM Potassium Cyanide, pH 6.0

Combine 70 mL of 200 mM dibasic sodium phosphate, 430 mL of 200 mM monobasic sodium phosphate, 1000 mL 1 M sodium chloride, 20.0 mL of 200 mM potassium cyanide, and 480 mL water.

CONDITIONS

Column: ProPac SCX-10, 4 × 250 mm

Flow Rate: 1 mL/min

Detection: Absorbance, 220 nm

Mobile Phase: A: 50 mM sodium phosphate, 2 mM potassium cyanide, pH 6.0
B: 50 mM sodium phosphate, 2 mM potassium cyanide, 0.5 M sodium chloride, pH 6.0

Gradient: Method 1: 0 min, 3% to 12% B in 20 min to 40% B at 30 min
Method 2: 0–50% B in 30 min

Method

Method 1

Separation of glycated hemoglobin variants (Figure 1)

Time	A (%)	B (%)	Comments
Initial	97.00	3.00	Equilibration
0.00	97.00	3.00	Injection
20.00	88.00	12.00	
30.00	60.00	40.00	End Gradient
30.10	97.00	3.00	Re-equilibration
40.10	97.00	3.00	

Method 2

Separation of hemoglobin sequence variants (Figure 2)

Time	A (%)	B (%)	Comments
Initial	100.00	0.00	Equilibration
0.00	100.00	0.00	Injection
30.00	50.00	50.00	End Gradient
30.10	100.00	0.00	Re-equilibration
40.00	100.00	0.00	

SAMPLE PREPARATION

Protein samples were prepared by dissolving hemoglobin variants in Mobile Phase A at a concentration of 0.5 mg/mL.

RESULTS AND DISCUSSION

Figure 1 shows the separation of hemoglobin variants on the ProPac SCX-10 column. This strong cation-exchange resin column produced a rapid, high resolution separation of hemoglobin variants found in a sample known to contain elevated levels of glycated hemoglobin. About 10% of the total hemoglobin was glycated. The peaks are labeled in accordance with established conventions.[2,5] Figure 1 reveals the presence of numerous glycated

forms of hemoglobin. Hemoglobin glycation is a modification that occurs nonenzymatically between hemoglobin and sugars in the blood. In principle, nonenzymatic glycation can occur with any free amino group in the hemoglobin protein (e.g., at the N-terminus of the protein chains or on the side chains of lysine residues). The major glycated component is formed when the N-terminal residues of the protein chains react with glucose to produce HbA_{1c}, although other forms have been identified and described elsewhere.[6]

The separation of several hemoglobin sequence variants, including sickle cell hemoglobin, fetal hemoglobin, and hemoglobin C, is shown in Figure 2. The high rate of mass transfer associated with the pellicular resin resulted in narrow, efficient peaks and, consequently, very high levels of resolution. The high peak efficiency observed in this separation and the relatively steep nature of the gradient profile indicate that the ProPac SCX-10 column has the potential to resolve a large number of hemoglobin variants. Two peaks (1 and 4) are tentatively identified in the chromatogram as HbA_{1c} and HbA_2.

CONCLUSION

These results demonstrate that the ProPac SCX-10 column has a high degree of selectivity for hemoglobin variants. The ability to separate these variants makes the ProPac SCX-10 column a powerful tool for the analysis of hemoglobin in clinical and research laboratories.

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4. Weitzhandler, M.; Farnan, D.; Horvath, J.; Rohrer, J. S., Slingsby, R. W.; Avdalovic, N.; and Pohl, C., J. *J. Chromatogr. A.* **1998**, *828*, 365–372.
5. Flickiger, R. and Mortensen, H. B. *J. Chromatogr. B.*, **1988**, *429*, 279–292.
6. Shapiro, R.; McManus, M. J.; Zalut, C.; and Bunn, H. F. *J. Biol. Chem.*, **1980**, 350, 3120–3127.

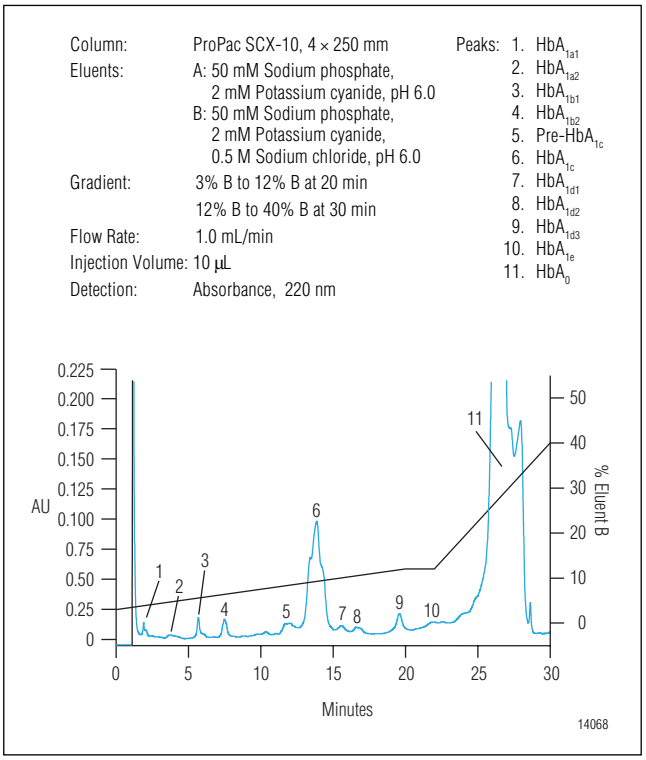

Figure 1. Separation of hemoglobin variants in a sample with elevated levels of glycated hemoglobins. Sample concentration is 0.5 mg/mL.

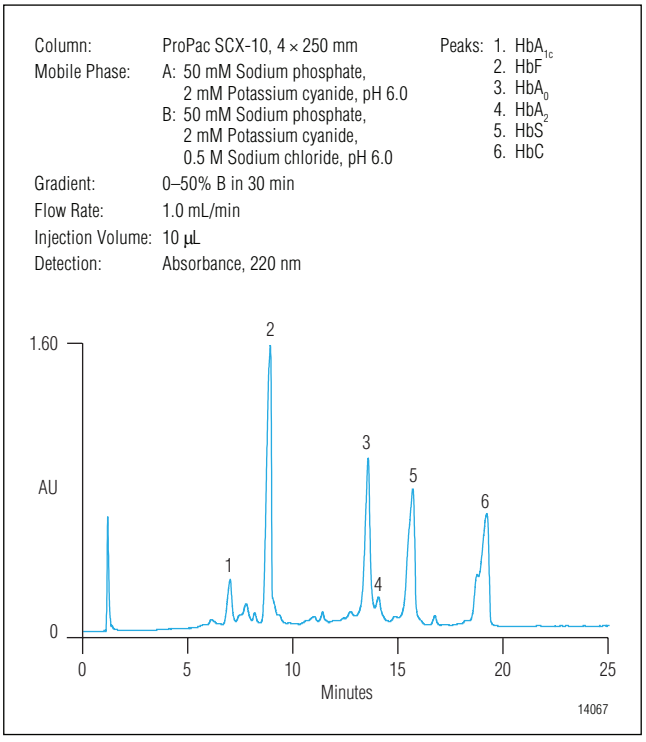

Figure 2. Separation of hemoglobin variants, including fetal (HbF), sickle cell (HbF), normal (HbA), and C-type (HbC) hemoglobins. Sample concentration is 0.5 mg/mL.

LIST OF SUPPLIERS

Millipore Corporation, 80 Ashby Road, Bedford, MA 01730, USA. Tel: (800) 645-5476

J. T. Baker Incorporated, 222 Red School Lane, Phillipsburg, NJ 08865, USA. Tel: (800) 582-2537

Fluka Chemika-BioChemika, Fluka Chemie AG, Industriestrasse 25, CH-9471 Buchs, Switzerland. Tel: +81 755 25 11

Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178, USA. Tel: (800) 325-3010

Helena Labs, 1530 Lindbergh Dr., Beaumont, TX 77707, USA. Tel: (409) 842-3714

Thermo SCIENTIFIC

Application Note 127

Monitoring Monoclonal Antibody Heterogeneity by Cation-Exchange Chromatography

INTRODUCTION

During the development and production of therapeutic proteins, characterization of structural variants is a critical challenge. C-terminal processing of lysine residues on the heavy chain of monoclonal antibodies (MAbs) is a common structural variation that demands careful analysis.[1-7] As a result of this processing, C-terminal lysine or arginine residues are often absent in proteins isolated from mammalian cell cultures, even though their presence may be expected on the basis of the gene sequence. This discrepancy, which is common in plasma-derived proteins, results from the activity of one or more basic carboxypeptidases. Incomplete protein processing results in charge heterogeneity which is readily identified by cation-exchange chromatography on the Dionex ProPac® WCX-10, a weak cation-exchange column. The packing in this column is a unique pellicular resin with a hydrophilic coating and carboxylate functional groups on grafted linker arms. The physicochemical properties of this support eliminate secondary (nonionic) interactions between the protein analytes and the stationary phase, affording minimal band broadening and high selectivity.[8] The UltiMate® 3000 Titanium System is an HPLC whose flow path is ensures that neither solvents nor sample are in contact with stainless steel materials, removing concerns about iron and other transition metals contaminating the column and samples. This application note describes a method for analyzing a humanized IgG_1 MAb for C-terminal lysine variants. These variants are baseline resolved from the native antibody using the ProPac WCX-10 column and a 4-morpholineethanesulfonic acid (MES)-based mobile phase. Other acidic and basic variants are also better resolved than had been observed with traditional phosphate-based mobile phases buffered at pH 7, enabling the detection of additional MAb variants, each with heavy chain C-terminal lysine heterogeneity.

EQUIPMENT

Dionex UltiMate 3000 Titanium System consisting of:

SRD-3600 Solvent Rack with 6 Degasser Channels (P/N 5035.9230) and Eluent Organizer, including pressure regulator, and 2-L glass bottles for each pump

LPG 3400AB Quaternary Analytical Pump (P/N 5037.0015) or DGP-3600AB Dual Ternary Analytical Pump (P/N 5037.0014) for dual gradient capability

WPS-3000TBPL Biocompatible Analytical Autosampler (P/N 5823.0020) with 50 µL biocompatible sample loop

TCC-3000 Column Compartment without Switching Valves (P/N 5722.0000) or TCC-3200B Column Compartment with 2 PEEK ten-port two-position valves (P/N 5723.0025) for added productivity

VWD-3400 Variable Wavelength Detector (P/N 5074.0010) or PDA-3000 Photodiode Array Detector (P/N 5080.0020)

Biocompatible Analytical Flow Cell for VWD (P/N 6074.0200) or Biocompatible Analytical Flow Cell for PDA (P/N 6080.0220)

Chromeleon® Chromatography Data System

Helium; 4.5-grade, 99.995%, <5 ppm oxygen (Praxair)

Filter unit, 0.2 µm Nylon (Nalgene 90-mm Media-Plus, Nalge Nunc International, P/N 164-0020 or equivalent Nylon filter)

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent; for degassing eluents)

0.3 mL polypropylene (Vial Kit, P/N 055428) injection vials with caps

Microcentrifuge tubes with detachable screw caps (polypropylene, 1.5 mL, Sarstedt, P/N 72.692.005; or equivalent)

REAGENTS AND STANDARDS

Deionized water, 18 MΩ-cm resistance or higher

4-Morpholineethanesulfonic acid (MES) hydrate, minimum 99.5% titration (Sigma-Aldrich; P/N M8250)

Sodium chloride, crystal (J.T. Baker; P/N 4058-05)

Sodium hydroxide solution, 50% W/W (Thermo Fisher Scientific; P/N SS254)

Carboxypeptidase B, chromatographically purified, ≥ 170 U/mg, 5.0 mg/mL (Worthington Biochemical Corporation; P/N LS005305) or equivalent

Humanized monoclonal IgG_1 antibody (a generous gift from a biotechnology company)

CONDITIONS

Method

Column: ProPac WCX-10 Analytical 4 × 250 mm (P/N 054993)

Flow Rate: 1.00 mL/min

Inj. Volume: 10 µL (partial loop)

Sampler Temp: 5 ºC

Column Temperature: 30 °C

Detection: Absorbance, 280 nm (absorbance at 214 and 254 nm also collected)

Data Collection Rate: 1.0 Hz

Noise: 12-24 µAU

Typical System Operating Backpressure: ~ 125 bar (~1830 psi)

Mobile Phase: A: 20 mM MES, 60 mM sodium chloride, pH 5.6
B: 20 mM MES, 240 mM sodium chloride, pH 5.6

Gradient: Linear, 20% B for 2 min, 20–50% B from 2–52 min

Gradient Method

Time (min)	A(%)	B(%)	Comments
–20.00	80.0	20.0	Equilibration
0.00	80.0	20.0	Sample Injection
2.00	80.0	20.0	Sample Binding
52.00	50.0	50.0	End Gradient
52.10	0.0	100.0	Column Regeneration
57.00	80.0	20.0	Re-equilibration

PREPARATION OF SOLUTIONS AND REAGENTS

All mobile phases are filtered through a 0.2 µm nylon filter under vacuum to remove particulates and to degas prior to their use. The mobile phases are blanketed under 34–55 kPa (5-8 psi) of helium headspace to reduce the growth of opportunistic microorganisms, and maintain a low concentration of dissolved air.

20 mM MES, 60 mM Sodium Chloride, pH 5.6 (Mobile Phase A)

Combine 7.81 g of MES, 7.01 g of sodium chloride, and 1900 mL of DI water. Adjust the pH of the resulting solution to 5.6 with 50% sodium hydroxide solution (~500 µL needed). Carefully pour the resulting solution into a 2 L volumetric flask and fill to the mark with DI water.

20 mM MES, 240 mM Sodium Chloride, pH 5.6 (Mobile Phase B)

Combine 7.81 g of MES, 28.04 g of sodium chloride, and 1900 mL of DI water. Adjust the pH of the resulting solution to 5.6 with 50% sodium hydroxide solution (~500 µL needed). Carefully pour the resulting solution into a 2 L volumetric flask and fill to the mark with DI water.

Sample Preparation

MAb samples were diluted to 10 mg/mL using filtered, degassed water. One 100 µL aliquot of the MAb solution was combined in a microcentrifuge tube with 0.5 µL of thawed carboxypeptidase B solution using a micropipette while another 100 µL aliquot was combined with 0.5 µL of DI water. Both sample and control were incubated at 37 ºC for 2 h. After the 2-h incubation period, sample and control were diluted 5-fold with Mobile Phase A and analyzed.

RESULTS AND DISCUSSION

The ProPac WCX-10 weak cation-exchange column was assessed for its ability to separate humanized IgG_1 variants. As shown in Figure 1 this antibody not only produces three major peaks but also separates a number of other variants, both more acidic as well as more basic variants compared to the three major peaks. The MES-based buffers resolved additional details not seen when using phosphate-based buffers, an observation sometimes reported when separating other protein variants.[9]

To verify that the three major peaks were due to variations in C-terminal lysine content, the IgG_1 sample was treated with carboxypeptidase B, an exopeptidase that specifically cleaves C-terminal lysine residues. A comparison of the two chromatographic traces in Figure 2 shows a disappearance of the second and third major peaks, which are proposed to contain one and two terminal heavy chain lysine residues, respectively. The disappearance of peaks 2 and 3 was accompanied by a corresponding increase in the area under the first major peak, the variant with no terminal lysine. Additionally, the pattern for the minor peaks eluting before the first major peak is maintained, suggesting that these peaks do not contain terminal heavy chain lysine residues. The complex chromatographic pattern of peaks eluting after the three major peaks is also simplified with carboxypeptidase B incubation, showing MAb variants, each with heavy chain C-terminal lysine heterogeneity, are present in the original sample.

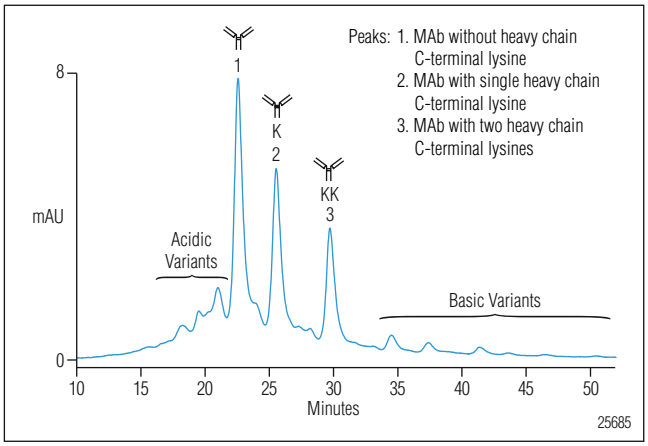

Figure 1. Separation of acidic and basic terminal lysine variants of an IgG_1 monoclonal antibody using the ProPac WCX-10 column and MES-based buffers on the UltiMate 3000 Titanium System.

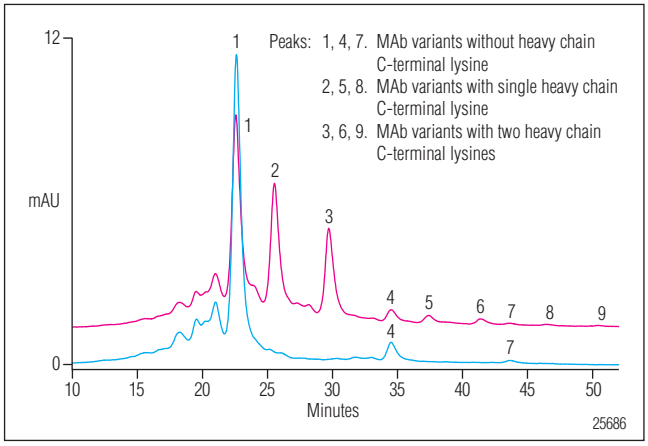

Figure 2: Analysis of IgG_1 monoclonal antibody before (upper trace) and after (lower trace) treatment with carboxypeptidase B for 2 h at 37 ºC.

CONCLUSION

The ProPac WCX-10 weak cation-exchange column, used with MES-based buffers on an inert titanium system, produced detailed chromatograms of variants of an IgG_1 MAb, including baseline separation of C-terminal lysine variants. These results demonstrate that this chromatographic system is well suited for analyses such as quality control monitoring of protein-based therapeutics that require high efficiency, high resolution separation of closely eluting proteins.

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8. Weitzhandler, M.; Farnan, D.; Horvath, J.; Rohrer, J.S.; Slingsby, R.W.; Avdalovic, N.; Pohl, C. Protein variant separations by cation-exchange chromatography on tentacle-type polymeric stationary phases. *J. Chromatogr. A.* **1998,** *828,* 365–372.
9. Dionex Corporation. Monitoring Protein Deamidation by Cation-Exchange Chromatography, Application Note 125 (LPN 2168, January, 2009); Sunnyvale, CA.

LIST OF SUPPLIERS

Gast Manufacturing Corp., 2550 Meadowbrook Road, Benton Harbor, MI 49022, U.S.A. Tel: 269-926-6171, http://www.gastmfg.com.

Mallinckrodt Baker, 222 Red School Lane, Phillipsburg NJ 08865, U.S.A. Tel: 800-582-2537 http://www.mallbaker.com

Nalge Nunc International, 75 Panorama Creek Drive, Rochester, NY 14625, U.S.A. Tel: 800-625-4327, http://www.nalgenunc.com

Praxair, 39 Old Ridgebury Road, Danbury, CT 06810-5113, U.S.A. Tel: 877-772-9247, http://www.praxair.com

Sarstedt AG & Co., Rommelsdorfer Straße, Postfach 1220, 51582 Nümbrecht, Germany
+49-2293-305-0, http://www.sarstedt.com

Sigma-Aldrich Chemical Company, P.O. Box 14508, St. Louis, MO 63178, U.S.A. Tel: 800-325-3010, http://www.sigma.sial.com

Thermo Fisher Scientific, 4500 Turnberry Drive, Hanover Park, IL 60133, U.S.A. Tel: 800-766-7000, http://www.fishersci.com .

Worthington Biochemical Corp., 730 Vassar Ave., Lakewood, NJ 08701, U.S.A. Tel: 800-445-9603 http://www.worthington-biochem.com

Thermo SCIENTIFIC

Application Note 128

Monitoring Monoclonal Antibody Stability by Cation-Exchange Chromatography

INTRODUCTION

Protein microheterogeneity can be attributed to a variety of post-translational modifications including glycosylation, oxidation, phosphorylation, amino-terminal modifications (e.g., to pyroglutamate), asparagine (Asn) deamidation,[1] and incomplete C-terminal processing.[2] Variations in protein composition can impact a protein's activity and stability as a biotherapeutic.[3–7] Monitoring stability of therapeutic proteins and peptides is regarded as essential for demonstrating safety and efficacy of these drugs, and is expected by the FDA and other regulating agencies.

Recombinant monoclonal antibodies (MAb) have been shown to have heterogeneity with either arginine (Arg) or lysine (Lys) at the C-terminus of the heavy chain(s). When a MAb is treated with carboxypeptidase B, an exopeptidase, Arg and Lys are cleaved from the C-terminus, eliminating C-terminal heterogeneity.[8–14] Proteins and peptides containing Asn adjacent to glycine (Gly) are particularly susceptible to Asn deamidation, converting Asn to aspartic acid (Asp) or isoaspartic acid to varying extents.[15–18] Monitoring the extent of deamidation is of interest to quality control and process development chemists concerned with product quality and stability.

Shelf-life studies of proteins and peptides typically monitor a variety of degradation products. This evaluation commonly uses ion-exchange chromatography. In this application note a sample of humanized MAb was first treated with carboxypeptidase B to remove C-terminal lysine charge heterogeneity. This modified antibody was subsequently subjected to forced deamidation conditions.[19] Changes in MAb heterogeneity were monitored using the Dionex ProPac® WCX-10 weak cation-exchange column housed in the UltiMate® 3000 Titanium HPLC System. The combination of a bio-inert HPLC system with a high resolution cation-exchange column is well suited for the analysis of protein microheterogeneity.[20]

EQUIPMENT

Dionex UltiMate 3000 Titanium System consisting of:

SRD-3600 Solvent Rack with 6 Degasser Channels (P/N 5035.9230) and Eluent Organizer, including pressure regulator, and 2-L glass bottles for each pump

LPG 3400AB Quaternary Analytical Pump (P/N 5037.0015) or DGP-3600AB Dual Ternary Analytical Pump (P/N 5037.0014) for dual gradient capability

WPS-3000TBPL Biocompatible Analytical Autosampler (P/N 5823.0020)

TCC-3000 Column Compartment without Switching Valves (P/N 5722.0000) or TCC-3200B Column Compartment with 2 PEEK ten-port two-position valves (P/N 5723.0025) for added productivity

VWD-3400 Variable Wavelength Detector (P/N 5074.0010) or PDA-3000 Photodiode Array Detector (P/N 5080.0020)

Biocompatible Analytical Flow Cell for VWD (P/N 6074.0200) or Biocompatible Analytical Flow Cell for PDA (P/N 6080.0220)

Chromeleon® Chromatography Data System

Helium; 4.5-grade, 99.995%, <5 ppm oxygen (Praxair)

Filter unit, 0.2 µm Nylon (Nalgene 90-mm Media-Plus, Nalge Nunc International, P/N 164-0020 or equivalent Nylon filter

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent; for degassing eluents)

SpeedVac Evaporator System (Thermo Scientific Savant or equivalent) consisting of:

SpeedVac microcentrifuge, model SVC100

Refrigerator Vapor Trap, Model RVT400

Vacuum Gauge, Model VG-5

Welch Duo-Seal Vacuum Pump, Model 1402 capable of pulling 0.2 Torr (200 µm Hg) vacuum

0.3 mL polypropylene (Vial Kit, P/N 055428) injection vials with caps

Microcentrifuge tubes with detachable screw caps (polypropylene, 1.5 mL, Sarstedt, P/N 72.692.005; or equivalent)

REAGENTS AND STANDARDS

Deionized water, 18 MΩ-cm resistance or higher

MES hydrate, minimum 99.5% titration (Sigma-Aldrich; P/N M8250)

Sodium chloride, crystal (J.T. Baker; P/N 4058-05)

Hydrochloric acid, Ultrapure Reagent (J.T. Baker, P/N 6900-05)

Sodium hydroxide solution, 50% W/W (Thermo Fisher Scientific; P/N SS254)

Carboxypeptidase B, chromatographically purified, ≥ 170 U/mg, 5.0 mg/mL (Worthington Biochemical Corporation; P/N LS005305) or equivalent

Ammonium carbonate, HPLC Reagent grade (J.T. Baker; P/N 0651-08)

Sodium azide (Sigma-Aldrich; P/N S8032)

Protease inhibitor cocktail, general use, containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), trans-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), bestatin, leupeptin, aprotinin, and sodium EDTA (Sigma-Aldrich; P/N P2714) or equivalent

Ribonuclease A, Type XII-A from bovine pancreas, minimum 90% (Sigma-Aldrich; P/N R5500)

Humanized monoclonal antibody (IgG_1, 0 mg protein/mL) was a generous gift from a biotechnology company)

CONDITIONS

Method

Column:	ProPac WCX-10 Analytical 4 × 250 mm (P/N 054993)
Flow Rate:	1.00 mL/min
Inj. Volume:	10 µL (partial loop)
Autosampler Temp:	5 ºC
Column Temp:	30 °C
Detection:	Absorbance, 280 nm (absorbance at 214 and 254 nm also collected)
Data Collection Rate:	1.0 Hz
Noise:	12-24 µAU
Typical System Operating Backpressure:	~ 125 bar (~1830 psi)

Mobile Phase:	A: 20 mM MES, 60 mM sodium chloride, pH 5.6 B: 20 mM MES, 240 mM sodium chloride, pH 5.6
Gradient:	Linear, 20% B for 2 min, 20–50% B from 2–52 min

Gradient Method

Time (min)	A(%)	B(%)	Comments
0.0	80.0	20.0	Sample Injection
2.0	80.0	20.0	Sample Binding
52.0	50.0	50.0	End Gradient
52.1	0.0	100.0	Column Regeneration*
57.0	80.0	20.0	Start Re-equilibration
77.0	80.0	20.0	End Re-equilibration

**Need for column regeneration has not been demonstrated*

PREPARATION OF SOLUTIONS AND REAGENTS

Prepare mobile phases as described below. Slightly different volumes of sodium hydroxide may be required to produce 2 L volumes of mobile phases A and B. Filter mobile phases through a 0.2 µm nylon filter under vacuum to remove particulates and to degas. Blanket the mobile phases under 34–55 kPa (5–8 psi) of helium headspace to reduce the growth of opportunistic microorganisms, and maintain a low concentration of dissolved air.

Buffers

20 mM MES, 60 mM Sodium Chloride, pH 5.6 (Buffer A)

Combine 7.81 g of MES, 7.01 g of sodium chloride, and 1900 mL of DI water. Adjust the pH of the resulting solution to 5.6 with 50% sodium hydroxide solution (~650 µL needed). Carefully pour the resulting solution into a 2 L volumetric flask and fill to the mark with DI water.

20 mM MES, 240 mM Sodium Chloride, pH 5.6 (Buffer B)

Combine 7.81 g of MES, 28.04 g of sodium chloride, and 1900 mL of DI water. Adjust the pH of the resulting solution to 5.6 with 50% sodium hydroxide solution (~650 µL needed). Carefully pour the resulting solution into a 2 L volumetric flask and fill to the mark with DI water.

Stock Standards

10% Ammonium Carbonate Buffer, pH 8.2

Dissolve 1.5 g ammonium carbonate in 12.0 mL water. Adjust pH to 8.2 with concentrated HCl.

Adjust total volume to 15.0 mL with additional water.

1% Sodium Azide

Dissolve 10 mg sodium azide in 1.0 mL water

Caution: sodium azide is very toxic. Use necessary precautions to protect against exposure

10× Protease Inhibitor Cocktail

Reconstitute the protease inhibitor cocktail vial with 10 mL DI water. Cocktail concentrate consists of 20 mM AEBSF, 14 µM E-64, 1.3 mM Bestatin, 10 µM Leupeptin, 3 µM Apotinin, and 10 mM EDTA.

Ammonium Carbonate-Azide-Protease Inhibitor Cocktail Buffer (ACAPIC buffer)

Combine:

1.05 mL 10% ammonium carbonate
0.53 mL 1% sodium azide
0.11 mL 10× protease inhibitor cocktail
8.31 mL water

Adjust pH to 8.2, if necessary.

Monoclonal Antibody

Dilute with mobile phase A to yield a 20 mg/mL solution. Use immediately after dilution.

Ribonuclease A (positive control)

Prepare 100 µL of a 20 mg/mL solution in mobile phase A. Use immediately after preparation.

SAMPLE PREPARATION

Combine 35 µL of 20 mg/mL MAb with 1.0 µL of carboxypeptidase B, and incubate at 37 ºC for 2 h. Remove a 2.0 µL aliquot, dilute to 1.0 mg/mL MAb with mobile phase A, and analyze 10 µL of the solution to ensure the removal of MAb heavy chain C-terminal lysines by carboxypeptidase B. Concurrently incubate positive controls (ribonuclease A) and negative controls (mobile phase A), each with and without carboxypeptidase B. Combine the remaining 34 µL of carboxypeptidase B-treated MAb sample with 627 µL of ACAPIC. Final deamidation conditions consist of 1.0 mg/mL protein (MAb or ribonuclease A positive control), 1% ammonium carbonate, 0.05% sodium azide, 210 µM AEBSF, 150 µM E-64, 14 µM Bestatin, 0.1 µM Leupeptin, 0.03 µM Aprotinin, and 100 µM EDTA. Incubate at 37 ºC, remove 100 µL aliquots at 0, 25, and 50 h, add 900 µL of DI water, and freeze these samples. When ready to analyze the samples, remove water and other volatile components by placing frozen samples into a SpeedVac evaporator system. Add 300 µL of water to the dried samples, freeze, and repeat the evaporation process. Dissolve the remaining solid in 100 µL of mobile phase A, transfer to a 0.3 mL autosampler vials, and analyze a 10 µL injection. The final protein concentration is 1.0 mg/mL. Controls include ribonuclease A (positive control) with and without carboxypeptidase B, and reagent blank with and without carboxypeptidase B.

RESULTS AND DISCUSSION

The MAb solution was treated with carboxypeptidase B to make the results of antibody exposure to deamidation conditions simpler to interpret. Heavy chain C-terminal lysine heterogeneity (the three major peaks between 20-30 min retention time in Figure 1-panel A) is eliminated after carboxypeptidase treatment (Figure 1-panel B).[2] Ribonuclease A, tested in parallel as a positive control, did not show any change upon addition of carboxypeptidase B (data not shown). The carboxypeptidase B-treated antibody and treated controls were then incubated for up to 50 h at 37 ºC in 1% ammonium carbonate containing sodium azide to eliminate microbial contamination, and a broad spectrum protease inhibitor cocktail to stop proteolytic cleavage from contaminating proteases that may be present in the carboxypeptidase B preparation.

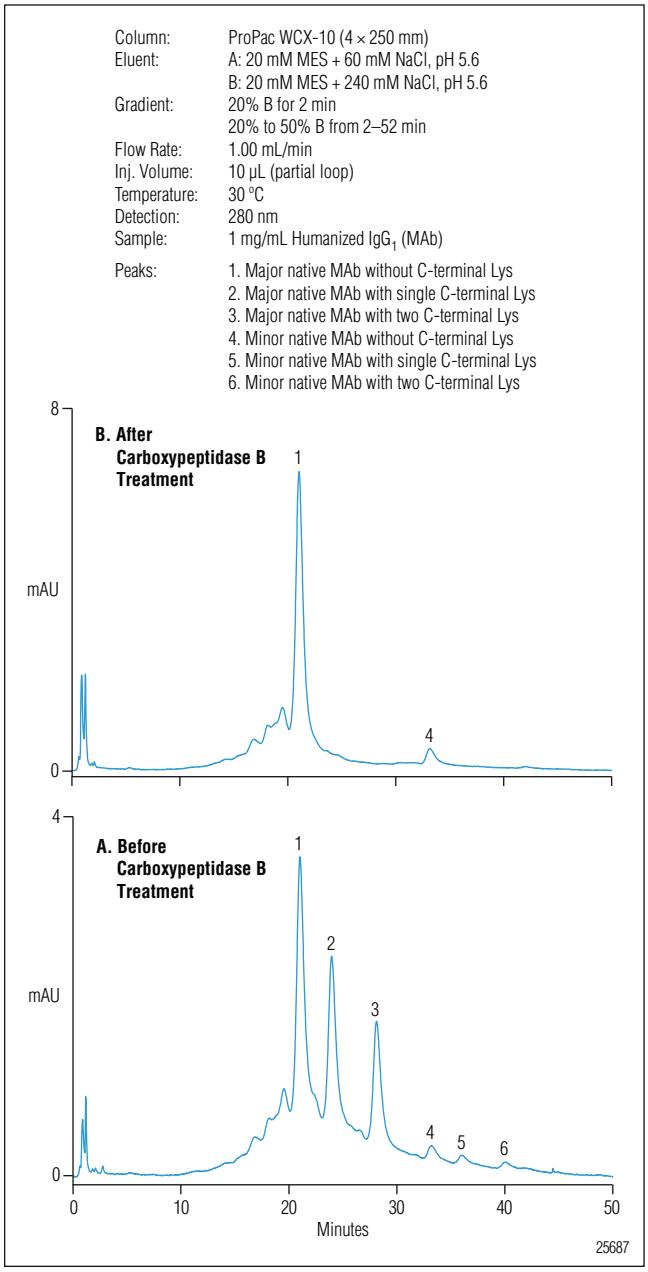

Figure 1. Monoclonal antibody (MAb) separations before (panel A) and after (panel B) carboxypeptidase B treatment (2 h at 37 ºC).

Figure 2 shows chromatograms from aliquots taken 0, 25, and 50 h after adding the ACAPIC mixture and incubating at 37 ºC. Several incompletely resolved peaks (#1-4 in Figure 2) eluting before the major native MAb peak increase in abundance at longer incubation times. The appearance of these multiple peaks is expected as a consequence of deamidation.[1,5,19] Having Asn adjacent to Gly makes the Asn susceptible to conversion to either Asp or isoaspartic acid. While the MAb's primary sequence was not disclosed, the complexity of the chromatogram implies the presence of multiple deamidation sites. The ribonuclease A positive control for deamidation showed the predicted formation of new peaks[1], confirming that this experiment replicated forced deamidation conditions. Peak 6, eluting after the major native MAb, appears immediately at time zero and is not present in the buffer control sample. Its area decreases with increasing incubation time. The source of this peak is unknown but may be the result of a rapid initial degradation. Peaks 1–4, and 6 are broadly defined as decomposition products because there is no additional analysis to confirm deamidation of the monoclonal antibody.

CONCLUSION

This study highlights the high efficiency of the ProPac WCX-10 column, coupled with an inert, titanium HPLC system, for the analysis of protein charge microheterogeneity. This system is an alternative to reversed-phase or affinity chromatography for characterizing proteins.

Caution: Sodium azide and some ingredients in the protease inhibitor cocktail are toxic. Consult the relevant MSDS and use necessary precautions to protect against exposure.

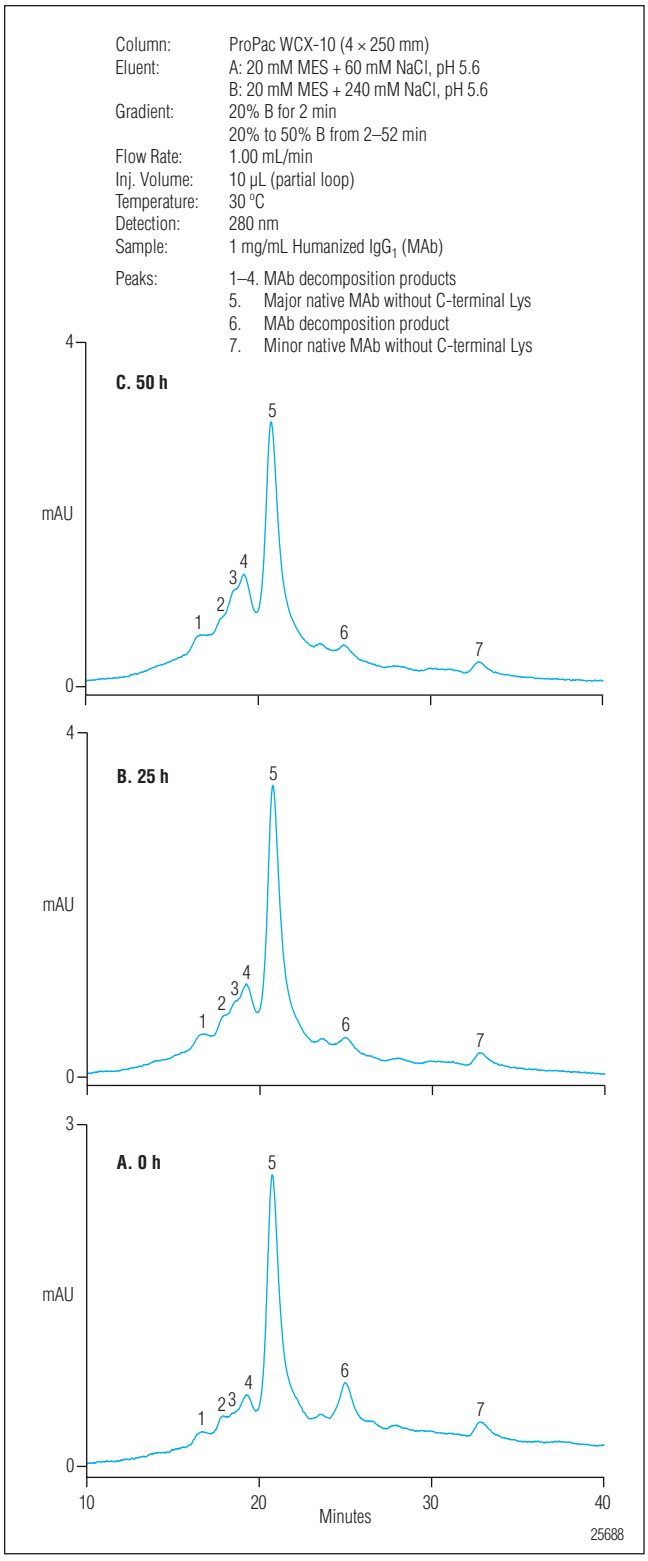

Figure 2. Forced deamidation of carboxypeptidase B-treated MAb (1 mg/mL) incubated at 37 ºC with 1% ammonium carbonate – 0.05% sodium azide – 0.1× protease inhibitor cocktail mixture for 0 h (panel A), 25 h (panel B), and 50 h (panel C).

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LIST OF SUPPLIERS

Gast Manufacturing Corp., 2550 Meadowbrook Road, Benton Harbor, MI 49022, U.S.A. Tel: 269-926-6171, http://www.gastmfg.com

Mallinckrodt Baker, 222 Red School Lane, Phillipsburg, NJ 08865, U.S.A. Tel: 800-582-2537, http://www.mallbaker.com

Nalge Nunc International, 75 Panorama Creek Drive, Rochester, NY 14625, U.S.A. Tel: 800-625-4327, http://www.nalgenunc.com

Praxair, 39 Old Ridgebury Road, Danbury, CT 06810-5113, U.S.A. Tel: 877-772-9247, http://www.praxair.com

Sarstedt AG & Co., Rommelsdorfer Straße, Postfach 1220, 51582 Nümbrecht, Germany, Tel: +49-2293-305-0, http://www.sarstedt.com

Sigma-Aldrich Chemical Company, P.O. Box 14508, St. Louis, MO 63178, U.S.A., Tel: 800-325-3010, www.sigma.sial.com

Thermo Fisher Scientific, 4500 Turnberry Drive, Hanover Park, IL 60133, U.S.A., Tel: 800-766-7000, www.fishersci.com

Worthington Biochemical Corporation, 730 Vassar Ave., Lakewood, NJ 08701, U.S.A. Tel: 800-445-9603, http://www.worthington-biochem.com

Routine analysis:
HIC methods

Protein therapeutics applications notebook

Hydrophobic Interaction Chromatography for Separation of Tryptophan and Methionine Oxidized Peptides from Their Native Forms

INTRODUCTION

Hydrophobic interaction chromatography (HIC) is a technique used to separate peptides, proteins, and other biological molecules based on their degree of hydrophobicity. The HIC mobile phase comprises a high-concentration salting-out agent—typically ammonium sulfate—that increases the hydrophobic interaction between the solute and the stationary phase.[1]

HIC and reversed-phase chromatography are closely related techniques. Both are based upon interactions between solvent-accessible nonpolar groups (hydrophobic patches) on the surface of the solute and the hydrophobic ligands of the stationary phase. In practice, however, HIC and reversed-phase chromatography are different, as reversed-phase stationary phases are more highly substituted with hydrophobic ligands than HIC stationary phases. The techniques also use different mobile phases. Protein binding to reversed-phase stationary-phases is usually very strong, and requires polar solvents for elution, which can denature the proteins during the separation. Reversed-phase HPLC has found extensive application in analytical and preparative separations of peptides and low molecular weight proteins that have stable primary structure in aqueous-organic mobile phases. HIC is an alternative to exploiting the hydrophobic properties of proteins, working with nonpolar, non-denaturing mobile phases.[2]

Proteins and peptides are sensitive to oxidative damage. Oxidizing proteins and peptides can alter their biological activity, half-life, and immunogenicity. Natural biological and environmental oxidants have been suggested as causative or contributory factors in many diseases. Oxidation of proteins has also been reported as a natural posttranslational event mediated enzymatically by amines and oxidases.[3] Methionine, cysteine, histidine, tryptophan, and tyrosine residues are most susceptible to this oxidation.[4] Methionine is easily oxidized by atmospheric oxygen to form methionine sulfoxide, and tryptophan can be oxidized into four different oxidation products by peroxide. Peroxide is an impurity generated during protein storage, or from polysorbates commonly used for protein purification and solubilization. Other peroxide-contaminated materials include polyethylene glycol (PEG) or silicon rubber from vial stoppers. When proteins are used as pharmaceutical ingredients, methionine and tryptophan oxidation during processing or storage can affect protein activity.[5]

In Application Note 129, the authors used a weak cation-exchange chromatography method with the ProPac® WCX-10 to separate oxidized and native Luteinizing Hormone-Releasing Hormone (LH-RH)—a tryptophan-containing peptide, and oxidized and native α-Melanocyte Stimulating Hormone (MSA)—a methionine-containing peptide. Here, the authors demonstrate separation of these oxidized and non-oxidized variants on the ProPac HIC-10 (hydrophobic interaction) column and the ProPac WCX-10 (weak anion-exchange) column using ICS-3000 PEEK™ and UltiMate® 3000 Titanium systems. The ProPac HIC-10 column separates LH-RH isoforms not found using weak the cation-exchange column and chemistry, and the resolution of the oxidized variants is improved over the aforementioned method.

EQUIPMENT

ICS-3000 Liquid Chromatography System
- ICS-3000 SP (P/N 079820)
- ICS-3000 VWD (P/N 064653)
- ICS-3000 TC (P/N 064444)
- AS Autosampler (P/N 056859)
- ProPac HIC-10 column, 4.6 × 100 mm, (P/N 063655)
- ProPac WCX-10 column, 4.6 × 100 mm, (P/N 054993)

UltiMate 3000 Titanium Liquid Chromatography System
- SRD-3600 Solvent Rack with six degasser channels (P/N 5035-9230)
- LPG-3400AB Quaternary Analytical Pump (P/N 5037.0015), or
- DPG-3600AB Dual Ternary Analytical Pump (P/N 5037.0014)
- WPS-3000TBPL Biocompatible Analytical Autosampler (P/N 5823.0020)
- TCC-3200B Column Compartment with two PEEK 10-port 2-position valves (P/N 5723.0025), or
- TCC-3000 Column Compartment (P/N 5722.0000)
- Detector VWD-3400 Variable Wavelength (P/N 5074.0010), or
- PDA-3000 PhotoDiode Array Detector (P/N 5080.0020)
- Biocompatible Analytical Flow Cell (P/N 6074.0200), or
- Biocompatible Analytical Flow Cell for PDA (P/N 6080.0200)

Chromeleon® Data Management Software

MSQ Plus™ Mass Spectrometer with Data System (P/N 063116)

AXP-MS Auxiliary Pump Kit (P/N 060684)

Chromeleon MS Support (MSQ MS control software) (P/N 060726)

REAGENTS AND SAMPLES

Deionized water 18.2 (MΩ-cm)

Ammonium sulfate, Molecular biology grade (Sigma-Aldrich, A4418)

Sodium phosphate, dibasic, anhydrous, powder (J.T. Baker, 3826)

Sodium phosphate, monobasic, monohydrate (Sigma-Aldrich, S8282)

Ammonium bicarbonate (Sigma-Aldrich, S9638)

Ethylenediaminetetraacetic acid (EDTA), disodium dihydrate (Sigma-Aldrich, E1644)

Hydrogen peroxide, 30% (Sigma-Aldrich, H1009)

Dimethyl sulfoxide (DMSO), ACS reagent (Sigma-Aldrich, 472301)

Hydrochloric acid (HCl), 11–12 N, Ultrex® II Ultrapure Reagent (J.T. Baker, 6900-05)

Acetic acid, glacial, HPLC grade (J.T. Baker 9515-03)

Luteinizing hormone-releasing hormone (LH-RH) Human (Sigma-Aldrich, L7134), 97% of the peptide material contains the following sequence:
p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

α-Melanocyte Stimulating Hormone (α-MSH), (Sigma-Aldrich, M-4135), 78% of dry weight is peptide, 98% of the peptide material contains the following sequence

N-Acetyl-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

CONDITIONS

Conditions for Hydrophobic Interaction Chromatography

olumn:	ProPac HIC–10 column, 4.6 × 100 mm (P/N 063655)
Flow Rate:	1.0 mL/min
Temperature:	30 °C
Inj. Volume:	10 µL
Detection:	UV, 214, 254, and 280 nm

Eluents: A: 2 M Ammonium sulfate in 0.1 M sodium phosphate (pH 7.0)
B: 0.1 M Sodium phosphate, monobasic (pH 7.0)

Gradient Program:

Time (min)	A%	B%	Comments
–10.0	100.0	0.0	Precondition column before sample injection
0.0	100.0	0.0	Sample injection
2.0	100.0	0.0	Isocratic mobile phase during sample injection
17.0	0.0	100.0	Gradient
20.0	0.0	100.0	Wash
22.0	100.0	0.0	Re-equilibration

Prewash column with 100% B for 15 min prior to the first injection.

Conditions for Weak Cation-Exchange Chromatography

Column: ProPac WCX–10 column, 4 × 250 mm (P/N 054993)
Flow Rate: 1.0 mL/min
Temperature: 30 °C
Inj. Volume: 10 µL
Detection: UV, 214, 254, and 280 nm
Eluents: A: 10 mM Sodium phosphate (pH 6.0)
B: 10 mM Sodium phosphate with 500 mM sodium chloride (pH 6.0)

Gradient Program:

Time (min)	A%	B%	Comments
0.0	98.0	2.0	Sample injection
30.0	84.0	16.0	Gradient
30.1	98.0	2.0	Wash
45.0	98.0	2.0	Re-equilibration

Mass Spectrometry Conditions

An AXP-MS auxiliary pump was used to manually infuse the oxidized and non-oxidized LH-RH samples at a flow rate of 0.2 mL/min for optimization in the MSQ Plus mass spectrometer.

During optimization, various cone voltages were examined, with 70V determined as optimal. As these were charged species, ESI was the preferred ionization mode, with positive ionization providing the best response.

Ion Source: Positive ESI
Needle voltage: 3000V
Cone voltage: 70V
Probe temp: 350 °C
Scan mode: Full scan at 1000 *m/z* per second
Scan range 15~2000 *m/z*
AXP-MS flow 0.2 mL/min CH_3CN/H_2O (50/50, v/v)
Infused Volume 10 µL

PREPARATION OF SOLUTIONS AND REAGENTS

2 M Ammonium Sulfate in 0.1 M Sodium Phosphate, Monobasic, pH 7.0

Dissolve 264.2 g ammonium sulfate and 12 g sodium phosphate monobasic in 650 mL DI water (18.2 MΩ cm) in a 1 L volumetric flask. Adjust the pH to 7.0 with approximately 2.5 mL 50% sodium hydroxide and bring to volume with DI water. Filter through a 0.22 µm filter.

0.1 M Sodium Phosphate, Monobasic, pH 7.0

Dissolve 12 g sodium phosphate monobasic in 900 mL DI water in a 1 L volumetric flask. Adjust the pH to 7.0 with approximately 1.5 mL of 50% sodium hydroxide and bring to volume with DI water. Filter through a 0.22 µm filter.

1.0 M Ammonium Bicarbonate, pH 8.8

Combine 7.91 g ammonium bicarbonate with 90 mL DI water in a 100 mL volumetric flask. Adjust the pH to 8.8 with 11-12 N HCl solution. Bring to volume with DI water. Filter through a 0.22 µm filter.

75 mM EDTA, pH 8.0

Combine 78.8 g EDTA disodium dihydrate with 900 mL DI water in a 1 L volumetric flask. Adjust the pH to 8.0 using a 0.05% NaOH (w/w) solution. Bring to volume with DI water. Filter through a 0.22 µm filter.

400 mM Hydrogen Peroxide

Combine 0.455 mL hydrogen peroxide (30%, 8.79 M) with 9.55 mL water in a 10 mL volumetric flask. Make this solution fresh daily.

1 M Sodium Chloride

Dissolve 58.45 g sodium chloride in deionized water in a 1 L volumetric flask and bring to volume. Filter through a 0.22 µm filter.

200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate in 1 L DI water in a volumetric flask. Filter through a 0.22 µm filter.

200 mM Sodium Phosphate, Monobasic

Dissolve 27.60 g of monohydrate monobasic sodium phosphate in 1 L DI water in a volumetric flask. Filter through a 0.22 µm filter.

10 mM Sodium Phosphate, pH 6.0

Combine 14 mL 200 mM dibasic sodium phosphate, 86 mL 200 mM monobasic sodium phosphate, and 1900 mL water in a 2 L volumetric flask. Adjust the relative proportions of 200 mM dibasic and monobasic sodium phosphate used to achieve a pH of 6.0 while maintaining a total volume of 100 mL.

10 mM Sodium Phosphate with 500 mM Sodium Chloride, pH 6.0

Combine 35 mL 200 mM dibasic sodium phosphate, 65 ml 200 mM monobasic sodium phosphate, 1000 mL 1 M sodium chloride, and 900 mL DI water in a 2 L volumetric flask. Adjust the relative proportions of 200 mM dibasic and monobasic sodium phosphate to achieve a pH of 6.0 while maintaining a total volume of 100 mL.

SAMPLE PREPARATION

Oxidation of Tryptophan in LH-RH

A vial containing 1 mg LH-RH was reconstituted with a solution consisting of 48.6 µL glacial acetic acid, 6.5 µL 11–12 N HCl, and 1.3 µL DI H_2O, and labeled Non-Oxidized LH-RH. Another vial containing 1 mg LH-RH was reconstituted with a solution consisting of 48.6 µL glacial acetic acid, 6.5 µL 11–12 N HCl, and 1.3 µL DMSO and labeled Oxidized LH-RH. A non-oxidized buffer control was prepared by combining 41.7 µL glacial acetic acid, 5.6 µL 11-12 N HCl, and 1.1 µL water. An oxidized buffer control was prepared by combining 41.7 µL glacial acetic acid, 5.6 µL 11–12 N HCl, and 1.1 µL DMSO. All vials were incubated at room temperature for 15 min. After incubation, 94.7 µL DI H_2O was added to each vial. The samples were diluted 100-fold by adding 10 µL of sample to 495 µL of eluent B, then adding 495 µL of eluent A. The samples were maintained at room temperature and analyzed within 48 h. When stored at 4 ºC, the injection solution can be used for up to one week. Samples can be stored in a -40 °C freezer for up to one month, with one thaw cycle. Samples stored frozen should be stored in single-use size aliquots.

Oxidation of Methionine in αMSH

A vial containing 1 mg MSH was reconstituted with 533 µL water to make a 1.5 mg/mL MSH solution. A 1 mg/mL non-oxidized MSH control was prepared by combining 105 µL 1.5 mg/mL MSH solution with 15 µL 1M ammonium bicarbonate, 10 µL 75 mM EDTA, and 20 µL water. A 1 mg/mL Met-Oxidized MSH sample was prepared by combining 105 µL 1.5 mg/mL MSH solution with 15 µL of 1M ammonium bicarbonate, 10 µL 75 mM EDTA, and 20 µL 400 mM hydrogen peroxide. A buffer control was prepared by combining 105 µL water with 15 µL 1M ammonium bicarbonate, 10 µL 75 mM EDTA, and 20 µL 400 mM hydrogen peroxide. All the oxidized samples had a final concentration of 0.1 M ammonium bicarbonate, 5 mM EDTA, and 53 mM hydrogen peroxide. All samples were incubated for 30 min in an ice water bath. After incubation, each sample was diluted 10-fold by adding 30 µL to 135 µL eluent B, then adding 135 µL eluent A. The samples are stable for 48 h at room temperature, one week at 4 °C, and one month at -40 °C (with one thaw cycle).

RESULTS AND DISCUSSION

Figure 1A shows the elution of two non-oxidized LH-RH peaks at 11 and 18 min (peaks 5 and 6) on the HIC-10 column at 214 nm. (Note, data was collected at 254 and 280 nm as well, but 214 nm provided optimum response for this analysis.) The peak eluting within the first 3 min was also seen in the buffer control (data not shown). Mass spectrometric analysis of the non-oxidized LH-RH (Figure 2B) revealed only a single major component with a mass-to-charge ratio (*m/z*) equal to that expected for LH-RH. Figure 2B shows the presence of two MS peaks. The first MS peak was identified as LH-RH $[M+2H]^{2+}$, and the second peak was identified as an acetonitrile adduct of LH-RH.

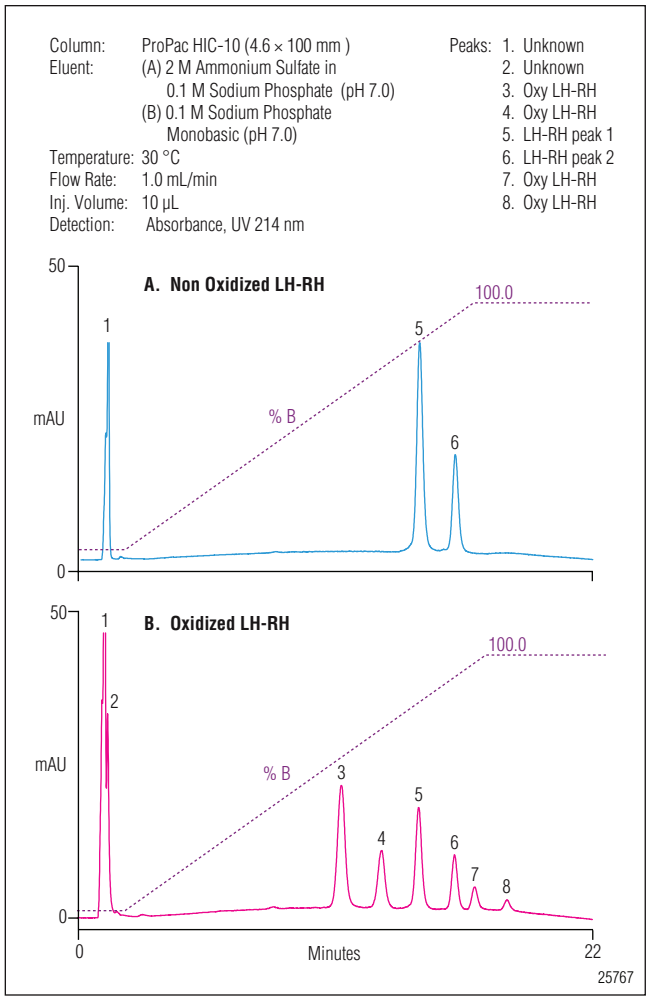

Figure 1: The separation of (A) non-oxidized LH-RH and (B) oxidized LH-RH using the ProPac HIC-10 column.

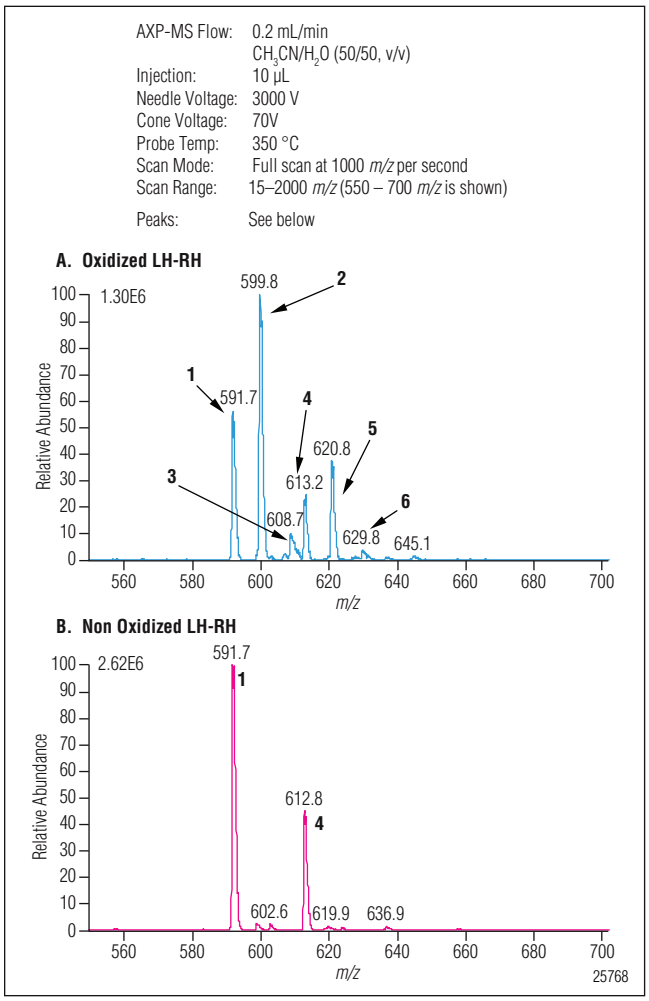

Figure 2. Mass spectra of (A) oxidized LH-RH and (B) non-oxidized LH-RH.

Fractions of peaks 5 and 6 in Figure 1A were collected, dialysed against water, vacuum dried to concentrate, and infused for MS analysis. The *m/z* of these two peaks were identical (data not shown). The source of the two impurity peaks observed on the HIC-10 column is uncertain, as the supplier reports the product is 97% pure by reversed-phase chromatography.

The Trp in LH-RH was forcibly oxidized with DMSO and HCl. Figure 1B shows four additional peaks, or two oxidation products for each retained peak in the non-oxidized LH-RH chromatogram. The presence of peaks 5 and 6, with reduced peak areas in the oxidized LH-RH indicates incomplete oxidation. All six peaks were well resolved using the HIC-10 column. Figure 3 shows a variety of Trp oxidation products described by E.L. Finley et al.[7] The expected products were: hydroxy-tryptophan (HTRP), N-formylkyurenine (NFK), kynurenine (KYN), and 3-hydroxykyurenine (3OH-KYN). The authors were able to identify two of the products using MS detection.

Figure 2B shows the presence of four additional major ions, three of which were identified. The following assignments were made based on mass/charge ratios.

Peak 1 LH-RH, $[M+2H]^{2+}$

Peak 2 Hydroxy-tryptophan (HTRP), oxidized product of LH-RH, $[(M+16)+2H]^{2+}$

Peak 3 N-formylkyurenine (NFK), oxidized product of LH-RH, $[(M+32)+2H]^{2+}$

Peak 4 Acetonitrile adduct of LH-RH, $[LH\text{-}RH+CH_3CN+2H]^{2+}$

Peak 5 Acetonitrile adduct of Hydroxy-tryptophan (HTRP), oxidized product of LH-RH, $[HTRP+CH_3CN+2H]^{2+}$

Peak 6 Acetonitrile adduct of N-formylkuyrenine, oxidized product of LH-RH, $[NFK+CH_3CN+2H]^{2+}$

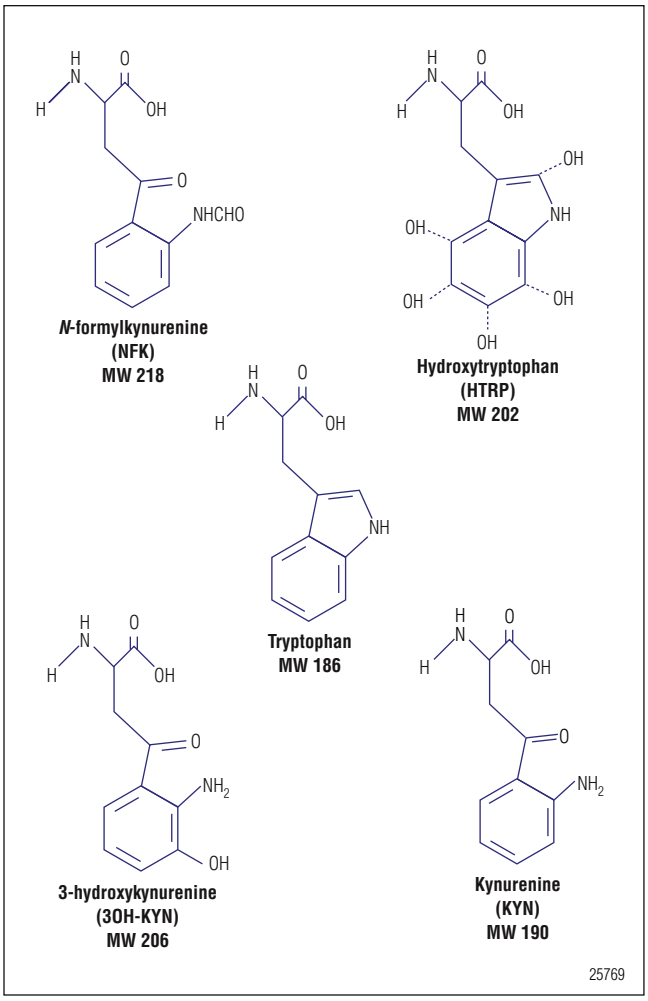

Figure 3. The chemical structure of tryptophan and its major oxidation products.[7]

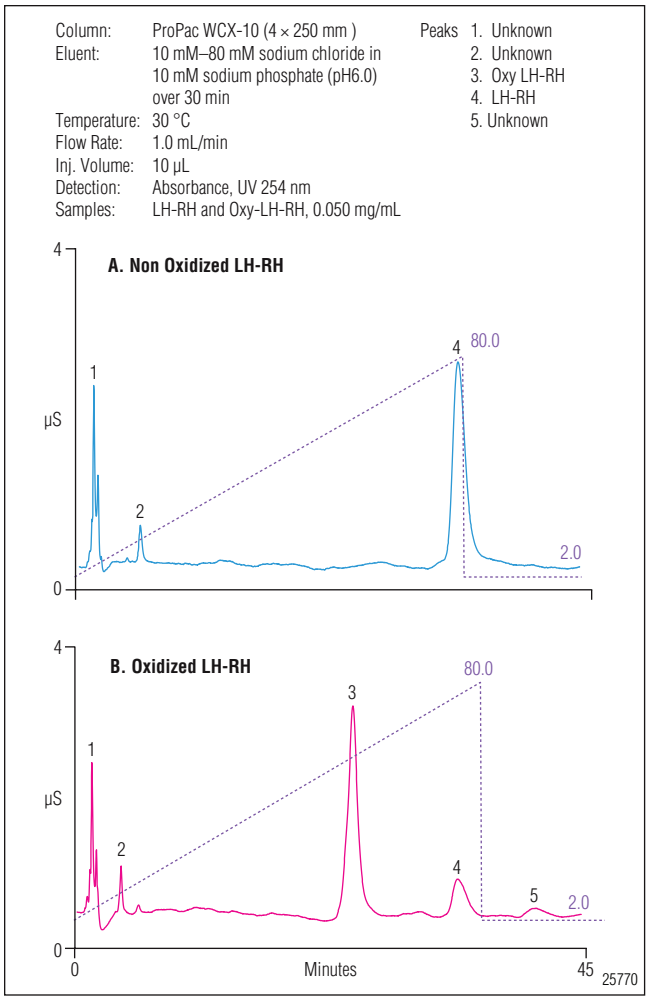

Figure 4. Separation of (A) non-oxidized LH-RH and (B) oxidized LH-RH using the ProPac WCX-10 column.

The mass spectroscopy results support our interpretation of the chromatography results for the oxidation of LH-RH.

Figure 4A shows the elution of non-oxidized LH-RH at 22 min on the ProPac WCX-10 column at 254 nm. These results show a single non-oxidized LH-RH at all wavelengths (214, 254, and 280 nm) used in this study, confirming the data previously published in AN129. The ProPac WCX-10 did not resolve the two forms of this peptide observed on the HIC column. After tryptophan oxidation, oxidized LH-RH isoforms eluted earlier at 16–17 min, as a single peak (Figure 4B). As observed with the HIC column, the presence of a small non-oxidized LH-RH component (peak 4, with a retention time of 22 min) remaining in the oxidized LH-RH sample suggested that LH-RH was not completely oxidized.

Figure 5A shows non-oxidized α-MSH (peak 1) at 26 min on the HIC-10 column. After forced oxidation of the methionine in α-MSH, a new peak for the oxidized α-MSH (peak 2) was observed at 20 min (Figure 4B). The presence of a remaining non-oxidized α-MSH peak at 26 min indicated that -α MSH was not completely oxidized. Although -α MSH also contains tryptophan residue, forcible oxidation was not attempted. These results show an orthogonal method to AN 129, which uses the ProPac WCX-10 column for separation of methionine oxidation variants.

PRECAUTIONS

LH-RH and α-MSH are bioactive peptides. Observe all safety precautions when handling these materials. Review the material safety data sheets (MSDS) for these materials prior to handling, use, and disposal.

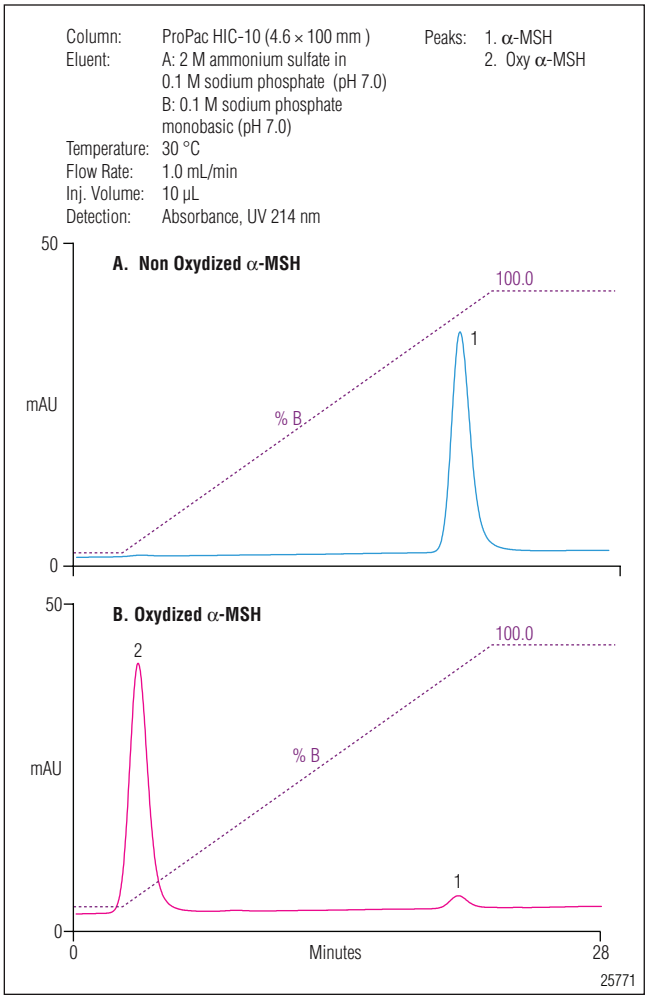

Figure 5. Separation of (A) non-oxidized α-MSH and (B) oxidized α–MSH using the ProPac HIC-10 column.

SUMMARY

The method described herein demonstrates separation of peptides with differences as small as the oxidation of a single amino acid residue using the ProPac HIC-10 column. The separations occur in a non-denaturing environment, and further demonstrate that HIC is a viable alternative to reversed-phase HPLC for separation of peptide variants. This technique is also an alternative to weak cation-exchange chromatography for separation of peptide variants as shown in AN 129, and may provide improved sensitivity and selectivity over that method.

SUPPLIERS

Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103, Tel: 800-521-8956, www.sigmaaldrich.com

Mallinckrodt Baker, Inc., 222 Red School Lane, Phillipsburg, NJ 08865, Tel: 908-859-2151, www.solvitcenter.com

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Routine analysis:

Reverse phase methods

Protein therapeutics applications notebook

Thermo SCIENTIFIC

Application Note 230

Purity Analysis of Synthetic Thymosin α1 by Reversed-Phase HPLC with an Acclaim 300 C18 column

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) is an efficient separation mode extensively applied to the purification and analysis of proteins and peptides. The stationary phases typically used for these applications are C18, C8, and C4, with C18 being the phase of choice for peptide analysis.[1] The Acclaim® 300 C18 column was designed for protein, peptide, and other biological macromolecule separations. This column contains a resin consisting of 3 µm diameter silica particles with 300 Å diameter pores, bonded with a highly characterized C18 phase. The unique bonding chemistry, which includes exhaustive alkyl group bonding for a high-density, highly uniform phase coverage, and extensive endcapping on 3 µm silica particles, results in fast, high-resolution separations.[2]

In this Application Note (AN), we establish HPLC methods for analyzing thymosin a1 and its synthetic precursors using an Acclaim 300 C18 column. These methods feature high-resolution separations and efficient peaks. Thymosin α1 is a 28 amino acid hydrophilic peptide with a sequence of Ac-S-S-A-A-V-D-T-S-S-E-I-T-T-K-D-L-K-E-K-K-E-V-V-E-E-A-E-N-OH. Its molecular formula is $C_{129}H_{215}N_{33}O_{35}$ (molecular weight 3108.37 after acetylation), and its isoelectric point (pI) is 4.25 (before acetylation). Thymosin α1 is derived from the 113 amino acid polypeptide prothymosin alpha, which is produced by the thymus gland. Medicinally, thymosin α1 is used to treat Hepatitis B either alone or in combination with other drugs. Thymosin α1 is also used as a vaccine adjuvant to enhance a vaccine's effectiveness (for example, it has been used in the influenza vaccine), and is used to treat other diseases, including Hepatitis C and certain types of cancers.

EQUIPMENT

Dionex UltiMate® 3000 HPLC system consisting of:

- HPG 3400A pump / DGP3600A pump / DGP3600M pump*
- WPS-3000TSL autosampler
- TCC-3200 thermostatted column compartment
- VWD-3400 UV-vis detector

MSQ Plus™ Mass Spectrometer with electrospray ionization (ESI) source

Chromeleon® 6.80 SP5 Chromatography Data System

*Any of these pumps can be used, but for best performance, we recommend the HPG 3400A. The DPG3600M is optimized for working with smaller column diameters.

REAGENTS

Water Milli-Q® Gradient A10

Methanol (CH_3OH) (HPLC grade, Fisher Scientific)

Acetonitrile (CH_3CN) (HPLC grade, Fisher Scientific)

Formic acid (FA) (analytical grade, SCRC, China)

Ammonium acetate (NH_4Ac) (analytical grade, SCRC, China)

Trifluoroacetic acid (TFA) (HPLC grade, Sigma-Aldrich)

SAMPLES

Three samples were obtained from a biotechnology company: a purified thymosin α1 product, a raw (unpurified) product, and a mixture of six synthetic precursors of thymosin α1, p-5, p-10, p-15, p-20, p-25 and p-28, which contain 5, 10, 15, 20, 25, and 28 amino acids respectively. Precursor p-28 was thymosin α1 without acetylation. All samples were dissolved in water.

CONDITIONS

Chromatographic Conditions

Analytical Column:	Acclaim 300 C18, 3 µm, 4.6 × 150 mm, P/N 060266 Acclaim 300 C18, 3 µm, 2.1 × 150 mm, P/N 060264, for MS
Column Temp.:	25 °C
Mobile Phase / Gradient:	See Figures
Flow Rate:	1.0 mL/min for the Acclaim 300 C18, 3 µm, 4.6 × 150 mm column 0.2 mL/min for the Acclaim 300 C18, 3 µm, 2.1 × 150 mm column
Inj. Volume:	10 µL
Detection:	UV Absorbance at 214 nm

MSQ-Plus Conditions

Ionization Mode:	ESI
Operating Mode:	Positive Scan
Probe Temperature:	400 °C
Needle Voltage:	3500 V
Mass Range:	400 ~ 1200 amu
Scan Time:	0.5 sec
Cone Voltage:	50 V
Nebulizer Gas:	Nitrogen at 75 psi

RESULTS AND DISCUSSION

Method Development

The Acclaim 300 C18 column can be used successfully with the different types of eluents typically used for reversed-phase separation of peptides and proteins with UV or MS detection. To achieve efficient peaks and high-resolution peptide and protein separations, TFA is widely used. It is typically used at 0.1 or 0.05%. The Acclaim 300 C18 column's unique bonding chemistry delivers efficient peptide peaks with TFA levels as low as 0.04%, and sometimes 0.01%.[2] On the Acclaim 300 C18 column, other reagents can be substituted for TFA (e.g. formic acid and ammonium acetate) while maintaining good peak shape and chromatographic efficiency. We used these features to develop methods for the analysis of thymosin a1 and its synthetic precursors.

In early method development, it is useful to proportion the mobile phase from three different eluent bottles: water, acetonitrile, and 0.5% TFA (or another ion-pairing reagent). Figure 1 shows seven consecutive separations of thymosin a1 and five synthetic precursors of thymosin a1 developed and executed with proportioning from three eluent bottles. The separation uses 0.05% TFA and there is some positive baseline drift due to the increased refractive index of TFA in CH_3CN compared to water. Peptides are detected at 214 nm, the absorbance maximum of the peptide bond. Sometimes 280 nm is useful for peptide detection, but because thymosin a1 has no aromatic amino acids, it has no UV absorption at 280 nm.

After the appropriate chromatographic conditions have been determined, premixing mobile phase components and using a high-pressure gradient pump can deliver a better baseline and the best reproducibility for routine analysis. We used two eluent bottles with each bottle containing a mixture of mobile phase components (Figure 2). Bottle A contained 0.05% TFA and bottle B contained 50% CH_3CN and 0.04% TFA. Because the highest amount of organic solvent in the mobile phase used in our separation is 20%, we can make an eluent B

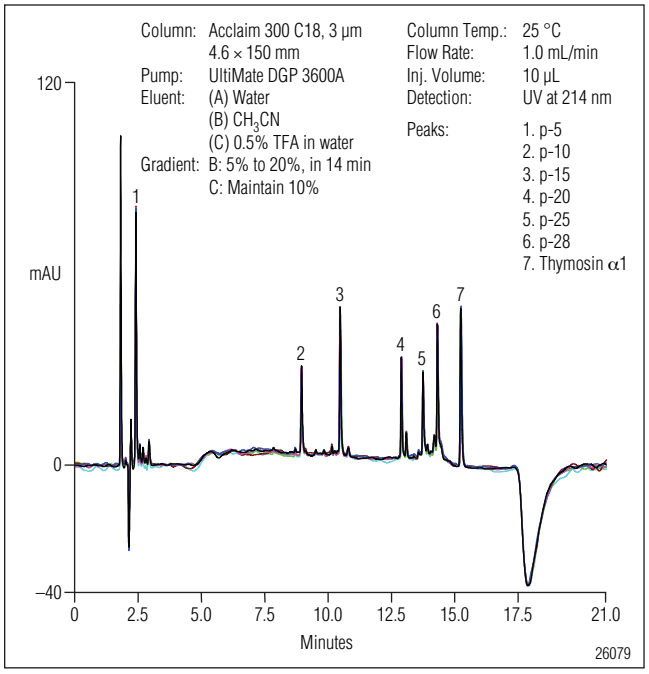

Figure 1. Overlay of chromatograms of seven consecutive injections of a mixture of thymosin α1 and its synthetic precursors.

containing 50% CH_3CN. Many samples will require more than 50% organic solvent to eluent more highly retained peptides and proteins. In those cases, 90% CH_3CN should be used. Using 50% organic solvent instead of 90% also allows a shallower solvent gradient to be used in a fixed time frame, which can deliver better and more reproducible separation. Additionally, by putting less TFA in bottle B compared to bottle A (0.04% vs. 0.05%), we were able to compensate for the change in refractive index as the percentage of organic solvent increases. An enlargement of the baseline (Figure 2B) shows only a small increase. Table 1 lists retention time RSD values of each analyte when the pump prepares the mobile phase from (a) three eluent bottles each containing a single mobile phase component, and (b) two eluent bottles containing premixed mobile phase components. Although the RSD values obtained by using three bottles are not as good as those obtained by using two bottles, they are acceptable for the method development phase of a project.

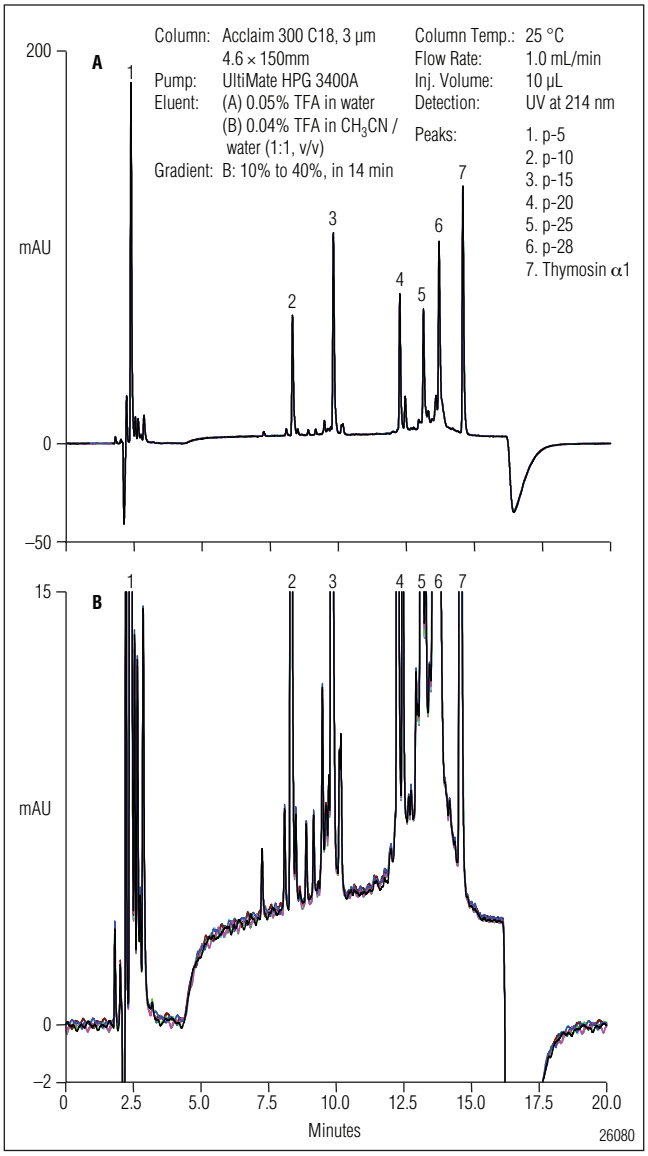

Figure 2. Overlay of (A) chromatograms and (B) enlarged chromatograms of eight consecutive injections of a mixture of thymosin α1 and its synthetic precursors.

Table 1. Retention Time Reproducibly Using Different Methods to Prepare the Mobile Phase

Peptide	RSD	
	Three-bottle mode	Two-bottle mode (premixing mobile phase components)
precursor p-5	0.104	0.032
precursor p-10	0.065	0.010
precursor p-15	0.132	0.016
precursor p-20	0.065	0.011
precursor p-25	0.063	0.017
precursor p-28	0.064	0.014
thymosin α1	0.086	0.016

MS Data for Peak Confirmation

Because TFA concentrations of 0.1% or higher have been shown to reduce analyte sensitivity when using electrospray ionization interfaces in LC-MS,[2] the 0.05% TFA was replaced with 0.1% FA. ESI interfaces work best at lower flow rates, therefore we replaced the 4.6 mm i.d. Acclaim 300 C18 column with a 2.1 mm i.d. column of the same length, reducing the flow rate from 1 to 0.2 mL/min. The pump prepared the mobile phase from three eluent bottles containing water, CH_3CN, and 1% FA in water. We used these three bottles for convenience. If we were seeking the best reproducibility, we would have used two bottles with one containing 0.1% FA and the other containing 50% CH_3CN and 0.1% FA. The molecular ions calculated from the observed mass to charge ratios matched the calculated values of the analyte molecular weights. For example, Figure 3 shows mass spectra of thymosin α1 and its synthetic precursors p-25 and p-28. The calculated values of molecular weights are the same as the theoretical values, 3108.3, 2794.9, and 3067.3, respectively.

Analysis of Unpurified Thymosin α1 Product

The analysis of unpurified thymosin a1 product was performed on the Acclaim 300 C18 (4.6 mm i.d. × 150 mm) column with UV detection and a mobile phase prepared from three eluent bottles containing water, CH_3CN, and 1% TFA or 100 mM ammonium acetate in water, respectively. It is difficult to separate thymosin α1 and the impurities in this preparation using TFA in the mobile phase (Figure 4A). When using ammonium acetate instead of TFA, the separation is significantly improved (Figure 4B). Seven impurity peaks were separated from thymosin α1. As ammonium acetate is MS compatible, we can use the MSQ for peak identification. Figure 5 shows the mass spectra of thymosin α1 and peak 3, an impurity. Assuming this impurity is a peptide, the MS data suggests it is Ac-S-S-A-A-V-D-T-S-S-E-I-T-T-K-D-L-K-E-K-K-E-V-V-E-E-A-OH, which is thymosin α1 with a loss of its two C-terminal amino acids. No synthetic precursors of thymosin a1 were found in the unpurified product.

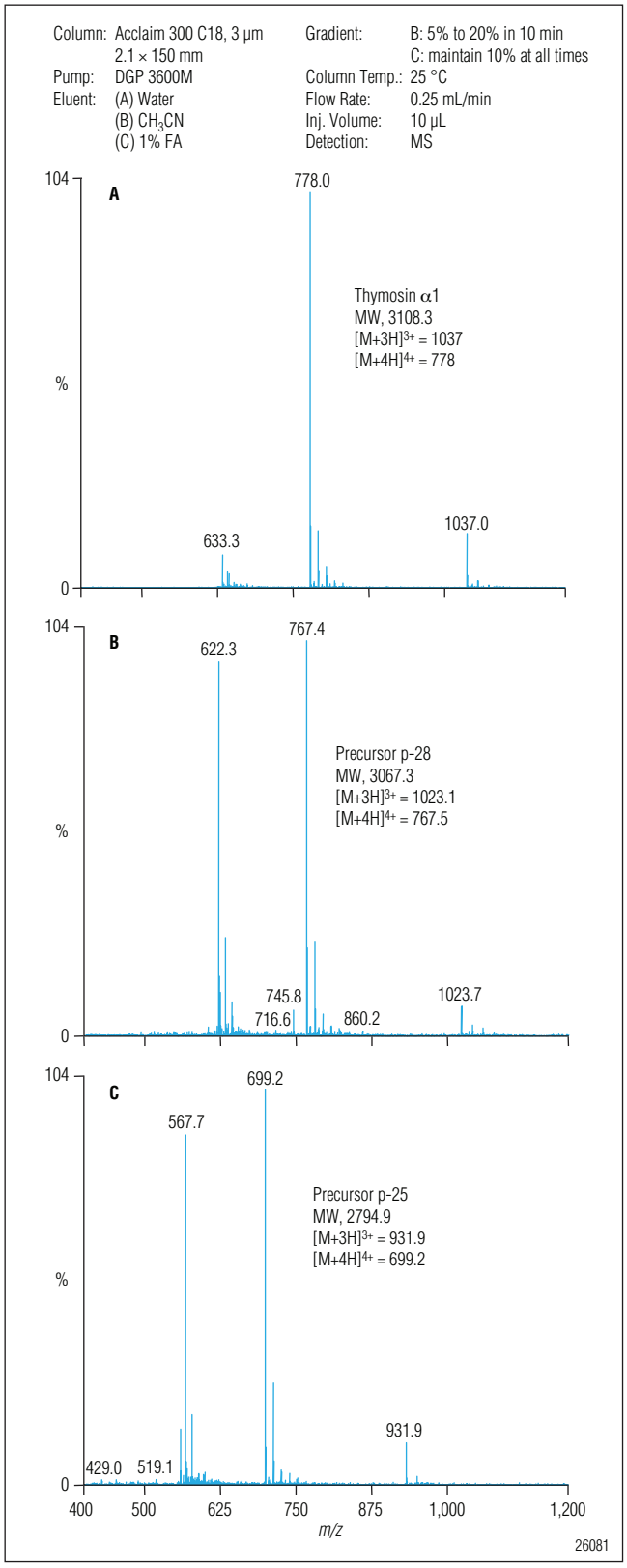

Figure 3. MS spectra of (A) thymosin α1, (B) precursor p-28, and (C) precursor p-25.

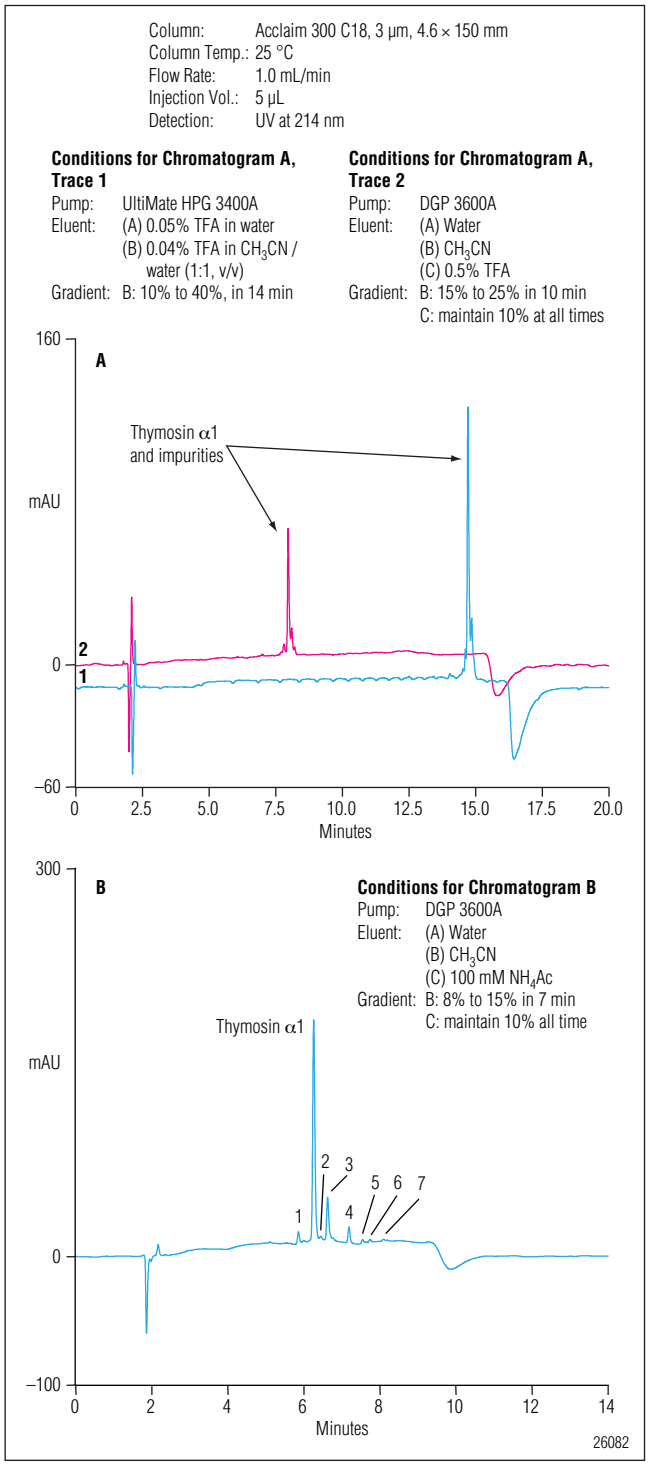

Figure 4. Chromatograms of the unpurified thymosin α1 product using (A) TFA and, (B) 100 mM NH_4Ac in the mobile phase.

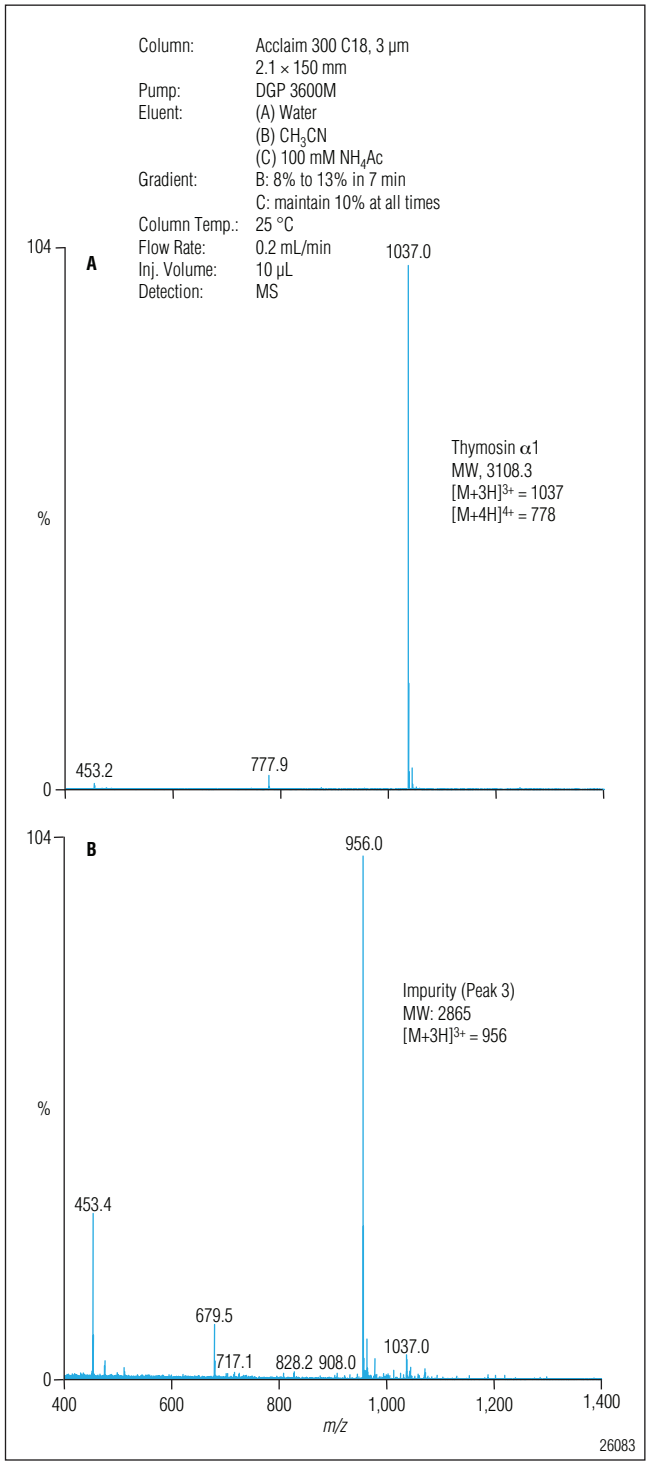

Figure 5. MS spectra of (A) thymosin α1 and (B) impurity (peak 3).

PRECAUTIONS

A smoother baseline can be delivered by filling the eluent bottles with solutions of the mobile phase components. To achieve better reliability with single mobile phase component solutions in eluent bottles A and B, a high-quality, high-pressure gradient HPLC pump (for example, UltiMate HPG 3400A system) is needed.

CH_3CN and TFA purities are critical for this application. Because TFA is light sensitive, eluents containing TFA greater than a week old may not be good, and should be discarded.

CONCLUSION

This application note describes an efficient LC method for analyzing peptides on the Acclaim 300 C18 column, using thymosin α1 and its synthetic precursors as an example. The Acclaim 300 C18 column delivers symmetrical efficient peptide peaks, and therefore high resolution with all the eluents typically used for reversed-phase analysis of peptides. Its bonding chemistry is compatible with MS detection. This makes the Acclaim 300 C18 column ideal for developing protein and peptide separations.

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High-resolution/accurate mass intact mass analysis

Protein therapeutics applications notebook

Application Note: 498

Utilizing a Hybrid Mass Spectrometer to Enable Fundamental Protein Characterization: Intact Mass Analysis and Top-Down Fragmentation with the LTQ Orbitrap MS

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Alexander Makarov, Thermo Fisher Scientific, Bremen, Germany

Key Words

- LTQ Orbitrap Velos
- LTQ Orbitrap XL
- Applied Fragmentation Techniques
- Electron Transfer Dissociation ETD
- Top-Down Proteomics

Introduction

A fundamental stage in protein characterization is to determine and verify the intact state of the macromolecule. This is often accomplished through the use of mass spectrometry (MS) to first detect and measure the molecular mass. Beyond confirmation of intact mass, the next objective is the verification of its primary structure, the amino acid sequence of the protein. Traditionally, a map of the macromolecule is reconstructed from matching masses of peptide fragments produced through external enzymatic digestion of the protein to masses calculated from an *in silico* digest of the target protein sequence. A more direct approach involves top-down MS/MS of the intact protein molecular ion.

To accommodate progressive stages of characterization, the choice of instrument for MS-based protocols should consider the diversity of required analyses, including further interrogation of protein sequence modifications, which can be very labile (glycosylation, phosphorylation). A hybrid linear ion trap-Orbitrap mass spectrometer offers extended versatility for this application, featuring both high-performance detection (high resolution, mass accuracy, and dynamic range) and distinctive flexibility in operation.[1-6] It offers several choices of fragmentation, to permit sophisticated experiments such as post-translational modification (PTM) analysis with preservation of labile modifications (electron transfer dissociation, ETD),[3-35] *de novo* sequencing (high mass accuracy and combined activation types),[36-39] and disulfide mapping to augment superior, sensitive MS/MS detection for peptide identification.[40-48]

This note describes protein characterization using the Thermo Scientific LTQ Orbitrap XL or LTQ Orbitrap Velos hybrid mass spectrometer, demonstrating intact protein and top-down fragmentation analysis (Figure 1).[48-58]

Experimental

Protein standards, including bovine carbonic anhydrase, yeast enolase, bovine transferrin and human monoclonal IgG, were purchased from Sigma-Aldrich. For direct infusion, proteins in solution were purified by either a Thermo Scientific Vivaspin centrifugal spin column or a size-exclusion column (GE Healthcare), employing at least two rounds of buffer exchange into 10 mM ammonium acetate. Protein solutions were at a concentration of least 1 mg/mL prior to clean-up. Samples were diluted into 50:50:0.1 acetonitrile:water:formic acid prior to infusion into the mass spectrometer. Instrument parameters were altered during infusion of protein solutions to optimize the signal-to-noise ratio and the accuracy of measurement. For LC/MS analysis, a polystyrene di-vinyl benzene (PSDVB) protein microtrap (Michrom Bioresources) was utilized to desalt proteins on-line.

Experiments were performed on LTQ Orbitrap XL™ and LTQ Orbitrap Velos™ hybrid mass spectrometers. Top-down experiments were conducted using direct infusion of a 5 pmol/uL solution of enolase. All data were acquired with external mass calibration. The Xtract feature within Thermo Scientific Xcalibur software was used to deconvolute isotopically resolved spectra to produce a monoisotopic mass peak list for high-resolution MS and MS/MS data. Thermo Scientific ProMass Deconvolution software was used to deconvolute intact protein spectra that contained unresolved isotopic clusters in production of an average zero charge mass of the intact protein. Thermo Scientific ProSightPC 2.0 software was used to map top-down fragmentation spectral data to associated sequences utilizing the single-protein mode search. Deconvoluted spectra produced from the Xtract algorithm within Xcalibur software were imported into ProSightPC software. To account for errors in the determination of the monoisotopic mass, a parameter tolerance of 1.01 Da was used to match fragments. However, only those fragments which corresponded to a mass error of 1.0076 Da ± 5 ppm were considered correct.

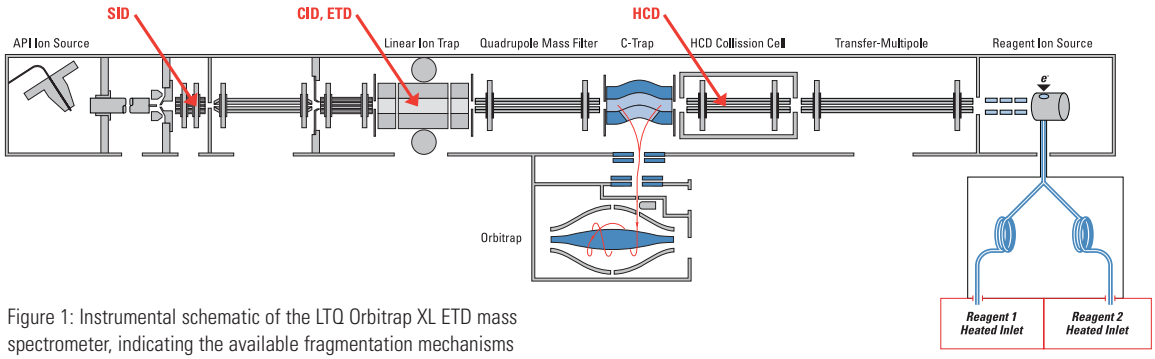

Figure 1: Instrumental schematic of the LTQ Orbitrap XL ETD mass spectrometer, indicating the available fragmentation mechanisms

Results and Discussion

Sample Preparation

Sample preparation prior to electrospray ionization (ESI) is critical for the generation of usable mass spectra, especially for intact protein analysis. A high concentration of salt or other adducts can suppress ionization and therefore sensitivity of analysis. It can also complicate spectra, broadening the observed MS peak to reduce sensitivity through division of analyte signal among ions of heterogeneous composition.

Sample purification can be accomplished through both on-line LC/MS chromatographic separation from salt or off-line clean-up followed by direct infusion. For protein mixtures, proteins should be chromatographically resolved as much as possible to maximize MS detection of individual compounds. It is optional to use formic acid as a modifier for reverse-phase (RP) separations to avoid ionization suppression induced by trifluoroacetic acid (TFA), though TFA can also improve the concentration of protein elution during chromatographic separation which can improve signal to noise for LC/MS separations.

Mass Spectrometry – Intact Analysis

The mass spectrum of large macromolecules is a composite of a distribution of naturally-occurring isotopes. As molecules increase in mass, the contribution of higher mass isotopes (^{13}C) increases proportionally. For large proteins, the natural distribution of these isotopes is nearly Gaussian, with the most abundant isotopes adjacent to the average mass of the protein. Electrospray ionization (ESI) produces a series of multiply-charged ions detectable by the mass spectrometer (Figure 2). Depending on the specification of the mass spectrometer, the isotopes of each charge state may or may not be resolved. The LTQ Orbitrap XL or LTQ Orbitrap Velos hybrid mass spectrometer is capable of resolving power in excess of 100,000 (FWHM) at *m/z* 400. This allows isotopic resolution of proteins close to 50,000 Da.

For best analysis of large macromolecules, it was determined that the initial portion of the FT transient produced during image current detection (containing first beat) contained the largest signal for transformation of large macromolecules with Orbitrap analyzer detection. This necessitates a reduction in the delay between excitation of the ion population (injection of ion cloud into the Orbitrap mass analyzer) and detection of the transient. To accomplish this on LTQ Orbitrap, LTQ Orbitrap XL, and LTQ Orbitrap Discovery mass spectrometers, a toggle for analysis of high-molecular-weight molecules is accessible from the instrument control window (Tune page) in instrument control software version 2.5.5 SP1 or later. The toggle is located under the diagnostics file menu under Tools→ Toggles→ FT detection delay, and should be set to High for large macromolecules. No changes are necessary for the LTQ Orbitrap Velos instrument.

If isotopic resolution of a protein is desired and achievable, a higher resolution setting should be used for detection – 60,000 or 100,000 resolving power. It is recommended that the pressure in the Orbitrap analyzer be less than 2×10^{-10} Torr. For larger proteins for which isotopic resolution is possible, it is recommended that sufficient spectral averaging is conducted to produce the best isotopic envelope, especially for broadband acquisition.

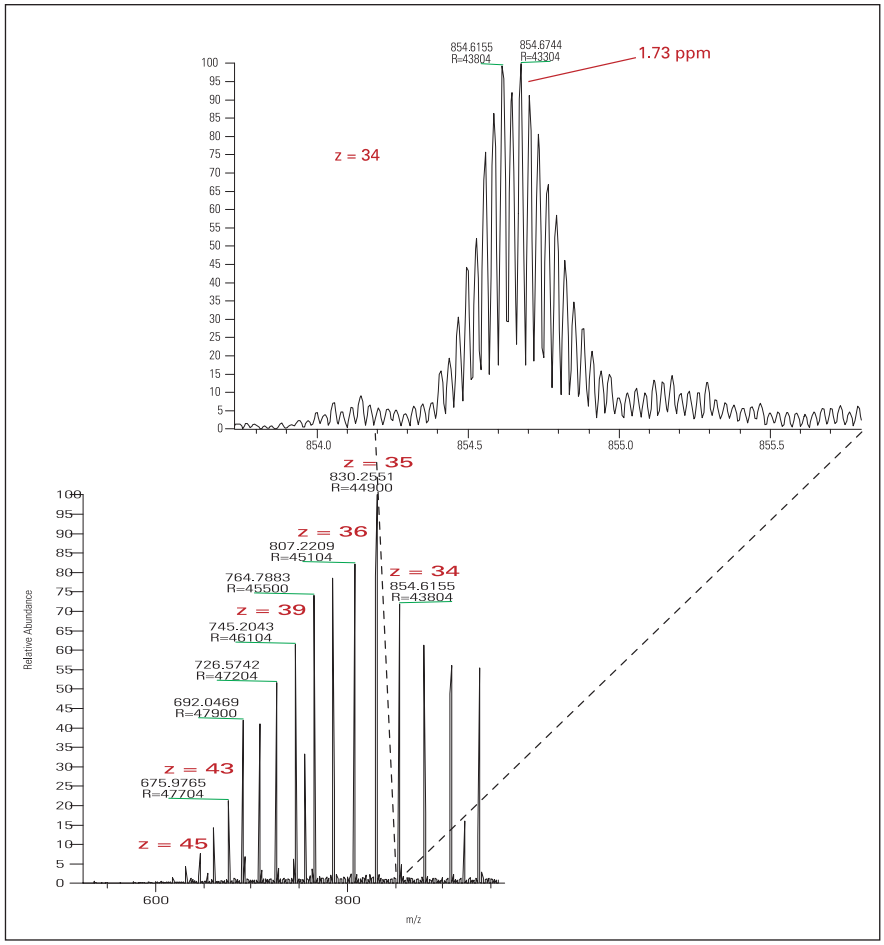

Figure 2: Mass spectrum of bovine carbonic anhydrase with one acetylation modification (29006.682 Da), depicting isotopic resolution of the z = 34 charge state. Data was acquired at 60,000 resolving power with Orbitrap analyzer detection. Accurate mass of the most abundant isotope is 1.73 ppm. HCD gas was turned off for acquisition on the LTQ Orbitrap XL instrument. Source voltage of 10 V was applied.

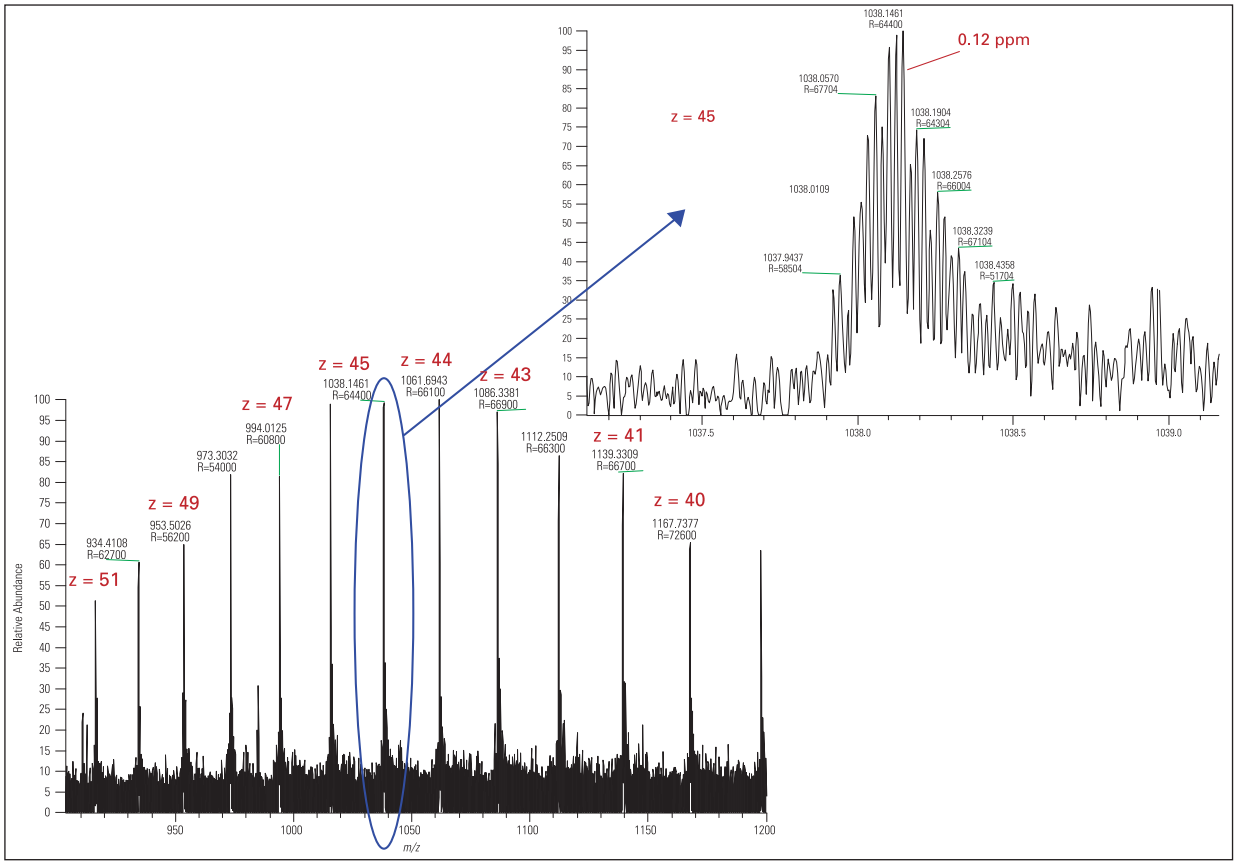

Figure 3: Mass spectrum from LTQ Orbitrap XL mass spectrometer yeast enolase (monoisotopic mass of 46642.214 Da) with broadband acquisition, depicting isotopic resolution. Inset depicts the z = 45 charge state with an accurate mass of 0.12 ppm for the most abundant isotope. Data was acquired at 100,000 resolving power with broadband Orbitrap analyzer detection (*m/z* 900-1200 shown), averaging ~600 scans. HCD gas was turned off for acquisition with the LTQ Orbitrap XL instrument. A tube lens of 110 V (LTQ Orbitrap XL instrument) or S-lens of 50% (LTQ Orbitrap Velos instrument) was used in combination with 10 V of source voltage.

Alternatively, a subset of charge states can be isolated in the ion trap and subjected to Orbitrap analyzer detection to increase ion statistics for production of a better quality isotopic envelope, potentially requiring less spectral averaging. Figure 3 depicts the mass spectrum of the z = 45 charge state of yeast enolase with isotopic resolution. For proteins for which isotopic resolution cannot be achieved (i.e. proteins >50 kDa), it is recommended to run at the 7500 or 15,000 resolution setting of the Orbitrap analyzer due to the interference effects as described in Makarov et al.[53] This results in a better signal-to-noise ratio and, because of the higher scan rate, allows for more scans averaged across a chromatographic peak (Figure 4). The accuracy of mass measurement for unresolved isotopic clusters is not dependent on the resolution setting.

It was also found that S/N is improved when the supply of external gas to the HCD collision cell is reduced. The collision gas can be turned off in the diagnostics file menu within the instrument control

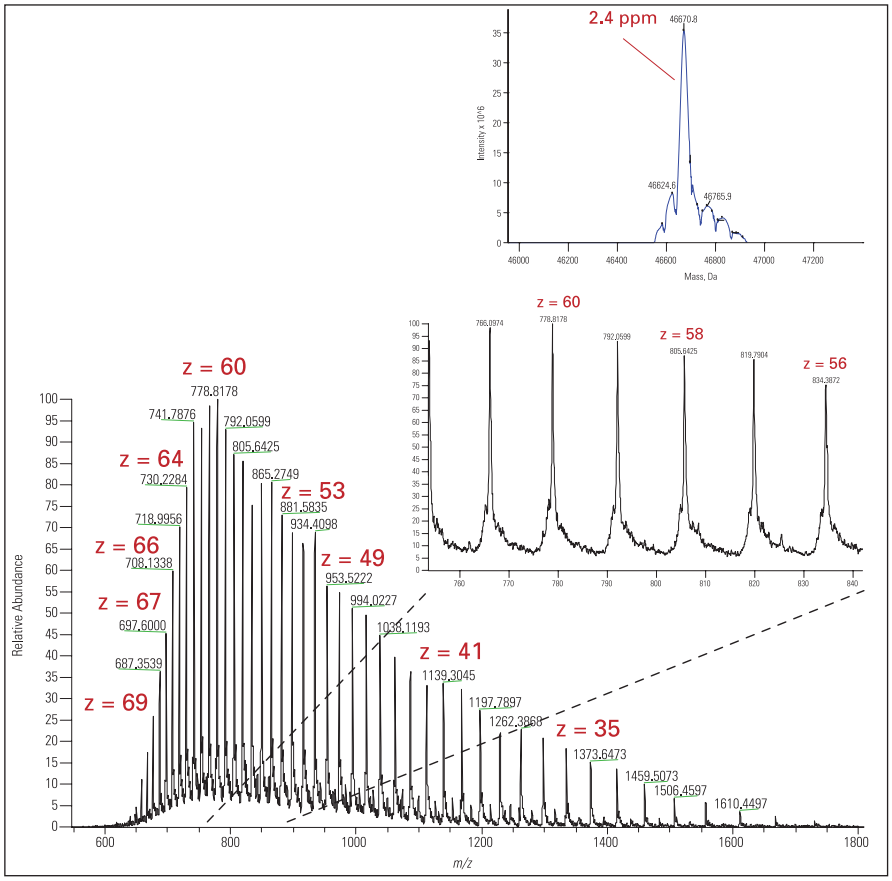

Figure 4: Mass spectrum of enolase acquired at 7500 resolving power. The spectrum is an average of ~100 scans across a chromatographic peak of 2 µg of enolase injected on a protein microtrap. Mass accuracy of the deconvoluted average mass (46670.6879 experimental) is 2.4 ppm. HCD gas was turned off for acquisition with the LTQ Orbitrap XL instrument. A tube lens of 110 V (LTQ Orbitrap XL instrument) or S-lens of 50% (LTQ Orbitrap Velos instrument) was used in combination with 10 V of source voltage.

window (tune software), under Tools→ Toggles→ FT HCD collision gas. The toggle should be set to "off". This toggle is available for the LTQ Orbitrap XL mass spectrometer. Due to a different layout of gas delivery, the LTQ Orbitrap Velos mass spectrometer offers a lower Orbitrap analytical pressure than previous systems, and the HCD gas does not need to be turned off.

As discussed previously, mass spectral protein signal is often complicated by the association of non-covalent adducts such as salt, acid and solvent adducts. It is best practice to achieve efficient desolvation of the protein upon electrospray ionization in addition to separation from salt. The sheath and auxiliary gas in the ESI source in conjunction with increased temperatures (capillary temperature, heated electrospray probe) aid in desolvation. These values should be set appropriate to the LC flow rate, and can be tuned manually with infusion of the analyte into the desired LC flow rate. During infusion of protein solutions for optimization of acquisition parameters, it is recommended that the number of microscans be increased to 5 or more for the full scan. Tuning of the source parameters should be done using the ion trap detector.

The application of source voltage (SID) can also be used to aid in desolvation or removal of undesired adducts. The amount of source voltage to apply is dependent on the size and stability of the protein and also on the strength of attraction between protein ions and adducts. A setting of 10-20 V is a good starting point, and can be increased upwards to promote a cleaner signal. For large macromolecules such as intact antibodies (~150 kDa), a source voltage of >35 V is recommended. It should be noted that the fragility of protein macromolecules and covalent modifications can vary. Too much energy deposition can also result in loss of fragile modifications or in fragmentation of the parent molecule.

Some adjustment of parameters, such as reduction of source voltage, temperature, or tube lens/S-lens voltage, may be required to minimize fragmentation. Alternatively, if a protein spectrum is pure, a high source voltage can be used to fragment the intact molecular ion (top-down analysis). This approach has the advantage of subjecting all charge states to fragmentation, which improves signal for the MS/MS spectrum, as the sum of signal from all contributing charge states is utilized. This also permits access to alternative fragmentation pathways from different charge states. A source voltage of up to 100 V can be applied to induce fragmentation of all ions and all charge states.

The voltage of the tube lens in LTQ Orbitrap XL or Discovery instruments, and the S-lens in LTQ Orbitrap Velos instruments affects ion transmission to the mass analyzer within a desired mass range. For proteins which ionize at higher *m/z*, a higher lens voltage is required. These values can be adjusted manually. For large macromolecules such as antibodies, the required voltage can be in excess of 200 V (tube lens) or 80% (S lens). Other ion optic voltages were found to be much less influential. Deviation from default values influenced signal less than 30% for the proteins analyzed in these experiments. It is recommended that they be left at default or as set during tuning with a small protein (ex: myoglobin).

For molecules such as intact antibodies analyzed in the high-mass range (up to *m/z* 4000), an additional calibration step is required in order to achieve proper mass accuracy with Orbitrap analyzer detection. This adjusts the ion population to the desired target when ions are ejected from the linear ion trap to the FTMS in high-mass mode, and does so by adjusting the calculated injection time. This procedure is also located in diagnostics, under Toggles → System evaluation → FT high mass range target compensation. Figure 5 depicts the mass spectrum for an immunoglobulin (IgG), with associated acquisition parameters listed. Again, a higher tube lens or S-lens voltage also promotes transmission of higher *m/z* ions for analysis in the high-mass range.

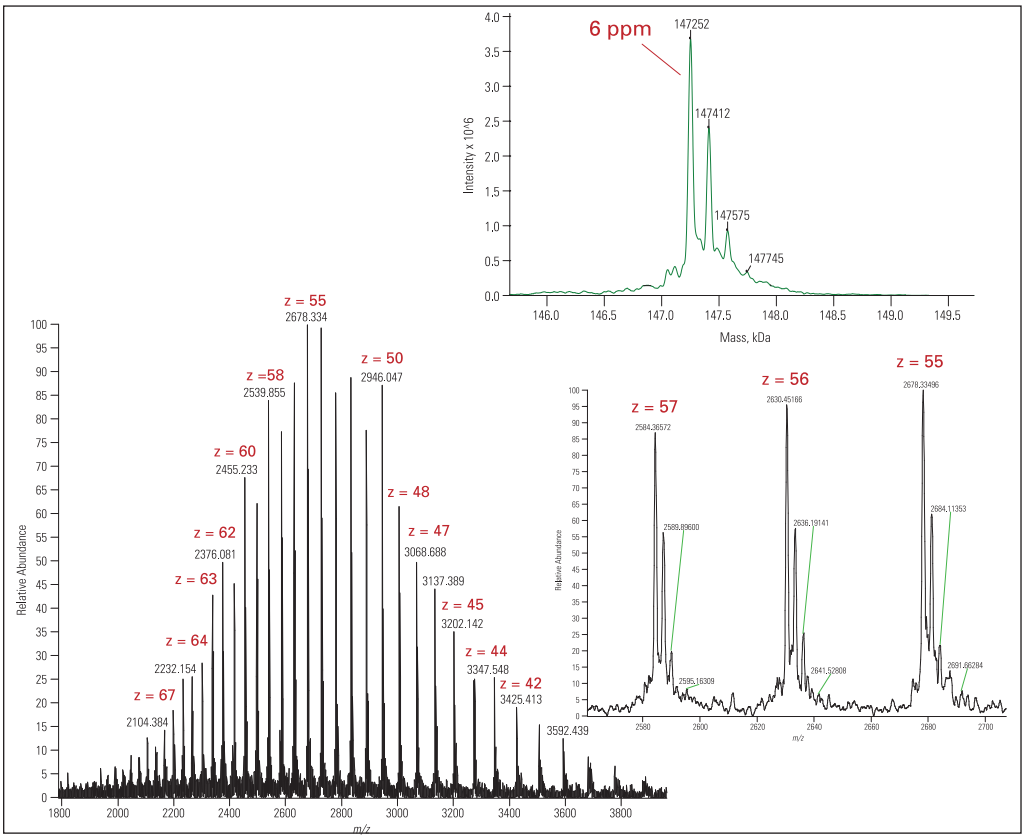

Figure 5: Mass spectrum of intact immunoglobulin (IgG, 147,251 Da) detected at 7500 RP by LC/MS. Mass accuracy of the average mass was 6 ppm. A tube lens of 230 V (LTQ Orbitrap XL instrument) or S-lens 90% (LTQ Orbitrap Velos instrument) was used. Source voltage of 40 V was used to remove adducts. HCD gas was turned off for the LTQ Orbitrap XL mass spectrometer.

Mass Spectrometry – Top-Down Analysis

Beyond routine intact mass verification lies the need to determine or confirm the identity of a protein's primary amino acid sequence. As described, the hybrid linear ion trap – Orbitrap system allows multiple activation strategies, which often provide complimentary information. The hybrid system can also resolve complex fragmentation spectra from high-molecular-weight precursors, which contain many fragments of large mass.

Whole protein fragmentation has the advantage of circumventing the limitations of traditional peptide mapping (such as *in vitro*-induced modifications, <100% sequence coverage and loss of information of the intact state). Alternate charge states of the same precursor can follow different fragmentation pathways to produce alternate fragments (different b/y or c/z ion pairs). For fragmentation of a pure protein, it is possible to isolate a range of charge states and subject all to fragmentation by CID, HCD or ETD. For example, an isolation width of *m/z* 100 was employed here to isolate multiple charge states. Because the initial signal is divided among multiple fragments, multiple charge states and multiple isotopes within those charge states, spectral averaging is required to achieve best sequence coverage with top-down fragmentation.

Collision-induced dissociation (CID) induces fragmentation through resonance excitation in the linear ion trap followed by detection of fragments in the Orbitrap mass analyzer. Resonance excitation prevents excitation of daughter ions that would induce secondary or tertiary fragmentation pathways, and has the advantage of limiting the production of internal fragments. Figure 6 shows the CID MS/MS spectrum for yeast enolase, isolated *m/z* 100 window around *m/z* 1010.

For Orbitrap mass analyzer detection, the HCD collision cell of the LTQ Orbitrap Velos mass spectrometer provides a significant improvement in the efficiency of ion extraction after fragmentation, especially for molecular ions of higher charge. Here, lower normalized collision energy resulted in more-optimal fragmentation (larger fragments) in the HCD collision cell. Excessively high collision energy resulted in over-fragmentation. For HCD, the voltage offset that should be applied versus the collision cell is dependent on the charge state of the precursor (collision energy is a normalized value in consideration of precursor charge, when the charge state is specified or determined by the system). For large proteins for which the instrument cannot determine the charge (not resolved), the assumed charge state can be chosen and input into the tune page for acquisition. A collision energy of 13 was used for fragmentation in the HCD collision cell of an LTQ Orbitrap Velos mass spectrometer, isolating a *m/z* 100 window around *m/z* 850 and a specified charge state of 50.

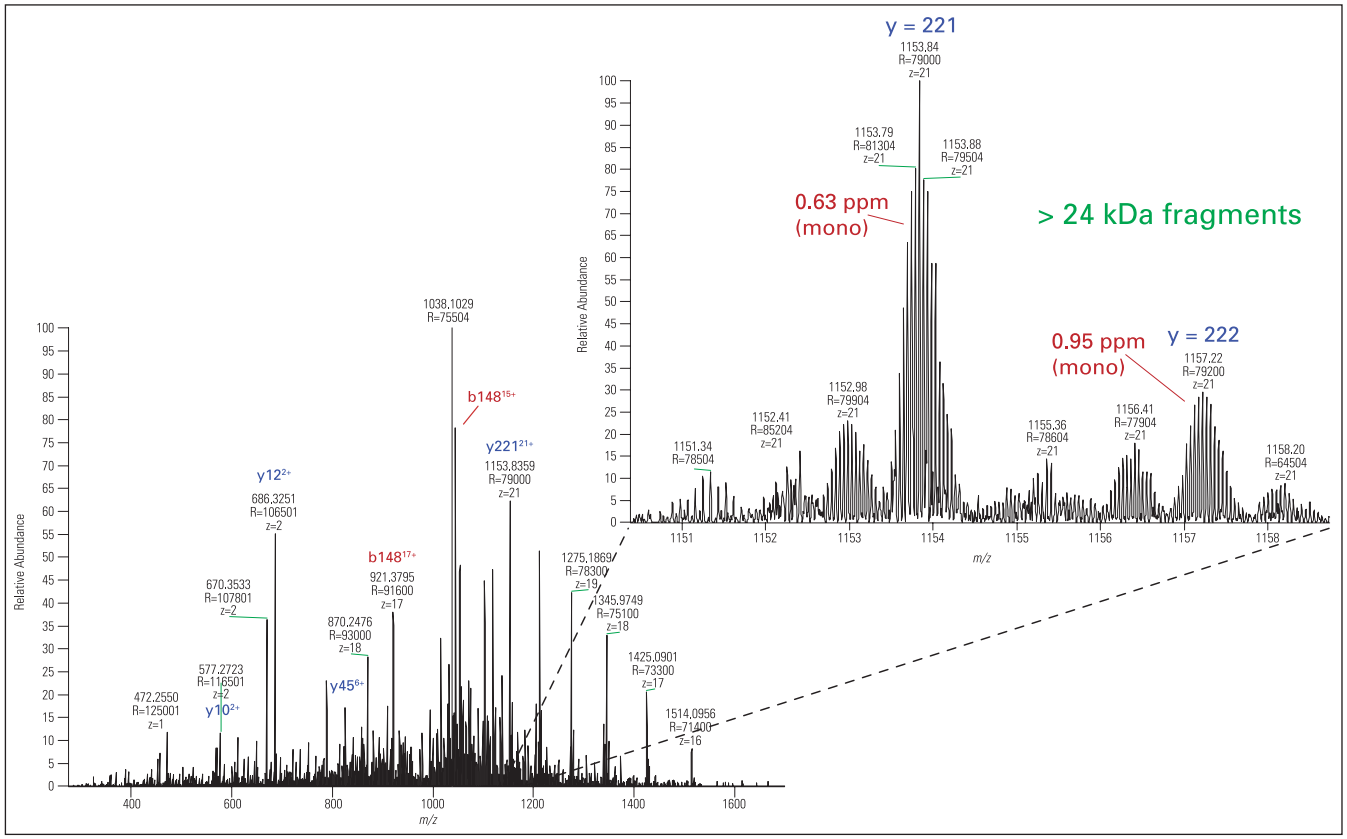

Figure 6: CID MS/MS spectrum for yeast enolase from an LTQ Orbitrap Velos mass spectrometer acquired at 100,000 resolving power, isolating a *m/z* 100 window around *m/z* 1010 with a normalized collision energy of 30. The inset shows resolution of large sequence fragments (y221 and y222) with masses greater than 20 kDa. Mass accuracy of the monoisotopic peaks for the labeled fragments, upon deconvolution with Xtract software, was less than 1 ppm. Approximately 2000 scans were averaged.

Electron transfer dissociation (ETD) is a kinetic reaction with the rate dependent on the charge of the precursor – the higher the charge state, the faster the reaction occurs. Shorter activation times should be used for highly-charged proteins to produce larger fragments. Activation time can also be manipulated to influence coverage in alternate regions of the protein sequence. For example, a longer activation time can influence the production of lower-charge fragments that correspond to the N- or C-terminus of a protein sequence.[48] Spectra were collected for alternative activation times – including 4 ms, 7 ms, 15 ms, 50 ms, and 100 ms. The shorter activation times resulted in richer fragmentation spectra, while longer activation times produced fragments corespondent to the termini of the protein sequence. Figure 7 displays ETD MS/MS fragmentation spectra produced using a 4 msec (A) and 100 msec activation time (B).

The combination of information from alternative fragmentation strategies produced the most comprehensive coverage of the protein sequence. The sequence map shown in Figure 8 illustrates the advantage of combining information garnered from multiple activation types to define the amino acid sequence, offering amino acid resolution for large parts of the N and C termini of the sequence. Complementary fragmentation mechanisms also provide increased evidence to confirm bond linkages, with the presence of b/y or c/z-type ions for the same breakage point, or coverage in alternative regions of the sequence. CID produced larger fragments representing cleavage in the middle of the sequence. Preservation of the intact state of the molecule through whole-molecule fragmentation avoids loss of information, which is a common consequence of protein digestion used in traditional peptide mapping characterization.

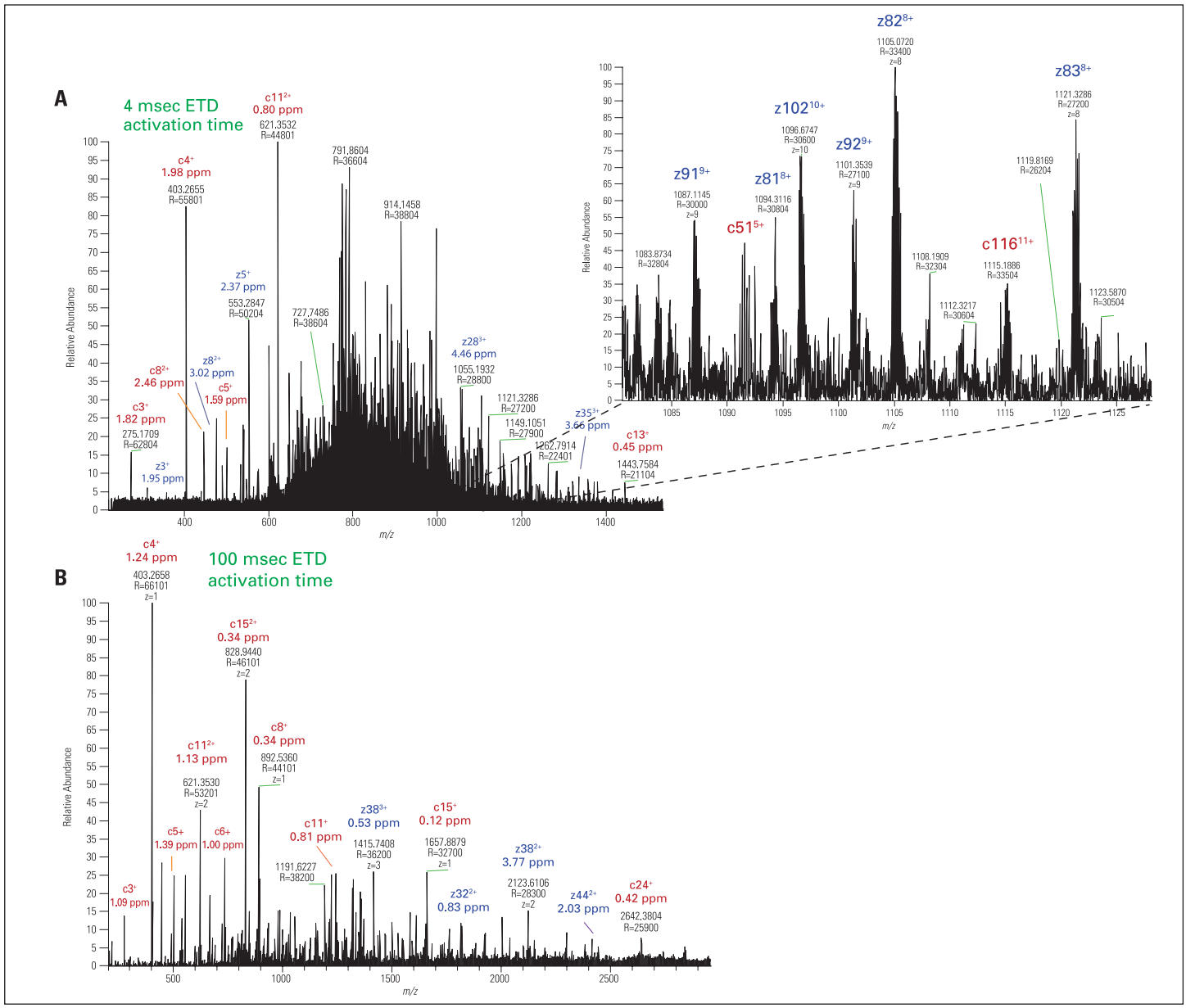

Figure 7: ETD MS/MS spectrum of enolase acquired at 60,000 resolving power, isolating a *m/z* 100 window around *m/z* 790, with an activation time of 4 msec. Approximately 1000 scans were averaged (A). ETD MS/MS spectrum of enolase acquired at 60,000 resolving power, isolating a *m/z* 100 window around *m/z* 790, with an activation time of 100 msec. Approximately, 600 scans were averaged (B). Some example sequence ions which could be labeled in the space of the figure are shown, with corresponding mass accuracies. A detailed map of coverage can be seen in Figure 8.

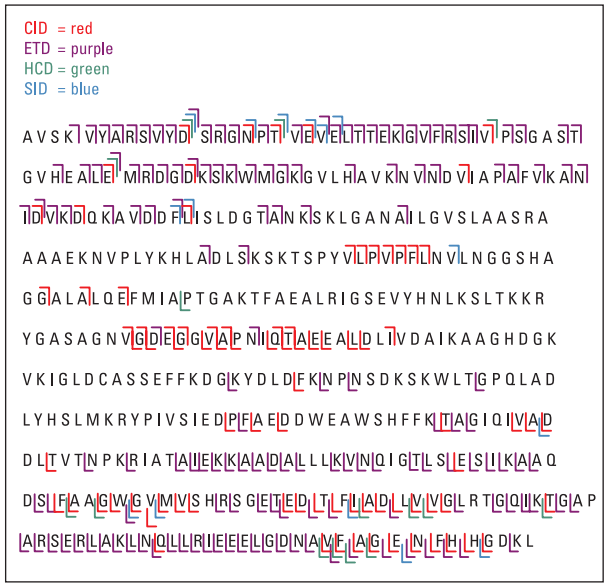

Figure 8: Coverage map combining multiple activation strategies to define the sequence of yeast enolase upon whole protein fragmentation. Xtract software was used to deconvolute fragmentation spectra to a monoisotopic peak mass list, which was then matched against the sequence with ProSightPC software. Mass accuracy of all matched fragments was less than 5 ppm. Respective fragments detected with each activation type are color-coded according to the legend.

Conclusions

- The high resolving power of Orbitrap analyzer detection allows isotopic resolution of proteins as large as 47 kDa as well as large fragments, facilitating top-down MS/MS experiments.
- For proteins larger than 50 kDa (for which isotopic resolution is not possible) a resolution setting of 7500 or 15,000 should be used for Orbitrap analyzer detection to improve the signal-to-noise ratio of the mass spectra.
- High-mass-accuracy detection offered by the Orbitrap analyzer allows more precise measurement of molecular masses of intact proteins.
- Proper desalting and desolvation are necessary for cleaner protein signals and better signal-to-noise ratios. Proper setting for temperatures, desolvation gases and source voltages are necessary for the removal of unwanted non-covalent adducts characteristic of large proteins.
- A reduced detection delay should be used for best detection of large macromolecules in LTQ Orbitrap mass spectrometers.
- The availability of multiple activation strategies in conjunction with high-performance detection characteristics offered by the hybrid systems allows top-down protein characterization with more complete sequence definition. ETD, in particular, offers amino acid resolution for large parts of the N and C termini of the sequence.
- Reducing the gas flow from the HCD collision cell for LTQ Orbitrap XL instruments (turning the gas off) results in improved detection of large proteins in the Orbitrap analyzer.

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Carbohydrate analysis: glycan, glycoform, and glycosylations

Protein therapeutics applications notebook

Analysis of Carbohydrates by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD)

INTRODUCTION

Methods for the liquid chromatographic analysis of carbohydrates have often employed silica-based amino-bonded or polymer-based, metal-loaded, cation-exchange columns, with refractive index (RI) or low-wavelength ultraviolet (UV) detection. These analytical methods require attention to sample solubility, sample concentration and, in the case of the metal-loaded cation-exchange columns, also require column heating. In addition, RI and low-wavelength UV detection methods are sensitive to eluent and sample matrix components. This usually precludes the use of gradients and often requires stringent sample cleanup prior to injection.

As a result, an improved chromatographic technique known as high-performance anion exchange (HPAE) was developed to separate carbohydrates. Coupled with pulsed amperometric detection (PAD), it permits direct quantification of nonderivatized carbohydrates at low-picomole levels with minimal sample preparation and cleanup. HPAE chromatography takes advantage of the weakly acidic nature of carbohydrates to give highly selective separations at high pH using a strong anion-exchange stationary phase. This technical note is intended as an introduction to HPAE-PAD carbohydrate analysis. The technique has been reviewed extensively,[1–4] and those articles should be consulted for more specific details.

HPAE-PAD is extremely selective and specific for carbohydrates because:

1. Pulsed amperometry detects only those compounds that contain functional groups that are oxidizable at the detection voltage employed (in this case, sensitivity for carbohydrates is orders of magnitude greater than for other classes of analytes).
2. Neutral or cationic sample components in the matrix elute in, or close to, the void volume of the column. Therefore, even if such species are oxidizable, they do not usually interfere with analysis of the carbohydrate components of interest.

ANION-EXCHANGE CHROMATOGRAPHY

I. Mechanism of Separation

Although anion-exchange chromatography has been used extensively to analyze acidic carbohydrates and glycopeptides, it has not been commonly used for analysis of neutral sugars. However, examination of the pK_a values of the neutral monosaccharides listed in Table 1 shows that carbohydrates are in fact weak acids. At high pH, they are at least partially ionized, and thus can be separated by anion-exchange mechanisms. This approach cannot be used with classical silica-based columns because these matrices dissolve at high pH. Anion exchange at high pH is, however, ideally suited to base-stable polymer anion-exchange columns.

Table 1. Dissociation Constants of Some Common Carbohydrates[5] (in water at 25 °C)

Sugar	pK_a
Fructose	12.03
Mannose	12.08
Xylose	12.15
Glucose	12.28
Galactose	12.39
Dulcitol	13.43
Sorbitol	13.60
α-Methyl glucoside	13.71

II. CarboPac™ Columns

A. CarboPac PA1 and PA-100 Columns

Dionex designed the CarboPac series of columns specifically for carbohydrate anion-exchange chromatography. These columns permit the separation and analysis of mono-, oligo-, and polysaccharides. The CarboPac PA1 and CarboPac PA100 are packed with a unique polymeric, nonporous, MicroBead™ pellicular resin. MicroBead resins exhibit rapid mass transfer, high pH stability (pH 0–14), and excellent mechanical stability that permits back pressures of more than 4000 psi (28 MPa). Column reequilibration after gradient analysis is fast, generally taking 10 min or less. A diagram of a typical pellicular anion-exchange resin bead is shown in Figure 1.

Both the CarboPac PA1 and the CarboPac PA100 are designed for the rapid analysis of mono- and oligosaccharides. The CarboPac PA1 is particularly well-suited to the analysis of monosaccharides and the separation of linear homopolymers, while the CarboPac PA100 is optimized for oligosaccharide resolution and separation. Several examples of separations obtained using these columns are shown in the "Applications" section of this technical note.

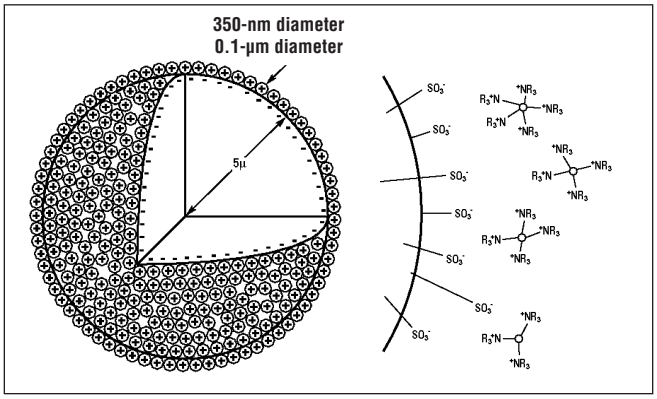

Figure 1. Pellicular anion-exchange resin bead.

B. CarboPac MA1 Column

Reduced carbohydrates (also called sugar alcohols) have traditionally been a difficult class of carbohydrates to separate by liquid chromatography. They are weaker acids than their nonreduced counterparts (compare the pK_as of glucose and sorbitol or galactose and dulcitol in Table 1), and are therefore poorly retained on the CarboPac PA1 and PA100 columns. The CarboPac MA1 was developed to address the challenge of retaining and separating extremely weak acids. This column is packed with a macroporous polymeric resin which has an ion-exchange capacity

Table 2. k´ Values of Selected Analytes on the CarboPac MA1 Column[a]

Analyte	Eluent Concentration (M NaOH)				
	0.05	0.14	0.25	0.38	0.50
Glycerol	1.13	0.99	0.89	0.80	0.72
m-Inositol	1.32	1.08	0.86	0.69	0.56
s-Inositol	1.63	1.30	1.02	0.81	0.64
GlcNol	1.81	1.40	1.09	0.89	0.75
Fucitol	1.94	1.63	1.40	1.18	1.05
Erythritol	2.02	1.71	1.44	1.25	1.13
GalNol	2.29	1.81	1.39	1.13	0.95
GalNAcol	2.35	1.81	1.38	1.12	0.95
GlcNAcol	2.61	1.96	1.48	1.18	0.95
Xylitol	3.09	2.48	1.95	1.59	1.35
Arabitol	4.69	3.62	2.76	2.24	1.92
Sorbitol	6.43	4.72	3.33	2.55	2.06
Dulcitol	6.52	5.04	3.73	2.87	2.26
Adonitol	7.09	5.31	3.83	2.96	2.43
Mannitol	8.98	6.38	4.37	3.28	2.63
Fucose	10.34	4.72	2.52	1.69	1.25
Isomaltitol	12.22	8.15	4.89	3.30	2.43
Lactitol	14.97	9.61	5.49	3.57	2.43
Gp-Man	15.66	10.36	6.18	4.15	3.05
GalN	18.56	7.16	3.39	2.13	1.48
GlcN	20.88	7.71	3.61	2.24	1.55
Maltitol	31.21	17.25	8.80	5.44	3.67
Glucose		15.70	7.19	4.31	2.91
Mannose		13.55	6.15	3.72	2.53
Galactose		17.82	8.25	4.99	3.43

[a] The capacity factor, k´, is defined as: $k' = (V_A - V_0)/V_0$, where V_A is the retention volume of the analyte on the column and V_0 is the void volume.

45 times that of the CarboPac PA1. As a result, weak anions bind more strongly to the column, requiring higher sodium hydroxide concentrations for elution. The increase in hydroxide ion concentration leads to greater ionization of the sugar alcohols, with greatly improved retention and resolution on the column.

Nonreduced neutral oligosaccharides can also be analyzed on the CarboPac MA1 column, although their analysis times are longer than on the CarboPac PA1 and PA100 columns. Retention of carbohydrates on the CarboPac MA1 can be manipulated by altering the sodium hydroxide concentration of the eluent (see Table 2). Note that the elution order of several of the compounds changes with the sodium hydroxide concentration. This can be used to design separation strategies for specific sets of analytes. Examples of separations obtained with the CarboPac MA1 column are shown in the "Applications" section of this technical note.

Table 3. Comparison of the CarboPac MA1, PA1, and PA100			
Characteristic	**CarboPac MA1**	**CarboPac PA1**	**CarboPac PA100**
Recommended applications	Mono- and disaccharide alcohol analysis in food products, physiological fluids, tissues, and reduced glycoconjugate saccharides[b]	Monosaccharide compositional analysis, linear homopolymer separations, saccharide purification	Oligosaccharide mapping and analysis
Resin composition	8.5-µm-diameter vinylbenzylchloride/divinylbenzene macroporous substrate fully functionalized with an alkyl quaternary ammonium group	10-µm-diameter polystyrene/divinylbenzene substrate agglomerated with 350-nm MicroBead quaternary amine functionalized latex	10-µm-diameter ethylvinylbenzene/divinylbenzene substrate agglomerated with 350-nm MicroBead quaternary amine functionalized latex
MicroBead latex cross-linking	N/A, no latex	5% cross-linked	6% cross-linked
Anion-exchange capacity	4500 µeq per 4 × 250-mm column	100 µeq per 4 × 250-mm column	90 µeq per 4 × 250-mm column
Recommended flow rate	0.4 mL/min (4 × 250-mm column)	1 mL/min (4 × 250-mm column)	1 mL/min (4 × 250-mm column)
pH compatibility	pH 0–14	pH 0–14	pH 0–14
Organic solvent compatibility	0%	0–2%	0–100%
Maximum back pressure	2000 psi (14 MPa)	4000 psi (28 MPa)	4000 psi (28 MPa)

[b] Note that sialylated and other acidic mono- and oligosaccharides may not be recovered from the CarboPac MA1 column. It is not recommended that this column be used with these analytes.

III. Guidelines for CarboPac Column Selection

Table 3 provides a comparison of the three CarboPac columns. The following guidelines are useful in selecting the right CarboPac column for a particular application.

A. Monosaccharides

For reducing monosaccharides, the recommended column is the CarboPac PA1, while the MA1 is recommended for sugar alcohols. The CarboPac MA1 column also generates excellent neutral monosaccharide separations, although retention times are longer than on the PA1. Amino-sugars are better resolved on the CarboPac PA1 than on the MA1, but the reverse is true for N-acetamido sugars.

B. Neutral Oligosaccharides

The CarboPac PA100 is the most appropriate column for the oligosaccharide mixtures characteristic of glycoprotein-derived oligosaccharides, although these compounds are only slightly less well-resolved on the CarboPac PA1 column than on the PA100. Neutral oligosaccharides up to nine monosaccharide units in size are separable on the CarboPac MA1. However, the CarboPac MA1 will usually have longer retention times than the PA100, and selectivities of the two columns are almost identical.

Oligosaccharides cleaved by reductive β-elimination from glycoproteins contain a reduced terminal and generally elute earlier than the same oligosaccharide with a reducing terminal. Reduced di- and trisaccharides will elute significantly earlier than their nonreduced counterparts, and may be poorly resolved on the CarboPac PA1 and PA100. These compounds are readily separated on the CarboPac MA1 column.

C. Charged Oligosaccharides

Charged oligosaccharides (for example, those that are sialylated, phosphorylated, sulfated, or contain carboxyl groups) are separated based on their composition, linkage, and the level of formal negative charge. They can be separated at both high (13) and low (4.6) pH. At low pH, the separations are largely dependent on the charge-to-mass ratio of the oligosaccharide but may also be influenced by linkage. Selectivity for sialylated oligosaccharides will change with pH as a result of oxyanion formation. The CarboPac PA100 is recommended for sialylated oligosaccharides, although in many cases the PA1 performs adequate separations.

D. Glycosaminoglycans

Oligosaccharides derived from glycosaminoglycans, such as nonsulfated chondroitin disaccharides, are separable on the CarboPac PA1.[6]

E. Linear Polysaccharides

Linear polysaccharides can be separated on the basis of length almost equally well on the CarboPac PA1 and PA100. The CarboPac PA1 has a slightly higher capacity than the PA100 and is the better column to use for linear homopolymers. The CarboPac PA100 was designed for nonlinear and heterogeneous polysaccharides. N, N – 1 resolution of linear polysaccharides has been demonstrated on the CarboPac PA1 and PA100 columns with inulin polymers to over 60 monosaccharide units. The CarboPac PA1 requires a higher sodium acetate concentration than the PA100 to elute species of the same length.

Table 4 summarizes the applications for which the three CarboPac columns are the most appropriate.

The CarboPac PA1 and PA100 are available in guard (4 × 50 mm), analytical (4 × 250 mm), semi-preparative (9 × 250 mm) and preparative sizes (22 × 250 mm). A guard column should be used in front of an analytical column to prolong the analytical column life. The CarboPac MA1 column is available in analytical and guard sizes. A partial list of column part numbers follows. Please contact your local Dionex office to order any column not listed below.

Part No.	Description
35391	CarboPac PA1 Analytical (4 × 250 mm)
43096	CarboPac PA1 Guard (4 × 50 mm)
39686	CarboPac PA1 Semipreparative (9 × 250 mm)
43055	CarboPac PA100 Analytical (4 × 250 mm)
43054	CarboPac PA100 Guard (4 × 50 mm)
44066	CarboPac MA1 Analytical (4 × 250 mm)
44067	CarboPac MA1 Guard (4 × 50 mm)

IV. Sample Stability at High pH

Carbohydrates undergo a number of well documented reactions at high pH that can potentially interfere with chromatography. However, in most cases these reactions are slow at room temperature and do not appear to occur to any noticeable extent over the time course of the chromatography. Some of these reactions are discussed below:

A. The Lowbry de Bruyn, van Ekenstein Transformations[7] (epimerization and keto-enol tautomerization)

D-fructose elutes as a single sharp peak with no evidence of formation of D-glucose or D-mannose via the Lowbry de Bruyn, van Ekenstein transformation. In addition, when glucose is left in 150 mM sodium hydroxide for four days at room temperature, there is no evidence for the presence of any mannose or fructose.

Table 4. CarboPac Columns Recommended by Application

	CarboPac PA1	CarboPac PA100	CarboPac MA1
Monosaccharides	+++	+/–	++
Sialylated branched oligosaccharides	++	+++	–
Neutral branched oligosaccharides	++	+++	+
Linear oligo- and polysaccharides	+++	+++	–
Reduced mono- and disaccharides	+	–	+++

+++ indicates most suitable

— indicates that the column is not recommended for this application.

Epimerization of *N*-acetyl glucosamine (GlcNAc) to *N*-acetyl mannosamine (ManNAc) has been demonstrated for solutions of GlcNAc in 100 mM sodium hydroxide. The equilibrium ratio of GlcNAc: ManNAc was 80:20 after 2–3 hours of exposure. This epimerization is not observed in separations using the CarboPac PA1 column, presumably because the sodium hydroxide concentration is 16 mM and the chromatography is sufficiently rapid (16 min) that exposure to alkali is minimized. Oligosaccharides are separated in 100 mM sodium hydroxide and are also retained longer on the column, particularly when sialylated. Under these conditions, oligosaccharides may exhibit 0 to 15% epimerization. As alditols do not epimerize in alkali, oligosaccharide epimerization can be eliminated if the oligosaccharide is reduced to the alditol prior to chromatography. For the same reason, monosaccharide alcohols are not epimerized in the high concentrations of alkali needed to elute them from the CarboPac MA1 column.

B. De-acetylation of N-acetylated Sugars

The hydrolysis of acylated sugars at high pH is another potential problem. Approximately 20% of a sample of *N*-acetylglucosamine is hydrolyzed to free glucosamine by exposure to 150 mM sodium hydroxide overnight at room temperature. However, chromatography of *N*-acetyl

glucosamine at high pH generates a single sharp peak with no evidence of formation of the (well resolved) free-base analog. Likewise, samples of *N*-acetyl neuraminic acid and *N*-glycolyl neuraminic acid are easily separated as sharp symmetrical peaks[8].

C. ß-Elimination or Peeling of 3-O-Substituents on Reducing Sugars

The ß-elimination of 3-O-substituents on reducing sugars is also a potentially serious side reaction that proceeds, in most cases, too slowly at room temperature to be a problem. The treatment of laminaribiose (glucopyranosyl ß-1-3 glucopyranose) with 150 mM sodium hydroxide for 4 h destroys more than 80% of the disaccharide, producing glucose and a second unidentified peak. However, laminaribiose generates a single peak during chromatography by HPAE with no evidence of glucose or other breakdown products.[8] Conversely, D-glucose-3-sulfate, which has a very good leaving group, decomposes rapidly during chromatography.

PULSED AMPEROMETRIC DETECTION

I. Theory of Operation

Pulsed amperometry permits detection of carbohydrates with excellent signal-to-noise ratios down to approximately 10 picomoles without requiring derivatization. Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of a gold electrode. The products of this oxidation reaction also poison the surface of the electrode, which means that it has to be cleaned between measurements. This is accomplished by first raising the potential to a level sufficient to oxidize the gold surface. This causes desorption of the carbohydrate oxidation products. The electrode potential is then lowered to reduce the electrode surface back to gold. The sequence of potentials is illustrated in Figure 2.

Pulsed amperometric detection thus employs a repeating sequence of three potentials. Current from carbohydrate oxidation is measured at the first potential, E_1. The second, E_2, is a more positive potential that oxidizes the gold electrode and cleans it of products from the carbohydrate oxidation. The third potential, E_3, reduces the gold oxide on the electrode surface back to gold, thus permitting detection during the next cycle at E_1.

The three potentials are applied for fixed durations referred to as t_1, t_2, and t_3. The step from one potential to the next produces a charging current that is not part of the analyte oxidation current, so the analyte oxidation current

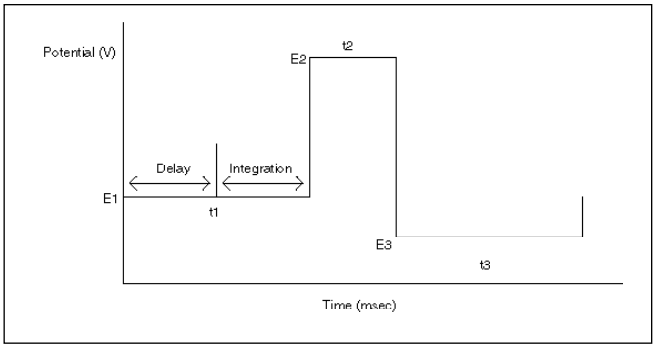

Figure 2. Diagram of the pulse sequence for carbohydrate detection.

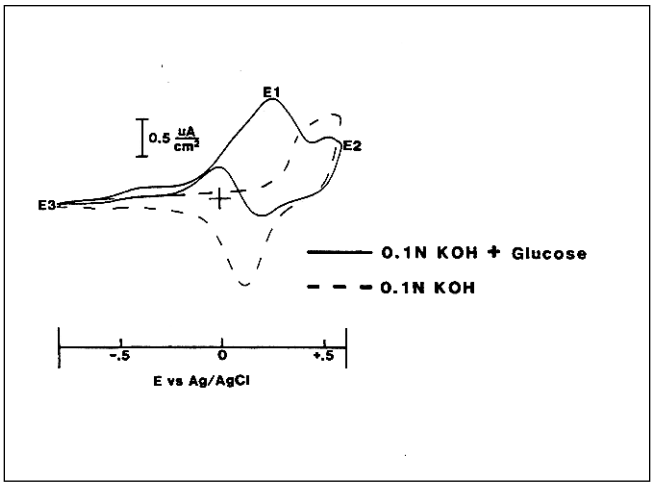

Figure 3. Cyclic voltammetry of glucose on a gold electrode.

is measured after a delay that allows the charging current to decay. The carbohydrate oxidation current is measured by integrating the cell current after the delay. Current integrated over time is charge, so the detector response is measured in coulombs. Alternatively, the average current during the integration period can be reported. In this case, the units used are amperes.

Optimal potentials can be determined by electrochemical experiments such as cyclic voltammetry, in which the applied potentials are slowly scanned back and forth between positive and negative potential limits. The resulting current is plotted on the Y-axis with oxidation (anodic) currents up and reduction (cathodic) currents down. Figure 3 shows the cyclic voltammogram of glucose in a 100 mM potassium hydroxide solution on a gold electrode. The dashed line is a background scan of a solution of 100 mM potassium hydroxide. As the potential is raised, the current starts to rise at about 0.2 V (see Figure 3, upper dashed line). This is caused by oxidation of the gold surface.

Reduction of the surface gold oxide back to gold occurs on the reverse scan (lower dashed line) with a cathodic (negative) current peak at about 0.1 V.

When glucose is present (solid line), its oxidation peaks at about 0.25 V (upper solid trace), which is also the potential at which formation of gold oxide begins. The glucose oxidation current drops as gold oxidation continues to increase, demonstrating that the formation of gold oxide inhibits oxidation of glucose. On the reverse scan, the current actually reverses from negative to positive at the onset of gold oxide reduction, further evidence of the inhibiting effect of gold oxide on the oxidation of glucose. It is thus important to use a measuring potential (E_1) below that required for gold oxidation.

All three potentials are important. However, the most important is E_1 — the potential at which the carbohydrate oxidation current is measured. A plot of detector response as a function of E_1 is shown in Figure 4. The background current is also shown. The maximum response is shown to occur at about 0.2 V for the three sugars tested, although the best signal-to-noise ratio actually occurs at a slightly lower potential. Figure 4 shows that the voltage at which the maximum response occurs is the same for three very different sugars: xylitol, a nonreducing sugar alcohol; glucose, a reducing monosaccharide; and sucrose, a nonreducing disaccharide. This is because the oxidation of the sugars at the electrode is catalyzed by the electrode surface. As a result, the amperometric response of a class of compounds is controlled primarily by the dependence of the catalytic surface state on the electrode potential and not on the redox potentials of the compounds themselves. Pulsed amperometric detection is thus a universal detection method for all carbohydrates, although derivatization of two or more hydroxyl groups will decrease (and may even abolish) detection.

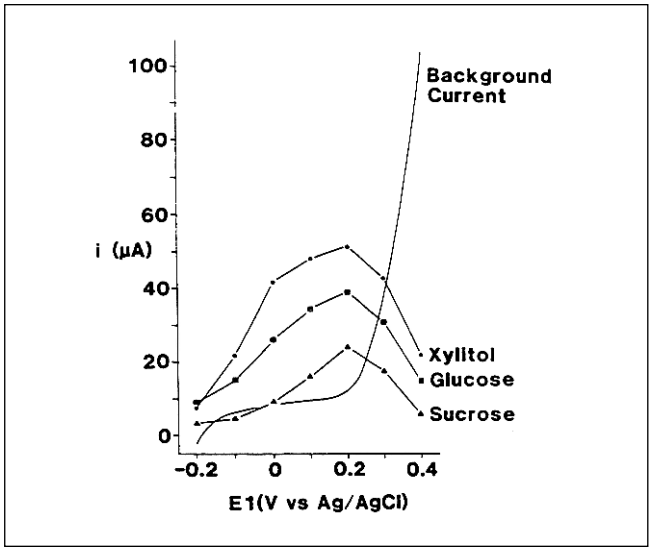

Figure 4. The oxidation current generated at different values of E^1 for three different carbohydrates.

Potential E_2 must be high enough and long enough to oxidize the electrode surface fully so that the carbohydrate oxidation products are completely removed. This potential cannot be too high, however, or excessive gold oxidation will occur and the electrode will wear too rapidly. The third potential, E_3, must be low enough to reduce the oxidized surface of the gold electrode completely without being so low that chemical reductions (for example, of oxygen to hydrogen peroxide) will occur. The results of these reactions may cause baseline disturbances during subsequent measurement at E_1.

Recommended pulse sequences for the Dionex pulsed amperometric detectors are given in Technical Note 21, which is available from your local Dionex representative.

APPLICATIONS

I. Eluent Preparation for Carbohydrate Analysis

When making eluents for carbohydrate analysis, it is important to use reagents of the grade listed:

- 50% (w/w) Sodium hydroxide solution
  Fisher Cat. No. SS254-1
- Anhydrous sodium acetate
  Fluka Cat. No. 71179

- Sodium Hydroxide: It is extremely important to minimize contamination of the eluent solutions with carbonate. Carbonate, being a divalent anion at pH ≥12, binds strongly to the columns and interferes with carbohydrate binding, causing a drastic decrease in column selectivity and a loss of resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should *not* be used. A 50% w/w sodium hydroxide solution is much lower in carbonate. Any carbonate present will precipitate to the bottom of the container and can be avoided. The concentration of the 50% sodium hydroxide solution is approximately 19.3 M, so diluting 20.8 mL of a 50% solution into 2 L of water yields a 0.2 M sodium hydroxide solution.

- Distilled Water: It is essential to use high-quality water. It is critical that there be as little dissolved carbon dioxide as possible in the water. It should also be of high resistivity (18 MΩ) and biological contamination should be absent. The use of fresh Pyrex® glass-distilled water is recommended. The still should be fed with high-resistivity (18 MΩ) water, and the use of plastic tubing should be avoided because it often supports microbial growth. Biological contamination is often the source of unexpected glucose peaks after acid hydrolysis.

A. Guidelines to Handling the 50% (w/w) Sodium Hydroxide Solution

1. To avoid mixing of the sodium carbonate precipitate into the solution, store the 50% sodium hydroxide close to where the eluent will be prepared. *Do not shake or stir this solution.*
2. Never pour the solution from the bottle.
3. Pipet sodium hydroxide from the center of the solution, not from edges or bottom. Do not allow the pipet to stir the solution at the bottom.
4. Use only plastic pipets, as sodium hydroxide leaches borate and silicate out of glass. Borate will complex with carbohydrates and will thus alter their chromatographic behavior.
5. Never return unused liquid to the bottle.
6. Close the bottle immediately after each use and leave open for the shortest time possible to avoid carbon dioxide absorption.
7. Discard the bottle of 50% sodium hydroxide when 2 to 3 cm or less of solution remains.

B. Eluent Preparation

It is impossible to completely eliminate all carbonate from eluents. Therefore, to ensure reproducible chromatography, it is essential to use the same methods consistently in preparing the solutions. Once eluents have been prepared, they should be kept blanketed under helium (5–7 psi/34–48 kPa) at all times.

i. Distilled Water

High-quality water should be degassed by one of the following two methods:

1. Sparging with helium for 20–30 min. Degassing is complete when all of the small bubbles first formed upon degassing disappear.
2. Sonication for 30–60 s while degassing with a water vacuum aspirator, followed by a 10-min helium sparge. Degassing is again complete when all of the small bubbles first formed upon degassing disappear.

ii. Sodium Hydroxide

Degas the required volume of water, as described above. After degassing is complete, use a plastic pipet to add the appropriate amount of 50% sodium hydroxide solution to give the required concentration. Avoid bubbling air into the eluent when expelling the 50% sodium hydroxide solution from the pipet. Rinse the pipet by drawing some of the sodium hydroxide/water mixture into the pipet and expelling it back into the solution. Repeat this several times. Add a stirring bar to the mixture and stir gently without agitating the surface for about 2 min.

As an alternative, the 50% sodium hydroxide can be pipetted directly into the distilled water as it is being sparged by the Dionex Eluent Degas Module (EDM). Sparge the water for 15 min, add the sodium hydroxide, rinse the pipet, and swirl the solution in the bottle to mix. Then sparge the solution for an additional 5 min. The sparging will complete the mixing.

Both methods work well. It is most important to be consistent in the method used. Store sodium hydroxide solutions in plastic containers, as they will leach borate and silicate out of glass.

iii. Sodium Hydroxide/Sodium Acetate Solutions

Degas the required volume of water as described above, and transfer it to a graduated cylinder. Add a stir bar and start stirring, while steadily adding the anhydrous crystalline sodium acetate. After the salt dissolves, retrieve the stir bar and add the appropriate volume of 50% (w/w) sodium hydroxide to the graduated cylinder in the same manner as described previously. Bring the volume to the requisite level (e.g., 1–2 L). Vacuum filter the mixture through a 0.2-µm nylon filter. Alternatively, sparge the filtered acetate solution for 15 min, add the sodium hydroxide, swirl the solution to mix, and continue to sparge for 5 min. Once again, it is important to be consistent in the method used to make up the solution.

Sodium acetate solutions should last about one week. The most consistent chromatography has been obtained using sodium acetate purchased from Fluka.

II. Sample Preparation

It is recommended that all samples other than pure standards be passed through a 0.45-µm nylon filter prior to injection to remove particulates. Cellulose acetate and other filters should be avoided because they may leach carbohydrates. Filters of a type not previously verified as "clean" should be evaluated for contribution of "PAD-active" components before use.

Sample preparation is obviously dependent on sample matrix complexity and, as such, the recommendations that follow should be considered as guidelines only. In particular, the effect of sample pretreatment cartridges on the carbohydrate analytes themselves should be predetermined using standard solutions. It may be found that some carbohydrates have a strong affinity for particular cartridge packing materials. This is obviously of importance for quantification and in the detection of low levels of carbohydrates.

A. Samples Containing High Levels of Protein or Peptides

Physiological fluids such as plasma, urine, or other samples containing high levels of proteins should be deproteinized first. This may be achieved by standard precipitation procedures or by passing the analyte solution through a hydrophobic filter cartridge such as the Dionex OnGuard® RP Cartridge (P/N 39595).

B. Samples Containing High Levels of Humic Acids or Phenolics

To remove the phenolic fraction of humic acids, tannic acids, or lignins found in food samples (such as wine), the sample may be passed through a polyvinylpyrrolidone (PVP) filter cartridge, such as the Dionex OnGuard-P Cartridge (P/N 39597).

C. Samples Containing Halides

To remove halides, the sample may be passed through a Dionex OnGuard-Ag cartridge (P/N 39637). This cartridge selectively removes Cl^-, Br^- and I^- in preference to other anionic species. This cartridge is, however, a cation exchanger, so amino sugars will be extracted unless they are *N*-acetylated.

D. Samples Containing Sulfate and Other Anions

Sulfate may be precipitated as the barium salt by addition of barium hydroxide solution. However, it should be noted that some carbohydrates may co-precipitate with the barium sulfate in this procedure, especially carbohydrates bearing sulfate esters. The Dionex OnGuard A cartridge (P/N 42102) is designed specifically to remove anion contaminants from sample matrices. OnGuard A cartridges contain styrene-based anion-exchange resin in the bicarbonate form. They should not be used with samples that contain sialic acids, or sugars with other acid substituents.

III. Standard Chromatography Conditions for the Analysis of Carbohydrates

The conditions described in this section have been found to give reliable separations of the common classes of carbohydrates using HPAE chromatography. Samples and their matrices vary, therefore these conditions are intended to be used as guidelines only.

A. Monosaccharides—Neutral and Amino Sugars

These sugars can be successfully separated on the CarboPac PA1 column using isocratic conditions with 16 mM sodium hydroxide as the eluent. A representative chromatogram is shown in Figure 5. Because the concentration of sodium hydroxide used for the separation is only 16 mM, the column should be regenerated after each run. Otherwise, carbonate will start to contaminate the column, irrespective of the care taken to eliminate it from eluents and samples. Regenerate the column by washing it with 200 mM sodium hydroxide for 10 min at a flow rate of 1.0 mL/min. This procedure will also remove other strongly bound contaminants such as peptides and amino acids. This step is extremely important and should not be omitted. After washing, the column should be reequilibrated with 16 mM sodium hydroxide at a flow rate of 1.0 mL/min for 10 min. It is very important to keep the rinse and reequilibration times consistent from run to run.

B. Sugar Alcohols

Mono- and oligosaccharide sugar alcohols can be separated using the CarboPac MA1 column with sodium hydroxide eluents. Examples of isocratic separations are shown in Figures 6 and 7. Gradients can be used to improve separations (Figure 8) or to accelerate the elution of late-eluting components (Figure 9). Table 2 shows that the elution order of certain carbohydrates may be altered by changing the sodium hydroxide concentration.

C. Sialic Acids, Sialylated, and Phosphorylated Oligosaccharides

The elution of acidic sugars from the CarboPac PA1 or the CarboPac PA100 columns requires stronger eluents than those used with neutral sugars. This is usually

accomplished by the addition of sodium acetate to the sodium hydroxide eluent. Sodium acetate accelerates the elution of strongly bound species without compromising selectivity and without interfering with pulsed amperometric detection. Sodium acetate/sodium hydroxide solutions can be used isocratically (Figure 10) or in gradients (Figure 11 and 12). Sodium acetate gradients are *not recommended* for the CarboPac MA1 column because column regeneration will require several hours. The use of sodium acetate gradients is not a problem with the CarboPac PA1 and CarboPac PA100, because these columns have a lower anion- exchange capacity and thus regenerate quickly.

To maintain baseline stability, it is helpful to keep the sodium hydroxide concentration constant during the sodium acetate gradient, because acetate has no buffering capacity at high pH. This is achieved by making eluents as follows:

Eluent A: x mM NaOH
Eluent B: x mM NaOH, y mM NaOAc

When devising gradients for the analysis of a carbohydrate sample of unknown composition, it is generally good practice to run a "scouting gradient". This gradient consists of a rapid linear gradient ($t \leq 15$ min) from a low to a sufficiently high acetate concentration that it will elute all of the components. It is then possible to fine tune the separation, with the assurance that the gradient is sufficiently broad to include all of the sample components.

Sialic acids can also be separated at neutral pH. This is particularly useful for O-acetylated species, which are unstable at high pH [9–11].

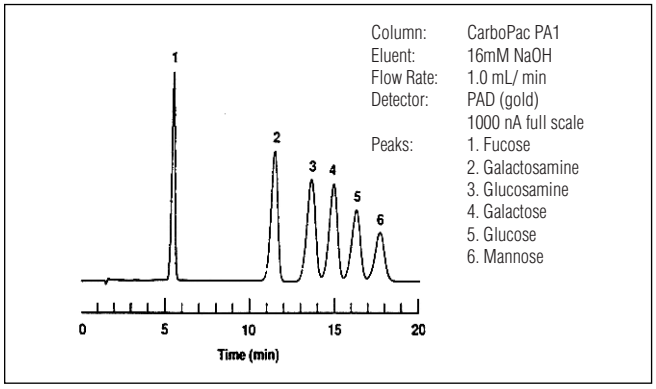

Figure 5. Separation of neutral and amino monosaccharides derived from glycoproteins.

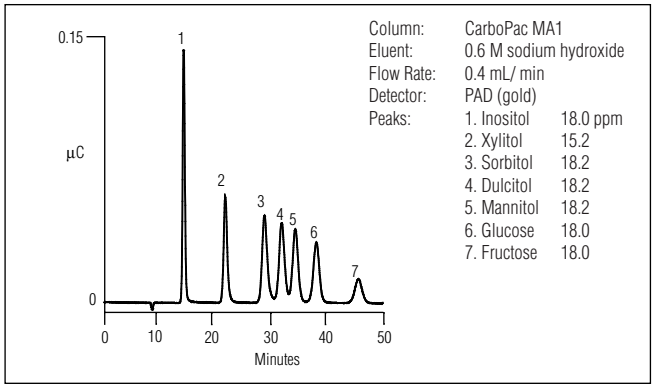

Figure 6. Isocratic separation of a group of alditols plus glucose and fructose on the CarboPac MA1 column.

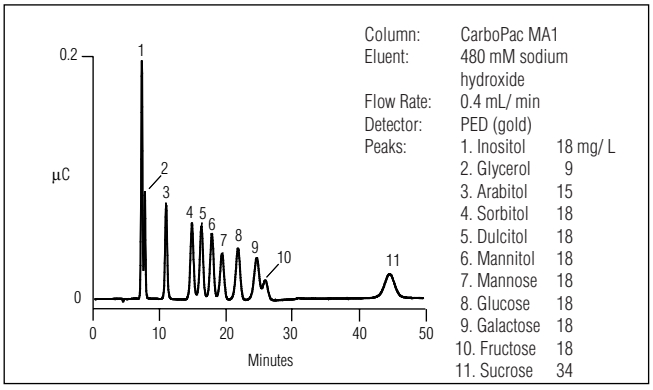

Figure 7. Separation of reducing and nonreducing carbohydrates. Food alditols and aldoses are separable under isocratic conditions on the CarboPac MA1.

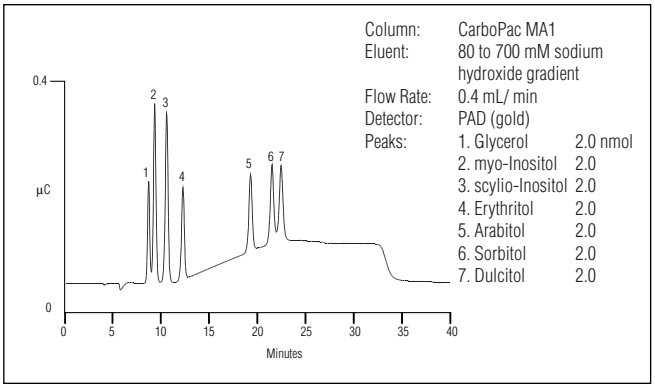

Figure 8. Separation of alditols found in biological fluids. The NaOH gradient improves the separation of sorbitol and dulcitol, which are poorly resolved at NaOH concentrations that permit resolution of glycerol from inositol.

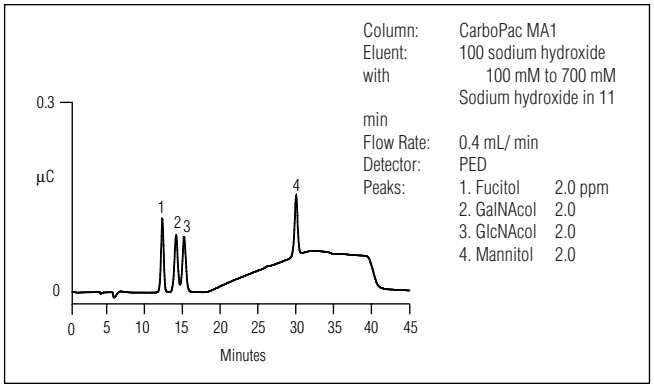

Figure 9. Separation of monosaccharide alditols released by direct ß-elimination from glycoproteins. The hydroxide gradient following the isocratic separation of the first three components accelerates the elution of mannitol as well as any oligosaccharide alcohols that may have been released during the ß-elimination process.

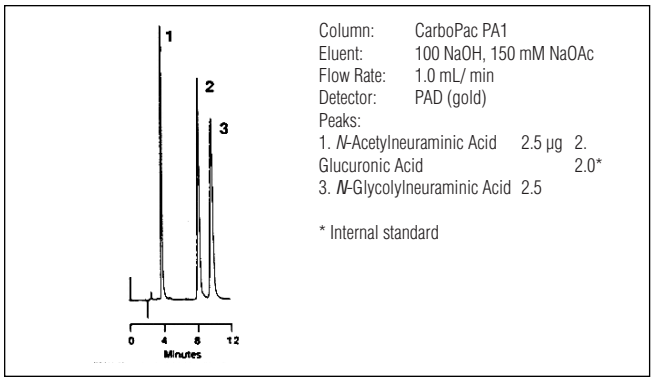

Figure 10. Isocratic separation of sialic acids using a sodium hydroxide/sodium acetate mixture.

D. Oligo- and Polysaccharides

Separations of high mannose, hybrid, and complex oligosaccharides are best accomplished using the CarboPac PA100 column. Linear homopolymers are successfully separated on the CarboPac PA1 or PA100. In all cases, separations are accelerated and improved by using sodium acetate gradients in sodium hydroxide. Sodium hydroxide gradients are also useful. Separation of several polysaccharides using either the CarboPac PA1 or the PA100 are shown in Figures 13 through 15. Figure 14 also shows the structure of inulin.

Figure 16 shows the separation of a group of neutral glycoprotein-derived oligosaccharides on the CarboPac PA100, while Figures 17 through 20 show examples of how HPAE-PAD analysis on the CarboPac PA100 column

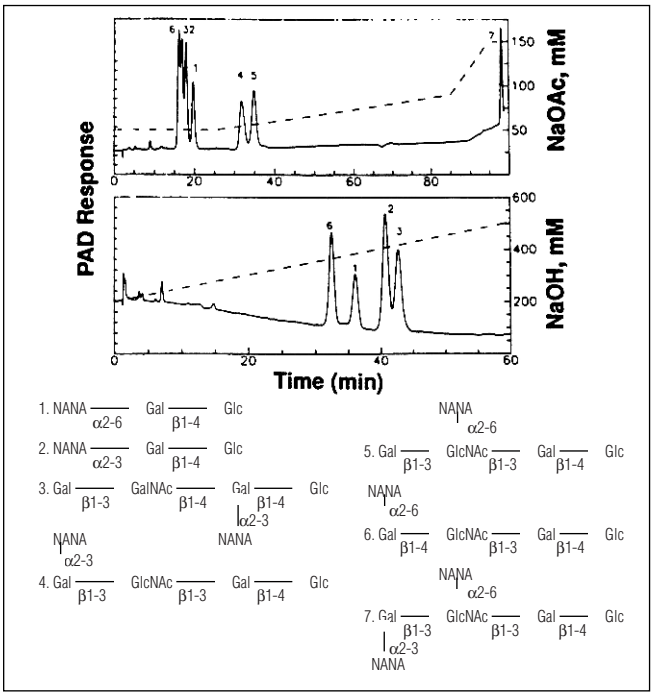

Figure 11. Gradient separation of sialylated oligosaccharides using the CarboPac PA1 column.

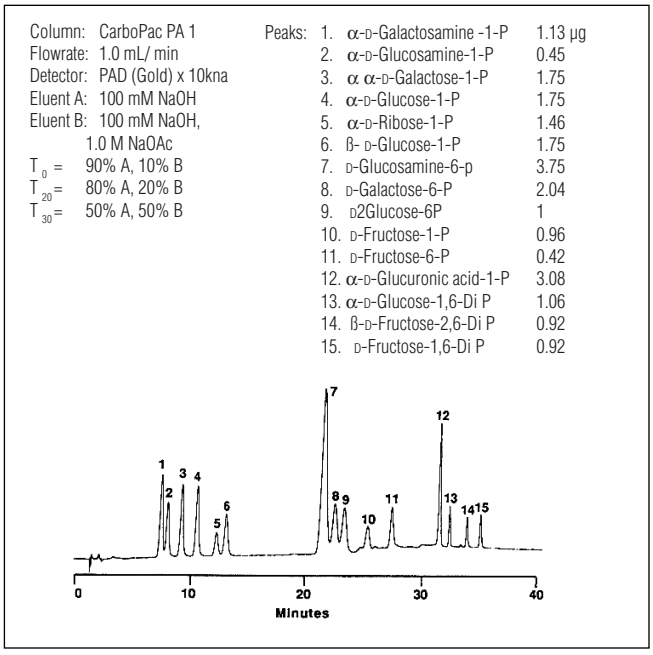

Figure 12. Analysis of mono- and diphosphorylated monosaccharides.

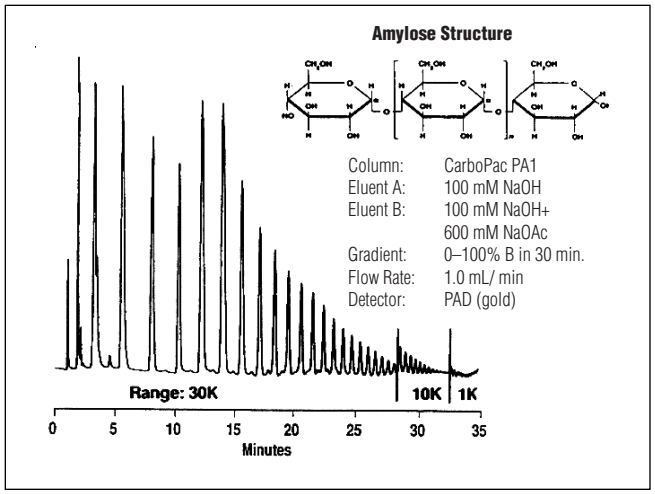

Figure 13. Analysis of "Dextrin 7" glucose polymer.

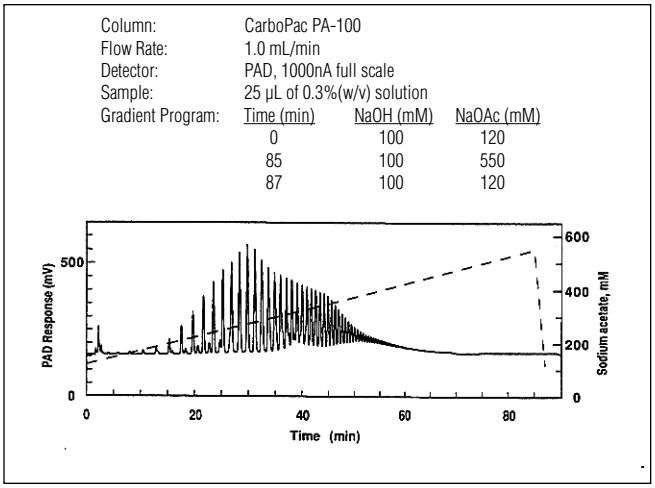

Figure 15. Gradient separation of chicory inulin using the CarboPac PA100.

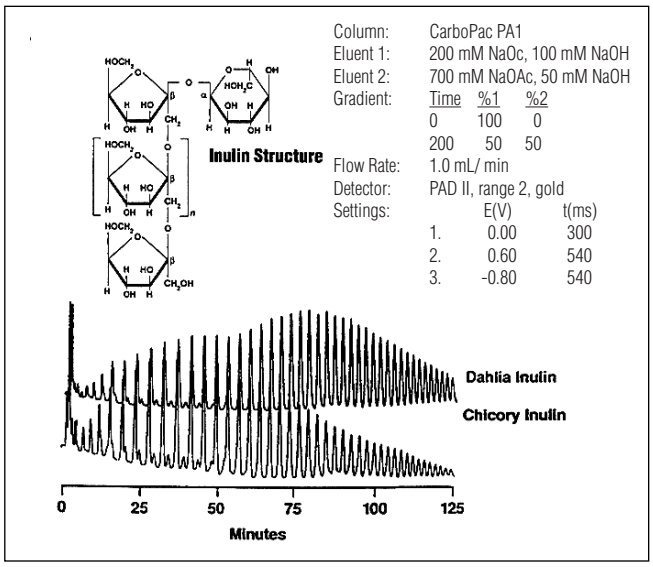

Figure 14. Comparison of water washed inulins (Cichorium intybus vs Dahlia sp.) using the CarboPac PA1.

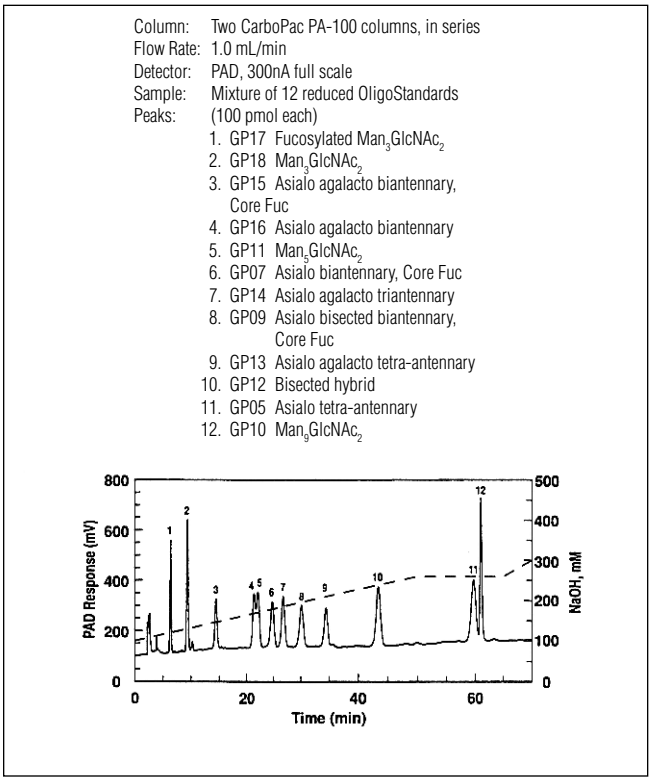

Figure 16. Separation of Dionex OligoStandard™ glycoprotein-derived oligosaccharides.

can be used to map oligosaccharides released from glycoproteins by enzyme digestion. Note that in Figure 18 the two oligosaccharide peaks marked 1 and 2 differ only in the linkage position of the sialic acid on one branch of the triantennary structure ($\alpha 2 \rightarrow 6$ versus $\alpha 2 \rightarrow 3$).

Neutral O-linked oligosaccharides released by reductive elimination are alditols and may be best separated on the CarboPac PA1 column (≥3 carbohy-drate units) or on the CarboPac MA1 column (<3 carbohydrate units).

V. Postcolumn Addition of Base

To optimize baseline stability and detector sensitivity, it is sometimes necessary to add strong base to the eluent stream postcolumn, particularly when using neutral pH eluents or when the eluent contains a low concentration sodium hydroxide. Postcolumn addition of base can help with quantification, in part by maintaining a more constant hydroxide concentration at the electrode. Users have found, however, that postcolumn addition of base is often unnecessary with routine isocratic and gradient separations at sodium hydroxide concentrations ≥15 mM.

A. Separations at Low pH

Sialic acids, sialylated oligosaccharides, and other carbohydrates bearing strongly acidic substituents can be separated by anion exchange at lower pH values.[10,11] This option is particularly useful when analyzing oligosaccharides that possess O-acetylated sialyl groups, because these groups are unstable at high pH. When low-pH eluents are used, sodium hydroxide must be added to the eluent after it has left the column and before it enters the detector, because carbohydrates are best detected at gold electrodes when pH ≥12.

B. Sodium Hydroxide Gradients

Changing the sodium hydroxide concentration alters the pH of the solution, which can affect the detector electrode response. While the PED and the solvent compatible PAD are affected very little by pH changes, the standard PAD-2 cell is fairly sensitive. A gradient from 10 mM sodium hydroxide to 100 mM sodium hydroxide results in an effective change of 1 pH unit during the gradient. The effect on detector response can be minimized by the use of optimized pulse sequence settings. Optimized pulse sequence settings are discussed in detail in Technical Note 21, which can be obtained from your local Dionex representative. If necessary, however, a solution of sodium hydroxide can be added postcolumn to minimize the pH shift. For example, the addition of 300 mM sodium hydroxide to the column effluent of a gradient of 10 to 100 mM sodium hydroxide would result in a total pH change of only 0.11 units (if the flow rates of the postcolumn base and eluent were equal). A pH change of this magnitude would generate a negligible baseline shift.

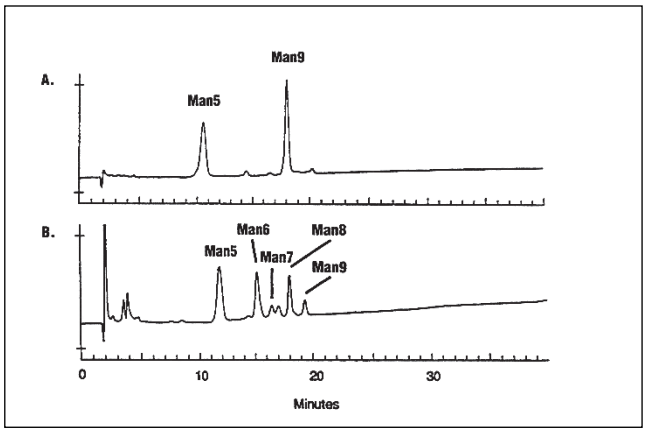

Figure 17. Separation of glycoprotein-derived mannose oligosaccharides using the CarboPac PA100 column. Panel A: Dionex OligoStandards GP11 and GP10, $Man_5GlcNAc_2$ and $Man_9GlcNAc_2$, respectively. Panel B: Endonuclease H digest of ribonuclease B.

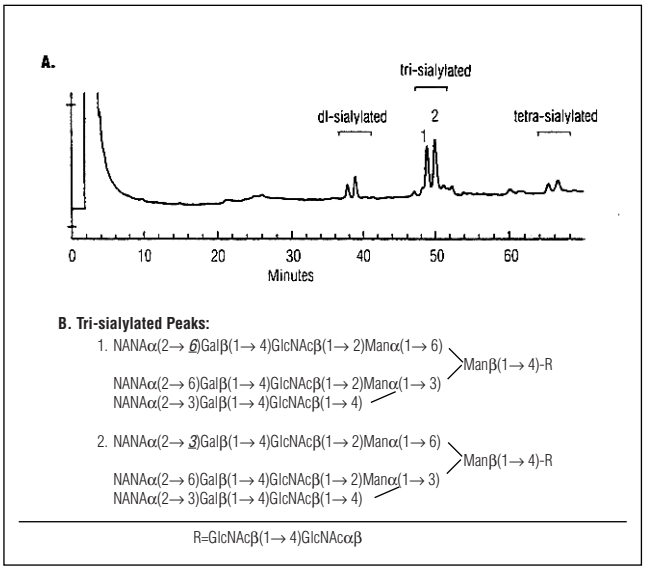

Figure 18. Separation of fetuin N*-linked oligosaccharides. Panel A: HPAE-PAD analysis using a CarboPac PA100. Panel B: Structures of the trisialylated species, peaks 1 and 2.*

Postcolumn base can be delivered through a mixing T using the Dionex Postcolumn Pneumatic Controller. To ensure run-to-run reproducibility, the Controller should be adjusted so that:

1. The flow rate is constant.
2. The mixture entering the detector has a pH ≈ 13.
3. The flow rate of the mixture of eluent plus postcolumn base stays the same from run to run.

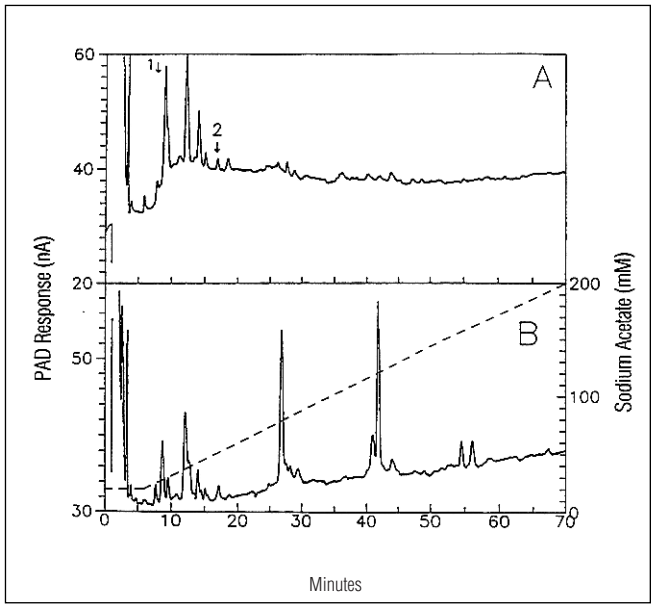

Figure 19. Gradient separation (using the CarboPac PA100 column) of oligosaccharides released by sequential enzyme digestion of recombinant tissue plasminogen activator (rtPA). Panel A: High-mannose oligosaccharides released from rtPA by digestion with endonuclease H. The elution positions of $Man_5GlcNAc_2$ and $Man_9GlcNAc_2$ are indicated by the numbered arrows. Panel B: Oligosaccharides released from rtPA by endonuclease F_2 (cleaves predominantly biantennary-type chains). (From Weitzhandler et al.[12] Reproduced with permission.)

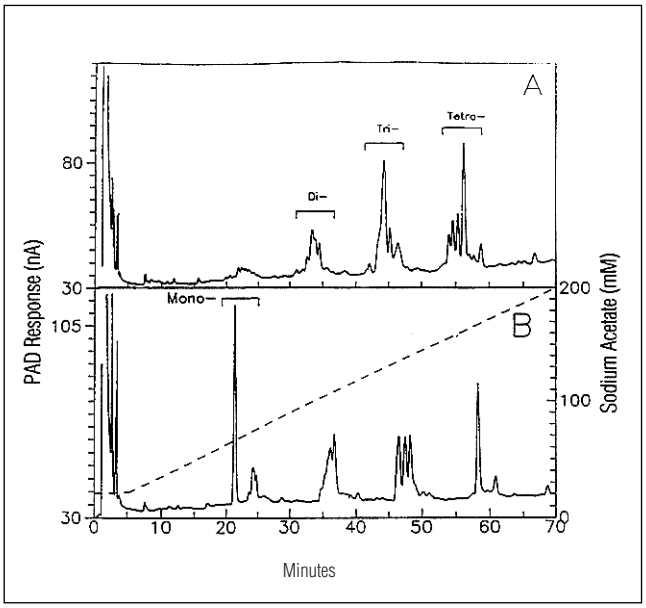

Figure 20. Gradient separation (using the CarboPac PA100 column) of oligosaccharides in recombinant erythropoietin (rEPO). Panel A: Oligosaccharides released by PNGase F digestion. Panel B: Digestion of the mixture in panel A by endo-ß-galactosidase shows that three of the four major tetrasialylated species contain polylactosamine structures. (From Weitzhandler et al.[12] Reproduced with permission.)

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Thermo SCIENTIFIC

Technical Note 36

Analysis of Exoglycosidase Digestions of *N*-Linked Oligosaccharides Using HPAE-PAD

INTRODUCTION

Analysis of protein glycosylation is an important part of glycoprotein characterization. Oligosaccharides can be linked to a protein through a serine or a threonine as O-linked glycans, or through an asparagine as *N*-linked glycans. To analyze the structure of oligosaccharides, various chromatographic and spectroscopic techniques are often required.

High-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) is one of the most powerful and commonly used techniques for protein glycosylation analysis. Using this technique, oligosaccharides and monosaccharides can be quickly separated and their structures can frequently be identified by comparing them with authentic carbohydrate standards. Separations of oligosaccharides are based on their fine structural differences such as the composition and the sequences of the oligosaccharides, linkage isomerism, degree of sialylation, and degree of branching. Separations of monosaccharides are mainly dependent upon the difference between their pK_a values. Such differences usually allow baseline resolution of common neutral and amino sugars found in mammalian glycoproteins.

HPAE-PAD, in combination with different endo- and exoglycosidases, and appropriate oligosaccharide standards, allows step-by-step structural analysis of oligosaccharides. Treatment of a glycoprotein with an endoglycosidase releases the attached oligosaccharides from the protein. Profiles of the released oligosaccharides can be obtained using HPAE-PAD. Subsequent digestion of these oligosaccharides with various speci-fic exoglycosidases generates structural information. Since intact enzymes are not commonly eluted at the regions where mono- and oligosaccharides are eluted, these enzymatic digestions can be injected directly into the analytical system for separation and identification of the released carbohydrates.

This technical note describes the use of exoglycosidases to obtain structural information about *N*-linked oligosaccharides commonly found in mammalian glycoproteins. HPAE-PAD is used to confirm the identity of the released monosaccharides, as well as the remaining di-, tri,- or oligosaccharides. Empirical rules used to predict oligosaccharide elution order are included in the last section of this technical note.

EQUIPMENT

Dionex DX 500 HPLC system consisting of:
- GP40 Gradient Pump
- ED40 Electrochemical Detector
- AS3500 Autosampler

PeakNet Chromatography Workstation

CONDITIONS AND METHODS

Columns: CarboPac™ PA1 Analytical Column (4 × 250 mm)
Guard Column (4 × 50 mm)
CarboPac PA-100 Analytical Column (4 × 250 mm)
Guard Column (4 × 50 mm)

Flow Rate: 1.0 mL/min

Pulse Setting for ED40 Detector:

Time	Potential	Integration
0.00	0.05	
0.20	0.05	Begin
0.40	0.05	End
0.41	0.75	
0.60	0.75	
0.61	–0.15	
1.00	–0.15	

Eluent A: 250 mM Sodium acetate

Eluent B: 500 mM Sodium hydroxide

Eluent C: Deionized water, 17.8 MΩ-cm resistance or better

Eluent D: 100 mM Sodium hydroxide

Methods:

Method*	Column	Time (min)	A (%)	B (%)	C (%)	D (%)
1	CarboPac PA-100	0	8	20	72	0
		5	8	20	72	0
		65	80	20	0	0
		65.05	8	20	72	0
		70	8	20	72	0
2	CarboPac PA-100	0	0	50	50	0
		4	0	50	50	0
		27	32	50	18	0
		32	32	50	18	0
		32.1	0	50	50	0
		37	0	50	50	0
3	CarboPac PA1	0	0	0	84	16
		3	0	0	84	16
		28	0	0	40	60
		28.05	0	50	50	0
		38	0	50	50	0
		38.05	0	0	84	16
		48	0	0	84	16

* Method 1: For separations of sialylated oligosaccharides
Method 2: For separations of neutral oligosaccharides
Method 3: For separations of mannose, GlcNAc, chitobiose, and fucosylated chitobiose

REAGENTS AND STANDARDS

Sodium hydroxide, 50% w/w (Fisher Scientific, Fair Lawn, New Jersey, USA)

Anhydrous sodium acetate (Fluka BioChemica, Ronkonkoma, New York, USA)

The following exoglycosidases and standards were obtained from Oxford GlycoSystems, Rosedale, New York, USA:

Chitobiose

6-α-Fucosyl chitobiose (fucosylated chitobiose)

α-Fucosidase from Bovine epididymi

β-Galactosidase from Streptococcus pneumoniae

β-N-Acetylhexosaminidase from Streptococcus pneumoniae

α-Mannosidase from Jack bean

β-Mannosidase from Helix pomatia

Neuraminidase from Vibrio cholerae

The following monosaccharide and oligosaccharide standards were obtained from Dionex Corporation, Sunnyvale, California, USA:

GP 03, GP 05, GP 07, GP 10, GP 11, GP 12, GP 14, GP 15, GP 16, GP 17, GP 18, FT 02, PI 01. (See Figure 1 for structures)

N-Acetylneuraminic acid (Dionex Standards: Mono, NANA)

The following monosaccharides were obtained from Sigma Chemical Company, St. Louis, Missouri, USA:

D(+)Galactose

N-Acetyl-D-Glucosamine (GlcNAc)

α-L(–)Fucose

D-Mannose, 99%, mixture of anomers (Aldrich Chemical Company, Milwaukee, Wisconsin, USA).

PREPARATION OF SAMPLES AND SOLUTIONS

Eluent A: 250 mM Sodium Acetate

Dissolve 20.5 g of anhydrous sodium acetate into a final volume of 1.0 L of deionized water. Filter the eluent through a 0.2 µm filter, and then degas the eluent for 5 minutes before use.

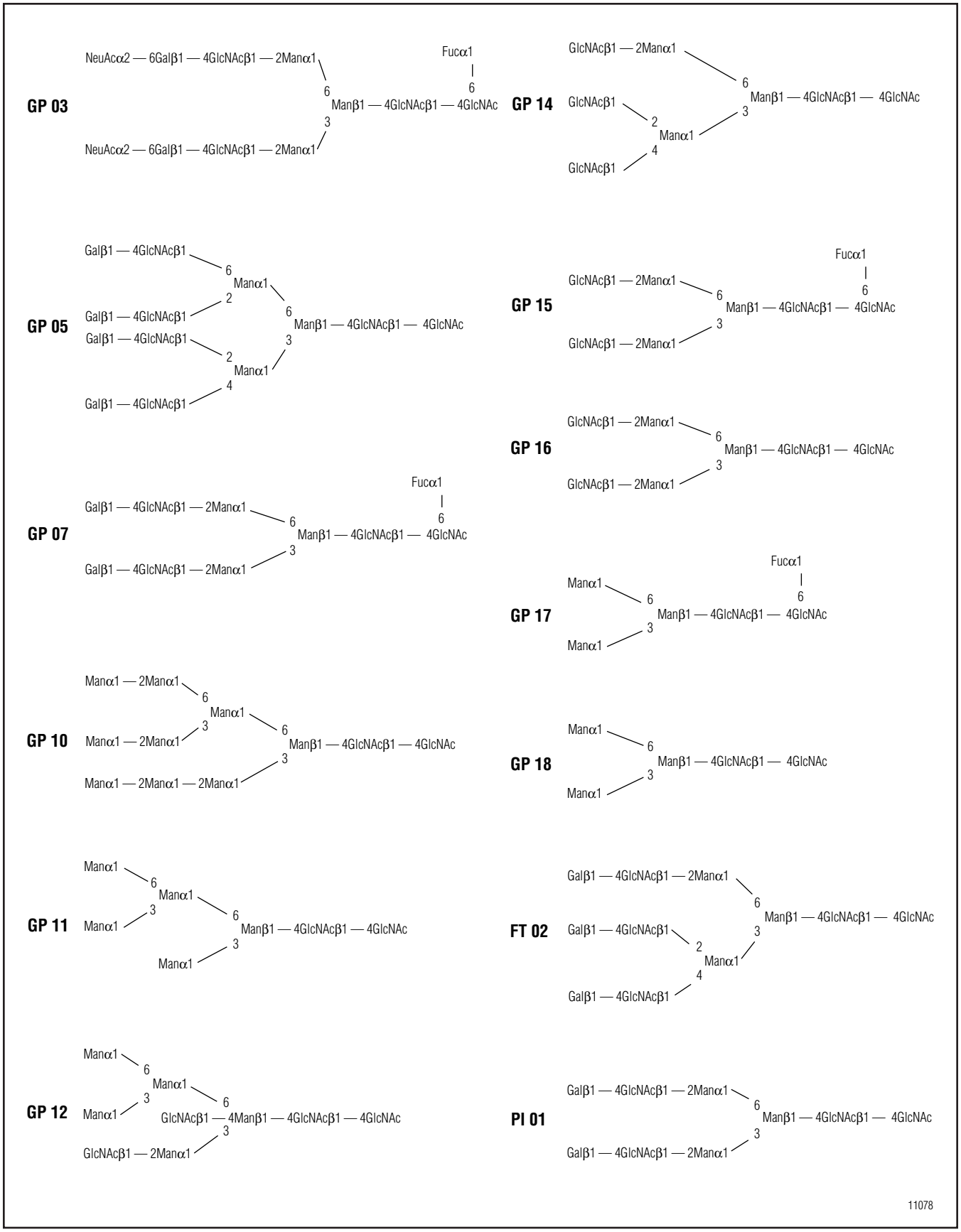

Figure 1. Structures of Dionex oligosaccharide standards.

Eluent B: 500 mM Sodium Hydroxide

Filter 1.0 L of deionized water through a 0.2 µm filter. Then degas the deionized water for 5 minutes. Add 26 mL of 50% w/w sodium hydroxide to a final volume of 1.0 L of the degassed water.

Eluent C: Water

Filter 1.0 L of deionized water through a 0.2 µm filter. Then degas the deionized water for 5 minutes before use.

Eluent D: 100 mM Sodium Hydroxide

Filter 1.0 L of deionized water through a 0.2 µm filter. Then degas the deionized water for 5 minutes. Add 5.2 mL of 50% w/w sodium hydroxide to a final volume of 1.0 L of the degassed water.

Exoglycosidase Stocks

α-Fucosidase from Bovine epididymis: Dissolve 0.1 U of the enzyme in 40 µL of the 5× incubation buffer (500 mM sodium citrate-phosphate, pH 6.0) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

β-Galactosidase from Streptococcus pneumoniae: Dissolve 40 mU of the enzyme in 40 µL of the 5× incubation buffer (500 mM sodium acetate, pH 6.0) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

β-N-acetylhexosaminidase from Streptococcus pneumoniae: Dissolve 30 mU of the enzyme in 40 µL of the 5× incubation buffer (500 mM sodium citrate-phosphate, pH 6.0) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

α-Mannosidase from Jack bean: Dissolve 2 U of the enzyme in 40 µL of the 5× incubation buffer (500 mM sodium acetate with 10 mM of Zn^{2+}, pH 5.0) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

β-Mannosidase from Helix pomatia: Dissolve 0.2 U of the enzyme in 40 µL of the 5× incubation buffer (500 mM sodium citrate-phosphate, pH 4.0) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

Neuraminidase from Vibrio cholerae: Dissolve 0.2 U of the enzyme in 40 µL of the 5× incubation buffer (50 mM sodium acetate with 4 mM $CaCl_2$, pH 5.5) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

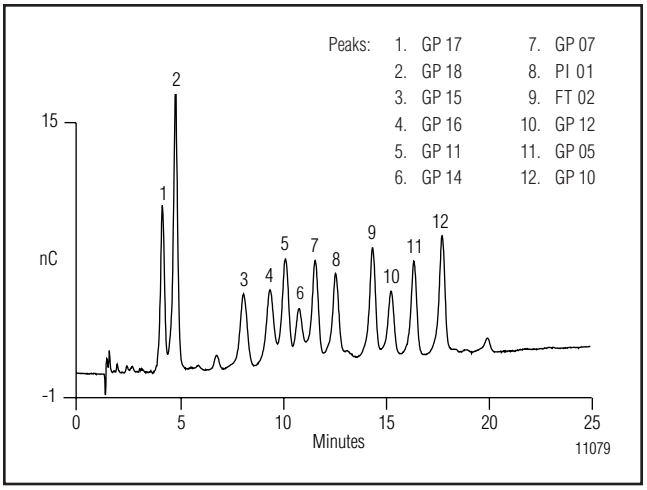

Figure 2. Separation of 12 neutral oligosaccharide standards. 1 µg of each standard was dissolved in 250 µL of water. Injection volume: 10 µL; separation: Method 2, Column: CarboPac PA-100.

Aliquots of the above enzyme stocks were used with different substrates to generate the desired digested products. See figure legends for digest conditions.

Carbohydrate Standards

Fucose, galactose, GlcNAc, and mannose (concentration: 1 mg/mL each): To prepare each sugar stock, dissolve 1 mg of the sugar in 1 mL of water.

N-acetylneuraminic acid (NANA, supplied as 25 nmol dry powder, final concentration: 50 nmol/mL): Add 500 µL of deionized water to 25 nmol of the sugar as supplied.

Chitobiose (supplied as 20 µg dry powder, final concentration: 100 µg/mL): Add 200 µL of deionized water to 20 µg of the sugar as supplied .

Fucosylated chitobiose (supplied as 20 µg dry powder, final concentration: 40 µg/mL): Add 500 µL of water to 20 mg of the sugar as supplied.

Dionex standards (supplied as 15 µg dry powder, final concentration: 30 µg/mL each): to prepare each oligosaccharide stock, add 500 µL of water to 15 µg of the sugar as supplied.

RESULTS AND DISCUSSION

1. Gradient Methods and Columns

Separations of sialylated oligosaccharides have previously been demonstrated using a CarboPac PA-100 column and a 20–200 mM sodium acetate gradient in the presence of 100 mM sodium hydroxide.[1–3] Using a

different linear gradient, a better separation of neutral oligosaccharides can be achieved, as shown in Figure 2. With this method, 12 neutral oligosaccharides, representing oligomannose-type, complex-type and hybrid-type oligosaccharides, were resolved.

Using a CarboPac PA1 column and Method 3, mannose, GlcNAc, fucosylated chitobiose and chitobiose can also be separated (mannose and GlcNAc are approximately 75% resolved). Method 3 is particularly useful for identification of the chitobiose core, and the released mannose and GlcNAc residues in an enzymatic digestion.

2. Exoglycosidase Digestions of Complex-Type Oligosaccharides

Neuraminidase

Both neutral and sialylated N-linked oligosaccharides can be released from glycoproteins using the amidase peptide-N-glycosidase F (PNGase F). A profile of the sialylated and the neutral oligosaccharides can be obtained using Method 1 and a CarboPac PA-100 column.[4] The terminal sialic acids can then be released from the nonreducing end of the sialylated oligosaccharides by subsequent treatment of the PNGase F digest with neuraminidase. The resulting neutral oligosaccharides can be separated from each other, and identified by comparing their retention times with appropriate oligosaccharide standards.

An example of a neuraminidase digestion and identification of the released products is shown in Figures 3a–3e. Figure 3b shows the separation of a digestion of a disialylated, core-fucosylated, biantennary complex oligosaccharide incubated with neuraminidase (from Vibrio Cholerae) at 37 °C for 24 hours. Peak 1 coelutes with an asialo, fucosylated biantennary oligosaccharide standard, as shown in Figure 3c. Thus, the peak represents the oligosaccharide substrate with its sialic acids removed. Peak 2, the released N-acetylneuraminic acid (NANA), is also identified as it coelutes with a NANA standard (see Figure 3d).

β-*Galactosidase*

Galactose is frequently present at the nonreducing end of neutral, complex-type oligosaccharides. These neutral oligosaccharides can be branched and the de-gree of galactosylation may vary. It has been demonstrated that neutral oligosaccharides with different degrees of galactosylation can be separated using the CarboPac PA-100 column.[5]

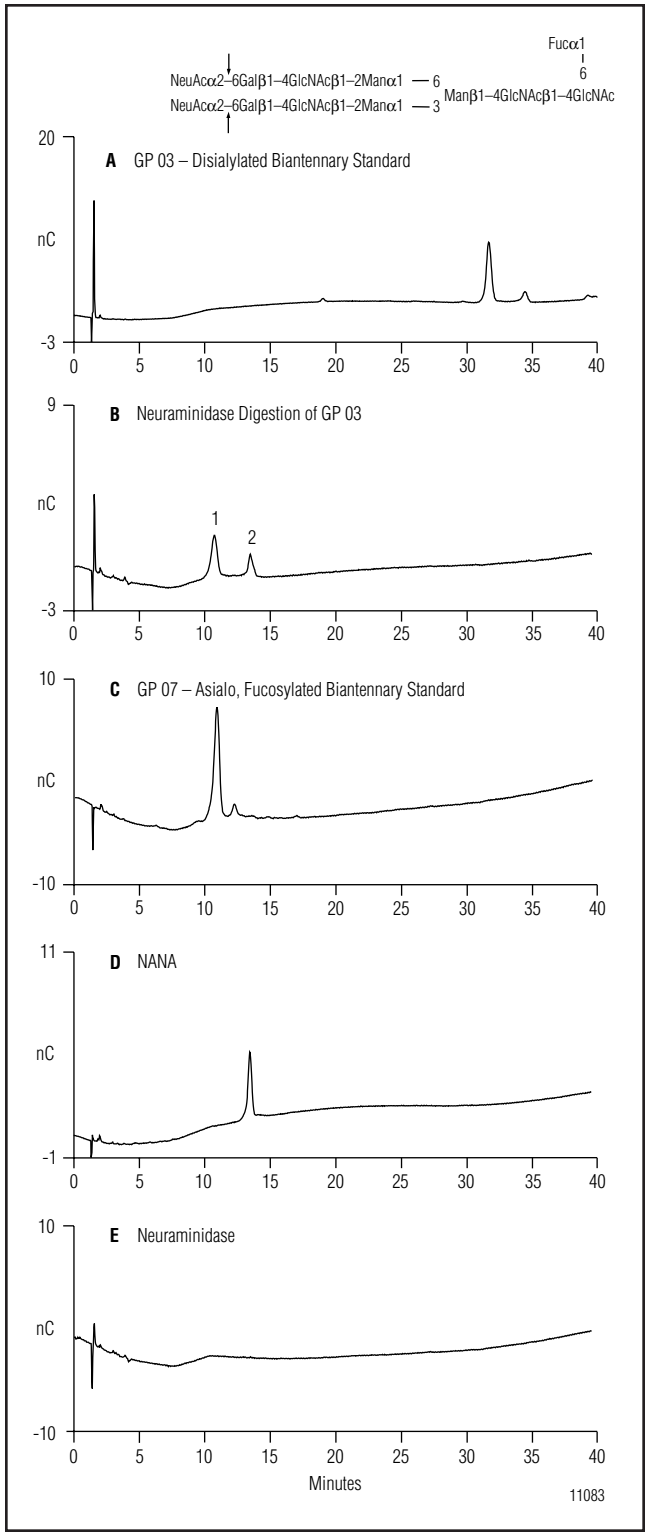

Figure 3. (A) Disialylated biantennary oligosaccharide stan-dard (Dionex standard GP 03); (b) separation of a neuraminidase digestion of a disialylated, biantennary complex type oligosaccharide with a fucosylated core (Dionex standard GP 03). Digest conditions: 30 µL of GP 03 was mixed with 5 mU of neuraminidase, and incubated at 37 °C for 24 hours. Injection volume: 10 µL; (c) an asialo, bi-antennary complex oligosaccharide with a fucosylated core (Dionex standard GP 07); (D) NANA; and (e) Neuraminidase. Separation: Method 1, Column: CarboPac PA-100.

Different galactosidases can be used to determine if an oligosaccharide is galactosylated at the nonreducing terminal and to identify the linkages of the galac-tose residues. For example, a terminal galactose linked to a GlcNAc (Galβ1—›4 GlcNAc) can be removed by treating the oligosaccharide with β-galactosidase from Streptococcus pneumoniae. After an asialo, fucosylated biantennary oligosaccharide was incubated with the β-galactosidase for 24 hours at 37 °C, two species were generated as shown in Figure 4b. Peak 2 coelutes with an asialo, agalacto, core-fucosylated, biantennary oligosaccharide standard shown in Figure 4c. The parent oligosaccharide and a galactose standard are shown in Figures 4a and 4d, respectively. The results indicate that peaks 1 and 2 represent the released galactose residues and the agalacto biantennary oligosaccharide product (with the galactose residues removed), respectively.

β-*N-Acetylhexosaminidase,* β-*Mannosidase, and* α-*Mannosidase*

All complex-type oligosaccharides have a common trimannosyl chitobiose core (see oligosaccharide structure in Figure 5) — a mannose residue linked (β1—›4) to the chitobiose (two GlcNAc residues) — with two additional mannose residues linked to the mannose (proximal to the chitobiose) through α1—›6 and α1—›3 linkages, respectively. Two additional GlcNAc residues can also be linked to the two terminal mannoses of the trimannosyl chitobiose core. To release the terminal GlcNAc residues and the mannose residues from the chitobiose core, several exoglycosidases can be used simultaneously. A terminal GlcNAc linked to a trimannosyl chitobiose core through a GlcNAcβ1—›2 Man linkage can be removed using β-N-acetylhexosaminidase from Streptococcus pneumonia (optimal incubation pH = 5). The mannose residues present in the trimannosyl chitobiose core can also be removed using β-mannosidase from Helix pomatia (optimal incubation pH = 4.5) and α-mannosidase from Jack bean (optimal incubation pH = 5).

Figure 5b shows an example of such a digestion —an asialo, agalacto, biantennary complex oligosaccharide was treated with all three enzymes at 37 °C for 24 hours. The substrate peak at 9.5 minutes disappears completely and several species appear in the region between 1.5 and 4 minutes. To identify these species, the digestion was also separated using Method 3 and a CarboPac PA1 column for resolution of the chitobiose, the mannose and the

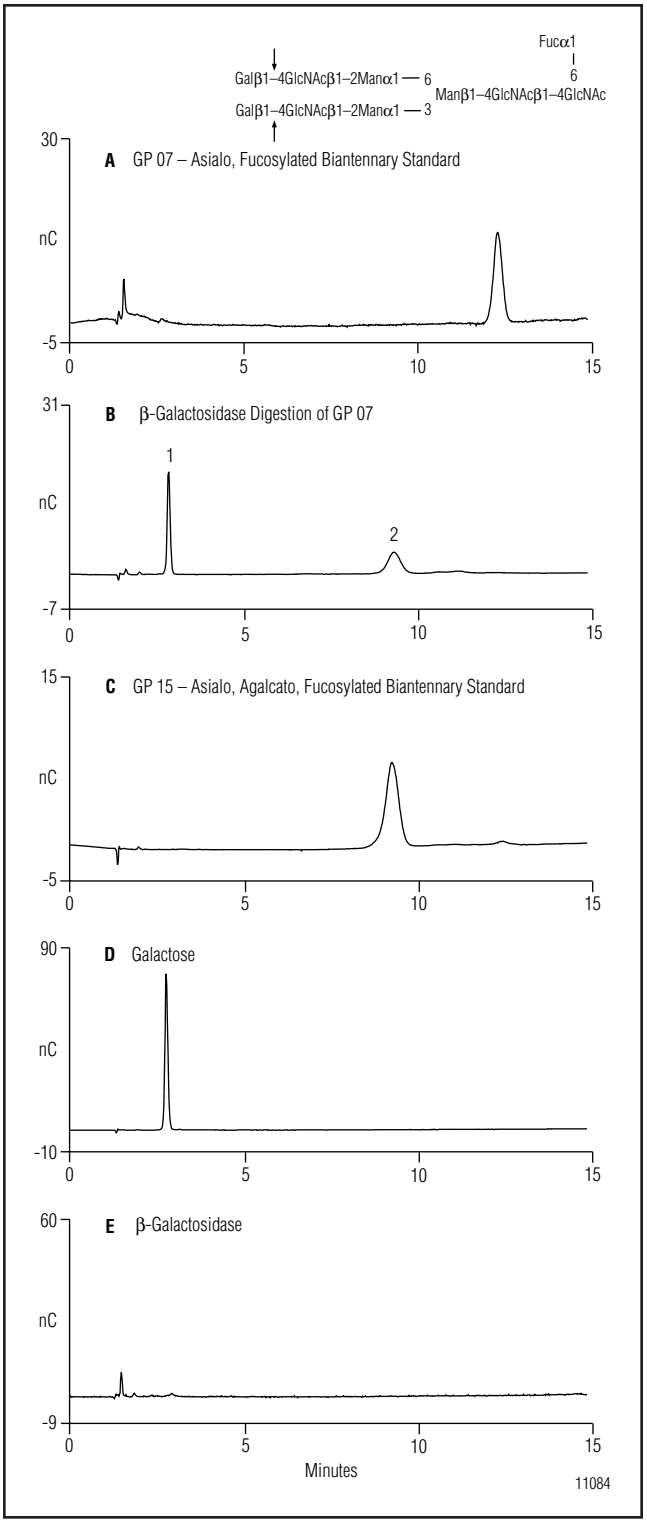

Figure 4. (A) Asialo, biantennary complex oligosaccharide with a fucosylated core (Dionex standard GP 07). (B) Separa-tion of a β-galactosidase digestion of an asialo, biantennary complex-type oligosaccharide with a fucosylated core (Dionex standard GP 07). Digest condition: 30 µL of GP 07 was mixed with 2 mU of β-galactosidase. The mixture was incubated at 37 °C for 24 hours. Injection volume: 10 µL. (C) Asialo, agalacto, fucosylated biantennary oligosaccharide (Dionex standard GP 15). (D) Galactose. (E) Galactosidase. Separation: Method 2, Column: CarboPac PA-100.

GlcNAc residues. As shown in Figure 5c, peaks 1 and 2 coelute with a GlcNAc standard shown in Figure 5d, and a mannose standard shown in Figure 5e, respectively. Peaks 1 and 2, thus represent the released GlcNAc and mannose residues, respectively. Peak 3 coelutes with a chitobiose standard shown in Figure 5f, indicating that it represents the released chitobiose.

Figure 5g shows a chromatogram of an incubation of β-N-acetylhexosaminidase, α-mannosidase, and β-manno- sidase at 37 °C for 24 hours. No peak was detected be-tween 5 to 30 minutes, indicating that none of the three exoglycosidases is acting as a hydrolysis substrate dur-ing the incubation.

3. Exoglycosidase Digestion of Fucosylated Oligosaccharides

α-Fucosidase

Frequently, an N-linked oligosaccharide (oligomannose-, complex- or hybrid-type) is fucosylated at the GlcNAc residue proximal to the asparagine at the glycosylation site of the protein backbone. This fucose can be removed independently using α-fucosidase without

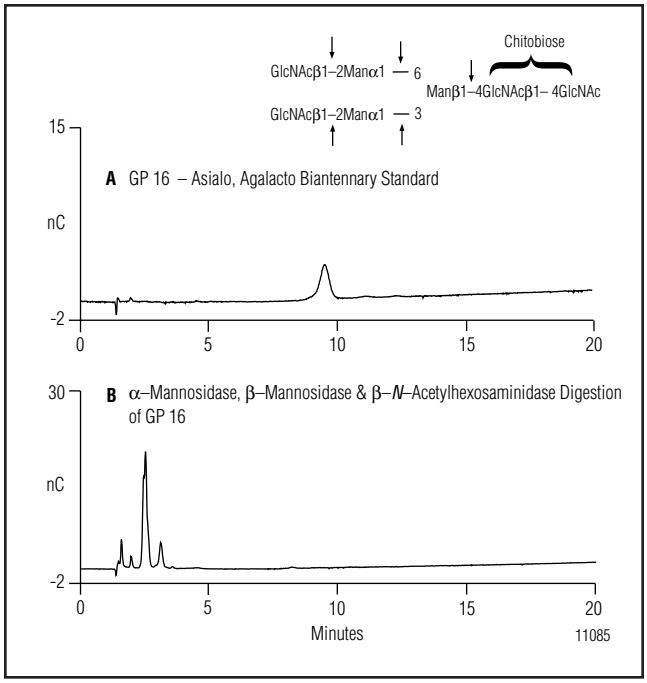

Figure 5. (A) Asialo, agalacto biantennary complex oligosaccharide (Dionex standard GP 16). (B) Separation of a β-N-acetylhexosaminidase, α-mannosidase, and β-mannosidase digestion of an asialo, agalacto, biantennary complex oligosaccharide (Dionex standard GP 16). Digest condition: 30 μL of GP 16 was mixed with 1.5 mU of β-N-acetylhexosaminidase, 20 mU of β-mannosidase, and 100 mU of α-mannosidase. The mixture was incubated at 37 °C for 24 hours. Injection volume: 18 μL; Separation: Method 2, Column: CarboPac PA-100.

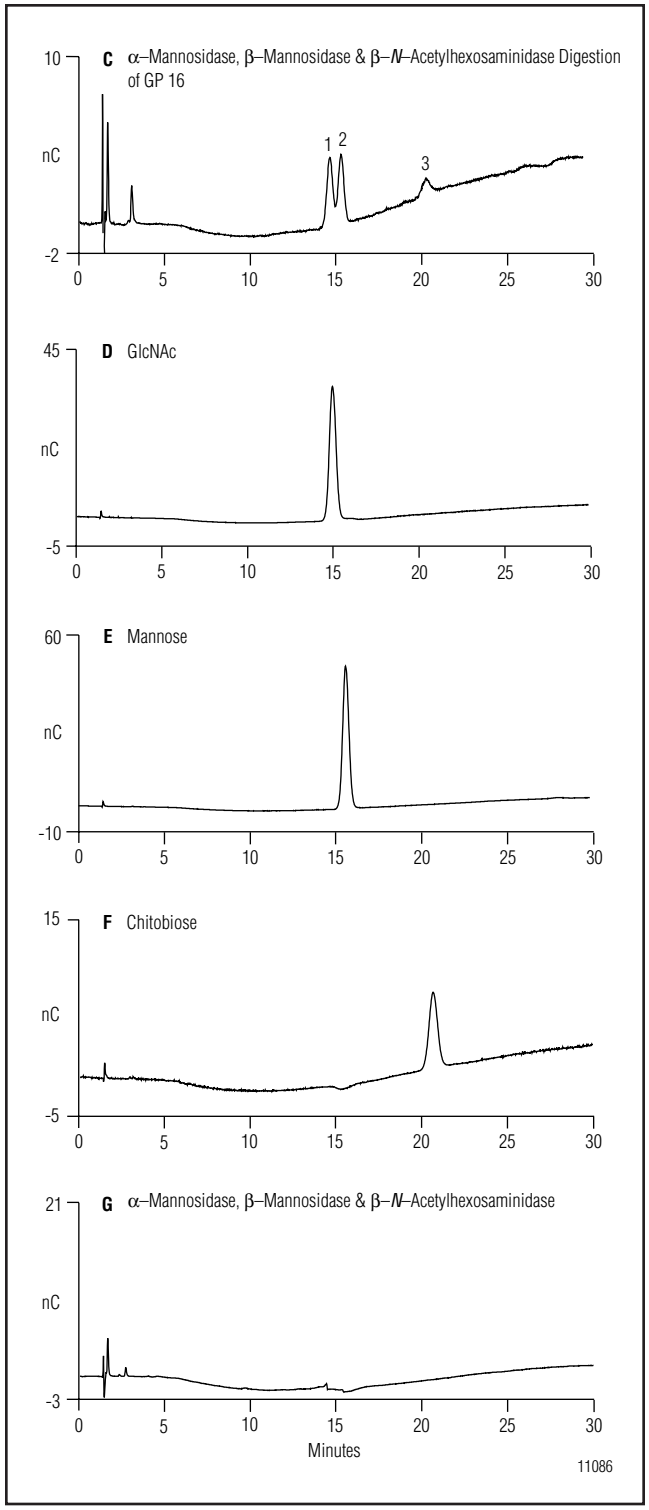

Figure 5 (cont.). (C) Separation of a β-N-acetylhexosaminidase, α-mannosidase, and β-mannosidase digestion of an asialo, agalacto, biantennary complex oligosaccharide (Dionex standard GP 16). Digest condition: Identical digest used for Figure 5b. Injection volume: 10 μL; (D) GlcNAc; (E) Mannose; (F) Chitobiose; (G) β-N-acetylhexosaminidase, α-mannosidase, and β-mannosidase. Separation: Method 3, Column: CarboPac PA1.

removing any monosaccharides attached to the nonreducing terminal of the glycan. Thus, using one exoglycosidase, analysts can determine if an oligosaccharide is fucosylated. This method is particularly useful when a collection of fucosylated and afucosylated oligosaccharides is present in the same sample. Disappearance of one or more peaks after fucosidase digestion, and identification of the afucosylated products and fucose allow an analyst to identify the fucosylated oligosaccharides present in the original sample.

Figure 6b shows the results of an α-fucosidase (from Bovine epididymis) digestion of a fucosylated $Man_3GlcNAc_2$ oligosaccharide (GP 17) after a 24-hour incubation at 37 °C. Peak 1 coelutes with a fucose standard shown in Figure 6d. Peak 2 coelutes with a $Man_3GlcNAc_2$ oligosaccharide standard shown in Figure 6c. The results suggest that the fucosylated $Man_3GlcNAc_2$ oligosaccharide was converted to its corresponding afucosylated analog in a 24-hour incubation.

4. Digestion of Oligomannose-Type Oligosaccharides α-*Mannosidase &* β-*Mannosidase*

Treatment of the oligomannose-type oligosaccharides with appropriate α- and β-mannosidases simultaneously can release all the mannose residues from the chitobiose core. Figure 7b shows a separation of a digestion of a fucosylated $Man_3GlcNAc_2$ oligosaccharide (GP 17) with β-mannosidase (from Jack bean) and α-mannosidase (from Helix pomatia). The substrate peak at 4.1 minutes disappears completely, and several peaks elute in the region between 1 to 4 minutes. This digest was also analyzed using method 3 and the CarboPac PA1 column for better separation of the fucosylated chitobiose and the released mannose residues. As shown in Figures 7c and 7d, peak 2 coelutes with a fucosylated chitobiose standard, and it also elutes approximately 1.5 minutes earlier than the chitobiose peak (see Figure 5f). This result agrees with the observation that fucosylated oligosaccharides elute earlier than their afucosylated analogs.

Figure 7f shows the chromatogram of an incuba-tion of α- and β-mannosidases at 37 °C for 24 hours. No peak was detected between 5 and 25 minutes, indicating neither exoglycosidase is acting as a hydrolysis substrate during the incubation.

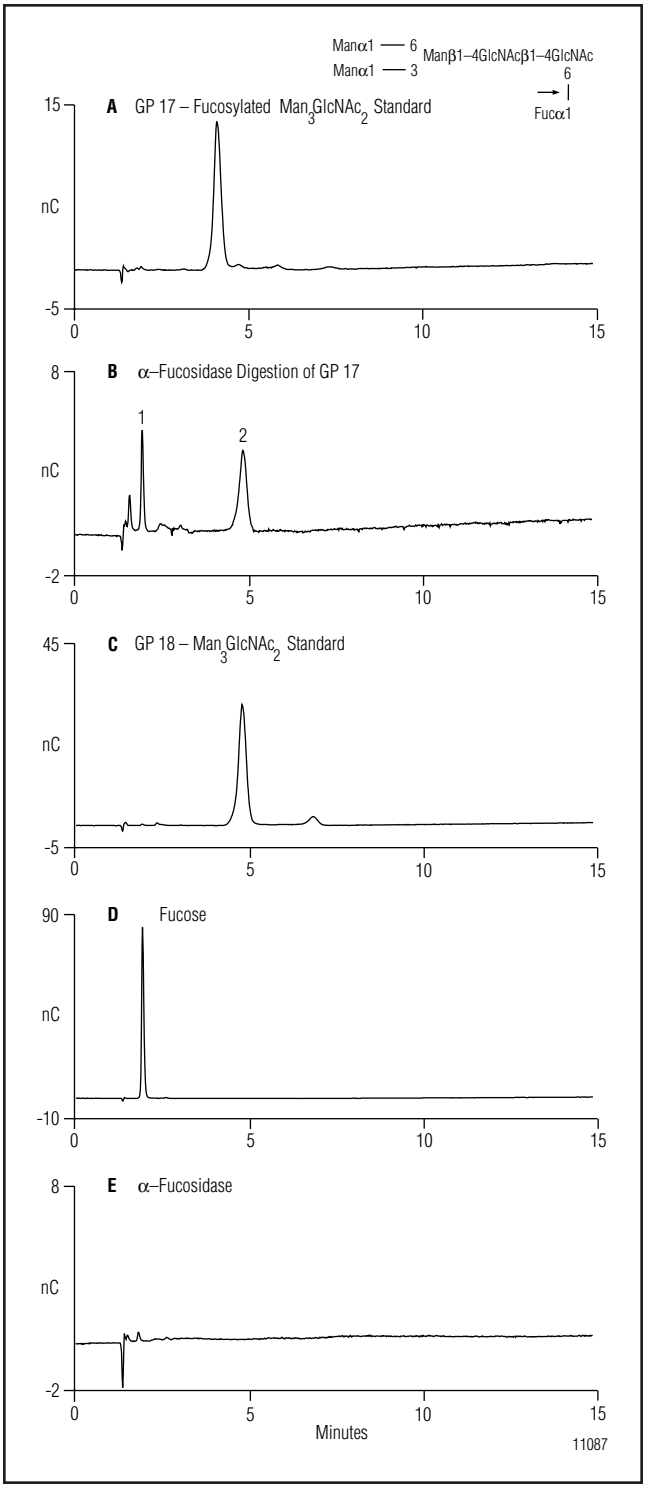

Figure 6. (A) Fucosylated $Man_3GlcNAc_2$ oligosaccharide (Dionex standard GP 17). (B) Separation of an α-fucosidase digestion of a core fucosylated $Man_3GlcNAc_2$ oligosaccharide (Dionex standard GP 17). Digest condition: 30 µL of GP 16 was mixed with 5 mU of α-fucosidase. The mixture was incu-bated at 37 °C for 24 hours. Injection volume: 8 µL. (c) Man_3-$GlcNAc_2$ (Dionex standard GP 18). (D) Fucose. (E) Fucosidase. Separation: Method 2, Column: CarboPac PA-100.

5. Factors Affecting Elution of Oligosaccharides Using the CarboPac PA-100 Column[1, 6–8]

Several factors affect the elution of these oligosaccharides (see Figure 2):

1. Fucosylated oligosaccharides (peaks 1, 3, 7) elute earlier than their afucosylated analogs (peaks 2, 4, 8).
2. As the number of mannose residues in a high mannose oligosaccharide increases, its retention time also increases (peaks 2, 5, 12).
3. As the degree of branching increases, the retention time of the oligosaccharide increases (peaks 8, 9,11).
4. Removal of terminal galactose residues from a complex oligosaccharide reduces its retention time (peaks 3, 4, 6 vs. peaks 7, 8, 9).

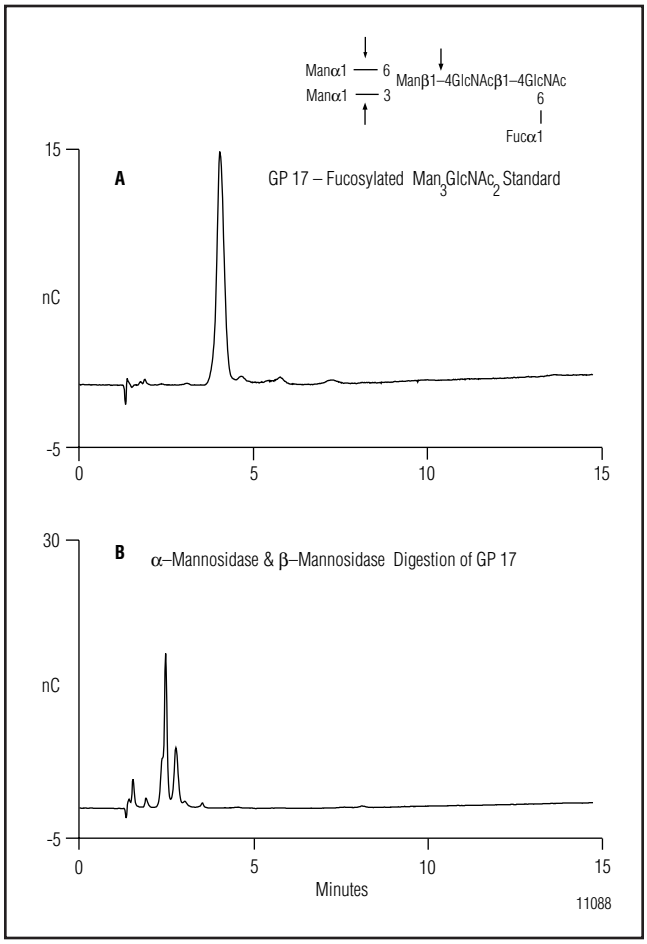

Figure 7. (A) Fucosylated $Man_3GlcNAc_2$ oligosaccharide (Dionex standard GP 17). (B) Separation of an α-mannosidase and β-mannosidase digestion of a core fucosylated $Man_3GlcNAc_2$ oligosaccharide (Dionex standard GP 17). Digest condition: 10 µL of GP 17 was mixed with 10 mU of β-mannosidase and 100 mU of α-mannosidase. The mixture was incubated at 37 °C for 24 hours. Injection volume: 7 µL. Separation: Method 2, Column: CarboPac PA-100.

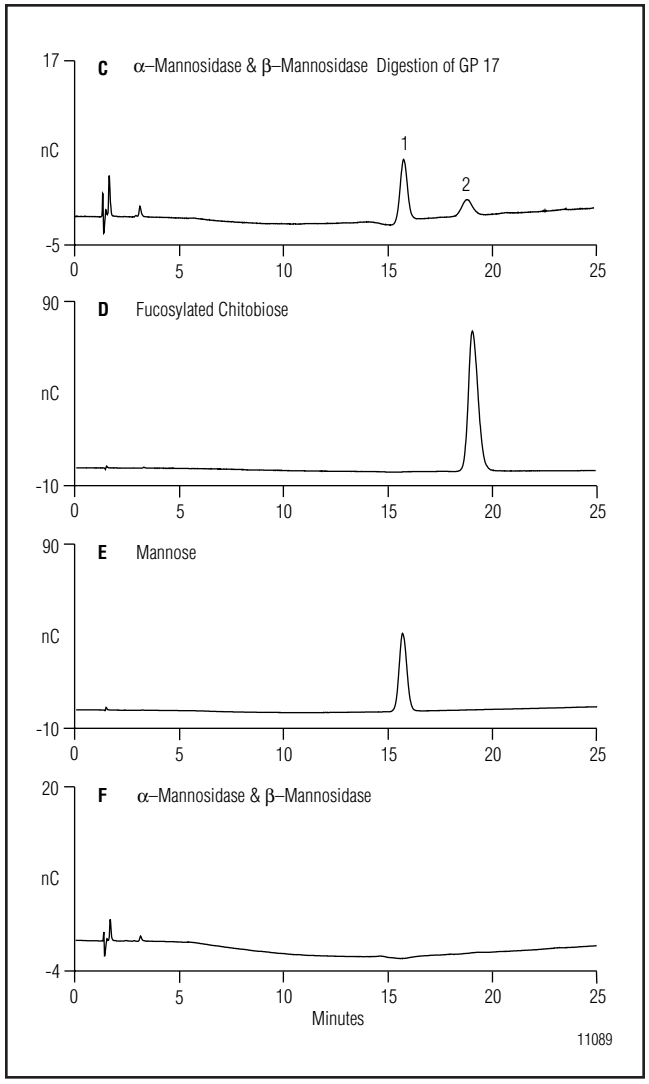

Figure 7 (cont.). (C) Separation of an α-mannosidase and β-mannosidase digestion of a core fucosylated $Man_3GlcNAc_2$ oligosaccharide (Dionex standard GP 17). Digest condition: Identical digest used for Figure 7b. Injection volume: 7 µL. (D) Fucosylated chitobiose. (E) Mannose. (F) α-mannosidase and β-mannosidase. Separation: Method 3, Column: CarboPac PA1.

Other factors have been known to affect the elution of oligosaccharides.[1, 6–8] These "rules" are summarized as follows:

1. A bisected oligosaccharide has a longer retention time compared to its non-bisected oligosaccharide analog.
2. Retention time of an oligosaccharide with a complete chitobiose decreases compared to its analog with one GlcNAc removed from the chitobiose.
3. An oligosaccharide with a Galβ1—›4 GlcNAc linkage elutes earlier than its analog with a Galβ1—›3 GlcNAc linkage; an oligosaccharide with a Neu5Acα2—›6 Gal linkage elutes earlier than its analog with a Neu5Acα2—›3 Gal linkage.
4. A nonreduced oligosaccharide has a longer reten-tion time compared to its reduced analog.

SUMMARY

Using a combination of six exoglycosidases, ter-minal monosaccharides were released from oligosaccharides for analysis. Using the Dionex CarboPac PA1 and PA-100 columns, these released monosaccharides and the remaining oligosaccharides were separated and identified using HPAE-PAD. These oligosaccharides also elute in a predictable manner and several empirical rules can be used to predict the relative times at which they elute.

In every digestion, the appearance of the oligo- and monosaccharide products could be monitored directly by injecting the digestion into the analytical system. Identities of these products were confirmed by a comparison with appropriate standards. Thus, the high resolution of the CarboPac columns coupled with pulsed amperometric detection allows analysts to obtain structural information of *N*-linked glycans without extensive sample clean up.

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Glycoprotein Monosaccharide Analysis Using HPAE-PAD with Eluent Generation

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Key Words

Carbohydrate Analysis, High Throughput, Bovine Fetuin, Human Immunoglobulin (IgG), Gold on PTFE Disposable Electrode

Goal

This study is intended as a guide for investigators interested in validating a high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)-based method for monosaccharide composition analysis. Described here are the configuration, typical performance, and precautions pertaining to use of an HPAE-PAD system for the monosaccharide analysis of glycoproteins. A Thermo Scientific Dionex ICS-5000 system with electrolytically generated eluent and a disposable gold on polytetrafluoroethylene (Au on PTFE) working electrode are used. Reproducibility, linearity, detection limits, and accuracy using common glycoprotein monosaccharides are evaluated.

Introduction

Characterization of glycoproteins routinely involves carbohydrate analysis. Minor variations in glycosylation can affect the efficacy of protein therapeutics.[1] Monosaccharide composition analysis can detect variations in glycosylation, and support quality control for process development and manufacturing procedures. There are over 30 approved glycoprotein-based biodrugs on the market and the number is increasing rapidly. Agencies such as the the U.S. FDA and the European Medicines Agency have increased pressure on biopharmaceutical manufacturers to demonstrate satisfactory programs for understanding, measuring, and controlling glycosylation in glycoprotein-based drugs.

Studies show that HPAE-PAD is a simple and effective way to determine glycoprotein monosaccharide composition without derivatization. In a typical method, monosaccharides are separated on a Thermo Scientific Dionex CarboPac PA10 or PA20 anion-exchange column, preceded by a Thermo Scientific Dionex AminoTrap guard column, using a hydroxide eluent prepared manually or generated by an eluent generator.[2]

Disposable Au on PTFE electrodes have longer lifetimes and can operate at higher hydroxide concentrations compared to earlier-generation disposable working electrodes. They also provide greater system-to-system reproducibility and require no laborious electrode polishing.

Equipment

- Dionex ICS-5000 Ion Chromatography system including:
  - SP Single Pump or DP Dual Pump, Gradient or Isocratic, with Vacuum Degas option
  - EG Eluent Generator Module
  - Potassium Hydroxide Eluent Generator Cartridge (Thermo Scientific Dionex EGC III KOH) (P/N 074532)
  - DC Detector/Chromatography Compartment
  - ED Electrochemical Detector (P/N 072042)
  - Gold on PTFE Disposable Electrode (6 pack, P/N 066480)
  - pH, Ag/AgCl Reference Electrode (P/N 061879)
  - AS or AS-AP Autosampler
  - EO Eluent Organizer and Accessories, including 2 L plastic bottles and pressure regulator
- Thermo Scientific Dionex Chromeleon Chromatography Data System software
- Vial Kit, 0.3 mL Polyprop with Caps and Septa, 100 each (P/N 055428)
- Polypropylene vials with detachable caps (1.5 mL, Sarstedt® P/N 72.692.005 or equivalent)
- Thermo Scientific Nalgene 250 mL HDPE Narrow-Mouth Bottle (P/N 2002-0008)
- Nalgene™ 1000 mL HDPE Narrow-Mouth Bottle (P/N 2002-0032)

Thermo SCIENTIFIC

- Nalgene 250 mL 0.2 µm nylon filter units (P/N 153-0020)
- Nalgene 1000 mL 0.2 µm nylon filter units (P/N 154-0020)
- Thermo Scientific SpeedVac Lyophilization System
- Heating Block (VWR® P/N 13259-005)

Reagents and Standards

- Deionized (DI) water, Type I reagent grade, 18 M -cm resistivity or above, filtered through a 0.2 µm filter immediately before use
- Thermo Scientific Dionex MonoStandard, Mixture of Six (P/N 43162) containing the following monosaccharides (in order of elution): fucose (Fuc), galactosamine (GalN), glucosamine (GlcN), galactose (Gal), glucose (Glc), and mannose (Man)
- IgG from human serum (Sigma-Aldrich® P/N I-4506)
- Fetuin from fetal calf serum (Sigma-Aldrich P/N F2379-250MG)
- Thermo Scientific Pierce Trifluoroacetic Acid (TFA), sequencing grade for making 0.1% v/v TFA solutions (P/N 28904)
- Thermo Scientific Pierce Hydrochloric Acid (P/N 24308)
- Thermo Scientific Pierce Micro BCA Protein Assay Kit (P/N 23235)

Conditions

Method

Columns:	Dionex CarboPac™ PA20 Analytical, 3 × 150 mm (P/N 060142) Dionex AminoTrap™, 3 × 30 mm (P/N 060146)
Eluent:	10 mM KOH
Eluent Source:	Thermo Scientific Dionex CR-ATC Continuously Regenerated Anion Trap Column EGC III KOH
Flow Rate:	0.5 mL/min
Inj. Volume:	10 µL (partial loop injection mode with a 4 µL cut volume)
Column Temp.:	30 °C
Cell Temp.:	30 °C
Backpressure:	2200 psi
Detection:	PAD
Background:	30–50 nC
Working Electrode:	Carbohydrate PTFE Disposable Au Working Electrodes
Reference Electrode:	Mode: Ag/AgCl mode Noise: 10–30 pC

Carbohydrate Waveform

Carbohydrate four-potential waveform for the ED:

Time (s)	Potential (V)	Gain	Ramp* Region*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	-2.0	Off	On	Off
0.42	-2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.50	-0.1	Off	On	Off

*Settings required in the Dionex ICS-3000/5000 systems but not used in older Dionex systems

Preparation of Solutions and Reagents

Eluent Solution

Generate the potassium hydroxide (KOH) eluent on line by pumping high-quality degassed DI water (no biological contamination) through the Dionex EGC III KOH cartridge. Chromeleon™ software tracks the amount of KOH used and calculates the remaining cartridge lifetime. Although eluents can be manually prepared if needed, reproducibility will be compromised because consistent preparation of a 10 mM hydroxide eluent is difficult due to variable carbonate contamination. The impact of carbonate contamination is significant when using low concentration hydroxide eluents. If eluents must be prepared manually, use NaOH rather than KOH and prepare according to the general instructions for hydroxide eluents in Dionex (now part of Thermo Scientific) Technical Note (TN) 71.[3] For this application, electrolytic eluent generation delivers superior performance. Keep the eluent water blanketed under 8–10 psi of nitrogen at all times to reduce diffusion of atmospheric carbon dioxide into the eluent water.

Carbohydrates Standards

Dissolve the contents of one Dionex MonoStandard™ 100 nmol vial in 1.0 mL of DI water and mix to prepare a stock standard solution containing 0.1 mM (100 pmol/µL) of each monosaccharide. Immediately freeze unused stock standard at <-10 °C. Avoid repeated freeze/thaw cycles. Deterioration can occur within 24–48 h at room temperature.

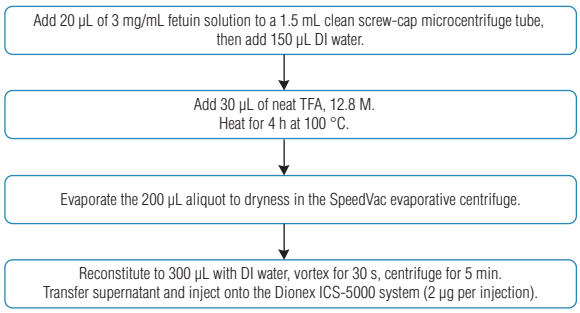
Working Standard Solutions

Use DI water to prepare appropriate dilutions of stock standard for calibration as needed. For example, prepare the 10 µM standard by diluting the stock (0.1 mM) 10-fold.

Glycoprotein Acid Hydrolysates

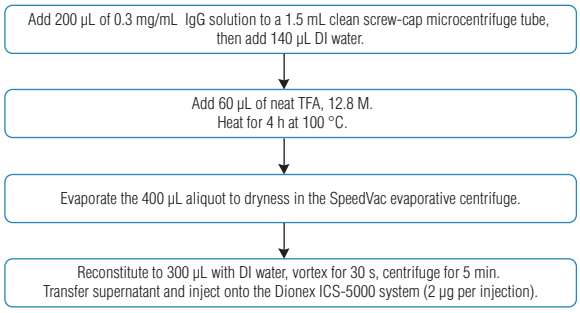
Determine the protein concentration by performing the Micro BCA™ assay.[4] The TFA hydrolysis is optimized for neutral sugar (Fuc, Gal, Glc, Man) recovery, whereas the HCl hydrolysis is optimized for amino sugar (GalN, GlcN) recovery.[5] Estimate the appropriate amount of protein to inject by considering the degree of glycosylation. As a guideline, the amount of protein to inject = (10 µg/x), where x = % glycosylation of the protein. For example, if the protein is 3% glycosylated, (10 µg/3) = 3.3 µg of protein. To inject 3.3 µg of hydrolyzed protein using a 10 µL injection requires a sample concentration of 0.3 µg hydrolyzed protein/µL (i.e., 0.3 mg/mL). It is convenient to prepare a stock standard that is 10 times this protein concentration (3 mg/mL). Once sample chromatography has been established, the best long-term results are achieved by injecting the lowest amount protein that yields monosaccharide peaks safely above the limit of quantification.

Schemes 1 and 2 describe the hydrolysis steps for fetuin and IgG. Prepare TFA hydrolysates of fetuin by combining 20 µL of 3 mg/mL fetuin solution, 150 µL DI water, and 30 µL of TFA (12.8 M) in a 1.5 mL microcentrifuge tube. For HCl fetuin hydrolysates, add 6 M HCl. Prepare HCl hydrolysates for IgG by combining 400 µL of 6 M HCl with 20 µL of 3 mg/mL IgG. Prepare TFA hydrolysates of IgG by combining 200 µL of 0.3 mg/mL IgG, 140 µL of DI water, and 60 µL of neat TFA. Heat the solutions for 4 h at 100 °C and then dry in a SpeedVac™ concentrator for ~3 h. Reconstitute each vial with 300 µL of DI water, vortexed for 30 s and centrifuged for 5 min. Inject 10 µL of the supernatant into the ion chromatography system.



Note: 6 M HCl also can be used for hydrolyzing fetuin. Combine 400 µL of 6 M HCl with 20 µL of 3 mg/mL fetuin solution. This is recommended for amino sugars and can improve their recovery by 30-57%; however, fetuin can potentially degrade neutral sugars.

Scheme 1.



Note: 6 M HCl also can be used for hydrolyzing IgG. Combine 400 µL of 6N HCl with 20 µL of 3 mg/mL IgG.

Scheme 2.

Guidelines and Precautions

Columns

To ensure that the column and system are operating correctly, the Dionex CarboPac PA20 Analytical column must be tested using the same conditions as described in the quality and reliability test report. Maintain the column set under a controlled temperature to reduce variations in peak area and retention times.

EG Module

To function properly, the EG module requires backpressure in the range of 2000 to 3000 psi.

Dionex CR-ATC Continuously Regenerated Anion Trap Column

The detector baseline signal typically will be between 30 and 50 nC at 0.5 mL/min of 10 mM KOH with the system described here. A high baseline may be due to malfunction or improper configuration of the degas module, or the presence of electrochemically active impurities in the water. Change the Dionex CR-ATC column when the KOH cartridge is changed.

Working Electrodes and Electrochemical Response

Replace the reference electrode every three to six months. Indications of a failed reference electrode are a pH reading >12.5 or the absence of any reading for 10 mM KOH eluent and the inability to calibrate the reference electrode. Disposable electrodes must always be replaced when they have been used with a failed reference electrode. Upon installation of a new working electrode, allow 1–2 h for the background to stabilize. Refer to Dionex (now part of Thermo Scientific) TN 110[6] for lifetime, background, and noise characteristics of the disposable Au on PTFE working electrodes.

System conditions resulting in low peak area (i.e., lower method sensitivity) can be due to contaminated eluents, a fouled electrode, a failed reference electrode, or incorrect waveform. Other factors that are not related to the detection can also cause lower response, such as incorrectly prepared standards or a malfunctioning autosampler.

Dionex AminoTrap Column

The Dionex AminoTrap column delays the elution of amino acids and small peptides found in glycoprotein hydrolysates and is used in place of a guard column before the Dionex CarboPac PA20 column. Install the Dionex AminoTrap column after the injection valve and condition by flushing with 100 mM KOH at 0.5 mL/min for 2 h. Although slight peak broadening and longer retention times are expected with the addition of the Dionex AminoTrap column (compared to those obtained with the analytical column), good resolution of the six monosaccharides still will be observed. *Do not pump water through the Dionex AminoTrap column; it will damage the column irreversibly.*

Acid Hydrolysis

Include at least one blank, substituting reagent water for protein sample, each time the hydrolysis procedure is performed.

Autosampler

Dionex ICS-5000 autosamplers have three modes of injection. For the highest peak area or height precision, the Full-Loop mode of injection is recommended; however, sample use is higher (e.g., 50 µL for a 10 µL injection) than the Partial-Loop mode of injection. For highest conservation of sample, use the Partial-Loop Limited-Sample mode of injection (e.g., 10 µL sample for a 10 µL injection). All results presented here were obtained using the Partial-Loop mode of injection with a 4 µL cut volume. When Partial-Loop mode of injection is chosen, the sample loop volume must be at least twice the injection volume for best accuracy. These modes of injection are available with the Dionex ICS-5000 AS and AS-AP autosamplers.

To prevent baseline disturbances and electrode fouling, do not use organic solvents in the wash reservoir. Replace rinse water (DI water) frequently. The injector syringe and the transfer line must be free of bubbles to ensure accurate injection volumes.

Results and Discussion

Separation

Figure 1 shows a typical separation of a 10 µL injection of the Dionex MonoStandard containing Fuc, GalN, GlcN, Gal, Glc, and Man at 10 µM each. The peaks are baseline resolved and elute within a window of 13 min. The total run time is 24 min to allow for stabilization of the baseline after the column regeneration step. When protein hydrolysate samples are analyzed, the Dionex CarboPac PA20 and AminoTrap columns must be regenerated with 100 mM KOH at 0.5 mL/min for ≥3 min following each run. A periodic 2 h wash with 100 mM KOH at 0.5 mL/min removes the more strongly retained compounds expected in these samples. In certain cases, complete restoration of column capacity will require the use of stronger premade eluents (for example, 200 mM NaOH for 2 h) in an off-line system mode.

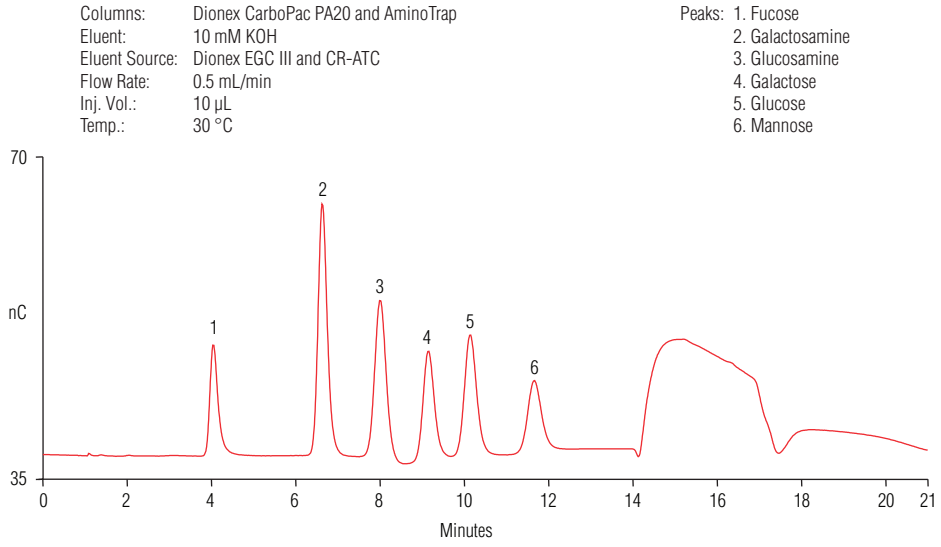


Figure 1. Monosaccharide standards on the Dionex CarboPac PA20 and AminoTrap columns

Figure 2 presents the HPAE-PAD chromatograms of bovine fetuin acid hydrolysates. The monosaccharide peaks are baseline resolved, the neutral monosaccharides are seen at higher concentrations in the TFA hydrolysate, and the amino sugars at a higher concentration in the HCl hydrolysate, as expected. These examples have much more protein injected than is needed. Five- to 10-fold less protein can be injected with good results.

Figure 3 shows a typical injection of IgG acid hydrolysates (TFA and HCl). Human serum IgG has lower carbohydrate content relative to most mammalian glycoproteins. The low concentration of carbohydrate makes monosaccharide determination more challenging due to the higher concentration of peptides and amino acids in the acid hydrolysate relative to glycoproteins with higher percentages of glycosylation.

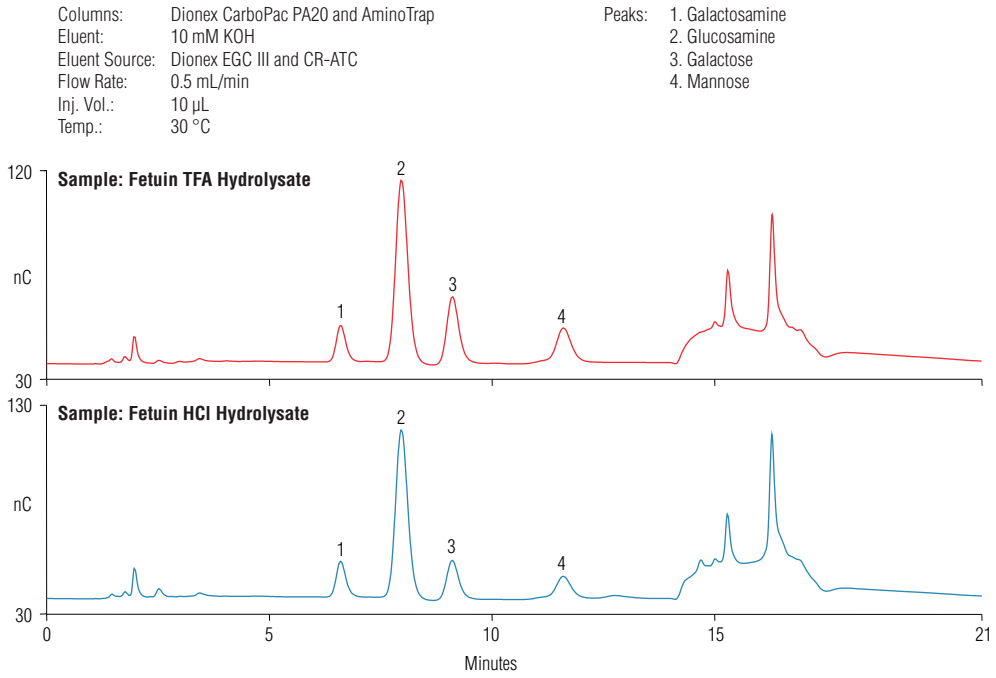


Figure 2. Monosaccharide composition analysis of bovine fetuin: the HCl hydrolysis condition was milder than Scheme 1 (20 µL of 3 mg/mL bovine fetuin with 30 µL of 6 M HCl).

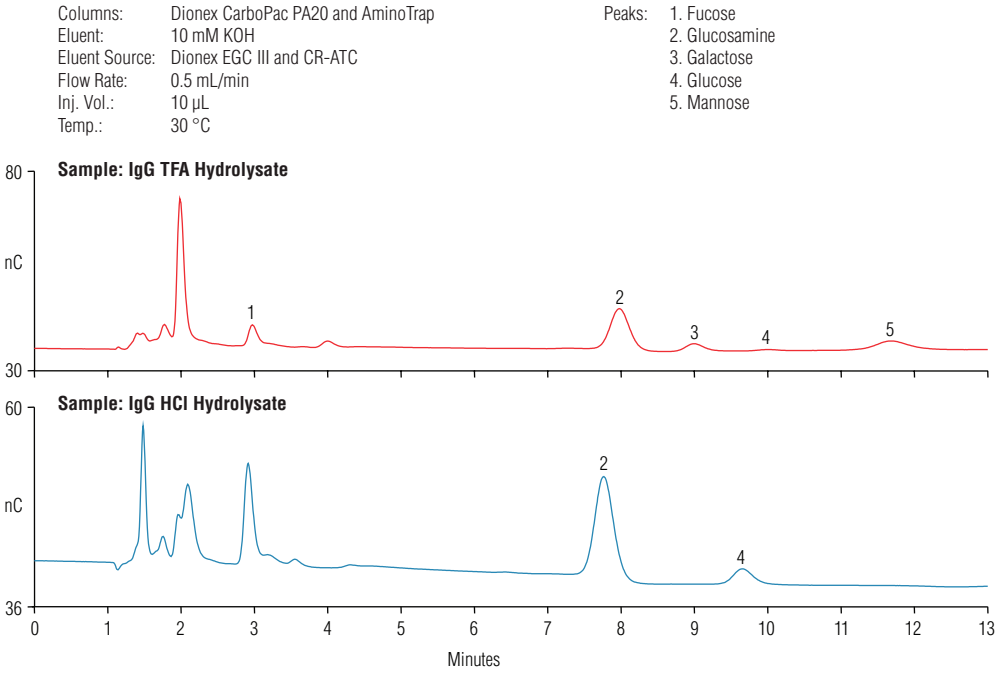


Figure 3. Monosaccharide composition analysis of human serum IgG

Reproducibility with Interspersed Samples

The ruggedness of the system was tested by evaluating the peak area and retention time stability of each monosaccharide in a 10 µM injection of the Dionex MonoStandard interspersed with ten injections of hydrolysates (2 µg protein load per injection). The sequence of one injection of 10 µM Dionex MonoStandard, five injections of fetuin TFA hydrolysates, five injections of fetuin HCl hydrolysates, and one injection of 10 µM Dionex MonoStandard was repeated for seven days (Figure 4). Figures 4 and 5 show selected chromatograms of the monosaccharide standard from the seven-day analysis interspersed with 2 µg protein/injection of fetuin and IgG acid hydrolysates, respectively.

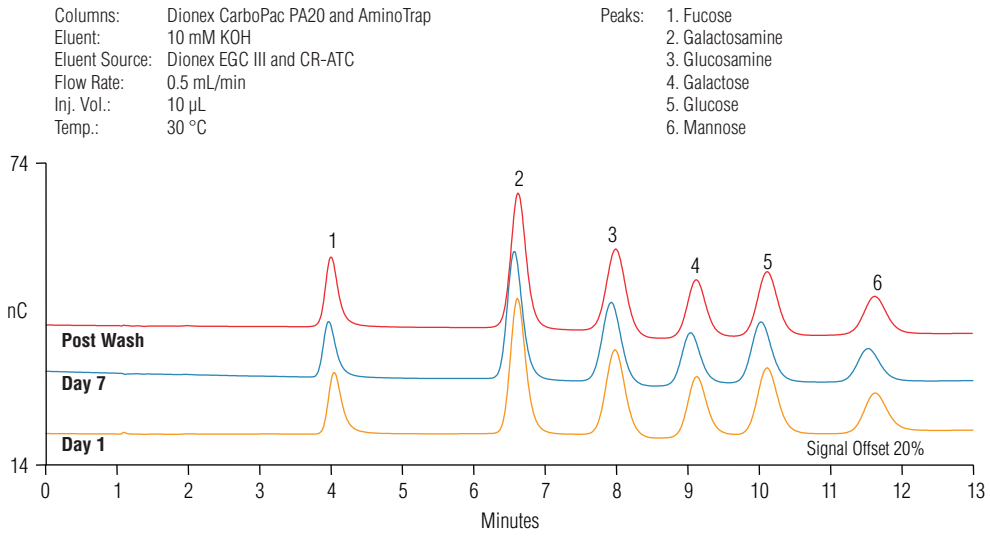


Figure 4. Monosaccharide standards: seven-day analysis interspersed with 2 µg/injection load of fetuin acid hydrolysates

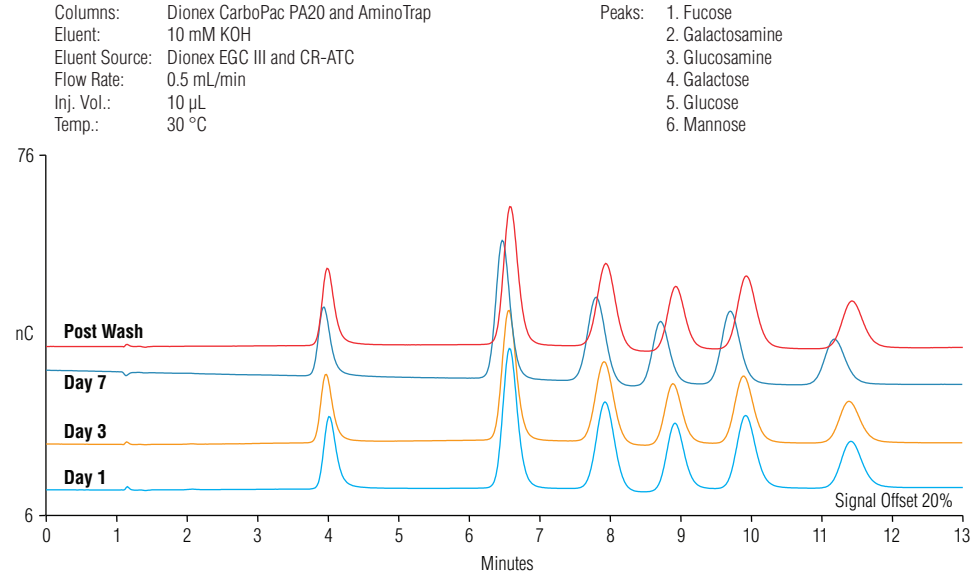


Figure 5. Monosaccharide standards: seven-day analysis interspersed with 2 µg/injection load of IgG acid hydrolysates

Table 1. Precision and percent change of retention time over seven days of fetuin hydrolysate analysis

	Retention Time RSD			Retention Time (min)			% Change
Analyte	Day 1	Day 7	Over 7 Days	Day 1	Day 7	After Wash on Day 7	After Wash[a]
Fucose	<0.01	0.15	0.69	4.04	3.95	4.00	-1.04%
Galactosamine	0.09	0.09	0.35	6.61	6.55	6.62	0.09%
Glucosamine	0.07	0.08	0.35	7.98	7.90	7.99	0.10%
Galactose	0.06	0.07	0.49	9.13	8.99	9.11	-0.15%
Glucose	0.06	0.06	0.44	10.11	9.97	10.11	-0.02%
Mannose	0.05	0.05	0.45	11.63	11.46	11.62	-0.09%

[a]2 h 100 mM KOH

Table 2. Precision and percent change of peak area over seven days of fetuin hydrolysate analysis

	Peak Area RSD			Peak Area			% Change
Analyte	Day 1	Day 7	Over 7 Days	Day 1	Day 7	After Wash on Day 7	After Wash[a]
Fucose	0.80	3.30	4.43	2.80	2.68	2.90	3.57%
Galactosamine	1.66	0.45	2.15	6.81	6.90	7.09	4.10%
Glucosamine	1.14	0.32	2.42	5.26	5.42	5.53	5.13%
Galactose	1.49	0.93	3.46	3.46	3.38	3.54	2.47%
Glucose	1.13	0.35	2.57	4.20	4.23	4.29	2.25%
Mannose	1.90	0.22	4.27	2.80	2.64	2.99	6.68%

[a]2 h 100 mM KOH

Tables 1 and 2 summarize the precision and percent change of retention time and peak area data over seven days of a protein load of 2 µg/injection of fetuin hydrolysates. Decrease in retention time ranged from -1.0 to -2.2% over time. The loss of up to 2.2% of retention time is extremely low and all peaks were separated and easily quantified, even after over 300 injections. Single-day and seven-day retention time RSDs are presented, but with the note that their value is limited because of the slight negative trend in the data. Peak area changes ranged from 0.65 to -5.82%. The peak area RSD ranged from 2.15 to 4.43%. Figure 4 shows chromatography from the reproducibility studies with fetuin hydrolysates, including a chromatogram after a 2 h 100 mM KOH column wash that was executed to restore the retention time. As noted in the previous archived version of TN 40, partial retention time restoration is achieved with a 100 mM base wash, and complete restoration of column capacity will require the use of premade eluents in an off-line system mode.

Table 3. Precision and percent change of retention time over seven days of IgG hydrolysate analysis

	Retention Time RSD			Retention Time (min)			% Change
Analyte	Day 1	Day 7	Over 7 Days	Day 1	Day 7	After Wash on Day 7	After Wash[a] on Day 7
Fucose	0.24	0.00	0.62	3.99	3.93	3.99	0.08%
Galactosamine	0.15	0.00	0.76	6.58	6.45	6.59	0.08%
Glucosamine	0.12	0.00	0.80	7.94	7.77	7.94	0.04%
Galactose	0.14	0.63	1.02	8.95	8.67	8.93	-0.16%
Glucose	0.15	0.61	0.80	9.95	9.65	9.93	-0.16%
Mannose	0.11	0.60	0.98	11.45	11.12	11.43	-0.19%

[a]2 h 100 mM KOH

Table 4. Precision and percent change of peak area over seven days of IgG hydrolysate analysis

	Peak Area RSD			Peak Area			% Change
Analyte	Day 1	Day 7	Over 7 Days	Day 1	Day 7	After Wash on Day 7	After Wash[a] on Day 7
Fucose	0.60	2.90	3.94	3.06	2.96	3.20	4.67%
Galactosamine	0.29	1.24	2.50	6.47	6.41	6.75	4.28%
Glucosamine	0.56	0.89	2.92	5.16	5.15	5.17	0.06%
Galactose	0.89	0.89	4.43	3.76	3.58	3.64	-3.23%
Glucose	0.81	0.76	1.13	4.45	4.46	4.58	2.83%
Mannose	0.35	0.01	3.80	3.14	3.14	3.33	5.86%

[a]2 h 100 mM KOH

Tables 3 and 4 summarize the precision and percent change of retention time and peak area data over seven days of a protein load of 2 µg/injection of IgG hydrolysates. Similar to the fetuin data, retention time decrease ranged from -1.5 to -3.0% over the seven days. Peak area changes ranged from 0.9 to -4.8%. The peak area RSD ranged from 1.13 to 4.43%. As noted for fetuin, single-day and seven-day retention time RSDs are presented, but with the note that their value is limited because of the slight negative trend in the data.

In both hydrolysate experiments (fetuin and IgG), retention time gradually decreased over seven days. This could be due to the accumulation of a portion of the hydrolysate sample matrix on the column, resulting in a slight loss of column capacity. Decreases in retention times ranged from 1 to 3%. As shown in Tables 1 and 3, the largest actual increase in retention time was 0.33 min (mannose in the IgG experiment), which did not result in any peak coelution (Figure 5).

Overall, the data is similar to or better than data reported in the previous archived version of TN 40, which was collected using a Dionex ICS-3000 system. Note that the Au on PTFE disposable working electrodes provide the advantage of increased lifetime compared to the previously used disposable Au on polyester (P/N 060139) working electrode. Disposable Au working electrodes on the polyester substrate are limited to 100 mM hydroxide as the maximum eluent strength, and have a lifetime of two weeks. After two weeks of use, there is a possibility of delamination of the electrode from the substrate, resulting in a loss in response. Gold on PTFE electrodes do not delaminate from the substrate at hydroxide concentrations >100 mM and have consistent response for at least four weeks and excellent electrode-to-electrode reproducibility.[6]

Ruggedness

The variance due to different columns was tested by comparing results from columns from two different lots. Both column sets showed similar precisions and trends in retention time and peak area.

Linearity

Response curves were generated for the six monosaccharides between 1 and 700 µM (Figure 6). The upper limit of linearity for each monosaccharide is shown in Table 5. Fucose and galactosamine are linear through 170 µM, glucosamine is linear up to 210 µM, galactose up to 420 µM, glucose up to 210 µM, and mannose up to 420 µM. Above these concentrations, a >10% decrease in response (relative to response predicted by the other concentrations) was observed. These concentrations reflect the upper limits found when a mix of monosaccharides was tested. The upper limits result due to exceeding either the column capacity or the limit of available electrolytic sites on the Au electrode.

Detection Limits

Lower limits of detection were determined for monosaccharides by injecting small quantities (0.025, 0.25, 0.5, and 1 µM) of Dionex MonoStandard. At 0.08–0.17 µM, 10 µL injection (i.e., at 0.8–1.7 pmol), signal-to-noise ratios for the monosaccharides were at least 3:1. Detection limits may be improved through larger injection volume (provided the column is not overloaded).

Conclusion

The HPAE-PAD-based method on a Dionex ICS-5000 chromatography system for monosaccharide composition analysis is fast and capable of providing reproducible retention time and detector response for hundreds of samples over several days. The disposable Au working electrode contributes to the reproducibility of PAD between electrodes and between laboratories. Overall, the method has high sample throughput, high precision, and performance ruggedness for glycoprotein monosaccharide analysis.

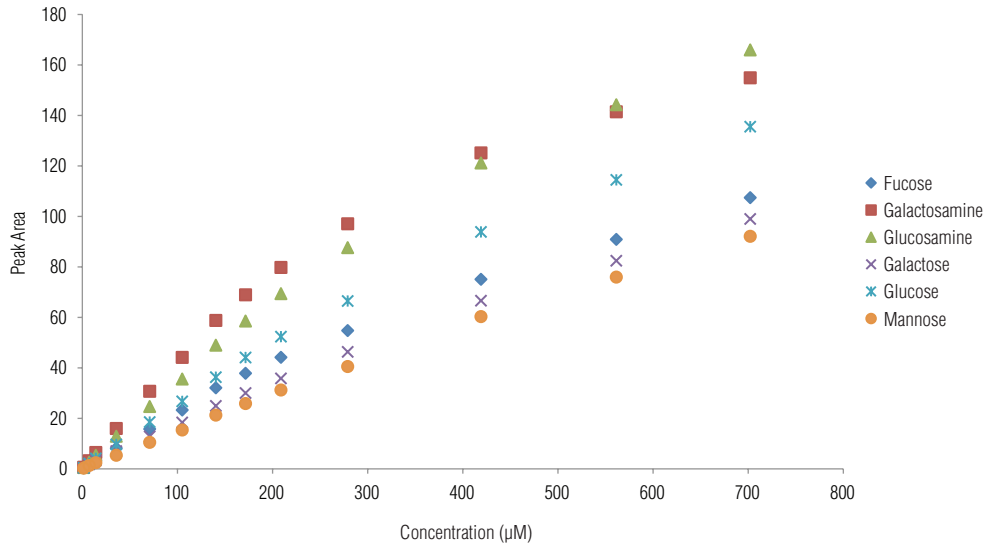


Figure 6. Plot of concentration vs response for the monosaccharides

Table 5. Method lower limit of detection and upper limit of linearity

	Lower Limit of Detection (µM)	Upper Limit of Linearity (µM)
Fucose	0.17	170
Galactosamine	0.08	170
Glucosamine	0.10	210
Galactose	0.14	420
Glucose	0.11	210
Mannose	0.16	420

Suppliers

VWR, 1310 Goshen Parkway, West Chester, PA 19380, U.S.A., Tel: 800-932-5000.

Sigma-Aldrich Co., P.O. Box 2060, Milwaukee, WI 53201, U.S.A., Tel: 800-558-9160.

Sarstedt Inc., 1025 St. James Church Road, P. O. Box 468, Newton, NC 28658-0468, U.S.A., Tel: 828-465-4000.

References

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Glycoprotein Oligosaccharide Analysis Using High-Performance Anion-Exchange Chromatography

INTRODUCTION

The development of recombinant-derived glycoproteins for therapeutic use has led to an increasing demand for methods to characterize their carbohydrate structures. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) has been widely used to characterize carbohydrates because the method allows rapid and direct quantification of underivatized samples. HPAE-PAD not only separates oligosaccharides according to charge, but can also resolve oligosaccharides with the same charge according to size, sugar composition, and linkage of monosaccharide units.

Oligosaccharides can be characterized through coelution with standards or through retention time comparison. Empirical relationships between oligosaccharide structure and chromatographic retention for HPAE-PAD have been described.[1] Oligosaccharides separated by HPAE-PAD are frequently collected postcolumn for further structural analysis using mass spectrometry and NMR.

In the pharmaceutical and biotechnology industries, HPAE-PAD mapping techniques for glycoprotein carbohydrate structures have been used: 1) during initial characterization to separate and identify the oligosaccharide structures present; 2) to monitor consistency of glycosylation and identify changes that may have resulted from alterations in cell culture conditions or during the manufacturing process; and 3) to monitor changes in glycosylation that occur as a result of expression in different cell lines. A short list of articles in which HPAE-PAD is used for mapping therapeutic glycoproteins is provided in the Appendix section.

In this Technical Note, accuracy, precision, linearity, and limits of detection are documented for a commercially available mix of bovine fetuin N-linked oligosaccharide alditols using an HPAE-PAD method currently in use for quality control at a biotechnology company. These results are intended as a guide for investigators who need to validate the performance of HPAE-PAD for oligosaccharide mapping.

EQUIPMENT

Dionex DX-500 BioLC® system consisting of:

- LC30 Chromatography Module
- GP40 Gradient Pump with On-line Degas
- ED40 Electrochemical Detector, gold electrode
- AS3500 Autosampler or an 8880 Autosampler

PeakNet Chromatography Workstation

GP40 pump performance can be validated using the GP40 Validation Kit (Dionex P/N 50809), while ED40 detector performance can be validated with the ED40 Validation Kit (P/N 49046).

The system should be properly installed according to relevant operating manuals for each component. The accuracy and precision of the autosampler is validated using a 100 µL loop and injecting fourfold 5, 10, 15, 25, 50 µL of a solution of 10 µM glucose onto a CarboPac™ PA10 or PA-100 column followed by elution with isocratic 100 mM sodium hydroxide at 1.5 mL/minute. The coefficient of determination (r^2) generated from a linear regression analysis of a plot of peak area versus volume should indicate linearity ($r^2 > 0.99$). The CarboPac PA-100 performance should be validated by reproducing the test chromatograms provided with the column.

REAGENTS AND STANDARDS

Deionized water, HPLC-grade

Sodium Hydroxide, 50% (w/w) (Fisher Scientific)

Fetuin Oligosaccharide Alditol Standards (Dionex, P/N 43064)

Asialo- and Disialylated Galactosylated Biantennary Oligosaccharide Standards (Oxford GlycoSciences)

CONDITIONS

Column: CarboPac™ PA-100 Analytical Column (4 × 250 mm) with guard

Flow rate: 1.0 mL/min

Detection: ED40 Electrochemical Detector, gold electrode

Inj. Vol.: 10 µL

Waveform: Carbohydrate waveform (reference Dionex Technical Note 21 for most current waveform)

Eluent: A: 100 mM Sodium hydroxide

B: 100 mM Sodium hydroxide/0.5 M Sodium acetate

Method:

Time (min)	% A	% B
Initial	99	1
0.0	99	1
0.2	99	1
10.0	90	10
50.0	55	45
50.1	0	100
55.0	0	100
55.1	99	1
70.0	99	1

In this separation method, the initial conditions include 1% eluent B to prevent a baseline disturbance that occasionally occurs when the column is converted from the hydroxide to the acetate form. This method was developed in a quality control laboratory at a biotechnology company. We believe this method will be more reproducible if eluent A is changed from 100 mM NaOH to 100 mM NaOH/5 mM NaOAc and all method times that use 99% A and 1% B are changed to 100% A.

A used, gold electrode that had not been recently polished was used for the experiments in this Technical Note.

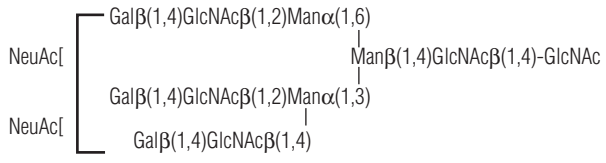
Gradient Optimization

The gradient shown above efficiently separates sialylated fetuin oligosaccharide alditols. Because HPAE separations are by ion exchange, the method is very flexible and gradients should be optimized for the particular separation. The development of two "optimized" gradients, one for the separation of sialylated N-glycans and a second gradient for the separation of asialo N-glycans, has been reported.[2] For the separation of neutral N-linked oligosaccharides, an increase in eluent pH has been reported to improve the separation.[3]

HPAE-PAD commonly uses strongly alkaline eluents. However, there are occasions when neutral or low pH eluents are preferable. For example, low pH eluents provide better resolution of sialylated and phosphorylated oligosaccharides.[4] Use of low pH eluents necessitates the addition of postcolumn base for detection by pulsed amperometry. It has been reported that high pH conditions are required for the optimum separation of fetuin oligosaccharides, while low pH significantly improves resolution of oligosaccharides obtained from orosomucoid, human chorionic gonadotropin, platelet-derived growth factor, and kallikrein.[5] A gradient in which only 0.5 mM sodium hydroxide and 3% sodium acetate (3% acetic acid titrated with 50% sodium hydroxide

Table 1 Major carbohydrate structures of bovine fetuin

1.



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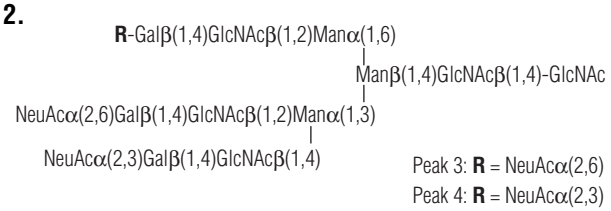

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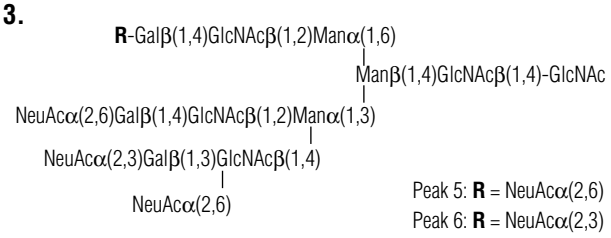

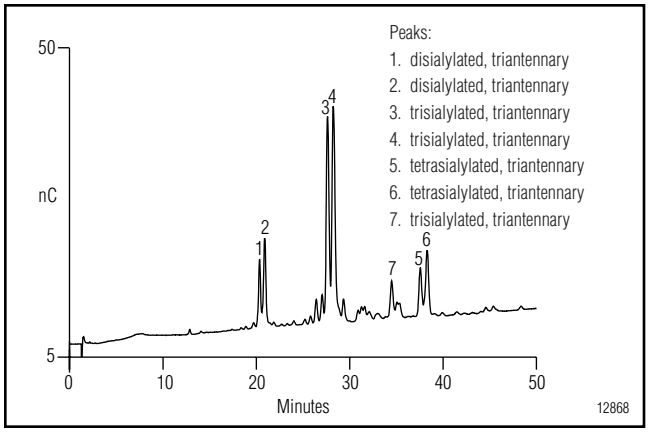

Figure 1. Fetuin N-linked oligosaccharide alditols separated on the CarboPac PA-100 column.

to pH 5.5) were used for gradient generation, has been shown to be useful for the separation of mono- as well as oligosaccharides.[6]

Reducing vs. Reduced Oligosaccharides

In 100 mM sodium hydroxide, a condition frequently used for HPAE elution, there may be measurable epimerization of terminal GlcNAc to ManNAc. Thus, some highly retained oligosaccharides with GlcNAc or GalNAc at the reducing terminus may suffer epimerization during the course of HPAE-PAD chromatography. Although epimerization is usually insignificant, some investigators choose to reduce oligosaccharides to the corresponding alditol prior to HPAE-PAD chromatography because they have observed better-defined peak shapes.[7–8]

PREPARATION OF SAMPLES AND SOLUTIONS

Eluents

0.1 M Sodium Hydroxide

It is essential to use high-quality water. It should be of high resistivity (18 MΩ-cm or better) and should contain as little dissolved carbon dioxide as possible. Biological contamination should be absent. Additionally, borate, a water contaminant that can break through water purification cartridges (prior to any other indication of depletion of the cartridge), should be removed by placement of a BorateTrap™ cartridge (Dionex Corporation) between the pump and injection valve. The use of plastic tubing in the high-purity water system should be avoided or minimized, as plastic tubing often supports microbial growth, which can be a source of carbohydrate contamination.

It is extremely important to minimize contamination with carbonate, a divalent anion at pH ≥12, because it binds strongly to the columns and interferes with carbohydrate chromatography to cause a loss of resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. A 50% (w/w) sodium hydroxide solution is much lower in carbonate and is the preferred source for sodium hydroxide.

Dilute 10.4 mL of a 50% (w/w) sodium hydroxide solution into 1990 mL of water to prepare a 0.1 M sodium hydroxide solution. After preparation, keep the eluent blanketed under helium at 34 to 55 kPa (5 to 8 psi) at all times.

0.5 M Sodium Acetate/0.1 M Sodium Hydroxide

Dispense approximately 800 mL of water into a 1-L graduated cylinder. Add a stir bar and begin stirring. Weigh out 41.0 g of anhydrous, crystalline sodium acetate. Add the solid sodium acetate steadily to the briskly stirring water to avoid the formation of clumps, which are slow to dissolve. After the salt dissolves, remove the stir bar with a magnetic retriever. Using a plastic pipette, measure 5.2 mL of 50% (w/w) sodium hydroxide, and add it to the acetate solution. Rinse the pipette by drawing up the acetate solution into the pipette and dispensing it back into the graduated cylinder several times. Add water to the solution to a final level of 1000 mL. Replace the stir bar and stir briefly to mix. Vacuum filter through a 0.2 µm nylon filter. This may take a while, as the filter may clog with insolubles from the sodium acetate. This eluent should also be kept blanketed under helium at 34 to 55 kPa (5 to 8 psi) at all times.

RESULTS AND DISCUSSION

Chromatography of Fetuin Oligosaccharides

The separation of bovine fetuin oligosaccharides by HPAE-PAD is shown in Figure 1. The carbohydrates of bovine fetuin have been intensively studied.[9–13] HPAE-PAD has been used to characterize ten sialylated oligosaccharides from bovine fetuin. Structures corresponding to peaks 1–6 in Figure 1 are shown in Table 1. Peak 7 is a trisialylated triantennary complex oligosaccharide. Under alkaline conditions the technique resolves these species not only by sialic acid content, but also according to the combination of α(2,3)- and α(2,6)-linked sialic acids within each charge class.[11] Oligosaccharides with the greatest proportion of α(2,6)- to α(2,3)-linked sialic acids are the least-retained. The neutral components of the oligosaccharides also influence separation. Of the oligosaccharides studied, those containing a Galβ(1,3)GlcNAc sequence are retained more strongly than those with Galβ(1,4)GlcNAc.

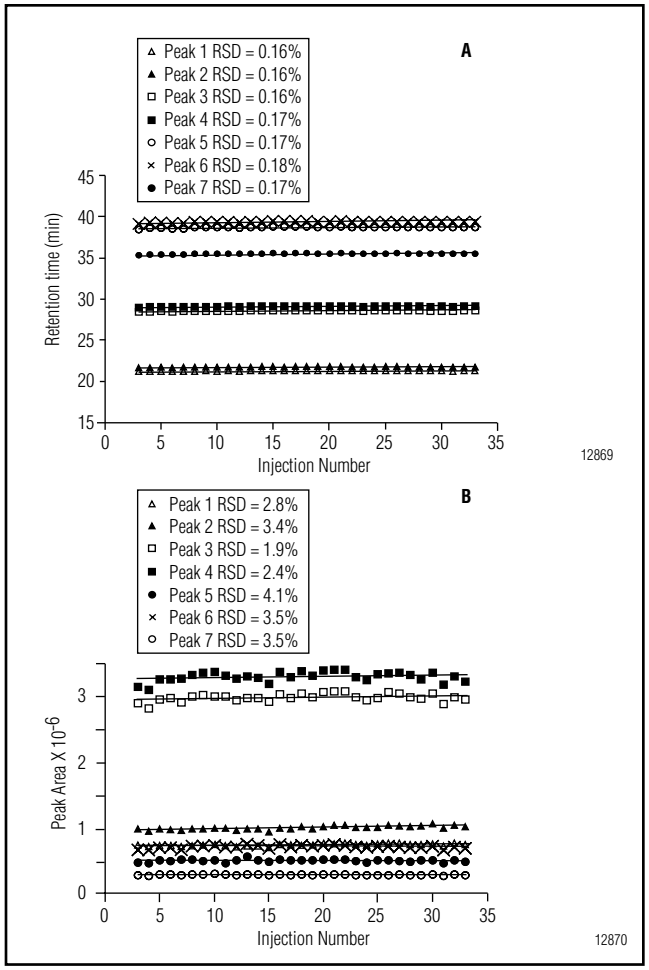

Figure 2. Retention time reproducibility (A) and peak area reproducibility (B) for fetuin oligosaccharide alditols on the CarboPac PA-100 column.

Accuracy and Precision

Retention time and peak area stability for the bovine fetuin oligosaccharide separation shown in Figure 1 were assessed by determining retention time and peak area RSDs of 33 consecutive injections of 250 picomoles of the commercial fetuin oligosaccharide alditol mix. The temperature of the CarboPac PA-100 column was held constant at 30 ˚C.

Retention time and peak area RSDs for each of the 7 major peaks in Figure 1 are plotted in Figures 2A and 2B. Data from the first two injections were omitted because it was found that detector response had not stabilized until the third injection. Retention time RSDs were less than 0.2%. Peak area RSDs fell in the range of 1.9% to 4.1%. Repetition of the identical schedule on a second system where the separations were done at ambient temperatures gave retention time RSDs of <0.5% and similar peak area RSDs. Retention time RSDs of <0.5% are similar to those reported for separations of the sialylated and desialylated N-linked carbohydrates isolated from recombinant human erythropoietin expressed in BHK cells.[2] The reported RSDs were obtained from multiple runs on different days using two internal standards [N-acetylneuraminic acid-Neu5Ac, and $(Neu5Ac)_3$] per run.[2]

Linearity

Detector response for the mix of N-linked fetuin oligosaccharide alditols was assessed. Across a broad concentration range (250 picomoles to 5 nanomoles), the peak area response for the seven major oligosaccharides was not linear (Figure 3A). However, over a narrower concentration range (100 picomoles to 1 nanomole), the response approached linearity (Figure 3B; r^2 >0.990 for peaks 1, 2, 3, 4, 6, 7 and r^2 >0.943 for peak 5). Repetition of this set of experiments on a second system generated similar results. HPAE-PAD quantitative analysis of the N-linked sialylated oligosaccharides of recombinant erythropoietin expressed in CHO cells has been reported. In that case, the response factors for each oligosaccharide were nearly the same.[14] Relative molar electrochemical responses for di-, tri-, tetra-, and pentasialylated oligosaccharides were reported to be 4.8 ± 14% relative to glucose.[11]

Limits of Detection

Limits of detection were determined for two authentic, commercially available oligosaccharide standards: an asialo galactosylated biantennary standard and a disialylated galactosylated biantennary standard. Low level determinations of the asialo galactosylated biantennary standard are shown in Figure 4A and reveal that limits are approached at low picomole levels. At 1.2 picomole the signal-to-noise ratio is 13.0. Limits of detection for the disialylated galactosylated biantennary standard are shown in Figure 4B. Limits of detection are similarly in the low (<3) picomole range. At 1.8 picomole, the signal-to-noise ratio is 11.8. Baseline drift from 0–50 minutes was found to be approximately 5 nC.

CONCLUSION

We have characterized the accuracy, precision, linearity, and lower limits of detection for an HPAE-PAD oligosaccharide mapping method. These results demonstrate "expected performance" and are intended for the use of investigators who need to validate HPAE-PAD oligosaccharide mapping methods.

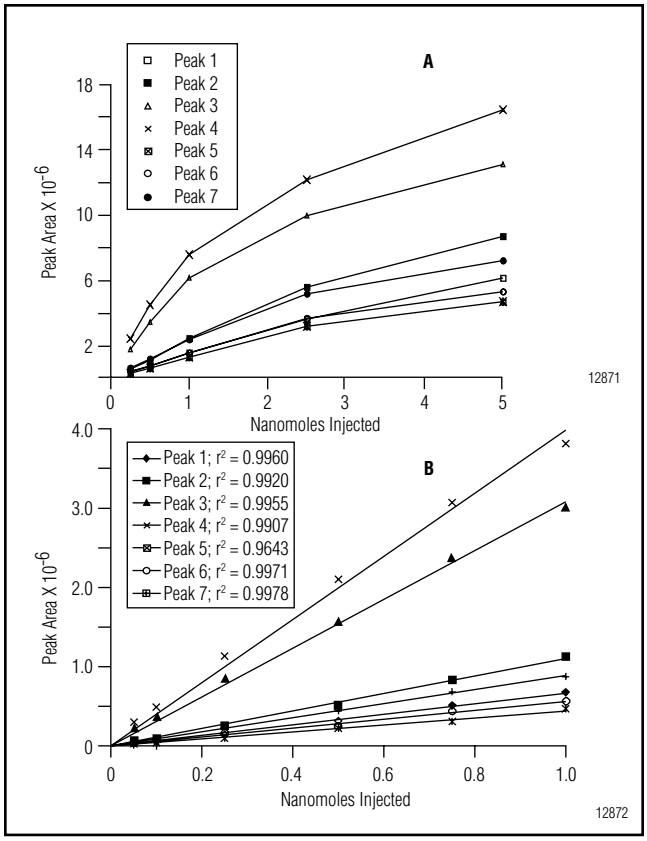

Figure 3. (A) Detector response for fetuin oligosaccharide alditols over a broad concentration range. (B) Linearity of response for fetuin oligosaccharide alditols from 50 to 1000 picomoles.

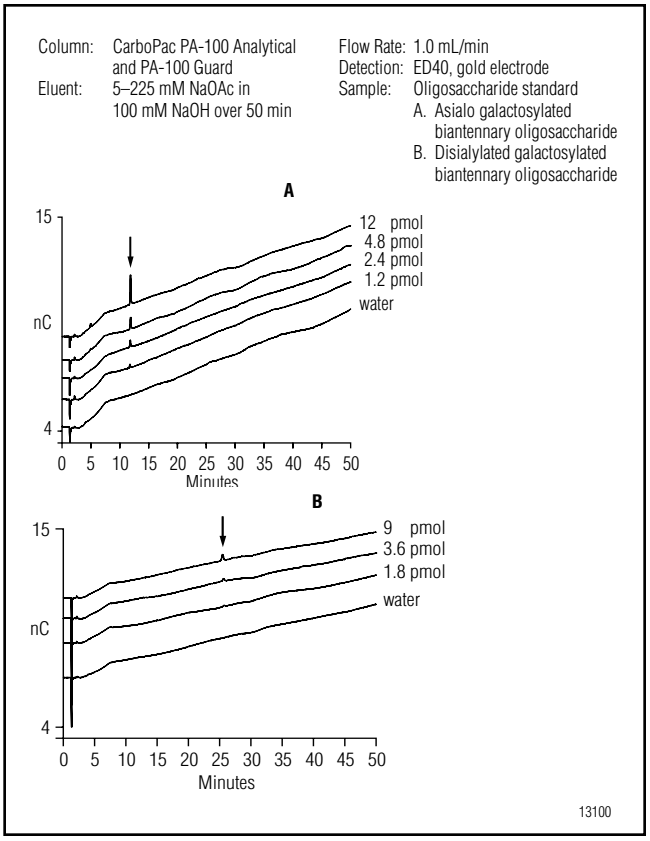

Figure 4. Oligosaccharide standards—low level determinations.

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LIST OF SUPPLIERS

Fisher Scientific
711 Forbes Ave.
Pittsburgh, Pennsylvania, 15219-4785, U.S.A.
Tel.: 800-766-7000.

Oxford GlycoSciences
75 Wiggins Ave.
Bedford, MA, 01730, U.S.A.
Tel.: 800-722-2597.

APPENDIX

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Determination of Glycoprotein Monosaccharide Composition by HPAE-PAD Using On-Line Electrolytically Generated Eluents

INTRODUCTION

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) at a gold electrode is a convenient and sensitive technique for carbohydrate analysis. HPAE-PAD provides picomole-level detection with no sample derivatization and simple isocratic elution. However, carbonate contamination of manually prepared hydroxide eluents inevitably causes retention time shifting that degrades performance. To ensure reproducibility, the column must be regenerated with 200 mM NaOH after every injection to elute strongly retained carbonate.

This Technical Note describes the setup and typical performance of an HPAE-PAD system for the monosaccharide analysis of glycoproteins that uses a high-purity eluent generated on-line with the EG40 Eluent Generator. L-Fucose, D-galactosamine, D-glucosamine, D-galactose, D-glucose and D-mannose are separated in 20 min on a CarboPac™ PA10 column by using an isocratic 18 mM KOH eluent. The column is regenerated by stepping to 100 mM KOH for 5 min and equilibrated for 15 min, for a cycle time of 40 min. The carbonate-free hydroxide regenerant produced by the EG40 accomplishes regeneration in less time than manually prepared hydroxide and eliminates the need for users to prepare the alkaline eluents.

This Technical Note describes general hydrolysis procedures to release monosaccharides from glycoproteins, and provides guidelines to optimize the hydrolysis procedures for success with most proteins. Several different proteins are analyzed, ranging from light to heavy glycosylation and spanning a wide range of monosaccharide composition.

EQUIPMENT

Dionex DX-600 chromatography system consisting of:

- GP50 Gradient Pump with vacuum degas option
- EG40 Eluent Generator
  EGC-KOH cartridge (P/N 053921)
- EG40 Vacuum Degas Conversion Kit (P/N 055431)
- ED40/50 Electrochemical Detector
- ED40/50 Electrochemical Amperometry Cell with Au Working Electrode (P/N 055290)
- AS50 Automated Sampler with thermal compartment
- Stainless steel sampling needle assembly (P/N 054273)

PeakNet® 6.1 Chromatography Workstation

Screw Cap Micro Tubes (Sarstedt #163.204)

Autosampler vials (P/N 055428)

SpeedVac Evaporator System (SpeedVac SVC100)

Heating Block (VWR 13259-005)

REAGENTS AND STANDARDS

Trifluoroacetic acid (Pierce 53102 HPLC-grade TFA)

Hydrochloric acid (Pierce 24309 constant-boiling 6 N HCl)

MicroBCA Protein Assay (Pierce 23235)

MonoStandard® Mixture of Six (Dionex P/N 043162)

SAMPLES

Fetuin, fetal bovine serum (Calbiochem 341506)

IgG, from human serum (Sigma I 4506)

Mucin, Type II from porcine stomach (Sigma M 2378)

Ribonuclease B, Type III-B from bovine pancreas (Sigma R 5750)

Thyroglobulin, bovine (Sigma T 1001)

Human serum transferrin, (Sigma T 0519 and Boehringer Mannheim 14877000)

CHROMATOGRAPHIC CONDITIONS

Columns: CarboPac PA10 Analytical 4 × 250 mm (P/N 046110)
BorateTrap, 4 × 50 mm (P/N 047078)
AminoTrap, 4 × 50 mm (P/N 046122)
ATC-1 4mm (P/N 037151, 2 each)

Eluent: KOH (EG40 as the source)

Flow Rate: 1.0 mL/min

Program:

Time (min)	mM KOH	Comment
0.00	100	
5.00	100	
5.10	18	
21.0	18	Inject
40.0	18	End

Temperature: 0 ˚C

Injection: 10 µL partial loop with 10 µL cut volume from 25 µL sample loop

Detection: Pulsed amperometry

Background: ~25–35 nC

Noise: ~30–100 pC peak-to-peak

Backpressure: ~2800 psi

Run Time: 40 min

PREPARATION OF SOLUTIONS AND REAGENTS

Reagent Water

Distilled or deionized water of specific resistance ≥ 17.5 MΩ-cm, preferably UV irradiated, filtered through a 0.2-µm filter immediately before use.

Eluent Solution

Generate 18 mM KOH eluent on-line by pumping reagent water through the EG40/EGC-KOH. Sparge the water prior to use with helium or sonicate under vacuum for 10 min. Maintain 3–5 psi head pressure of helium on the water reservoir to reduce contamination from atmospheric carbon dioxide.

Stock Standard Solutions

Dissolve the contents of one Dionex MonoStandard Mix of Six 100 nmol vial in 1.0 mL of reagent water and mix to prepare a stock standard solution containing 0.1 mM (100 pmol/µL) of each monosaccharide. Immediately freeze unused stock standard at < -10 ˚C. Avoid repeated freeze-thaw cycles. Deterioration has been observed at room temperature within 24–48 h.

Working Standard Solutions

Use reagent water to prepare appropriate dilutions of the stock standard for calibration as needed.

Sample Preparation

Estimate the appropriate amount of protein to inject by considering the degree of glycosylation of the protein. As a guideline, the amount of protein to inject = (10 µg/x), where x = % glycosylation of the protein. For example, if the protein is 3% glycosylated, (10 µg/3) = 3.3 µg protein. To inject 3.3 µg of hydrolyzed protein by using a 10-µL injection requires a sample concentration of 0.3 µg hydrolyzed protein/µL (i.e., 0.3 mg/mL).

It is convenient to prepare a stock standard that is nominally 10× this protein concentration: 10 x 0.3 mg/mL = 3 mg/mL. Transfer 0.030 ± 0.001 g protein to a small bottle on a tared analytical balance. Record the mass, remove from the balance, and add 10.0 mL of reagent water. Dissolve by gentle swirling to avoid denaturing the protein.

Next, because protein preparations typically include an unknown amount of water and buffer salts, determine the actual protein concentration by performing the MicroBCA assay on a portion of the solution.

Dilute the stock standard as necessary with reagent water to prepare a 0.3 mg/mL solution for 2 N TFA hydrolysis. For 6 N HCl hydrolysis, either use the 3 mg/mL stock standard, or dry an aliquot of the dilute sample in the hydrolysis tube before adding 6 N HCl, as discussed below.

Hydrolysis Procedures

TFA hydrolysis for neutral monosaccharides: Add 200 µL of a protein solution to a clean Sarstedt vial. Add 140 µL reagent water. Add 60 µL of neat TFA from a freshly opened 1-mL ampoule. Cap, mix, and place vial in a heating block at 100 ˚C for 4 h. Include at least one blank, substituting reagent water for sample, each time the TFA hydrolysis procedure is performed.

HCl hydrolysis for amino monosaccharides: Add 20 µL of a concentrated protein solution to a clean Sarstedt vial. (Or, add 200 µL of a dilute protein solution and evaporate to dryness in a SpeedVac). Add 400 µL of 6 M HCl from a freshly opened 1-mL ampoule. Cap, mix, and place vial in a heating block at 100 ˚C for 4 h. Include at least one blank, substituting reagent water for sample, each time the HCl hydrolysis procedure is performed.

After 4 h, remove the vials from the heating block and let them cool before opening. Microfuge the vials and unite the condensate with the bulk liquid. Evaporate to dryness at ambient temperature in a SpeedVac. Drying time may range from 4 hours to overnight. Reconstitute by adding 200 µL of reagent water to each vial, vortex 30 s to mix, and centrifuge at 5000 rpm for 5 min. Transfer supernatant to a limited volume autosampler vial. Cap the vial and inspect the vial for bubbles; dislodge any bubbles that might interfere with injection.

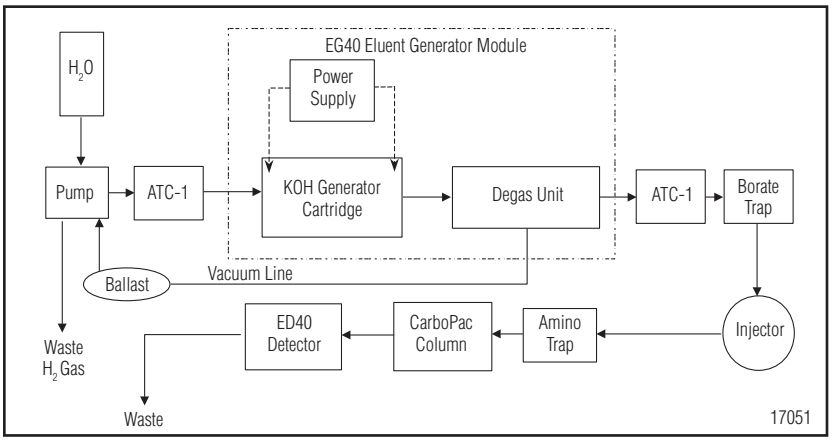

Figure 1. HPAE-PAD system using EG40-generated eluent.

These are the specific procedures followed for this Technical Note. The procedures may be adjusted as described in "Results and Discussion" on page 5.

SYSTEM PREPARATION AND SETUP

Figure 1 shows the placement of each component. Use 0.01-in. ID PEEK tubing to plumb 4-mm systems (0.005-in. tubing for 2-mm systems). Cut tubing square with a tubing cutter and avoid excess lengths of tubing to minimize extra column band broadening. Use the following installation sequence to ensure that contaminants flushed from each new column do not contaminate the next column in line.

Condition two ATC-1 columns with 2 N KOH or 2 N NaOH as described in the Installation Instructions and Troubleshooting Guide for the IonPac ATC-1 Anion Trap Column (Document No. 032697). Place one conditioned ATC-1 column between the pump and the EG40 to scavenge carbonate from the source water. Connect the Pump Out end of the black PEEK tubing to this ATC-1 and the EGC In end to the inlet of the EGC-KOH.

Connect the tubing labeled EGC Out that originates from the bottom front of the EG40 Degas Module to the EGC Out port of the EGC-KOH. For carbohydrate applications, modify the EG40 Degas Module to further reduce hydrogen gas generated by the EG40 at high KOH concentrations to the low level required for sensitive amperometric detection. Connect the EG40 Degas Module to the GP40/50 Vacuum Degas system by using the Degas Conversion Kit (P/N 055431) as directed in Document No. 031521. Turn on the pump and go to the Degas Options screen (<Menu> <4>). Set the start-up duration to at least 5 min, the cycle duration to at least 60 s, and the time between cycles to 5 min or less. Set the pump front control panel to "Degas Status" and monitor the degas reading to ensure that there are no leaks in the high-vacuum side of the Degas Module. A major vacuum leak will cause both a rapid drop in the degas reading after the vacuum pump cycles off and baseline oscillations in the chromatogram with a period corresponding to the vacuum pump cycle. A minor leak will cause a smaller drop in the vacuum reading and smaller baseline oscillations.

Connect the EG40 to the LAN and configure it with the PeakNet chromatography data system, then condition the EluGen® cartridge as directed in the EG40 manual. Cut and temporarily couple a 24-in. piece of 0.003-in. PEEK tubing to the Injection Valve In tubing exiting the bottom rear of the Vacuum Degas Module. Trim the length to provide backpressure of 2000 psi at 1 mL/min. Perform the following gradient to condition the EluGen cartridge: 1 to 60 mN KOH in 20 min, then 60 mN for 40 min at 1 mL/min.

Remove the 0.003-in. PEEK backpressure tubing temporarily installed during conditioning of the EluGen cartridge and connect the tubing labeled Injection Valve In to the second ATC-1 column. Connect the second ATC-1 column to the BorateTrap column, and connect the BorateTrap to port 2 of the injection valve. The BorateTrap column will scavenge borate from the source water. Borate is one of the first ions to break through water purification systems and will cause peak tailing, especially for mannose. Typical peak asymmetry for a well performing system is 1.0 ± 0.3. Replace the BorateTrap column when tailing of the mannose peak exceeds this limit.

Install an AminoTrap™ column after the injection valve and condition as directed in the AminoTrap instructions by flushing with 100 mN KOH at 1 mL/min for 1 hour. The AminoTrap delays the elution of amino acids and small peptides found in glycoprotein hydrolysates and is used in place of a guard column before the CarboPac PA10. *Do not pump pure water through the AminoTrap column; it may damage the column irreversibly.*

Install a 4 × 250 mm CarboPac PA10 immediately after the AminoTrap column. With the second ATC-1, the AminoTrap, and the CarboPac PA10 columns now installed, monitor the system pressure displayed by the pump when 18 mM KOH is delivered at 1 mL/min. The EG40 Vacuum Degas Module requires at least 2000 psi backpressure to efficiently remove hydrolysis gas from the eluent. If necessary, install the backpressure coils supplied with the EG40 shipkit to bring the system pressure to between 2300 and 2800 psi (2300 psi accounts for about 300 psi contributed by the ATC-1 and the EGC-KOH). Condition the CarboPac PA10 by flushing the column with 100 mN KOH at 1 mL/min for 1 h as directed in the column manual. Program the EG40 to deliver the gradient program shown in the "Conditions" section and run the gradient, monitoring the backpressure during the entire run. Trim the backpressure coil if necessary to maintain backpressure less than 3000 psi. Caution: Because the backpressure can rise over time, trim the backpressure coil as needed to keep the backpressure under 3000 psi. *Do not exceed 3000 psi or the EG40 Degas Module tubing may rupture.*

Assemble and install the ED40/50 Electrochemical Detector Amperometry Cell with a gold working electrode. From the front control panel of the ED40/50, go to the Detail screen for the Integrated Amperometry mode (<Menu> <2>). Select the Ag/AgCl reference electrode in the REF field.

Program the data acquisition software to deliver the gradient shown in "Chromatographic Conditions". In the program, use the quadruple potential waveform in Table 1 and program a 2-Hz data acquisition rate.[1] Make replicate injections of a reagent water blank until two successive runs resemble the blank run shown in Figure 4A. An equilibrated, contaminant-free system has a background signal of 25–35 nC, with peak-to-peak noise of 30–100 pC and no significant peaks in the 22-40 min window.

Table 1. Waveform for the ED40/ED50

Time (sec)	Potential (V vs. Ag/AgCl)	Integration
0.00	+0.1	
0.20	+0.1	Begin
0.40	+0.1	End
0.41	-2.0	
0.42	-2.0	
0.43	+0.6	
0.44	-0.1	
0.50	-0.1	

AUTOSAMPLER QUALIFICATION

Peak area precision and accuracy depend on autosampler performance. For best performance, inspect the AS50 sample syringe and its tubing daily for bubbles. Remove any bubbles by following the instructions in the AS50 manual. Replace the water in the flush reservoir daily with freshly filtered and degassed water or apply 5 psi helium pressure to the AS50 syringe reservoir. Use a stainless steel sampling needle assembly (P/N 054273) for monosaccharide analysis to reduce adsorptive loss of aminosugars caused by worn Teflon®-coated sampling needles.

The precision and accuracy of the AS50 will vary depending on the mode of injection. The most accurate and precise injections can be made with a calibrated sample loop in the Full Loop injection mode. For loop sizes less than 17 µL, 2.5 times the loop volume plus 25 µL is delivered to the valve. Peak area precision for full loop injections of 25 µL is typically 0.3% RSD. A 10-µL Full Loop injection uses 50 µL of sample, allowing three injections from a 200 µL sample.

To conserve sample, use either the Partial Loop or the Partial Loop, Limited Sample (Partial LS) injection mode. In the Partial Loop mode, the AS50 draws the volume to be injected from the sample vial, plus two times the cut segment volume. (The cut segment volume is a portion of the sample that is discarded from each end of the aspirated sample to improve accuracy.) The middle portion of the sample is positioned in the loop and injected. For the best precision and accuracy when using one of the partial loop injection modes, install a sample loop that is at least two times the injected volume. Peak area precision for Partial Loop injections of 10 µL is typically 1% RSD. A 10-µL Partial Loop injection with a cut volume of 10 µL uses 30 µL of sample, allowing six injections from a 200-µL sample.

The AS50 in Partial LS mode wastes no sample and usually provides precision of 1–2% RSD. See the AS50 reference manual for a complete discussion of the different injection modes.

In this Technical Note, we describe the use of an AS50 in the Partial Loop injection mode, which allows six 10-µL injections from a 200-µL glycoprotein hydrolysate sample with good precision and accuracy. Be sure to enter the correct sample loop size and sample syringe volume in the AS50 Plumbing Configuration screen. Reconstitute the MonoStandard Mix of Six with 1.00 mL of reagent water, dilute 1:1 with reagent water, and inject 10 µL from a 25-µL sample loop with the cut volume set to 10 µL. Confirm that the resulting chromatogram resembles that shown in Figure 2.

Qualify the autosampler before proceeding by injecting three more replicates of the 2X dilution of the MonoStandard Mix of Six. The relative standard deviation of the peak area of the four replicates should be ≤ 2%. See the autosampler reference manual for troubleshooting techniques if the autosampler fails this test.

RESULTS AND DISCUSSION

Chromatography

Figure 2 shows a typical separation of a 2× dilution of the MonoStandard Mix of Six containing L-fucose, D-galactosamine, D-glucosamine, D-galactose, D-glucose, and D-mannose at 500 pmol each. The peaks are baseline resolved and elute within a window of 15 min. The total run time is extended to 40 min to allow complete stabilization of the baseline after the column regeneration step.

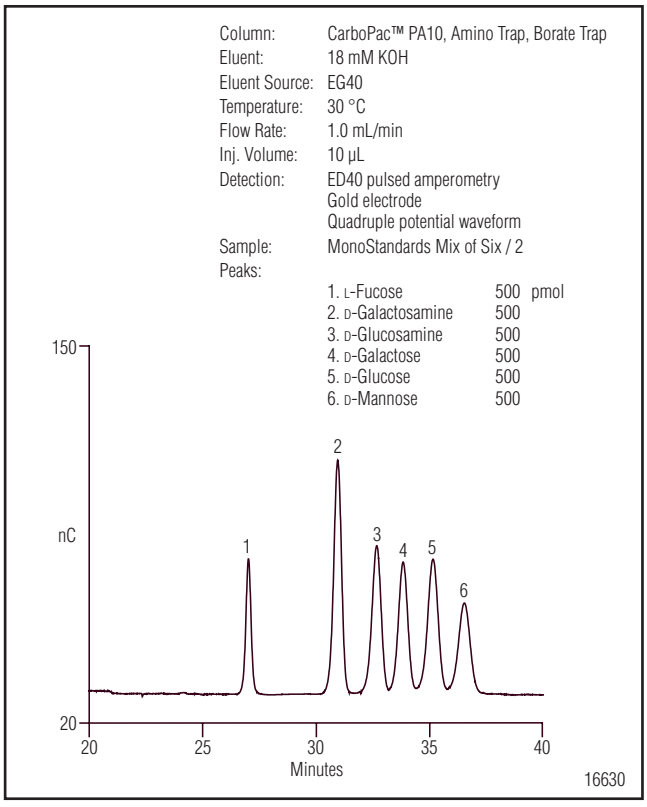

Figure 2. 500 pmol monosaccharide standard.

In the blank runs in Figure 4, the large flat-topped peak eluting between 2 and 10 min results from the column cleanup step. The height and appearance of this peak will vary depending on the amount of material from the previous injection that is flushed off the column. A trough that lasts until t = 25 min may follow this peak, whereupon the baseline should increase to its normal, stable value. (A second broad hump may elute between 10 and 20 min as hydrogen gas, a byproduct of eluent generation, elutes from the column.) The sample is injected at t = 21 min so that the peaks of interest elute on a stable baseline.

Performance

Table 2 summarizes the calibration data and method detection limits (MDLs) obtained in this study for the six monosaccharides. The MDL for each analyte is established by making seven replicate injections of a monosaccharide standard with peak height for each analyte of three to five times the estimated baseline noise. We used seven replicates of a 0.75 pmol standard. Pulsed amperometric detection with the quadruple waveform allows quantification of these monosaccharides at the low picomole level if a 10-µL sample is injected. It is possible to lower the detection limit by injecting a larger volume of sample, depending on the ionic strength and nature of the sample matrix.

EG40 eluent generation is highly reproducible. Actual performance of the EG40 is demonstrated in Figure 3, a summary of the retention time of monosaccharide standards injected over a period of seven days. The variation in retention time of each monosaccharide is less than 0.3% RSD for over 200 injections. When retention times become significantly shorter or more variable,

Table 2. Linear Range and MDLs for Monosaccharides

Monosaccharide	Range (pmol)	r^2	MDL Sample (pmol)	Calculated MDL* (pmol)
L-Fucose	0.5–500	0.9989	0.75	0.17
D-Galactosamine	0.5–500	0.9996	0.75	0.21
D-Glucosamine	0.5–500	0.9999	0.75	0.30
D-Galactose	0.5–500	0.9992	0.75	0.34
D-Glucose	0.5–500	0.9997	0.75	0.34
D-Mannose	0.5–500	0.9992	0.75	0.25

*MDL = (t) x (S) where t = Student's t value for a 99% confidence level and a standard deviation estimate with n - 1 degrees of freedom [t = 3.14 for seven replicates of the MDL Standard] and S = standard deviation of the replicate analysis.

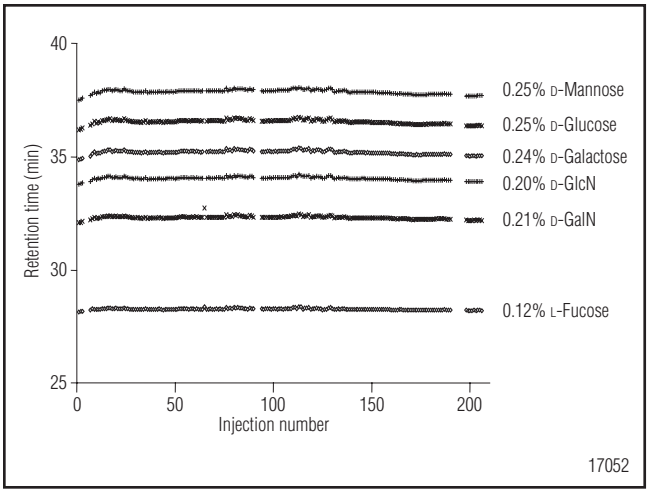

Figure 3. Long-term reproducibility (% RSD) of monosaccharide standards.

recondition the ATCs as described in the Installation Instructions and Troubleshooting Guide for the IonPac ATC-1 Anion Trap Column (Document No. 032697). The EGC-KOH cartridge lifetime under the conditions used here is approximately 2000 injections, or 1300 hours of continuous operation. PeakNet 6 software tracks the amount of KOH used and calculates the remaining lifetime. Be prepared to replace the EGC-KOH cartridge when the remaining lifetime is less than 10%.

The next several figures illustrate the performance of this method with various glycoprotein hydrolysates, beginning with the TFA and HCl hydrolysis blanks depicted in Figure 4. The hydrolysis acid, the reagent water, and the labware used in the hydrolysis procedure can contaminate the sample with monosaccharides or coeluting interferants. The level of contamination will affect the detection limit. A reasonable guideline for the detection limit is 10 times the background level of contaminating monosaccharides, so for trace level determinations significant sources of contamination must be eliminated. The hydrolysis procedure blanks are an important means of assessing contamination and qualifying the reagents. The level of glucose contamination in Figure 4B is typical and is acceptable for the glycoproteins analyzed here (none of which is known to contain glucose). However, for analysis of glycoproteins that contain glucose, removal of contaminating glucose may be important.

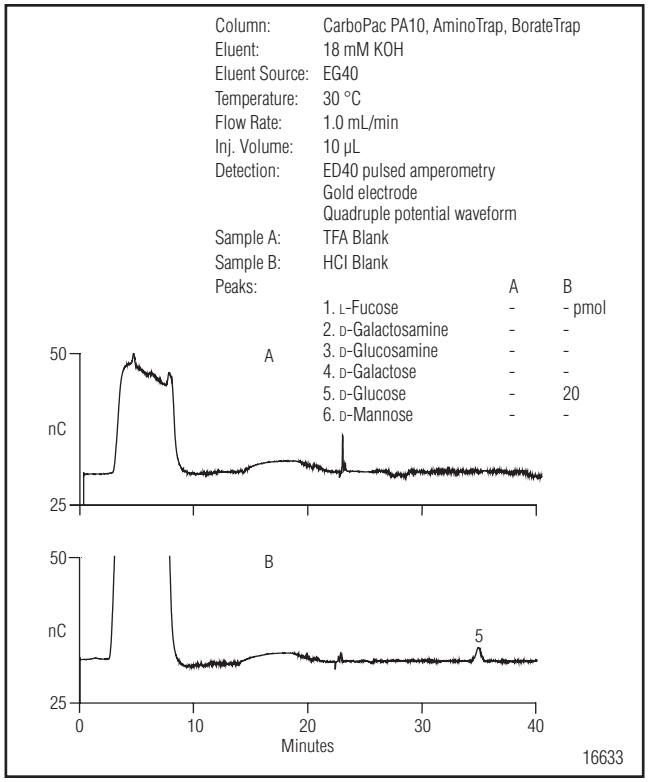

Figure 4. Hydrolysis procedure blanks: deionized water.

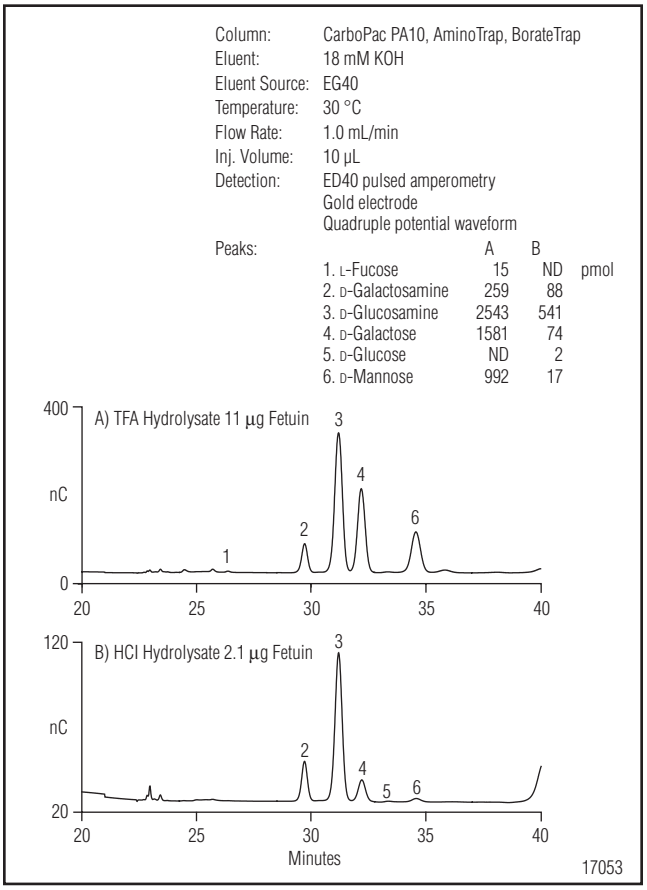

Figure 5. Glycoprotein hydrolysates: fetuin.

Figure 5 shows the HPAE-PAD chromatogram of a fetal bovine fetuin hydrolysate. The monosaccharide peaks are baseline-resolved and elute well away from the early baseline disturbances caused by the column cleanup step, even though the amount of protein injected was about 10X the recommended amount. The neutral monosaccharides are seen at higher concentrations in the TFA hydrolysate, and the amino sugars at higher concentrations in the HCl hydrolysate, as expected.

When injecting glycoprotein hydrolysates, strongly retained hydrolysis products can accumulate on the column and impair its performance. Signs of a fouled column include an increase in unidentified peaks, baseline disturbances, peak tailing, and shortened retention times. This method includes a step change to 100 mM KOH for 5 minutes to flush strongly retained material off the column. Table 3 highlights the efficacy of the column cleanup step to prevent column fouling when glycoprotein hydrolysates are analyzed. Every six injections of a glycoprotein hydrolysate sample were followed by three injections of the MonoStandard Mix of Six containing 500 pmol of each monosaccharide. Compare the retention time reproducibility of monosaccharide standards alone to that obtained when the standards are interspersed with injections of various protein hydrolysates. The good RT stability of the standards alone is maintained not only with typical protein hydrolysates (3 µg IgG), but also with injections of more concentrated protein hydrolysates (30 µg IgG) that challenge the method. Good RT stability is also observed with a mix of glycoproteins representing a range of glycosylation types and amounts, including transferrin, fetuin, ribonuclease B, thyroglobulin, and mucin.

Table 3. Retention Time Precision (% RSD) of Monosaccharide Standards Interspersed with Glycoprotein Hydrolysates

Sugar	Standards Alone	Standards with 3 µg IgG	Standards with 30 µg IgG	Standards w/ Various Proteins
L-Fucose	0.10	0.14	0.09	0.08
D-Galactosamine	0.19	0.19	0.08	0.12
D-Glucosamine	0.20	0.17	0.09	0.14
D-Galactose	0.15	0.17	0.11	0.18
D-Glucose	0.18	0.17	0.11	0.19
D-Mannose	0.19	0.17	0.12	0.19

The column cleanup step, together with the AminoTrap guard column, helps maintain the stability of the electrode response. Protein hydrolysis products such as amino acids and peptides, which can poison the gold working electrode, are strongly retained by the AminoTrap column until they are eluted by the 100 mM KOH cleanup step. Under these conditions, working electrode performance is maintained.

Stable electrode response is demonstrated in Table 4, which summarizes the peak area reproducibility of monosaccharide standards injected alone or interspersed with injections of various glycoprotein hydrolysates. Although the peak area variability does increase when glycoprotein hydrolysates are analyzed compared to standards alone, even under the most challenging conditions peak area reproducibility remains acceptable (i.e., less than 10% RSD).

Table 4. Peak Area Precision (% RSD) of Monosaccharides Standards Interspersed with Glycoprotein Hydrolysates

Sugar	Standards Alone	Standards with 3 µg IgG	Standards with 30 µg IgG	Standards w/ Various Proteins
L-Fucose	1.74	2.35	4.28	5.53
D-Galactosamine	1.17	1.72	2.56	3.24
D-Glucosamine	1.56	1.95	2.34	6.82
D-Galactose	1.17	2.31	1.34	3.72
D-Glucose	1.09	2.71	1.68	3.34
D-Mannose	1.91	4.36	4.18	4.90

Hydrolysis Procedures

Two different hydrolysis procedures are available to release monosaccharides from glycoproteins. Hydrolysis with 2 N TFA is recommended for neutral sugars, but is too mild to release completely the amino sugars galactosamine and glucosamine. Hydrolysis with 6 N HCl is recommended for the amino sugars, but partially degrades the neutral sugars; both procedures must be used in tandem to accurately measure the monosaccharide composition of a glycoprotein.

To hydrolyze with 2 N TFA, dilute neat (13 N) TFA with a combination of protein solution and reagent water. Here we add 60 µL of neat TFA to 200 µL of protein solution plus 140 µL of reagent water. The final TFA concentration is calculated to be: (13 M TFA) * (60 µL TFA/400 µL total) ≈ 2 M TFA. Different ratios of protein solution/reagent water can be used, depending on the concentration of the protein solution, to produce 2 M TFA.

Evaporate the hydrolysate to remove residual TFA and water and then reconstitute the hydrolysate with reagent water. Here we add 200 µL to provide a sufficient volume for replicate injections. Vortex to mix, centrifuge, and transfer the supernatant to an autosampler vial for analysis.

When hydrolyzing with 6 N HCl, use a smaller volume of protein solution than with 2 N TFA to minimize dilution of the 6 N HCl by the sample. For example, we use 20 µL of protein solution and 400 µL of 6 N HCl for a total volume of 420 µL. The final HCl concentration is: (6 N HCl) * (400 µL 6 N HCl/420 µL total) ≈ 6 N HCl. (If the protein solution is too dilute, a larger volume can be added and then dried prior to the addition of HCl.)

Evaporate the hydrolysate to remove residual HCl and water. Then reconstitute the hydrolysate with reagent water, vortex to mix, centrifuge, and transfer the supernatant to an autosampler vial for analysis.

These are good general hydrolysis conditions for determining the monosaccharide content of a glycoprotein. The hydrolysis procedure may be optimized for any specific glycoprotein that is going to be analyzed routinely. Fan et al. have provided a good framework for optimizing glycoprotein hydrolysis conditions for monosaccharide analysis.[2]

Variations in hydrolysis reproducibility add to the overall variability of this analysis, along with such factors as water quality, autosampler performance, column condition, and electrode performance. We evaluated hydrolysis reproducibility by comparing the variability of replicate injections from a single hydrolysate vial to the variability between vials of replicate hydrolysates of the same protein sample. Results are summarized in Table 5 (TFA hydrolysis) and Table 6 (HCl hydrolysis) for several different glycoproteins.

Two trends are evident in Tables 5 and 6. First, the peak area variability (% RSD) of injections from a single hydrolysate is less than the variation from vial to vial. Single-vial precision of 1–2% is what we expect for a well performing autosampler. However, several factors can cause higher variability between hydrolysates. For example, it was difficult to get uniform samples from the mucin solution, which was viscous and contained suspended solids. Because the hydrolysis procedure is an important source of variability, we recommend performing triplicate hydrolyses for each protein. Second, the peak area variability increases as analyte amounts approach the minimum detection limits of the method. Peak area RSD of about 10–15% is expected for a peak with S/N = 3. The peak area reproducibility for replicate hydrolyses is good enough to assure us that we have a robust hydrolysis technique and to allow us to calculate the relative amount of each monosaccharide that was present in the original glycoprotein.

Table 5. Peak Area Precision of Replicate Injections of Glycoprotein TFA Hydrolysates

Sugar	% RSD for Replicate Injections[a] Vial 1	Vial 2	Vial 3	% RSD of replicate Hydrolyses[b]
Thyroglobulin				
L-Fucose	1.65	1.86	1.84	6.64
D-Galactose	0.92	0.51	0.80	3.76
D-Mannose	1.08	2.34	1.63	2.13
Ribonuclease B				
L-Fucose	ND	ND	ND	ND
D-Galactose	7.03	14.0	7.68	10.8
D-Mannose	2.37	2.8	0.70	3.45
Mucin				
L-Fucose	1.44	0.55	0.86	1.59
D-Galactose	1.28	0.45	1.00	0.82
D-Mannose	6.97	5.54	4.67	26.0

[a]Number of injections was six
[b]Number of hydrolyses was three

Table. 6 Peak Area Precision of Replicate Injections of Glycoprotein HCl Hydrolysates

Sugar	% RSD for Replicate Injections[a] Vial 1	Vial 2	Vial 3	% RSD of Replicate Hydrolyses[b]
Thyroglobulin				
D-Galactosamine	ND	ND	ND	ND
D-Glucosamine	2.33	0.99	0.80	1.80
Ribonuclease B				
D-Galactosamine	ND	ND	ND	ND
D-Glucosamine	1.50	2.34	1.80	2.73
Mucin				
D-Galactosamine	0.51	0.55	1.02	4.87
D-Glucosamine	0.37	0.42	0.46	5.71

[a]Number of injections was six
[b]Number of hydrolyses was three

Composition Analysis

Calculate the monosaccharide content of a glycoprotein in mol monosaccharide/mol glycoprotein as follows. First calculate the amount of protein analyzed by HPAE-PAD. Begin with the actual protein concentration of the solution that was hydrolyzed and account for dilution when reconstituted. (It is very important to measure the actual protein content of the solution hydrolyzed; otherwise, only the relative proportion of each sugar can be determined, and not the actual monosaccharide composition or percent glycosylation. An assay such as the BCA assay will help account for an unknown amount of water, salts, or other components present in the solid protein.)

For example, the fetuin sample concentration determined by using the BCA assay was 2.1 g/L. The fetuin HCl hydrolysate was diluted tenfold upon reconstitution. Therefore, the mass of hydrolyzed fetuin injected was:

$$(2.1\ g/L) * (20\ \mu L/200\ \mu L) * (10 \times 10^{-6}\ L) = 2.1 \times 10^{-6}\ g$$

The number of moles of fetuin injected was:

$$(2.1 \times 10^{-6}\ g) / (48{,}000\ g\ fetuin/mol) = 44 \times 10^{-12}\ mol$$

Next, determine the amount of each monosaccharide found in the glycoprotein hydrolysate. Use the results from the 2 N TFA hydrolysate for neutral sugars and the 6 N HCl hydrolysate for the aminosugars. For example, the amount of D-galactosamine found in the fetuin 6 N HCl hydrolysate was 93×10^{-12} mole. The D-galactosamine content of fetuin is:

$$\frac{(93 \times 10^{-12}\ mol\ D\text{-galactosamine})}{44 \times 10^{-12}\ mol\ fetuin} = \frac{2.1\ mol\ D\text{-galactosamine}}{mol\ fetuin}$$

Monosaccharide composition analysis of the various glycoprotein hydrolysates (Table 7) yields monosaccharide ratios consistent with expected ranges. For example, the monosaccharide ratios obtained for bovine fetuin are consistent with the ranges determined by a multicenter study of quantitative carbohydrate analysis.[3] The slightly low values obtained for galactose and mannose may be a result of injecting about 10× more fetuin than the recommended amount.

Table 7. Glycoprotein Monosaccharide Composition

Protein	Protein Injected (μg)	Monosaccharide	Amount Found (pmol)	mol Sugar/ mol Protein
Transferrin				
	13.5	L-Fucose	24	0.1
	13.5	D-Galactosamine		
	13.5	D-Glucosamine	1827	5.4 (6.8)*
	13.5	D-Galactose	645	1.9 (2.4)*
	13.5	D-Mannose	816	2.4 (3.0)*
BM Transferrin				
	15.3	L-Fucose	33	0.1
	15.3	D-Galactosamine		
	15.3	D-Glucosamine	2196	5.2 (6.5)*
	15.3	D-Galactose	740	1.9 (2.4)*
	15.3	D-Mannose	912	2.4 (3.0)*
Fetuin				
	10.7	L-Fucose	30	0.1
	2.1	D-Galactosamine	926	2.1
	2.1	D-Glucosamine	5549	12.4
	10.7	D-Galactose	3147	7.1
	10.7	D-Mannose	1962	4.4
Thyroglobulin				
	12.4	L-Fucose	320	8.5
	6.2	D-Galactosamine		
	6.2	D-Glucosamine	2469	65.5
	12.4	D-Galactose	896	23.7
	12.4	D-Mannose	1848	49.0
Ribonuclease B				
	7.8	L-Fucose		
	7.8	D-Galactosamine		
	7.8	D-Glucosamine	2362	1.0
	7.8	D-Galactose	94	0.0
	7.8	D-Mannose	5882	2.6
Mucin				
	0.5	L-Fucose	3930	9198
	0.5	D-Galactosamine	5999	14040
	0.5	D-Glucosamine	11507	26931
	0.5	D-Galactose	6439	15070
	0.5	D-Mannose	283	662

*Normalized to mannose = 3

SUMMARY

HPAE-PAD using an EG40-generated hydroxide eluent simplifies and improves monosaccharide composition analysis of glycoproteins. It can provide reproducible retention times and detector response for hundreds of samples over several days. Pulsed amperometric detection provides sensitivity at the low-picomole level and a linear range of 0.5–500 pmol for monosaccharides commonly found in mammalian glycoproteins.

REFERENCES

1. Dionex TN21: Analysis of Carbohydrates by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD); **1993**, Dionex Corp.
2. Fan, J.-Q.; Namiki, Y.; Matsuoka, K.; Lee, Y.C. *Anal. Biochem.* **1994**, *219,* 375-378.
3. Townsend, R.R.; Manzi, A.; Merkle, R.K.; Rohde, M.F.; Spellman, M.; Smith, A.; Carr, S.A. www.abrf.org/ABRFNews/1997/March1997/mar97/monosach.html.

SUPPLIERS

Pierce Chemical Co., P.O. Box 117, Rockford, IL 61105, USA. Tel: 800-874-3723. www.piercenet.com.

VWR Scientific Products, 3745 Bayshore Blvd., Brisbane, CA 94005, USA. Tel: 800-932-5000. www.vwrsp.com.

Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, USA. Tel: 800-325-3010. www.sigma-aldrich.com.

Calbiochem, P.O. Box 12087, La Jolla, CA 92039-2087, USA. Tel: 800-854-3417. www.calbiochem.com.

Sarstedt Inc., P.O. Box 468, Newton, NC 28658-0468, USA. Tel: 828-465-4000. www.sarstedt.com.

Savant Instruments/E-C Apparatus, 100 Colin Drive, Holbrook, NY 11741-4306 USA. Tel: 800-634-8886. www.savec.com.

Sun International, 3700 Highway 421 North, Wilmington, NC 28401, USA.Tel: 800-522-8425. www.sun-intl.com.

Glycosylation Analysis of Human Serum Transferrin Glycoforms Using Pellicular Anion-Exchange Chromatography

INTRODUCTION

Glycoforms and Protein Sialylation

Glycoproteins are proteins with a carbohydrate attached to the polypeptide backbones through one or more glycosylation sites. Oligosaccharides can be linked to a protein through a serine or a threonine as O-linked glycans, or through an asparagine as N-linked glycans. Glycoprotein glycoforms contain identical polypeptide backbones and differ from one another in the oligosaccharides attached to the glycosylation sites. To complicate the analytical task, the oligosaccharides attached to glycoproteins can also be sialylated at the nonreducing end of the glycans and the degree of sialylation can vary. Thus, the variation in glycoprotein sialylation can also create a collection of glycoforms.

Use of Pellicular Anion Exchange Columns for Fractionation of Glycoforms

The DNAPac™ PA-100 column, a pellicular anion-exchange column, has been successfully used to fractionate sialylated glycoprotein and sialylated glycopeptide glycoforms.[1,2] Separations of glycoforms are dependent upon the difference in the degree of sialylation of the glycoforms. The greater the degree of sialylation, the longer the glycoforms are retained on the column; thus, allowing the glycoprotein to be separated into different glycoform populations.

Examination of fetuin glycopeptide separations using the DNAPac PA-100 column indicated that glycopeptide glycoforms can also be fractionated based on the structure of the attached sialylated oligosaccharides.[2] The selectivity based upon structural differences in the carbohydrate moiety may allow separation of glycoforms with oligosaccharides that are sialylated on different oligosaccharide branches, but contain the same number of sialic acid residues.

The DNAPac PA-100 column is available in analytical (4 × 250 mm), semipreparative (9 × 250 mm), and preparative (22 × 250 mm) formats. The analytical column can be used to obtain a preliminary separation of glycoforms using a small amount of sample. The semi-preparative and the preparative columns can then be used to fractionate a larger quantity of the glycoprotein so that column fractions can be collected for further analysis.

Use of HPAE-PAD for Glycosylation Analysis of Fractionated Glycoforms

Once the DNAPac PA-100 fractions are obtained, PNGase F digestions can be performed. PNGase F, an amidase, removes N-linked oligosaccharides attached to glycoproteins. The released oligosaccharides from the different PNGase F digested fractions can be analyzed using HPAE-PAD (High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection) using a CarboPac™ PA-100 column.[3–5] Because the retention of sialylated oligosaccharides on the CarboPac PA-100 column is primarily based on the number of sialic acid residues attached to the glycan, the distribution of the sialylated species of a particular glycoform fraction can be estimated based on retention time and peak areas. Treatment of these digests with neuraminidase (an exoglycosidase that removes terminal sialic acid from sialylated oligosaccharides) and subsequent analysis of the digests with HPAE-PAD, confirms the presence of sialylated oligosaccharides in the glycoform fractions.

Human Serum Transferrin

Transferrin, an iron-binding glycoprotein, is found in serum and extravascular fluids in a variety of vertebrates.[6] Transferrin glycosylation varies depending on the species and on the tissues from which the protein is isolated. In addition, transferrin from the same tissue of the same species may exhibit variations in glycan structures, particularly with respect to the degree of sialylation.[7–9]

Human serum transferrin (HST), a serum β-globulin, has been shown to contain two N-linked glycosylation sites.[7] Prior studies found that HST contains 85% biantennary and 15% triantennary glycans.[10] One disialylated oligosaccharide and two different trisialylated oligosaccharides are believed to make up the majority of the biantennary and the triantennary species, respectively. In addition, 82% of HST has a biantennary oligosaccharide at both sites, 17% has a triantennary oligosaccharide at one site and a biantennary oligosaccharide at another site, and 0–1% has a triantennary oligosaccharide at each site.[11]

Clinical Significance of HST Sialylation

Variation of glycoprotein sialylation has significant clinical implications. For example, HST of cancer, rheumatoid arthritis, and haemochromatosis patients show an increased level of sialylation[12]; whereas patients with heavy alcohol consumption show a high incidence of asialo HST glycoforms.[13] Clearance of glycoproteins has also been shown to be related to sialylation. For example, catabolism of human serum transferrin in the liver has been found to be dependent on the interactions between the carbohydrate moiety and the asialoglycoprotein receptor. When the sialylation of HST is low, elimination from circulation is more rapid.[9]

Analytical Strategy

In this Application Note, procedures for glycoprotein sialylation analysis are described. HST is used as a model glycoprotein because of its clinical significance and its availability from commercial sources. The analytical strategy is summarized as follows:

1. Fractionation of HST into distinct populations of glycoforms according to the degree of sialylation.
2. Removal of N-linked oligosaccharides from the glycoform fractions.
3. Profiling of the removed glycans.
4. Identification of the sialylated glycans using neuraminidase.
5. Estimation of the relative distribution of the sialylated oligosaccharides.

EQUIPMENT

Dionex DX-500 BioLC® system consisting of:
GP50 Gradient Pump
AD20 Absorbance Detector
ED40 Electrochemical Detector
AS3500 Autosampler

PeakNet Chromatography Workstation

Savant Speed Vac® Concentrator, Model A290 (Savant Instruments-EC Apparatus, Inc.)

Spectra/Por® Membrane; molecular weight cut off: 1000; diameter: 29 mm, length: 5 m (Spectrum Medical Industries, Inc., available from VWR Scientific)

REAGENTS AND STANDARDS

Human serum transferrin, holo, iron saturated (Boehringer Mannheim)

Triton X-100, hydrogenated, protein grade, 10% (Calbiochem)

Anhydrous sodium acetate (Fluka Chemika-BioChemika)

Sodium hydroxide, 50% w/w (Fisher Scientific)

Acetonitrile, HPLC-grade (EM Science)

Ammonium acetate (EM Science)

Sodium phosphate, Na_2HPO_4 (Sigma)

β-Mercaptoethanol, approximately 98%, d = 1.11 g/mL (Sigma)

Trifluoroacetic acid (TFA), protein sequencing grade, 1-mL sealed ampules (Sigma)

Peptide-N-Glycosidase F (PNGase F), from Flavobacterium meningosepticum, recombinant in E. coli (Oxford Glycosciences)

Neuraminidase from Vibrio cholerae (Oxford Glycosciences)

N-Acetylneuraminic acid, NANA, P/N A9646 (Sigma)

Asialo biantennary, NA2, P/N C-024300 (Oxford Glycosciences)

CONDITIONS AND METHODS

Columns: CarboPac PA-100 Analytical Column, 4 × 250 mm, and Guard Column, 4 × 50 mm
DNAPac PA-100 Analytical Column, 4 × 250 mm, and Guard Column, 4 × 50 mm
DNAPac PA-100 Semi-Prep Column, 9 x 250 mm
Zorbax® RP300-C18 Analytical Column, 4.6 x 150 mm

Flow Rates: CarboPac PA-100 Analytical Column, 1 mL/min
DNAPac PA-100 Analytical Column, 1 mL/min
DNAPac PA-100 Semi-Prep Column, 5 mL/min
Zorbax RP300-C18 Analytical Column, 1 mL/min

Pulse Setting for ED40 Detector:

t (s)	E (V)	Integration
0.0	0.05	
0.2	0.05	Begin
0.4	0.05	End
0.41	0.75	
0.6	0.75	
0.61	–0.15	
1.0	–0.15	

Absorbance Detector: UV, 215 nm

Eluent A: 250 mM Sodium acetate

Eluent B: 500 mM Sodium hydroxide

Eluent C: Deionized water (DI H_2O), 17.8 MΩ-cm resistance or better

Eluent D: 500 mM Ammonium acetate

Eluent E: 0.1% TFA in deionized water

Eluent F: 0.085% TFA in 10% deionized water/90% acetonitrile

Methods:

Method	Column	Time (min)	A (%)	B (%)	C (%)	D (%)	E (%)	F (%)
1	CarboPac PA-100	0	8	20	72	0	0	0
		5	8	20	72	0	0	0
		65	80	20	0	0	0	0
		65.05	8	20	72	0	0	0
		70	8	20	72	0	0	0
2	DNAPac PA-100 Analytical & Semi-Prep	0	0	0	95	5	0	0
		3	0	0	95	5	0	0
		33	0	0	80	20	0	0
		36	0	0	80	20	0	0
		36.05	0	0	95	5	0	0
		41	0	0	95	5	0	0
3	Zorbax RP300-C18	0	0	0	0	0	95	5
		5	0	0	0	0	95	5
		60	0	0	0	0	40	60
		61	0	0	0	0	20	80
		64	0	0	0	0	20	80
		65	0	0	0	0	95	5
		90	0	0	0	0	95	5

PREPARATION OF SAMPLES AND SOLUTIONS

Eluent A: 250 mM Sodium acetate

Dissolve 20.5 g of anhydrous sodium acetate into a final volume of 1.0 L of deionized water. Filter the eluent through a 0.2-µm filter, and then vacuum degas the eluent for 5 minutes before use.

Eluent B: 500 mM Sodium hydroxide

Filter 1.0 L of deionized water through a 0.2-µm filter. Then vacuum degas the deionized water for 5 minutes. Dilute 26 mL of 50% (w/w) sodium hydroxide to a final volume of 1.0 L with the degassed water.

Eluent C: Water

Filter 1.0 L of deionized water through a 0.2-µm filter. Then vacuum degas the deionized water for 5 minutes before use.

Eluent D: 500 mM Ammonium acetate

Dissolve 38.5 g of ammonium acetate in deionized water to make up a 1-L solution. Filter the ammonium acetate solution through a 0.2-µm filter before use.

Eluent E: 0.10% TFA in Deionized water

Dilute 500 µL of TFA to 500 mL with deionized water and vacuum degas.

Eluent F: 0.085% TFA in 10% Deionized water/ 90% Acetonitrile

Add 425 µL of TFA to 50 mL of deionized water. Dilute to 500 mL with acetonitrile and vacuum degas.

EXOGLYCOSIDASE AND AMIDASE PREPARATIONS

Neuraminidase from *Vibrio cholerae*

Dissolve 0.2 U of the enzyme in 40 µL of the 5X incubation buffer (50 mM sodium acetate with 4 mM calcium chloride, pH 5.5) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

PNGase F (from *Flavobacterium meningosepticum*, recombinant in *E. coli*)

Dissolve 20 U of the enzyme in 40 µL of the 5X incubation buffer (20 mM sodium phosphate with 50 mM EDTA, 0.02% sodium azide, pH 7.5) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

CARBOHYDRATE STANDARDS

***N*-Acetylneuraminic acid (NANA, supplied as 25 nmol dry powder, final concentration: 50 nmol/mL)**

Add 500 µL of deionized water to 25 nmol of the carbohydrate as supplied.

NA2 (Asialo biantennary oligosaccharides)

To prepare each oligosaccharide stock, add 500 µL of water to 100 µg of the carbohydrate as supplied.

Fractionation

A quantity of 10 mg of HST was dissolved in 1 mL of 100 mM sodium phosphate, pH 7.2. An injection of 2 mg (200 µL) was then applied to the DNAPac PA-100 semi-prep column. Three column fractions designated F1, F2, and F3, as shown in Figure 2, were collected. F1 was collected between 18 and 20 minutes, F2 was collected between 22.5 and 24.5 minutes, F3 was collected between 28 and 30 minutes.

Preparation of Samples for the Zorbax C18 Reversed-Phase Separations

1. DNAPac PA-100 fractions of HST: 2 mg of HST was injected onto the semi-prep DNAPac PA-100 column. Fractions were collected as described above. Each fraction (in ammonium acetate buffer) was dried in a SpeedVac, redissolved in 200 µL of distilled water, and then dried again using the SpeedVac. This procedure was repeated four times to remove the residual ammonium acetate. These dried samples were finally redissolved in 200 µL of deionized water.
2. Unfractionated HST: 1 mg of HST was dissolved in 1 mL of water.

PNGase F Digestion

1. DNAPac PA-100 fractions of HST: Each fraction (in ammonium acetate buffer) was dried in a SpeedVac, redissolved in 200 µL of distilled water, and then dried again using the SpeedVac. This procedure was repeated four times to remove the residual ammonium acetate. These dried samples were then redissolved in 100 µL, 100 mM sodium phosphate, pH 7.2 with 10 mM β-mercaptoethanol and 4 µL Triton X-100. PNGase F, 1 U, was added to each sample, and the samples were incubated at 25 ˚C for 24 hours.
2. Unfractionated HST: 500 µg of HST were dissolved in 250 µL, 100 mM sodium phosphate, pH 7.2 with 10 mM β-mercaptoethanol and 10 µL Triton X-100. PNGase F, 2 U, were then added, and the sample was incubated at 25 ˚C for 24 hours.

Dialysis of the PNGase F Digests

The PNGase F digests were transferred into individual Spectra/Por dialysis tubing, and were dialyzed in distilled water for 27 hours. The distilled water was replaced four times (at 2, 4, 6, and 24 hours after the beginning of dialysis). The dialyzed samples were analyzed by HPAE-PAD as described in the Conditions and Methods section. Each dialyzed sample, 50 µL, was also retained for treatment with neuraminidase as described below.

Neuraminidase Digestions

A quantity of 50 µL from each of the PNGase F treated, dialyzed samples was dried in a SpeedVac. Each of the dried samples was then dissolved in 50 µL of 100 mM sodium acetate, pH 5.5, and incubated with 5 mU of neuraminidase at 37 ˚C for 24 hours. Samples were analyzed by HPAE-PAD as described in the Methods section.

RESULTS AND DISCUSSION

Fractionation of HST Glycoforms

HST was analyzed using an analytical DNAPac PA-100 column as shown in Figure 1A; three major peaks can be identified from 15 to 28 minutes. Treatment of the HST sample with neuraminidase (an exoglycosidase that removes sialic acid residues from the nonreducing ends of the attached oligosaccharides) collapses all three peaks into a broad peak eluting close to the void volume of the column, as shown in Figure 1B.

The results indicate that upon removal of terminal sialic acids, the retention of all three HST populations is significantly reduced. Prior work has indicated that upon removal of sialic acids, HST elutes at the void volume. The neuraminidase digestion apparently did not remove all the sialic acids from the HST glycans as the treated HST peak is not eluted at the void volume as shown Figure 1B. The results, however, provide strong evidence that the existence of the three peaks from the untreated HST is due to the sialylation of the HST glycans.

HST was also separated using a semi-preparative DNAPac PA-100 column. The goal was to inject a larger amount of HST (in this example, milligrams), and to collect fractions corresponding to the three peaks in a single separation. As shown in Figure 2, the semipreparative separation resembles the one using the analytical column. The three peaks were identified as F1, F2, and F3 (Fractions 1, 2, and 3 respectively), as shown in Figure 2, and the fractions were collected for further analysis.

Reversed-Phase HPLC of the DNAPac PA-100 Fractions

The DNAPac PA-100 fractions and an unfractionated HST were analyzed using a Zorbax C18 column. A single peak eluting at 44.2 minutes was obtained from a reversed-phase separation of the HST (before neuraminidase treatment), as shown in Figure 3A. A single peak with identical retention time is obtained from each of the three fractions, and these peaks also coelute with that of the unfractionated HST, as shown in Figures 3A–3D. The results confirm that all three DNAPac peaks originate from HST, and suggest that there is no detectable degradation of HST on the DNAPac column.

PNGase F Digestion of the DNAPac PA-100 Fractions

Treatment of the DNAPac PA-100 fractions with PNGase F released the N-linked oligosaccharides. The digestions were analyzed by HPAE-PAD to profile the released glycans, and to obtain a relative distribution of the sialylated oligosaccharides. The determination of the extent of sialylation of the oligosaccharides is possible

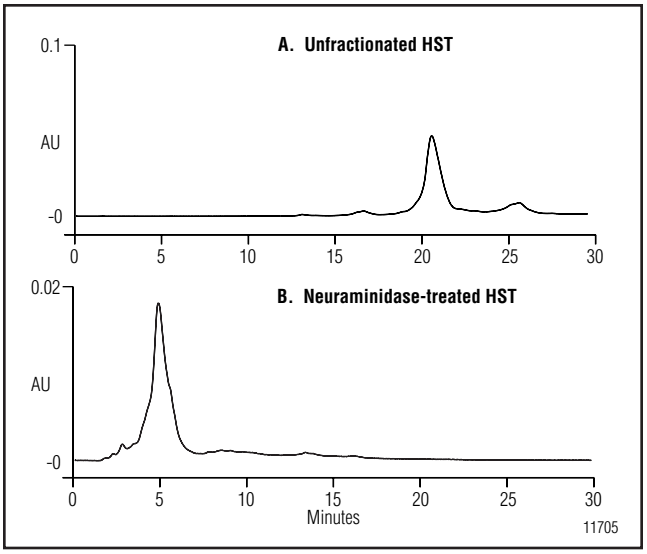

Figure 1. (A) Separations of unfractionated HST. Column: DNAPac PA-100 analytical column. Sample injected: 600 µg; (B) Separation of Neuraminidase-treated HST. Column: DNAPac PA-100 analytical column. Sample injected: 50 µg.

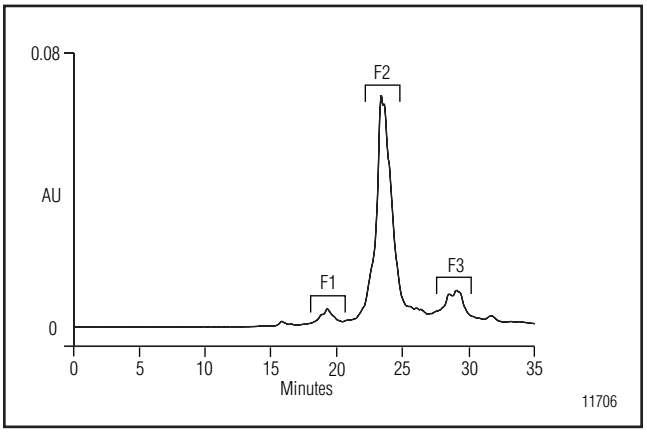

Figure 2. Semi-prep DNAPac PA-100 separation of unfractionated HST. Column: DNAPac PA-100 semi-prep column. Sample injected: 2 mg.

because mono-, di-, and trisialylated oligosaccharides elute in the regions between 20–30, 30–40, and 40–48 minutes, respectively, using the method (Method 1) suggested in this note.[1-5] These retention time windows can be reconfirmed using available Oxford Glycosciences oligosaccharides (C-124300, C-224300, and C-335300). The relative distribution of the three classes of oligosaccharides was determined based on peak areas.

Figures 4A–4D show the separations of the PNGase F digestions of the unfractionated HST, F1, F2, and F3, respectively. For the separation of the unfractionated HST, a main peak (Peak 3) eluted at approximately 32 minutes, and three minor peaks (Peaks 4, 5, and 6) eluted between 32 and 36 minutes, as shown in Figure 4A. The retention times of these features suggested that the majority of glycans in the unfractionated HST are disialylated species. As shown in Figure 4B, Peaks 3, 4, and 5, corresponding to the disialylated species present in the unfractionated HST, are also observed in the separation of F1. In addition, two peaks (Peaks 1 and 2) eluted in the region between 21–24 minutes, indicating that there are monosialylated species present in F1. Similarly, the disialylated species (Peaks 3–5) observed in F1 and the unfractionated HST are also present in F2, as shown in Figure 4C. The mono-sialylated species found in F1, however, are absent in F2. Peaks 3–6, corresponding to the disialylated species of the unfractionated HST, are present in F3, as shown in Figure 4D. In addition, three peaks (Peaks 7–9) eluted at the region between 41–45 minutes, indicating that a population of trisialylated species is also present in F3.

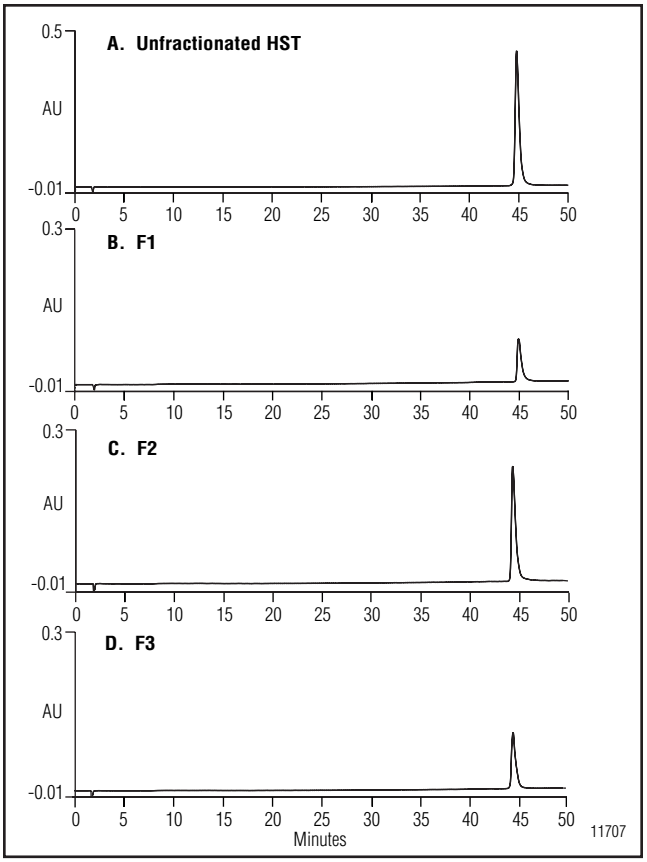

Figure 3. Reversed-phase separation of unfractionated HST, F1, F2, & F3. (A) Unfractionated HST. Sample injected: 30 µg. (B) F1. Sample injected: 9 µL from the 200 µL reconstituted fraction. (C) F2. Sample injected: 9 µL from the 200 µL reconstituted fraction; (D) F3. Sample injected: 9 µL from the 200 µL reconstituted fraction. Column: Zorbax RP 300-C18 analytical column.

Neuraminidase Treatment of the PNGase F Digestions

To confirm that the distribution of peaks observed in the separations of the different PNGase F digestions actually reflects oligosaccharide sialylation, neuraminidase digests were performed. Aliquots from each of the PNGase F digestions were treated with neuraminidase and then analyzed by HPAE-PAD. The retention times of the sialylated peaks observed in Figures 4 should be reduced significantly; whereas the nonsialylated oligosaccharides features should not be affected.

Separations of all three DNAPac fractions, the unfractionated HST, an asialo bi (NA2) standard, and a NANA standard, are shown in Figures 5A–5F. The sialylated oligosaccharides peaks (Peaks 1–9 of Figures 4A–4D) are no longer present in the four digestions shown in Figures 5A–5D, indicating that these peaks indeed represent sialylated oligosaccharides. The peaks eluting at approximately 13 minutes in Figures 5A–5D coelute with the NANA standard shown in Figure 5F. The peaks eluting at approximately 11.3 minutes from the unfractionated HST and F2 separations, as shown in Figures 5A and 5C, coelute with an asialo biantennary standard shown in Figure 5E. These results suggest that the main oligosaccharide species in HST is an N-linked, disialylated biantennary oligosaccharide. A broad peak eluting between 10 and 12 minutes is observed in F1 and F3, as shown in Figures 5B and 5D, respectively. Further investigation is needed to confirm the identities of these peaks.

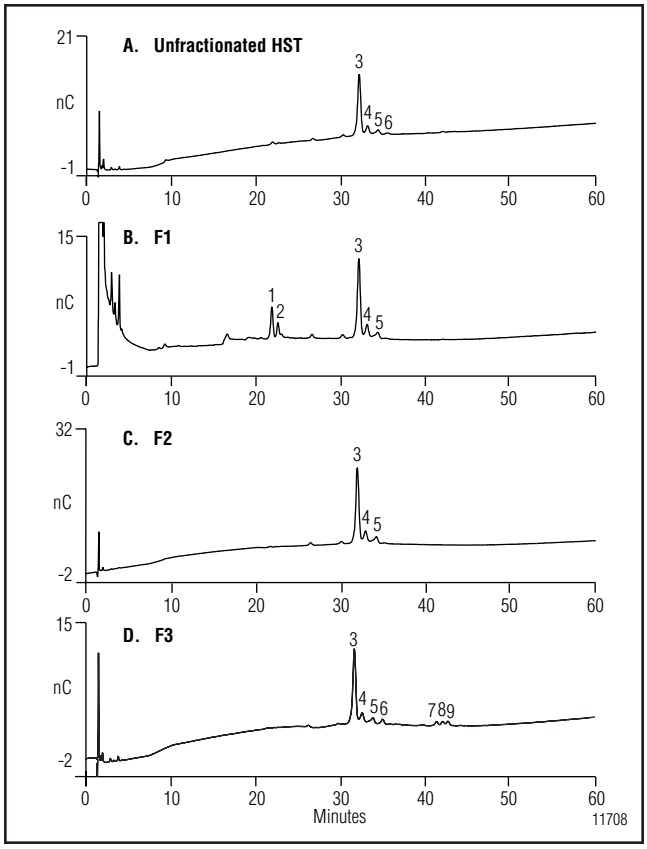

Figure 4. Separations of PNGase F digestions of unfractionated HST, F1, F2, & F3. (A) Unfractionated HST. Sample injected: 25 µL from the PNGase F digest; (B) F1. Sample injected: 25 µL from the PNGase F digest; (C) F2. Sample injected: 10 µL from the PNGase F digest; (D) F3. Sample injected: 20 µL from the PNGase F digest. Column: CarboPac PA-100 column.

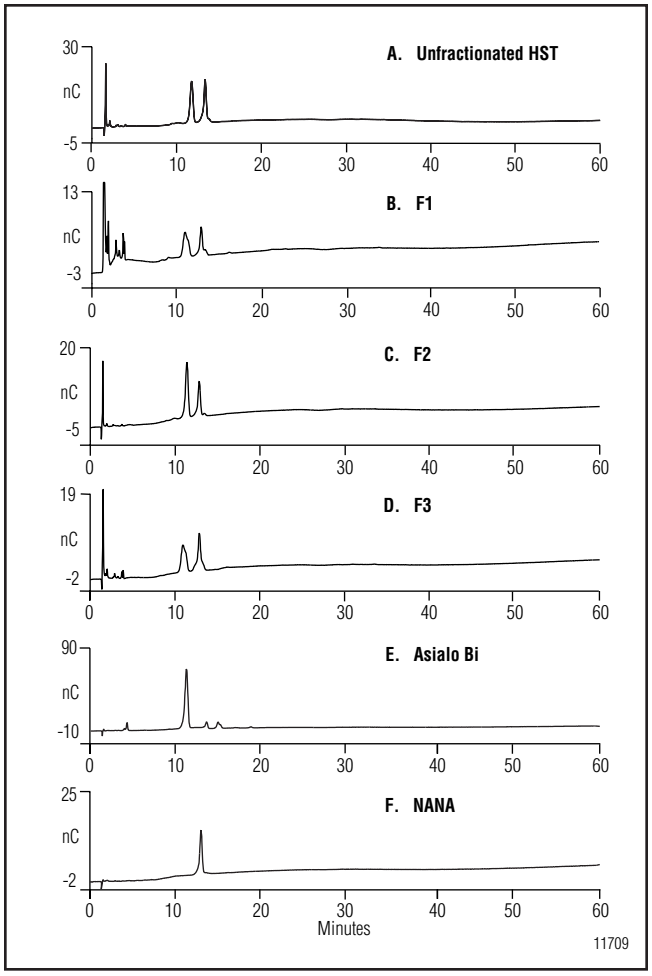

Figure 5. Separations of neuraminidase-treated PNGase F digestions of unfractionated HST, F1, F2, & F3. (A) Unfractionated HST. 10 µL from the neuraminidase + PNGase F digest; (B) F1. sample injected: 20 µL from the neuraminidase + PNGase F digest; (C) F2. sample injected: 10 µL from the neuraminidase + PNGase F digest; (D) F3. 20 µL from the neuraminidase + PNGase F digest; (E) Asialo Biantennary Standard (PI 01); (F) N-Acetyl neuraminic acid. Column: CarboPac PA-100 column.

Distribution of the PNGase F-Released, Sialylated Oligosaccharides

Peak area analysis of the different sialylated oligosaccharide peaks for the unfractionated HST and the HST fractions is shown in Table 1. Both the unfractionated HST and F2 contain more than 95% of disialylated oligosaccharides. F1, the fraction eluted before the main peak from the DNAPac PA-100 separation, contains approximately 30% and 70% of monosialylated and disialylated oligosaccharides, respectively. F3, the fraction eluted after the main peak from the DNAPac PA-100 separation, contained approximately 90% and 10% of disialylated and trisialylated oligosaccharides.

It has been reported that the relative molar electrochemical responses for di-, tri, tetra, and pentasialylated oligosaccharides are similar (4.8 ± 14% relative to glucose).[14] This peak area analysis thus provides a good approximation of the relative distribution of the sialylated glycans released form HST.

Table 1 Peak area of sialylated oligosaccharide peaks

Sample	Peak Area (%)		
	Mono-sialylated	Di-sialylated	Tri-sialylaed
Unfractionated HST	3	97	Below Detection Limit
F1	30	70	0
F2	1	99	0
F3	1	89	10

The peak area percentage of the disialylated species is higher compared to that of the monosialylated species, and to that of the trisialylated species in F1 (70% to 30%) and F3 (90% to 10%), respectively. These ratios indicate that there may be partially sialylated oligosaccharides present in the fractions. The glycoforms containing these partially sialylated oligosaccharides apparently were retained differently on the DNAPac column, compared to those with fully sialylated oligosaccharides and an identical number of sialic acids. Further investigation is needed to confirm the presence of these species in the two fractions.

The results support earlier observations that structural differences in the carbohydrate moiety also contribute to separation of the glycoforms on the DNAPac column.[2]

SUMMARY

- The DNAPac PA-100 column fractionates sialylated glycoforms based on the degree of sialylation and other structural features. Glyco-forms with a higher degree of sialylation are retained longer in the column.
- The analytical DNAPac PA-100 column permits analytical scale separation of sialylated glycoforms. The semi-prep DNAPac PA-100 column allows fractionation of larger quantity of proteins (milligrams, in this example) suitable for post-separation analysis.
- Minor glycoform populations can be separated by the semi-prep DNAPac PA-100 column in sufficient quantity for subsequent analysis.
- HPAE-PAD, with a CarboPac PA-100 column, can be used to analyze the released oligosaccharides and characterize the distribution of sialylated oligosaccharides from the different glycoform populations.

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LIST OF SUPPLIERS

Boehringer Mannheim Corporation, 9115 Hague Road, Indianapolis, Indiana, 46250-0414, USA, Tel: 1-800-262-1640.

CALBIOCHEM, P.O. Box 12087, La Jolla, California, 92039-2087, USA, Tel: 1-800-854-3417.

Dionex Corporation, P.O. Box 3603, Sunnyvale, California, 94088-3603, USA, Tel: 1-800-346-6390.

EM Science, P.O. Box 70, 480 Democrat Road, Gibbstown, New Jersey, 08027, USA, Tel: 1-800-222-0342.

Fisher Scientific, 711 Forbes Ave., Pittsburgh, Pennslyvania, 15219-4785, USA, Tel: 1-800-766-7000.

Fluka Chemika-BioChemika, Fluka Chemie AG, Industriestrasse 25, CH-9471 Buchs, Switzerland, Tel: +81 755 25 11.

Oxford Glycosciences, Cross Island Plaza, 133-33 Brookville Boulevard, Rosedale, New York, 11422, USA, Tel: 1-800-722-2597.

Savant Instruments-EC Apparatus Inc., 100 Colin Drive, Holbrook, NY 11741-4306, USA, Tel: 1-800-327-2643.

Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri, 63178, USA, Tel: 1-800-325-3010.

VWR Scientific, P.O. Box 7900, San Francisco, California, USA, Tel: 1-800-932-5000.

Thermo SCIENTIFIC

Application Update 180

Direct Determination of Sialic Acids in Glycoprotein Hydrolyzates by HPAE-PAD

INTRODUCTION

Sialic acids are critical in determining glycoprotein bioavailability, function, stability, and metabolism.[1] Although over 50 natural sialic acids have been identified,[2] two forms are commonly determined in glycoprotein products: *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Because humans do not generally produce Neu5Gc and have been shown to possess antibodies against Neu5Gc, the presence of this sialic acid in a therapeutic agent can potentially lead to an immune response.[3] Consequently, glycoprotein sialylation, and the identity of the sialic acids, play important roles in therapeutic protein efficacy, pharmacokinetics, and potential immunogenicity.

Sialic acid determination can be performed by many methods. Typically, sialic acids are released from glycoproteins by acid hydrolysis or by enzymatic digestion before analysis. Once the sialic acids are liberated, there are many options for quantification. Numerous spectroscopic methods exist, although interferences in these methods can cause overestimation of the concentration of sialic acids in many samples. Therefore, chromatographic methods that separate the sialic acids from potentially interfering compounds are preferred.[4] Among the chromatographic methods, there are thosethat require further sample derivatization for analyte detection, such as fluorescent labeling followed by high-performance liquid chromatography (HPLC), and direct detection methods such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD).[5] Of these methods, HPAE-PAD offers the advantage of direct analysis without sample derivatization.

In this work, sialic acids are determined in five representative glycoproteins by acid hydrolysis followed by HPAE-PAD. Sialic acid determination by HPAE-PAD on a CarboPac® PA20 column is specific and direct, eliminating the need for sample derivatization after sample preparation. The use of a disposable gold on polytetrafluoroethylene (Au on PTFE) working electrode simplifies system maintenance compared to conventional gold electrodes while providing consistent response with a four-week lifetime. The rapid gradient method discussed separates Neu5Ac and Neu5Gc in under 10 min with a total analysis time of 16.5 min, compared to 27 min using the CarboPac PA10 column by a previously published method.[6,7] By using the CarboPac PA20 column, the total analysis time is reduced, eluent consumption and waste generation are reduced, and sample throughput is improved.

EQUIPMENT

Dionex ICS-3000 or ICS-5000 Ion Chromatography system including:

SP Single Pump or DP Dual Pump module

DC Detector/Chromatography module

AS Autosampler

ICS-3000/5000 ED Electrochemical Detector (Dionex P/N 061719)

Electrochemical Cell (Dionex P/N 061757)

Disposable Gold Working Electrode, Au on PTFE (Dionex P/N 066480)

Reference Electrode (Ag/AgCl) (Dionex P/N 061879)

Chromeleon® 7 Chromatography Workstation

Polypropylene injection vials, 0.3 mL, with caps (Dionex P/N 055428)

Polypropylene injection vials, 1.5 mL, with caps (Dionex P/N 061696)

Nalgene® 1000 mL 0.2 µm nylon filter units (VWR P/N 28198-514)

Polypropylene microcentrifuge screw-cap tubes, 1.5 mL (Sarstedt P/N 72.692.005)

Dry block heater (VWR P/N 13259-005)

REAGENTS AND STANDARDS

Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better

Sodium hydroxide, 50% (w/w) (Thermo Fisher P/N SS254-500)

Sodium acetate, anhydrous (Dionex P/N 059326)

Acetic acid (JT Baker P/N 9515-03)

N-Acetylneuraminic acid (Neu5Ac, NANA), Ferro Pfanstiehl

N-Glycolylneuraminic acid (Neu5Gc, NGNA), Ferro Pfanstiehl

Micro BCA™ Protein Assay Kit (Thermo Scientific P/N 23235)

α (2→3,6,8,9) Neuraminidase, proteomics grade from *Arthrobacter ureafaciens* (Sigma P/N N3786)

SAMPLES

Five glycoproteins were selected for analysis:

Calf fetuin (Sigma P/N F2379)

Bovine apo-transferrin (b. apo-transferrin) (Sigma P/N T1428)

Human transferrin (h. transferrin) (Sigma P/N T8158)

Sheep α_1-acid glycoprotein (s. AGP) (Sigma P/N G6401)

Human α_1-acid glycoprotein (h. AGP) (Sigma P/N G9885)

CONDITIONS

Columns: CarboPac PA20, 3 × 150 mm (P/N 060142)
CarboPac PA20 Guard, 3 × 30 mm (P/N 060144)

Eluent Gradient: 70–300 mM acetate in 100 mM NaOH from 0–7.5 min, 300 mM acetate in 100 mM NaOH from 7.5–9.0 min, 70 mM acetate in 100 mM NaOH from 9.0–9.5 min, 7 min of equilibration at 70 mM acetate in 100 mM NaOH.

Eluents: A: NaOH, 100 mM
B: Sodium acetate, 1.0 M, in 100 mM NaOH

Flow Rate: 0.5 mL/min

Temperature: 30 °C (column and detector compartments)

Inj. Volume: 10 µL

Detection: Pulsed amperometric, disposable Au on PTFE electrode

Background: 18–25 nC (using the carbohydrate waveform)

Noise: ~15–30 pC

System Backpressure: ~3000 psi

Carbohydrate 4-Potential Waveform for the ED

Time(s)	*Potential (V)*	*Gain Region**	*Ramp**	*Integration*
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	-2.0	Off	On	Off
0.42	-2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.50	-0.1	Off	On	Off

*Settings required in the ICS-3000/5000, but not used in older Dionex systems.

Reference electrode in Ag mode (Ag/AgCl reference). See Application Update 141 for more information.[6]

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent Solutions

Prepare 1 L of 100 mM sodium hydroxide by adding 5.2 mL of 50% (w/w) NaOH to 994.8 mL of degassed DI water.

Prepare 1 L of 1 M sodium acetate in 100 mM sodium hydroxide by dissolving 82.0 g of anhydrous sodium acetate in ~800 mL of DI water. Filter and degas the acetate solution through a 0.2 µm nylon filter unit. Transfer the solution to a 1 L volumetric flask, add 5.2 mL of 50% (w/w) NaOH, and fill the flask with degassed DI water. See Dionex Technical Note 71 for detailed information on eluent preparation for HPAE-PAD applications.[8]

Acetic Acid, 4 M

Transfer 22.5 mL of glacial acetic acid to a polyethylene bottle containing 77.5 mL of DI water.

Sodium Acetate Buffer, 0.1 M, pH 5 for Neuraminidase Digestions

Prepare a 0.3 M sodium acetate stock solution by dissolving 12.31 g of sodium acetate in 500 mL of DI water. Transfer 68.21 g (68.3 mL) of 0.3 M sodium acetate to a 250 mL polypropylene bottle. Add 1.8 mL of 4 M actetic acid to the solution. Dilute to a total of 249.62 g (250 mL).

Stock Standard Solutions

Dissolve 149.8 mg of Neu5Ac in 50 mL DI water and 41.0 mg Neu5Gc in 50 mL of DI water. This results in 9.68 mM and 2.52 mM stock solutions, respectively. Dilute 500 µL of 9.68 mM Neu5Ac and 130 µL of 2.52 mM Neu5Gc to 48.4 mL total with DI water. Aliquot this mixed stock of 0.10 mM Neu5Ac and 6.8 µM Neu5Gc into 1.5 mL cryogenic storage vials and store at -40 °C.

Working Standard Solutions

Prepare calibration standards by diluting the standard stock solution as detailed in Table 1. For example, 10 µL of the stock solution were added to 990 µL of DI water to prepare a calibration standard of 1.0 µM Neu5Ac, or 10 pmol/10 µL injection. Prepare standards daily from the stocks stored at -40 °C.

Protein Stock Solutions, 4.0 mg/mL Nominal

Dissolve 3.44 mg of sheep α_1-acid glycoprotein in 860 µL of DI water. Gently swirl to thoroughly mix the solution. Prepare 200 µL aliquots of the solution in microcentrifuge vials to minimize freeze/thaw cycles when the stock is needed. Store all protein solutions at -40 °C. Repeat this process as follows. Dissolve 8.8 mg (b. apo-transferrin), 8.6 mg (h. transferrin), and 8.1 mg (fetuin) of the glycoprotein in individual aliquots of 2.0 mL DI water. Dissolve 2.2 mg of h. AGP in 0.56 mL of DI water. Each glycoprotein will be at a nominal concentration of 4 mg/mL.

Table 1. Sialic Acid Standards Used for Sample Analysis

Volume of Combined Stock Standard (µL) Diluted to 1000 µL	Neu5Ac Concentration (nM)	Neu5Gc Concentration (nM)	Neu5Ac Amount (pmol/10µL)	Neu5Gc Amount (pmol/10 µL)
1.0	100	7.8	1.0	<LOQ*
2.5	250	20	2.5	<LOQ*
5.0	500	39	5.0	0.39
10	1000	78	10	0.78
25	2500	200	25	2.0
50	5000	390	50	3.9
75	7500	580	75	5.8
100	10000	780	100	7.8

*Not used for Neu5Gc calibration

Table 2. Protein Hydrolyzate Concentrations						
Protein	**BCA Measured Working Soln. Conc. (µg/mL)**	**Volume Protein (µL)**	**Amount of Protein (µg)**	**Volume DI Water (µL)**	**Volume 4M Acetic Acid (µL)**	**Protein Conc. (µg/µL)**
Fetuin	280	50	14	50	100	0.07
h. Transferrin	400	50	20	50	100	0.10
b. apo-Transferrin	500	50	25	50	100	0.12
h. AGP	260	50	13	50	100	0.06
s. AGP	140	50	7.0	50	100	0.04

Working Stock Protein Solutions

Pipet 250 µL of a protein stock solution into 1750 µL DI water to prepare a working stock solution. Aliquot 400 µL of the working stock into individual microcentrifuge tubes and store the working stock solutions at -40 °C. Protein may be lost both during freeze/thaw cycles and by adsorption to surfaces. Therefore, it is important to measure the working stock protein concentrations before hydrolysis by using a colorimetric BCA protein assay kit. Values listed in Table 2 are results from BCA assay of the working stock solutions.

Acetic Acid Hydrolysis of Proteins

Add 14 µg (fetuin), 20 µg (h. transferrin), 25 µg (b. apo-transferrin), 13 µg (h. AGP), and 7 µg (s. AGP) of the glycoprotein to individual 1.5 mL microcentrifuge vials with a total of 200 µL of 2 M acetic acid as detailed in Table 2. For example, pipet 50 µL of the working fetuin stock, 50 µL of DI water, and 100 µL of 4 M acetic acid to prepare the solution for hydrolysis. Hydrolyze the protein solutions for 2 h by the method of Varki et al.[9] to preserve *O*-acetylated sialic acids for comparison to UHPLC-FLD method using DMB derivatization.[10] After hydrolysis, lyophilize and resuspend 50 µL of sample hydrolyzates in 500 µL of DI water; prepare more concentrated hydrolyzates (0.70 µg/µL of protein) by diluting the hydrolyzate 1:80 with DI water. Note that this acid hydrolysis method may not be optimized for complete release of all sialic acids without degradation of the free sialic acids. Optimization of the hydrolysis conditions for a given sample and analysis method is highly recommended. Additional hydrolysis conditions may be found in Technical Note 41.[7]

Neuraminidase Digestion of Proteins

Add 50 µL of DI water to a 25 mU vial of neuraminidase. Add 2 µL of this stock to 148 µL of 0.1 M sodium acetate buffer to prepare a 1 mU/mL neuraminidase solution. Add 14 µg, 20 µg, 25 µg, 13 µg, and 7 µg of fetuin, h. transferrin, b. transferrin, h. AGP, and s. APG, respectively, to individual 1.5 mL microcentrifuge vials with this solution and incubate at 37 °C for 18 h. After incubation, centrifuge the samples and dilute them with an additional 300 µL of DI water prior to analysis.

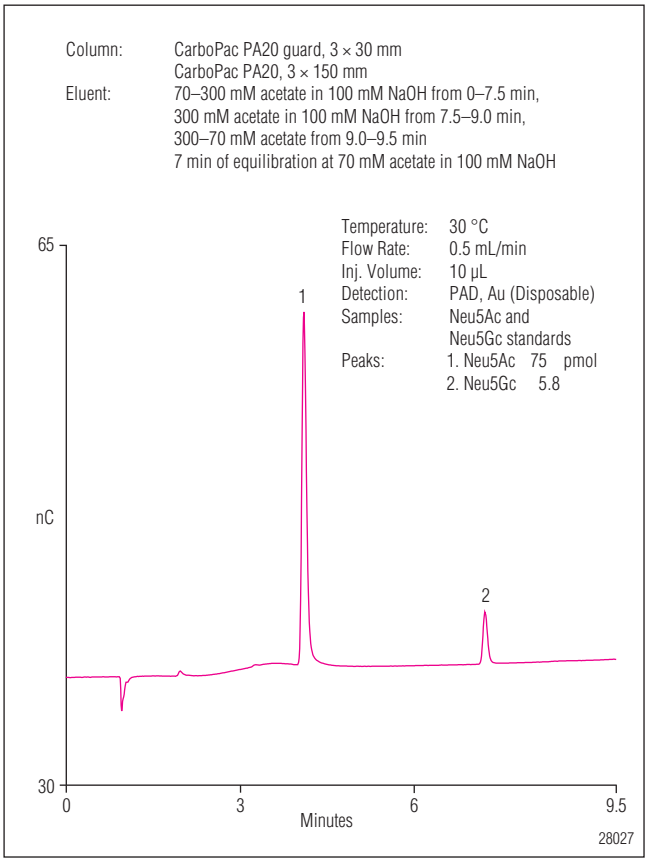

Figure 1. Separation of sialic acid standards on the CarboPac PA20 column.

RESULTS AND DISCUSSION

Figure 1 shows the separation of Neu5Ac and Neu5Gc on the CarboPac PA20 column with a 70–300 mM acetate gradient in 100 mM NaOH. The peaks are well separated and easily quantified. Additionally, the

Table 3. Linearity, LOD, LOQ, and Precision of Sialic Acid Determination							
Analyte	**Range (pmol)**	**Coeff. of Determination (r^2)**	**RT (min)**	**RT Precision (RSD)**	**Peak Area Precision[a] (RSD)**	**LOQ[b] (pmol)**	**LOD (pmol)**
Neu5Ac	1.0–100	0.9997	4.08	0.18	1.08	0.5	0.17
Neu5Gc	0.39–7.8	0.9995	7.18	0.09	1.01	0.3	0.08

[a]Precision was measured by 7 injections of 25 pmol Neu5Ac, 2.0 pmol Neu5Gc.

[b]LOD and LOQ are confirmed by injections at the concentrations listed and measuring response at 3× and 10× the noise, respectively.

Neu5Ac peak is well separated from the void, which is an important consideration when analyzing acid hydrolyzed samples that may contain additional poorly retained compounds.

Linear Range, Limit of Quantification, Limit of Detection, and Precision

Table 3 shows the calibration results for Neu5Ac and Neu5Gc. In both cases, response is linear for the range studied. The limit of detection (LOD) and limit of quantification (LOQ) were confirmed by standard injections that resulted in a response of 3× and 10× the noise, respectively. Neu5Ac was determined to have an LOD of 0.17 pmol on column and an LOQ of 0.50 pmol. Similarly, Neu5Gc limits were found to be 0.08 pmol and 0.30 pmol. During this work, 2 and 3 mil gaskets were installed with the disposable electrodes and evaluated in terms of analyte linearity and LOQ. The results listed were determined with a 3 mil gasket. Injections of LOD and LOQ standards with a 2 mil gasket installed yielded equivalent results. When establishing an assay, standardize on one gasket size and specify it in the standard operating procedure.

Retention time and peak area precisions of standards were determined by seven injections of a mid-range standard. In both cases, precision was excellent, with an RSD of <0.2 for retention time for both sialic acids and peak area RSDs of 1.08 and 1.01 for Neu5Ac and Neu5Gc, respectively.

Sample Analysis, Precision, and Accuracy

Figure 2 shows the separation of hydrolyzed and lyophilized protein samples. In each case, Neu5Ac is well separated from early eluting components of the hydrolyzed sample. In each case, Neu5Ac is present, and as expected, Neu5Gc is not detected in human forms of the glycoproteins. Using fetuin as an example, 0.07 µg/µL of protein were hydrolyzed. After lyophilizing 50 µL of hydrolyzate and dissolving it in 500 µL of DI water (a 10-fold dilution), a 10 µL full-loop injection loads the equivalent of 0.07 µg of protein on the column. After preparation by dilution, 10 µL of 0.75µg/µL hydrolyzate are diluted to a total of 800 µL of sample with the equivalent of 0.09 µg of protein injected with 10 µL. In both cases, there is enough sample for multiple sets of triplicate full-loop injections.

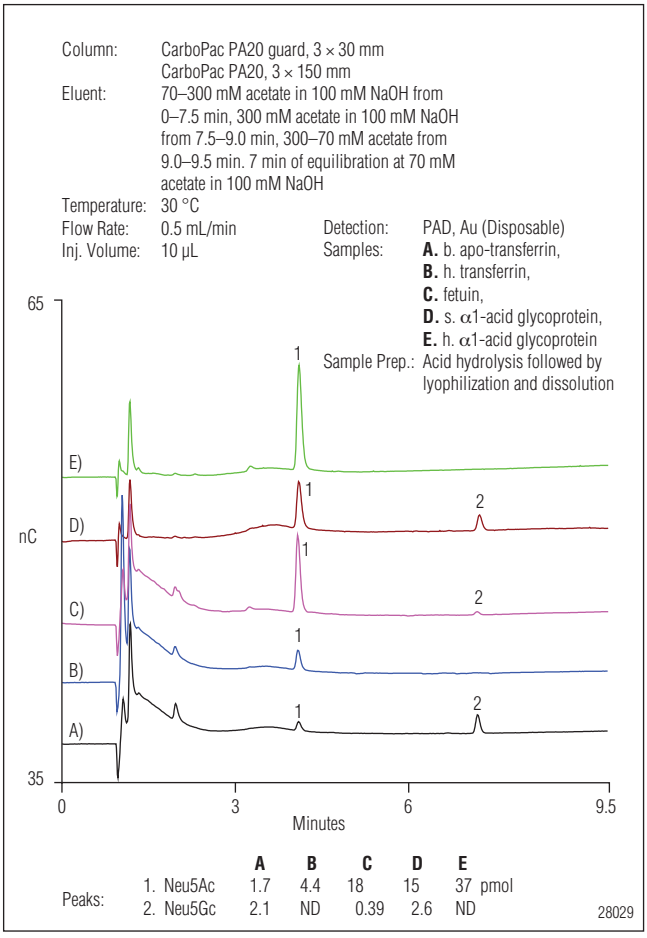

Figure 2. Sialic acid determination of five glycoprotein acid hydrolyzates. A 10% signal offset has been applied.

For glycoproteins that are highly glycosylated, such as α_1-acid glycoproteins, the amount of protein that is hydrolyzed can easily be reduced. In the example of s. AGP, 0.04 µg/µL of protein were hydrolyzed. Because of the high degree of glycosylation, the average determined amount for a single day of sample analysis was 20 pmol of Neu5Ac (Table 4). In comparison, an average of 19 pmol of Neu5Ac was determined in fetuin hydrolyzates, even though nearly twice as much protein was hydrolyzed. Based on the amount of Neu5Gc in the fetuin hydrolyzate and the determined LOQs for Neu5Ac and Neu5Gc, a lower concentration of fetuin is not recommended. However, the concentration of s. AGP can be reduced by a factor of 10 before reaching the LOQ of Neu5Gc and by a factor of 40 before reaching the LOQ of Neu5Ac. Depending on the degree of sialylation, the amount of protein hydrolyzed can be reduced to low ng/µL concentrations and still allow efficient sialic acid determination. Designing experiments that routinely release amounts of analyte near the LOQ is not recommended. However, this evaluation of potential conditions highlights both the sensitivity of the method and the importance of considering the approximate protein sialylation amount when designing acid hydrolysis experiments.

Table 4. Results from Analysis of Triplicate Protein Acid Hydrolysis, n=3 per Hydrolyzed Sample

Sample (replicate #)	Analyte	Amount (pmol)	Peak Area (nC*min)	Peak Area Precision (RSD)	RT (min)	RT Precision (RSD)
Fetuin (1)	Neu5Gc	0.45	0.017	1.76	7.18	0.13
	Neu5Ac	20	0.404	2.76	4.04	0.24
Fetuin (2)	Neu5Gc	0.54	0.020	2.20	7.18	<0.01
	Neu5Ac	23	0.479	0.54	4.04	<0.01
Fetuin(3)	Neu5Gc	0.35	0.014	1.44	7.19	0.12
	Neu5Ac	15	0.313	2.23	4.06	0.21
h. Transferrin (1)	Neu5Gc	ND				
	Neu5Ac	4.4	0.095	2.39	4.08	0.20
h. Transferrin (2)	Neu5Gc	ND				
	Neu5Ac	4.5	0.096	2.66	4.08	<0.01
h. Transferrin (3)	Neu5Gc	ND				
	Neu5Ac	3.9	0.084	2.25	4.07	0.12
b. Transferrin (1)	Neu5Gc	2.6	0.099	0.59	7.20	0.08
	Neu5Ac	2.0	0.044	2.66	4.09	0.14
b. Transferrin (2)	Neu5Gc	2.2	0.083	2.34	7.21	0.07
	Neu5Ac	1.6	0.036	2.22	4.09	0.12
b. Transferrin (3)	Neu5Gc	2.4	0.090	1.01	7.21	0.07
	Neu5Ac	1.8	0.039	2.19	4.10	0.12
h. AGP (1)	Neu5Gc	ND				
	Neu5Ac	42	0.876	1.55	4.10	<0.01
h. AGP (2)	Neu5Gc	ND				
	Neu5Ac	41	0.820	3.52	4.10	<0.01
h. AGP (3)	Neu5Gc	ND				
	Neu5Ac	42	0.865	1.60	4.10	<0.01
s. AGP (1)	Neu5Gc	3.7	0.139	1.31	7.21	0.07
	Neu5Ac	21	0.431	1.39	4.10	<0.01
s. AGP (2)	Neu5Gc	3.4	0.128	0.86	7.21	0.07
	Neu5Ac	19	0.396	0.73	4.10	<0.01
s. AGP (3)	Neu5Gc	3.4	0.131	0.52	7.21	0.07
	Neu5Ac	19	0.403	0.38	4.10	0.12

Table 5. Triplicate Sample Analysis Results Between-Day Precision Over 3 Days, n=3 per Hydrolyzed Sample					
Sample	**Analyte**	**Acid Hydrolysis Average (mol analyte/ mol protein)**	**Intraday Precision Between Replicates (RSD)**	**Between-Day Precision (RSD)**	**Neuraminidase Digestion Average (mol analyte/ mol protein)**
Fetuin	Neu5Gc	0.32	22	14	0.30
	Neu5Ac	14	21	13	19
h. Transferrin	Neu5Gc	ND	ND	ND	ND
	Neu5Ac	3.4	7.8	8.6	4.8
b. apo-Transferrin	Neu5Gc	1.6	8.6	7.9	1.4
	Neu5Ac	1.2	9.5	9.4	1.9
h. AGP	Neu5Gc	ND	ND	ND	ND
	Neu5Ac	25	1.7	8.9	30
s. AGP	Neu5Gc	4.5	4.6	12	3.1
	Neu5Ac	26	4.6	13	25

Table 4 presents the results from one day of triplicate analysis. Retention time precision was similar to that determined by injecting standards, with retention time RSDs ranging from <0.01–0.24. Variability in the absolute retention time may be expected based on the batch of manual eluent prepared. Peak area precision for triplicate injections, as measured by RSD, is generally good, ranging from 0.38–3.52.

Table 5 shows the calculated results of sialic acid analysis for the proteins studied, as well as intraday precision for one day of analysis, as in Table 4, and between-day precision for three days of triplicate analysis. Variability between sample replicates can be large, with RSDs ranging from 1.7 to 22; therefore, optimization of the digestion for individual glycoproteins is highly recommended. Between-day precision, as RSD, ranges from 7.9 to 14, with an average of 11.

Comparison of the determined amounts between acid hydrolysis and neuraminidase digestion suggest that either the hydrolysis is not complete using the mild hydrolysis or that acid degradation of the free sialic acids has occurred. Acid hydrolysis is a complex balance between release of the sialic acids from the glycoprotein and degradation of the released analytes. The efficiency of the hydrolysis will depend on the hydrolysis temperature, acid concentration, type of sample being hydrolyzed, and the relative concentrations of acid and the sample. Because of these interdependent factors—which can impact the hydrolysis—variability between sample preparations can be expected. For the best accuracy, either optimized acid hydrolysis or neuraminidase digestion is recommended. For methodology to optimize acid hydrolysis, see Fan et al.[11] However, the amounts of sialic acids determined in the protein samples are consistent with literature results for the glycoproteins.[12-16]

Table 6. Recoveries, Triplicate Hydrolyzes				
Sample	**Analyte**	**Amount Added (pmol)**	**Average Recovery (Dilution) (%)**	**Average Recovery (Lyophilization) (%)**
Reagent blank	Neu5Gc	0.50	92.7	75.9
	Neu5Ac	5.0	91.3	78.7
Fetuin	Neu5Gc	0.60	99.0	86.4
	Neu5Ac	25	94.7	81.6
h. Transferrin	Neu5Gc	0.50	99.8	74.9
	Neu5Ac	5.0	77.4	74.6
b. apo-Transferrin	Neu5Gc	2.5	76.3	84.9
	Neu5Ac	2.5	83.2	78.9
h. AGP	Neu5Gc	0.50	98.8	74.9
	Neu5Ac	50	102	74.6
s. AGP	Neu5Gc	5.0	88.9	84.9
	Neu5Ac	30	87.2	78.9

Table 7. Stability of Samples Stored at -40 °C				
Sample (Replicate)	**Analyte**	**Initial Determined Amount (pmol)**	**Amount After 14 Days of Storage at -40 °C (pmol)**	**Difference (%)**
h. AGP (1)	Neu5Gc	ND	ND	
	Neu5Ac	42±0.6	45±0.8	7.4
h. AGP (2)	Neu5Gc	ND	ND	
	Neu5Ac	41±1.7	38±0.8	-6.9
h. AGP (3)	Neu5Gc	ND	ND	
	Neu5Ac	42±0.7	35±1.4	-15
s. AGP (1)	Neu5Gc	3.7±0.5	3.6±0.04	-2.3
	Neu5Ac	21±0.3	20±0.2	-4.4
s. AGP (2)	Neu5Gc	3.3±0.03	3.2±0.03	-3.8
	Neu5Ac	19±0.1	18±0.07	-4.4
s. AGP (3)	Neu5Gc	3.4±0.02	3.4±0.03	<0.1
	Neu5Ac	19±0.07	19±0.3	<0.1

Method accuracy was investigated by spiking protein acid hydroylzates with known amounts of Neu5Ac and Neu5Gc equal to the determined amounts. For human glycoproteins, which did not contain Neu5Gc, 0.38 pmol of Neu5Gc was added. Recoveries were evaluated for both sample preparation by lyophilization and by dilution. Recoveries for Neu5Ac ranged from 83–103% by dilution and 75–82% by lyophilization (Table 6). Recoveries for Neu5Gc were similar, ranging from 76–100% by dilution and 75–86% by lyophilization. Where protein amounts are not limited, dilution is recommended for both ease of sample preparation and improved recoveries.

Glycoprotein Hydrolyzate Stability

A set of glycoprotein hydrolyzates was re-analyzed after 14 days of storage at -40 °C. These samples were stored in solution after lyophilization. The comparative results of these stored samples quantified with freshly prepared working standards are shown in Table 7. Values across replicates can be more variable after storage; however, overall, the determined amounts are generally within 10% of the original values.

CONCLUSION

In this work, sialic acids are determined in five representative glycoproteins by acid hydrolysis release and HPAE-PAD. Determination of the sialic acids Neu5Ac and Neu5Gc by HPAE-PAD on a CarboPac PA20 column is specific and direct. After sample hydrolysis or enzymatic treatment, there is no need for further sample derivatization. Disposable Au on PTFE working electrodes with a four-week lifetime simplify system maintenance compared to conventional gold electrodes. The gradient method discussed separates Neu5Ac and Neu5Gc with a total analysis time of 16.5 min, which is faster than previous methods, allowing greater sample throughput.

SUPPLIERS

VWR, 1310 Goshen Parkway, West Chester, PA 19380 U.S.A. Tel: 800-932-5000. www.vwr.com

Fisher Scientific, One Liberty Lane, Hampton, NH 03842 U.S.A. Tel: 800-766-7000. www.fishersci.com

Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178 U.S.A. Tel: 800-325-3010. www.sigma-aldrich.com

Ferro Pfanstiehl, 1219 Glen Rock Avenue, Waukegan, IL 60085 U.S.A. Tel: 800-383-0126. www.ferro.com

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Thermo SCIENTIFIC

Application Update 181

Rapid Screening of Sialic Acids in Glycoproteins by HPAE-PAD

INTRODUCTION

Glycoprotein sialylation has been shown to be critical to bioavailability, stability, metabolism, and immunogenicity of therapeutic proteins.[1-4] As a result, such proteins are routinely analyzed to determine sialylation amount and identity. Although over 50 forms of sialic acid have been identified,[5-6] two forms of this carbohydrate are routinely determined, *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Of these, Neu5Gc is generally not found in human proteins.[7] Due to this lack of Neu5Gc in healthy human tissue and the natural occurrence of antibodies against Neu5Gc, this sialic acid has the potential to cause an immune response in patients when present in a glycoprotein therapeutic.[8]

Many therapeutic proteins are produced via expression of the protein in a cell line chosen to maximize protein yield. Because the final glycoprotein sialylation amount and identity depend on the expression cell line and growth conditions for that cell line,[9-11] expression experiments and production optimization have the potential to generate large numbers of samples requiring analysis. In this case, high-throughput screening assays are valuable for quick product evaluation relative to expression cell lines and growth conditions.

Sialic acid determination can be performed by many methods. Typically, sialic acids are released from glycoproteins by acid hydrolysis or by enzymatic digestion before analysis. Once the sialic acids are liberated, there are multiple options for quantification. Numerous spectroscopic methods exist, although interferences in these methods can overestimate the sialic acid concentrations in many samples, and therefore, chromatographic methods that separate the sialic acids from potentially interfering compounds are preferred.[12] Among the chromatographic methods, some require further sample derivatization for analyte detection, such as fluorescent labeling followed by high-performance liquid chromatography (HPLC). Others use direct detection methods, such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD).[13] Of these two methodologies, HPAE-PAD offers the advantage of direct analysis without sample derivatization.

In this work, sialic acids are determined in five representative glycoproteins by acid hydrolysis followed by HPAE-PAD. Sialic acid determination by HPAE-PAD using the Thermo Scientific Dionex CarboPac™ PA20 Fast Sialic Acid column is specific and direct, eliminating the need for sample derivatization after hydrolysis. The use of a disposable gold on polytetrafluoroethylene (PTFE) working electrode simplifies system maintenance while providing consistent response with a lifetime of four weeks. The rapid gradient method discussed separates Neu5Ac and Neu5Gc in <3 min with a total analysis time of 4.5 min. By using the Dionex CarboPac PA20 Fast Sialic Acid column, total analysis time is reduced, per sample eluent consumption and waste generation are reduced, and sample throughput is improved.

EQUIPMENT

Thermo Scientific Dionex ICS-3000 or Dionex ICS-5000 Ion Chromatography system including:
SP Single Pump or DP Dual Pump module
DC Detector/Chromatography module
AS Autosampler

Electrochemical Detector (P/N 061719)

Electrochemical Cell (P/N 061757)

Disposable Gold Electrode, Au on PTFE (P/N 066480)

Reference Electrode (P/N 061879)

Thermo Scientific Dionex Chromeleon™ 7 Chromatography Workstation

Polypropylene injection vials with caps, 0.3 mL (P/N 055428)

Polypropylene injection vials with caps, 1.5 mL (P/N 061696)

Nalgene™ 1000 mL, 0.2 µm nylon filter units (Fisher Scientific P/N 09-740-46)

Polypropylene microcentrifuge screw cap tubes, 1.5 mL (Sarstedt P/N 72.692.005)

Dry block heater (VWR P/N 13259-005)

REAGENTS AND STANDARDS

Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better

Sodium hydroxide, 50% (w/w) (Fisher Scientific P/N SS254-500)

Sodium acetate, anhydrous (P/N 059326)

Acetic acid (JT Baker P/N 9515-03)

N-Acetylneuraminic acid (Neu5Ac, NANA) Ferro Pfanstiehl

N-Glycolylneuraminic acid (Neu5Gc, NGNA) Ferro Pfanstiehl

Micro BCA™ Protein Assay Kit, (Thermo Scientific P/N 23235)

SAMPLES

Five glycoproteins were selected for analysis:

Calf fetuin (Sigma P/N F2379)

Bovine apo-transferrin (b. apo-transferrin) (Sigma P/N T1428)

Human transferrin (h. transferrin) (Sigma P/N T8158)

Sheep α_1-acid glycoprotein (s. AGP) (Sigma P/N G6401)

Human α_1-acid glycoprotein (h. AGP) (Sigma P/N G9885)

CONDITIONS

Columns:	Dionex CarboPac PA20 Fast Sialic Acid Column, 3 × 30 mm (P/N 076381)
Eluent Gradient:	70–300 mM acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min. 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH
Eluents: A:	100 mM NaOH
B:	1.0 M sodium acetate in 100 mM NaOH
Flow Rate:	0.5 mL/min
Inj. Volume:	4.5 µL, (full loop)
Temperature:	30 °C (column and detector compartments)
Detection:	Pulsed amperometric, disposable Au on PTFE working electrode
Background:	18–25 nC (using the carbohydrate waveform)
Noise:	~15–30 pC
Sys. Backpress.:	~750 psi

Carbohydrate 4-Potential Waveform for the ED

Time(s)	*Potential (V)*	*Gain Region**	*Ramp**	*Integration*
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	-2.0	Off	On	Off
0.42	-2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.50	-0.1	Off	On	Off

*Settings required in the Dionex ICS-3000/5000, but not used in older Dionex systems. Reference electrode in Ag mode (Ag/AgCl reference). See Dionex Application Note 141 and Dionex Technical Note 41 for more information regarding sialic acid determination.[14,15]

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent Solutions

Prepare 1 L of 100 mM sodium hydroxide by adding 5.2 mL of 50% (w/w) NaOH to 994.8 mL of degassed DI water.

Prepare 1 L of 1 M sodium acetate in 100 mM sodium hydroxide by dissolving 82.0 g of anhydrous sodium acetate in ~800 mL of DI water. Filter and degas the acetate solution through a 0.2 µm nylon filter unit. Transfer the solution to a 1 L volumetric flask, add 5.2 mL of 50% (w/w) NaOH, and fill the flask with degassed DI water.

See Dionex Technical Note 71 for detailed information on eluent preparation for HPAE-PAD applications.[16]

Acetic Acid, 4 M

Transfer 22.5 mL of glacial acetic acid to a polyethylene bottle containing 77.5 mL of DI water.

Stock Standard Solutions

Dissolve 149.8 mg of Neu5Ac in 50 mL DI water and 41.0 mg Neu5Gc in 50 mL of DI water. This results in 9.68 mM and 2.52 mM stock solutions, respectively. Add 20 µL 9.68 mM Neu5Ac to 949 µL DI water to prepare a 0.20 mM solution, and 8.0 µL 2.52 mM Neu5Gc to 992 µL DI water to prepare a 0.020 mM solution of Neu5Gc. Add 500 µL of 0.20 mM Neu5Ac and 500 µL of 0.020 mM Neu5Gc to a 1.5 mL cryogenic storage vial to prepare a combined stock of 0.10 mM Neu5Ac and 10 µM Neu5Gc and store at -40 °C.

Working Standard Solutions

Prepare calibration standards by diluting the stock standard solution as detailed in Table 1. For example, add 5.0 µL of the stock solution to 195 µL of DI water to prepare a calibration standard of 2.5 µM Neu5Ac and 0.25 µM Neu5Gc (11 pmol Neu5Ac and 1.1 pmol Neu5Gc per 4.5 µL injection). Prepare working standards daily from the stocks stored at -40 °C.

Table 1. Sialic Acid Standards Used for Sample Analysis

Volume of Combined Stock Standard (µL) Diluted to 1000 µL	Neu5Ac Concentration (µM)	Neu5Gc Concentration (nM)	Neu5Ac Amount (pmol/4.5 µL)	Neu5Gc Amount (pmol/4.5 µL)
0.5	0.25	25	1.1	0.11*
1.0	0.5	50	2.3	0.23
5.0	2.5	250	11.0	1.1
10.0	5.0	500	23.0	2.3
20.0	10.0	1000	45.0	4.5
30.0	15.0	1500	68.0	6.8
40.0†	20.0	2000	90.0	9.0

*Not used for routine Neu5Gc calibration
† Not used for Neu5Ac calibration

Table 2. Protein Hydrolyzate Concentrations						
Protein	**Working Soln. Conc. (mg/mL)**	**Volume Protein (µL)**	**Amount of Protein (µg)**	**Volume DI Water (µL)**	**Volume 4 M Acetic Acid (µL)**	**Protein Conc. (µg/µL)**
Fetuin	2.3	35	80	65	100	0.40
s. AGP	1.0	35	35	65	100	0.18
h. AGP	2.9	35	100	65	100	0.51
b. apo-Transferrin	5.0	35	180	65	100	0.88
h. Transferrin	4.1	35	140	65	100	0.72

Protein Stock Solutions, 4.0 mg/mL Nominal

Dissolve 2.4 mg of sheep α_1-acid glycoprotein in 400 µL of DI water to prepare a 4 mg/mL solution. Gently swirl to thoroughly mix the solution. Prepare 200 µL aliquots of the solution in microcentrifuge vials to minimize freeze/thaw cycles when the stock is needed. Store all protein solutions at -40 °C. Repeat this process as follows. Dissolve 8.8 mg (b. apo-transferrin) and 8.6 mg (h. transferrin) in individual 2 mL aliquots of DI water. Dissolve 19.0 mg of fetuin in 4.75 mL of DI water. Dissolve 2.2 mg of h. AGP in 0.60 mL of DI water. Protein may be lost both during freeze/thaw cycles and by adsorption to surfaces. Therefore, it is important to measure the working stock protein concentrations before hydrolysis using a colorimetric BCA protein assay kit. Values listed in Table 2 are results from BCA assay of the working stock solutions.

Acetic Acid Hydrolysis of Proteins

Add 80 µg fetuin, 140 µg h. transferrin, 175 µg b. apo-transferrin, 100 µg h. AGP, and 35 µg s. AGP to individual 1.5 mL microcentrifuge vials with a total of 200 µL of 2 M acetic acid, as detailed in Table 2. For example, pipet 35 µL of the fetuin stock, 65 µL of DI water, and 100 µL of 4 M acetic acid to prepare the solution for hydrolysis. Hydrolyze the protein solutions for 3 h at 80 °C.[17] After hydrolysis, dilute the hydrolyzate 1:100 with DI water. Please note that this acid hydrolysis method may not be optimized for complete release of all sialic acids without degradation of the free sialic acids. Optimization of the hydrolysis conditions for a given sample and analysis method is highly recommended. Additional hydrolysis conditions may be found in Dionex Technical Note 41.[15]

Precautions

The Dionex CarboPac PA20 Fast Sialic Acid column has been tested for glycoprotein hydrolyzates only. More complex matrixes may not separate acceptably with the conditions presented here. For greater sensitivity and sample stability, lyophilization followed by dissolution in DI water is recommended. See Dionex Application Update 180 for more information on stability of lyophilized hydrolysates that have been dissolved in DI water.[18] To avoid underestimation of sialic acid content due to acid catalyzed degradation, perform analysis of samples prepared by dilution within 24 h of hydrolysis.

Absolute mass detection limits will depend on the sample injection volume. For this reason, a calibrated injection loop was used. Prepare a 4.5 µL sample loop by measuring approximately 3.7 in of 0.010 in. i.d. tubing. Verify the volume of the loop by first weighing the empty tubing, fill the tube with DI water, then reweigh the filled tube and calculate the volume. The total sample volume should be ~4.5 µL. Due to the high-throughput nature of this method, service requirements on the autosampler and injection valves will increase. If replicate injections show poor precision, check the autosampler needle assembly, transfer line, and the injection valve to ensure each is in good condition.

Table 3. Linearity, LOD, LOQ, and Precision of Sialic Acid Determination							
Analyte	**Range (pmol)**	**Coeff. of Determination (r^2)**	**Retention Time (min)**	**Retention Time Precision (RSD)**	**Peak Area Precision[a] (RSD)**	**LOQ[b] (pmol)**	**LOD (pmol)**
Neu5Ac	0.27–68	0.9995	0.745	0.88	1.36	0.34	0.11
Neu5Gc	0.23–11	0.9997	2.58	0.32	1.38	0.18	0.058

[a]Precision was measured by seven injections of 11 pmol Neu5Ac and 1.1 pmol Neu5Gc.

[b]LOD and LOQ are confirmed by injections at the concentrations listed and measuring response at 3× and 10× the noise, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the separation of Neu5Ac and Neu5Gc on the Dionex CarboPac PA20 Fast Sialic Acid column with a 70–300 mM acetate gradient in 100 mM NaOH. The peaks are well separated and easily quantified. Additionally, the Neu5Ac peak is well separated from the void, which is an important consideration because a large void volume peak can interfere with quantification. Neu5Gc elutes in <3 min under these conditions, allowing a short run time.

Linear Range, Limit of Quantification, Limit of Detection, and Precision

Table 3 shows the calibration results for Neu5Ac and Neu5Gc. In both cases, response is linear for the range studied. The limit of detection (LOD) and limit of quantification (LOQ) were confirmed by standard injections that resulted in a response of 3× and 10× the noise, respectively. Neu5Ac had an LOD of 0.11 pmol on column and an LOQ of 0.34 pmol. Similarly, Neu5Gc limits were 0.058 pmol and 0.18 pmol. Retention time and peak area precisions of standards were determined by seven injections of a mid-range standard. In both cases, precision was excellent, with an RSD of <0.9 and a standard deviation of <0.008 min for retention time for both sialic acids and peak area RSDs of 1.36 and 1.38 for Neu5Ac and Neu5Gc, respectively.

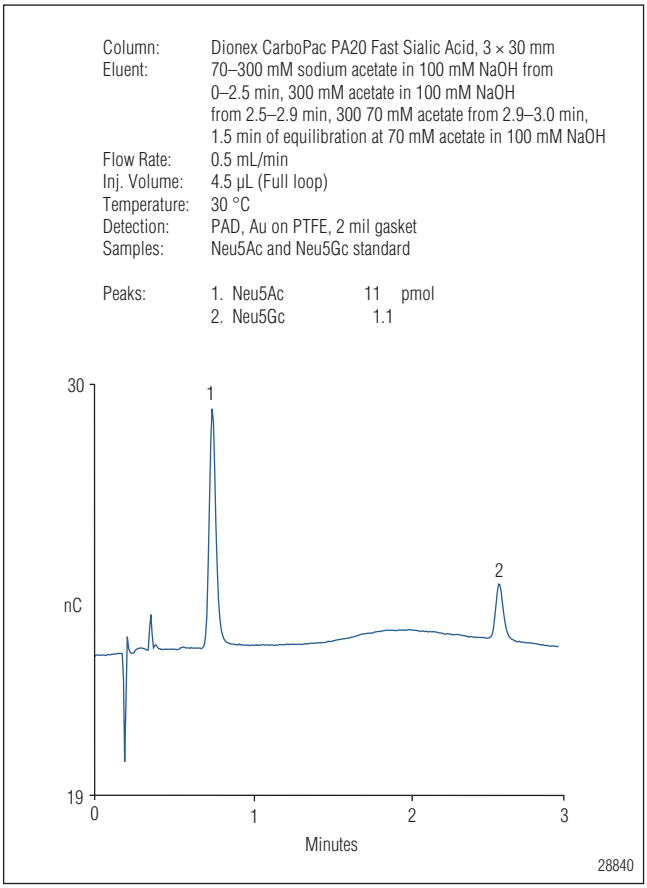

Figure 1. Separation of sialic acid standards on the Dionex CarboPac PA20 Fast Sialic Acid column.

Sample Analysis, Precision, and Accuracy

Figures 2 and 3 illustrate the separation of sialic acids from acid hydrolyzed and diluted protein samples. In each case, Neu5Ac is well separated from early eluting components of the hydrolyzed sample and, as expected, Neu5Gc is not detected in the human glycoproteins. The amount of protein necessary for sialic acid determination depends on the individual protein. For glycoproteins that are highly sialylated, such as α_1-acid glycoproteins, the amount of protein that is hydrolyzed easily can be reduced. In the example of s. AGP, 35 µL of 0.18 µg/µL protein solution are hydrolyzed, which is equivalent to 7.9 ng of protein per injection. The average determined amount for a single day of triplicate sample analysis in the hydrolyzate for s. AGP is 6.1 pmol of Neu5Ac and 1.1 pmol of Neu5Gc. Based on the determined LOQs for Neu5Ac and Neu5Gc, and because of the high degree of sialylation, the concentration of s. AGP can be reduced by a factor of 5 before reaching the LOQ of Neu5Gc and a factor of 17 before reaching the LOQ of Neu5Ac.

Conversely, b. apo-transferrin (39 ng per injection), as shown in Figure 3, contains less total sialic acid, and lower hydrolysis amounts are not recommended. Depending on the degree of sialylation, the amount of protein hydrolyzed can be reduced to low-ng/µL concentrations and still allow efficient sialic acid determination. Designing experiments that release amounts of analyte routinely near the LOQ is not recommended. However, this evaluation highlights both the sensitivity of the method and the importance of considering the approximate protein sialylation amount when designing acid hydrolysis experiments.

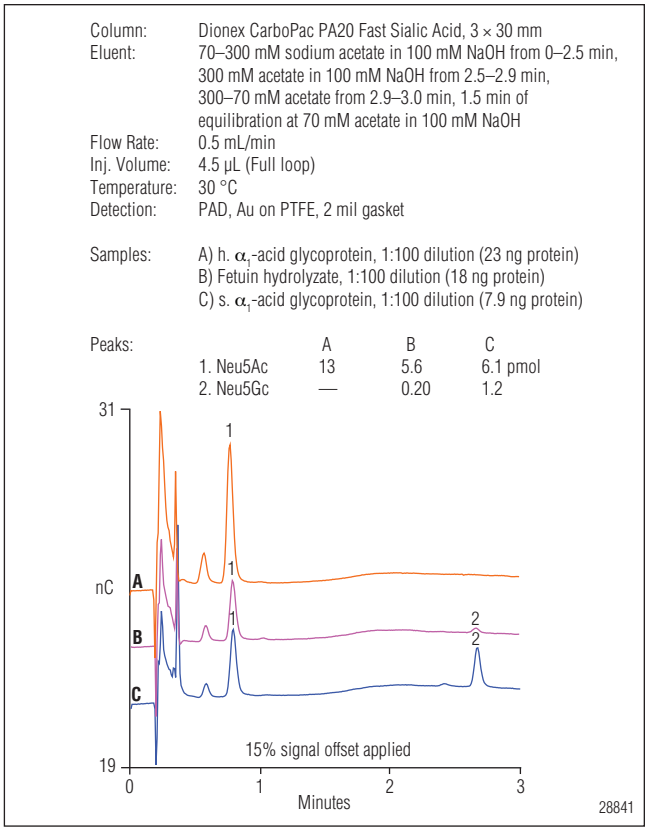

Figure 2. Separation of fetuin and AGP acid hydrolyzates on the Dionex CarboPac PA20 Fast Sialic Acid column.

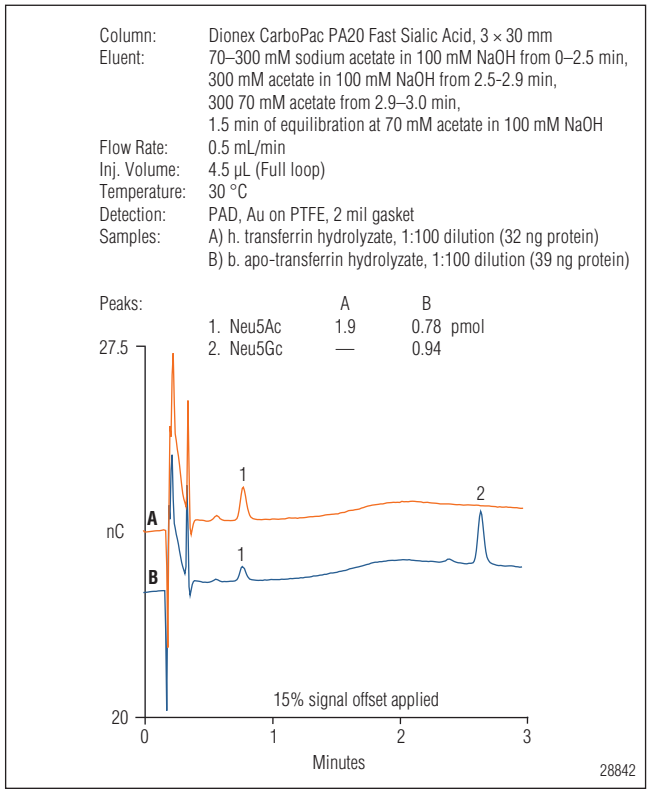

Figure 3. Separation of transferrin acid hydrolyzates on the Dionex CarboPac PA20 Fast Sialic Acid column.

Table 4. Sialic Acid Determination from Five Glycoprotein Acid Hydrolyzates					
Sample (replicate #)	**Analyte**	**Amount (pmol)**	**Retention Time (min)**	**Retention Time Precision (RSD)**	**Peak Area Precision (RSD)**
Fetuin (1)	Neu5Gc	0.18*	2.58	0.19	3.35
	Neu5Ac	5.78	0.74	0.65	2.59
Fetuin (2)	Neu5Gc	0.17*	2.58	0.19	5.25
	Neu5Ac	6.31	0.75	0.65	1.23
Fetuin (3)	Neu5Gc	0.18*	2.58	<0.01	6.14
	Neu5Ac	7.44	0.74	0.65	0.99
h. Transferrin (1)	Neu5Gc	ND			
	Neu5Ac	1.42	0.72	0.67	2.62
h. Transferrin (2)	Neu5Gc	ND			
	Neu5Ac	1.52	0.72	0.67	1.75
h. Transferrin (3)	Neu5Gc	ND			
	Neu5Ac	1.64	0.72	0.67	2.50
b. apo-Transferrin (1)	Neu5Gc	0.91	2.51	<0.01	2.75
	Neu5Ac	0.61	0.70	0.68	3.23
b. apo-Transferrin (2)	Neu5Gc	0.91	2.51	0.19	1.49
	Neu5Ac	0.63	0.71	<0.01	2.93
b. apo-Transferrin (3)	Neu5Gc	0.88	2.51	0.19	2.23
	Neu5Ac	0.61	0.71	<0.01	2.14
h. AGP (1)	Neu5Gc	ND			
	Neu5Ac	15	0.72	1.34	1.14
h. AGP (2)	Neu5Gc	ND			
	Neu5Ac	13	0.72	1.16	0.98
h. AGP (3)	Neu5Gc	ND			
	Neu5Ac	12	0.72	0.67	0.95
s. AGP (1)	Neu5Gc	1.0	2.52	0.57	1.18
	Neu5Ac	5.8	0.71	0.68	2.33
s. AGP (2)	Neu5Gc	1.2	2.52	0.57	1.70
	Neu5Ac	6.6	0.71	1.18	2.64
s. AGP (3)	Neu5Gc	1.0	2.52	0.33	0.13
	Neu5Ac	5.9	0.71	0.68	2.52

*Neu5Gc calibration range extended from 0.11 pmol–11 pmol, $r^2 = 0.9995$.

Table 4 presents the results from one day of triplicate sample analysis. Retention time precision is similar to that determined by injecting standards, with retention time RSDs ranging from <0.01–1.18. Differences in the absolute retention time can be expected, depending on the eluent preparation. Peak area precision for triplicate injections, as measured by RSD, is generally good, ranging from 0.13–6.14. As expected, larger peak area RSD is observed near the LOQ.

Table 5 lists the calculated results of sialic acid determination for the studied proteins, as well as both intraday precision for one day of analysis and between-day precision for three days of triplicate analysis. The amounts of sialic acids determined in the protein samples are generally consistent with literature results for the glycoproteins.[19-24] However, as shown in Table 5, sample replicate precision RSDs can be greater than chromatographic precision, with intraday RSDs ranging from 2.4–11 and between-day RSDs ranging from 3.9–17. For this reason, optimization of the acid hydrolysis for individual glycoproteins is highly recommended.

Table 5. Triplicate Sample Analysis Results Between-Day Precision over Three Days

Sample	Ana-lyte	Acid Hydrolysis Average (mol analyte/mol protein)	Intraday Precision Between Repli-cates (RSD)	Between-Day Precision (RSD)
Fetuin	Neu5Gc	0.33	7.2	7.3
	Neu5Ac	15	8.8	7.0
h. Transferrin	Neu5Gc	ND	—	—
	Neu5Ac	3.1	6.7	17
b. apo-Transferrin	Neu5Gc	1.4	2.4	3.9
	Neu5Ac	1.1	3.5	13
h. AGP	Neu5Gc	ND	—	—
	Neu5Ac	29	11	14
s. AGP	Neu5Gc	4.7	6.2	8.7
	Neu5Ac	26	6.4	9.0

Acid hydrolysis is a complex balance between release of the sialic acids from the glycoprotein and degradation of the released analytes. The efficiency of the hydrolysis depends on the hydrolysis temperature, acid concentration, type of sample being hydrolyzed, and the relative concentrations of acid and the sample. Because of these interdependent factors, which can impact the hydrolysis, variability between sample preparations must be expected. For the best accuracy, either an optimized acid hydrolysis or neuraminidase digestion is recommended. For methodology to optimize acid hydrolysis, see Fan et al.[25]

Method accuracy was investigated by spiking protein acid hydroylzates with known amounts of Neu5Ac and Neu5Gc in similar concentration as the determined amounts (Table 6). For human glycoproteins, which lack Neu5Gc, 0.22 pmol of Neu5Gc was added. Recoveries for Neu5Ac ranged from 81–96% and recoveries for Neu5Gc were similar, ranging from 82–106%.

Table 6. Accuracy of Analysis, as Measured by Recovery (n=3)

Sample	Analyte	Average Native Amount (pmol)	Added Amount (pmol)	Recovery (%)
Hydrolyzate blank	Neu5Ac	ND	2.2	94 ± 5.8
	Neu5Gc	ND	0.22	92 ± 7.1
Fetuin	Neu5Ac	5.8	2.2	84 ± 1.0
	Neu5Gc	0.18	0.22	86 ± 2.2
h. Transferrin	Neu5Ac	1.6	2.2	95 ± 4.1
	Neu5Gc	ND	0.22	94 ± 2.4
b. Apo-transferrin	Neu5Ac	0.35	1.8	87 ± 5.0
	Neu5Gc	0.45	0.90	95 ± 3.0
h. AGP	Neu5Ac	4.5	3.6	91 ± 1.5
	Neu5Gc	ND	0.36	89 ± 3.4
s. AGP	Neu5Ac	5.8	4.5	94 ± 1.9
	Neu5Gc	1.0	0.45	98 ± 6.9

CONCLUSION

In this work, sialic acids are determined in five representative glycoproteins by acid hydrolysis release followed by HPAE-PAD. The method is both specific and direct, eliminating the need for sample derivatization common in other chromatographic methods. Good recoveries, precision, and linear detection for Neu5Ac and Neu5Gc are demonstrated, indicating the method is appropriate for glycoprotein analysis. Using the Dionex CarboPac PA20 Fast Sialic Acid column, this rapid method separates Neu5Ac and Neu5Gc with a total analysis time of <5 min, providing high-throughput sample analysis while reducing eluent consumption and waste generation.

SUPPLIERS

VWR, 1310 Goshen Parkway, West Chester, PA 19380, U.S.A., Tel: 800-932-5000. www.vwr.com

Thermo Fisher Scientific, One Liberty Lane, Hampton, NH 03842, U.S.A., Tel: 800-766-7000. www.fishersci.com

Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178, U.S.A., Tel: 800-325-3010. www.sigma-aldrich.com

Ferro Pfanstiehl, 1219 Glen Rock Avenue, Waukegan, IL, 60085, U.S.A., Tel: 800-383-0126. www.ferro.com

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Thermo SCIENTIFIC

Application Note 215

Separation of Asparagine-Linked (*N*-Linked) Oligosaccharides from Human Polyclonal IgG Using the CarboPac PA200 Column

INTRODUCTION

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a widely used technique for determining an extensive set of carbohydrates including, but not limited to, monosaccharides, disaccharides, oligosaccharides, smaller polysaccharides, sugar acids, such as sialic acids, sugar alcohols, sugar phosphates, and sugar nucleotides. One of the first published applications of HPAE-PAD was the separation of *N*-linked oligosaccharides released from mammalian glycoproteins. These early separations used the CarboPac® PA1 column.[1] In 1992 Dionex introduced the CarboPac PA100 to improve oligosaccharide resolution. The improved resolution was mainly the result of increased peak efficiency. Dionex Application Note 67[2] shows the improved efficiency of the PA100 relative to the PA1 by making a direct comparison of separations of neutral linear oligosaccharides with the two columns. Since its introduction, the PA100 has become the standard for neutral and charged oligosaccharide separations. Dionex Technical Note 42[3] demonstrates the separation of sialylated *N*-linked oligosaccharides using the PA100.

The importance of characterizing the *N*-linked oligosaccharides from recombinant glycoproteins used as therapeutics and other applications necessitated even more oligosaccharide peak resolution.[4] To meet this demand, Dionex introduced the CarboPac PA200 column. The PA200 is a pellicular anion-exchange column that mostly differs from the PA100 by a reduction in the resin bead size from 8.5 to 5.5 µm and a decrease in the latex bead size from 275 nm to 43 nm. This resin is packed in a 3 × 250 mm column body rather than the standard 4 × 250 mm format. In addition to increased resolution, this column also reduces eluent consumption and waste generation (optimum flow rate is 0.5 mL/min compared to 1.0 mL/min for the PA100), and requires less acetate to elute a given oligosaccharide compared to the PA100, allowing faster separations and making subsequent acetate removal a bit easier. Dionex Application Update 150[5] shows the benefits of higher resolution and faster separations using the PA200 for the same maltodextrins separated with the PA1 and PA100 in Application Note 67.

This application note shows how the PA200 improves *N*-linked oligosaccharide separations. The PA200 is used to profile *N*-linked oligosaccharides released from human polyclonal IgG by the enzyme PNGase F or endoglycosidase H (Endo H). Human polyclonal IgG has a mixture of neutral and charged *N*-linked oligosaccharides that is generally more complex than the set of *N*-linked oligosaccharides found on a typical monoclonal IgG. This note also shows how subsequent exoglycosidase digestions can be used to assist in understanding and identifying oligosaccharide structure. The CarboPac PA200 is the new standard for achieving high resolution HPAE-PAD *N*-linked oligosaccharide separations and delivers the resolution necessary to ensure that the *N*-linked oligosaccharides from one lot of a recombinant glycoprotein are comparable to the *N*-linked oligosaccharides from a second lot.

EQUIPMENT

ICS-3000 chromatography system consisting of:

- SP single gradient pump module
- DC detector and chromatography module with single or dual heating zone and 6-port injection valve
- ED electrochemical detector equipped with cell containing a disposable Au working electrode and a combination pH–Ag/AgCl reference electrode
- AS Autosampler with Sample Tray Temperature Controlling option, and 1.5 mL sample tray

Chromeleon® 6.8 Chromatography Workstation

Centrifuge (Eppendorf® 5400 series)

SpeedVac™ evaporator

Heated water bath

Vacuum pump (for eluent preparation)

*This application can also be performed on older Dionex systems equipped for HPAE-PAD.

REAGENTS AND STANDARDS

Deionized water, Type 1 reagent-grade, 18.2 MΩ-cm resistivity

Sodium acetate, HPLC grade (CH_3COONa, Aldrich, P/N 71185; or Dionex, P/N 059326)

Sodium hydroxide, 50% (w/w) (NaOH, Fisher Chemicals, P/N SS254-500)

Glacial acetic acid (ACS Grade or better)

Neuraminidase, recombinant (cloned from Clostridium perfringens) (New England BioLabs) (P/N P0720S)

PNGase F, glycerol-free (New England BioLabs)

b-Galactosidase (*S. pneumoniae*)—(Oxford Glycosystems—currently available from other sources)

N-acetylneuraminic acid (Neu5Ac) (Ferro Pfanstiehl Laboratories—see Dionex Technical Note 41)

N-glycolylneuraminic acid (Neu5Gc) (Ferro Pfanstiehl Laboratories—see Dionex Technical Note 41)

OligoStandard™ Sialylated N-Linked Alditols (Dionex, P/N 043064)

Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarstedt 72.692.005)

Filter unit, 0.2 µm nylon (Nalgene® Media-Plus with 90 mm filter, Nalge Nunc International, P/N 164-0020) or equivalent nylon filter for eluent preparation.

1.5 mL polypropylene autosampler vials, with caps and slit septa (Dionex vial kit P/N 061696)

SAMPLE

Human Polyclonal IgG (Sigma-Aldrich)

CONDITIONS

Column:	CarboPac PA200 Analytical, 3 × 250 mm (P/N 062896)
	CarboPac PA200 Guard, 3 × 50 mm (P/N 062895)
Eluents:	A: 100mM Sodium hydroxide
	B: 100 mM Sodium hydroxide, 0.5 M sodium acetate
Method:	0–5 min 99% A, 1% B
	5–60 min 1 to 36% B
Flow Rate:	0.5 mL/min
Column Temperature:	30 ºC
AS Tray Temperature:	15 ºC
Inj. Volume:	9 µL
Inj. Loop:	10 µL
Detection:	PAD, conventional or disposable Au WE
Waveform:	See Table 1.
Run Time:	75 min (return to initial conditions for 15 min prior to injection).

Table 1. Waveform A, Four-Potential Carbohydrate Waveform[6]				
Time (sec)	Potential (V) (Ag/AgCl reference)	Gain*	Ramp*	Integration
0.00	+ 0.1	Off	On	Off
0.20	+ 0.1	On	On	On
0.40	+ 0.1	Off	On	Off
0.41	- 2.0	Off	On	Off
0.42	- 2.0	Off	On	Off
0.43	+ 0.6	Off	On	Off
0.44	- 0.1	Off	On	Off
0.50	- 0.1	Off	On	Off

*These parameters are not used on older model Dionex chromatography systems.

PREPARATION OF SOLUTIONS AND REAGENTS

100 mM Sodium Hydroxide (Eluent A)

It is essential to use high-quality water of high resistivity (18 MΩ-cm or better) that contains as little dissolved carbon dioxide as possible. Biological contamination should be absent. Dilute 10.4 mL of a 50% (w/w) sodium hydroxide solution into 2 L of water to prepare a 0.1 M sodium hydroxide solution. After preparation, keep the eluent blanketed under helium at 34 to 55 kPa (5 to 8 psi) at all times. See Dionex Technical Note 71 for more details on eluent preparation for HPAE-PAD carbohydrate analysis.[7]

100 mM Sodium Hydroxide/1 M Sodium Acetate (Eluent B)

Measure approximately 800 mL of water into a 1 L graduated cylinder. Add a stir bar and begin stirring. Weigh out 82.0 g of anhydrous, crystalline sodium acetate. Add the solid sodium acetate steadily to the briskly stirring water to avoid the formation of clumps, which are slow to dissolve. After the salt dissolves, remove the stir bar with a magnetic retriever. Using a plastic pipette, measure 5.2 mL of 50% (w/w) sodium hydroxide and add it to the acetate solution. Rinse the pipette by drawing up the acetate solution into the pipette and dispensing it back into the graduated cylinder several times. Add water to the solution to reach a final level of 1000 mL. Replace the stir bar and stir briefly to mix. Vacuum filter through a 0.2 µm nylon filter. This may be slow, as the filter may clog with insolubles from the sodium acetate. After preparation, keep this eluent blanketed under helium at 34 to 55 kPa (5 to 8 psi) at all times. See Dionex Technical Note 71 for more details on eluent preparation for HPAE-PAD carbohydrate analysis.[7]

25 mM Sodium Acetate Buffer pH 5.5

Prepare 200 mL of 0.025 M sodium acetate (0.41 g anhydrous sodium acetate dissolved in 200 mL water) and 200 mL 25 mM acetic acid (0.29 mL glacial acetic acid (17.4 M) added to 150 mL water and brought to a final volume of 200 mL). Prepare the pH 5.5 buffer by combining 89 mL of the 0.025 M sodium acetate and 11 mL of the 0.025M acetic acid.

PREPARATION OF SAMPLES

Human Polyclonal IgG *N*-linked Oligosaccharides

200 µL of 10 mg/mL human polyclonal IgG (in water) was treated with 20 µL 10X G7 buffer (included with the PNGase F purchase) and 20 µL PNGase F, which had been diluted 1:100 with water. This sample was incubated at 37 °C for 20 h, microcentrifuged, and the supernatant analyzed directly.

Desialylated Human Polyclonal IgG *N*-linked Oligosaccharides

20 µL of the PNGase F-digested IgG sample above was treated with 20 µL 25 mM sodium acetate buffer pH 5.5, and 2 µL neuraminidase. This sample was incubated at 37 °C for 20 h and the supernatant analyzed directly. Note: The sodium citrate buffer supplied with the enzyme was not used; instead, the above sodium acetate buffer was used.

Desialylated Human Polyclonal IgG *N*-linked Oligosaccharides and Degalactosylated Human Polyclonal IgG *N*-linked Oligosaccharides

20 µL of the PNGase F and neuraminidase-digested IgG sample and 40 µL of the PNGase F-digested IgG sample were treated with 5 µL and 10 µL of a 1 mU/µL galactosidase solution (vial containing 40 mU of lyophilized product was reconstituted in 40 µL 25 mM sodium acetate buffer pH 5.5), respectively. These samples were incubated at 37 °C for 25 h and the supernatants analyzed directly.

RESULTS AND DISCUSSION:

Figure 1 shows a comparison of the asparagine-linked (*N*-linked) oligosaccharides released from bovine fetuin, reduced to form the oligosaccharide alditols, and separated on the CarboPac PA100 and CarboPac PA200 columns. The smaller bead size of the PA200 resin results in more efficient peaks and therefore better oligosaccharide resolution compared to the PA100 column. The oligosaccharides are also eluted with less sodium acetate and due to the lower flow rate (0.5 mL/min rather than 1.0 mL/min) the efficiency of online desalting with carbohydrate membrane desalter should be improved. There is less eluent used and less waste generated. We have observed the same resolution improvement for *N*-linked oligosaccharides released from human transferrin, human alpha-1-acid glycoprotein, and bovine ribonuclease B.

The improvement in oligosaccharide resolution is more dramatic when the PA100 and PA200 separations of *N*-linked oligosaccharides released from human polyclonal IgG are compared (Figure 2). The improved resolution of the oligosaccharides between 5 and 15 min is especially beneficial for the oligosaccharides released from recombinant monoclonal antibodies, which have less heterogeneity than human polyclonal IgG.[8] The majority of the *N*-linked oligosaccharides from human IgG are biantennary.

HPAE-PAD used in combination with exoglycosidases can yield information about oligosaccharide structure (Figure 3). Treating the PNGase F digest of human polyclonal IgG with neuraminidase reveals that the later eluting peaks (18–22 min) contained Neu5Ac. The retention times of those peaks suggest they each contained a single Neu5Ac. The disappearance of small peaks between 27 and 29 min suggest that they are disialylated oligosaccharides. Chromatography of a standard mixture containing *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) shows that the only sialic acid released from these oligosaccharides is Neu5Ac, as is expected from a glycoprotein of human origin. A recombinant glycoprotein expressed in Chinese hamster ovary cells or a glycoprotein from another mammal may contain Neu5Gc.

Among the *N*-linked oligosaccharides known to occur on human IgG are those that have branches that terminate in a β-linked galactose. We treated the PNGase F digest of human polyclonal IgG with b-galactosidase to reveal these structures. Figure 4 shows that many of the neutral *N*-linked oligo-saccharides of human IgG have at least one terminal galactose. This also suggests that the two major monosialylated oligosaccharides are biantennary, where one oligosaccharide has one branch with a terminal *N*-acetylglucosamine and the other branch with a terminal

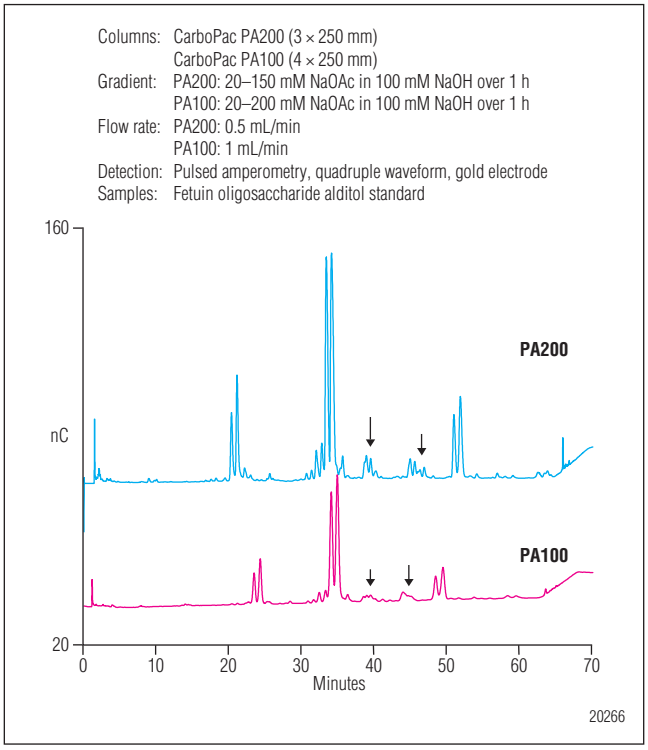

Figure 1. Fetuin oligosaccharide profiles: CarboPac PA200 vs CarboPac100 column.

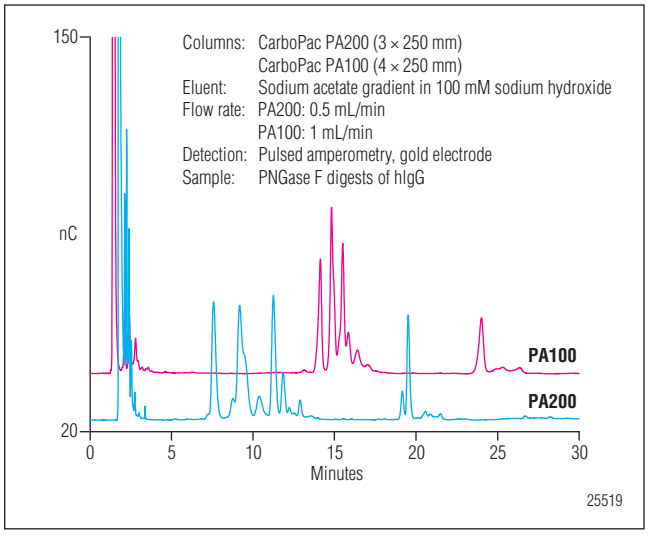

Figure 2. Human polyclonal lgG N*-linked oligosaccharides CarboPac PA200 vs CarbPac PA100 column.*

Neu5Ac (peak 8), and the second oligosaccharide has terminal galactose and terminal Neu5Ac on its two branches (peak 9). Other studies suggest that both structures fucose linked to the reducing terminal N-acetylglucosamine in an a1,6 linkage ("core fucose").[8] An oligosaccharide with core fucose will elute earlier than the same oligosaccharide without core fucose.9 Peak 2, eluting just after the main peak 1, in the degalactosylated sample may be the defucosylated version of the main peak. Treatment with an a-fucosidase either before or after galactosidase treatment should reveal the oligosaccharides containing fucose.

If we first treat the PNGase F digest of human polyclonal IgG with neuraminidase and then treat it with β-galactosidase (Figure 5), we get a similar result as Figure 4 with the notable absence of the sialylated oligosaccharides. Peaks 1 and 2 are undoubtedly the same oligosaccharides as peaks 1 and 2 in Figure 4. Either peak 3 or 4 is Neu5Ac. After treatment with the neuramindase, there are a number of neutral oligosaccharides eluting close together and some partial co-elutions. The released Neu5Ac elutes in the same region. Although resolution of this region of the chromatogram is improved compared to the CarboPac PA100, it is possible that increasing the sodium hydroxide concentration from 100 to 250 mM could offer further improvement.[10]

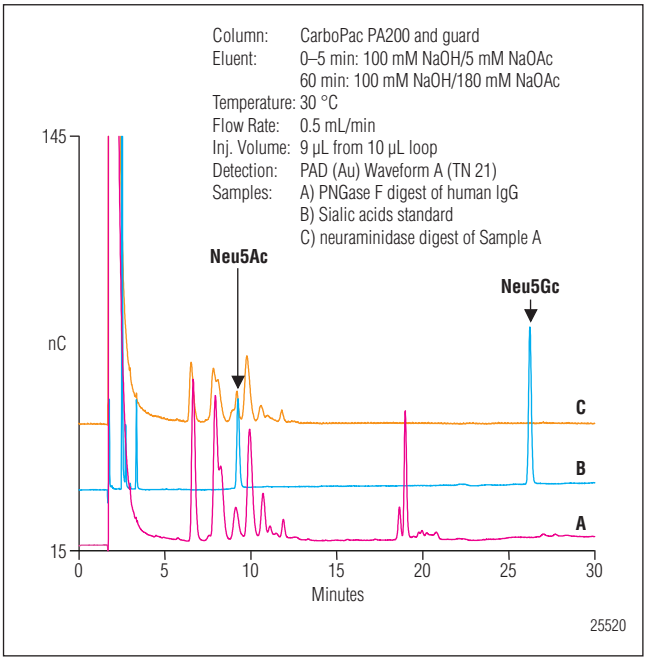

Figure 3. Monitoring release of sialic acids from human polyclonal lgG N*-linked oligosaccharides by HPAE-PAD.*

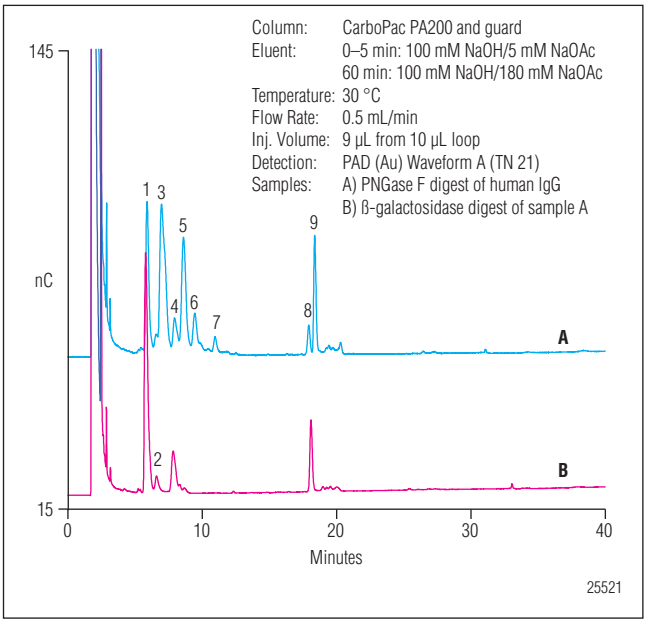

Figure 4. Degalactosylated N*-linked oligosaccharides from human polyclonal lgG separated on a CarboPac PA200 column.*

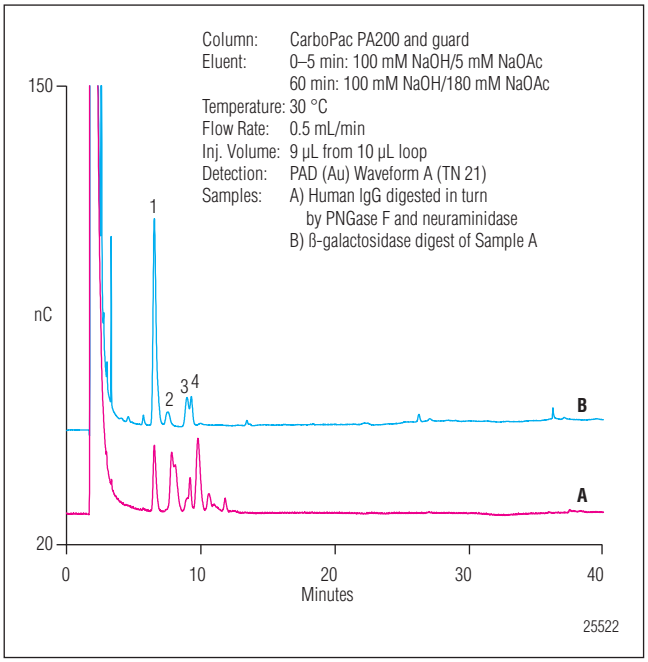

Figure 5. Desialylated and degalactosylated N*-linked oligosaccharides from human polyclonal lgG separated on a CarboPac PA200 column.*

CONCLUSION

The smaller bead size of the CarboPac PA200 column improves resolution of closely-eluting oligosaccharides. The improved resolution is beneficial for characterization of a variety of *N*-linked oligosaccharides, especially those released from recombinant monoclonal antibodies. Further optimization of hydroxide eluent concentration may improve resolution further. The smaller column diameter of the PA200 reduces eluent consumption and waste generation while improving online desalting.

PRECAUTIONS

PNGase F is sold in glycerol-containing and glycerol-free preparations. For HPAE-PAD glycerol-free preparations are recommended. Figure 6 shows chromatograms of the *N*-linked oligosaccharides released from human polyclonal IgG using PNGase F with and without glycerol. The peaks from the glycerol-containing PNGase F treatment elute a bit earlier than the same peaks from the glycerol-free PNGase F treatment, and the peaks are less efficient. This suggests that the glycerol overloads the column slightly.

Enzyme digests are performed in salt-containing buffers (e.g. the neuraminidase digest was executed in sodium acetate buffer). Injecting too large a volume of these digests will overload the column with the buffer anion, altering the chromatography. A slight column overload will be similar to the effect shown with the glycerol-containing PNGase F. For most buffers and samples, injection volumes under 50 µL should yield good chromatography. Injection volumes over 50 µL should be tested. Tests can use a Neu5Ac standard or similarly inexpensive compound rather than valuable sample or a more costly oligosaccharide standard.

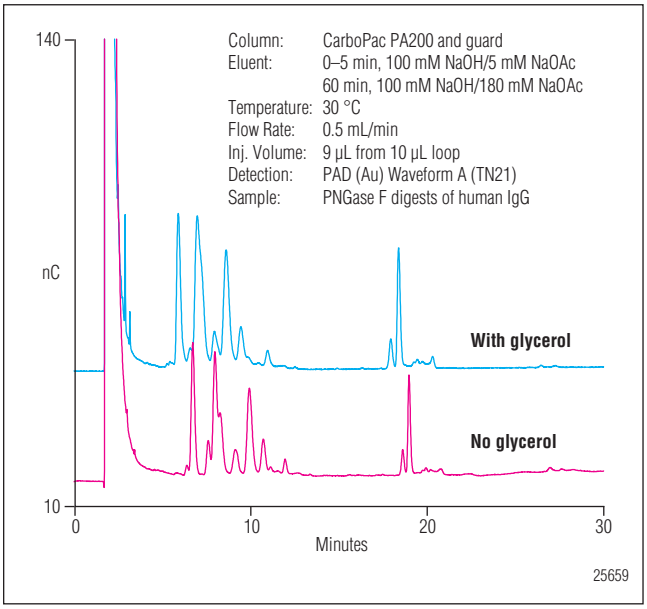

Figure 6. Effect of Glycerol on HPAE-PAD of human polyclonal IgG N-*linked oligosaccharides.*

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Characterization of post-translational modifications

Protein therapeutics applications notebook

Thermo SCIENTIFIC

Application Note 125

Monitoring Protein Deamidation by Cation-Exchange Chromatography

INTRODUCTION

A common structural modification of recombinant proteins is the deamidation of asparagine (Asn) residues.[1,2] This modification occurs in a variety of protein-based pharmaceuticals, including human growth hormone, tissue plasminogen activator, hirudin, monoclonal antibodies, acidic fibroblast growth factor, and interleukin-1, with varying effects on the activity or stability of the therapeutic protein.[3-10] Determining the deamidation of Asn residues in recombinant proteins is a significant challenge for analytical and protein chemists in the quality control and process departments at biotechnology and pharmaceutical companies.[11]

This application note describes the use of the ProPac® WCX-10 column in a bio-inert HPLC system to monitor the products of protein deamidation. The ProPac WCX-10, a weak cation-exchange column, is well suited for the separation of protein variants produced by posttranslational modifications. The packing in this column is a unique pellicular resin with a hydrophilic coating and carboxylate functional groups on grafted linker arms. The physicochemical properties of this support eliminate secondary (nonionic) interactions between the protein analytes and the stationary phase, affording minimal band broadening and high selectivity.[12] The UltiMate® 3000 Titanium System is an HPLC whose flow path ensures that neither solvents nor sample are in contact with stainless steel materials, removing concerns about iron and other transition metals contaminating the column and samples.

In the example presented here, deamidated variants of ribonuclease A (RNase A) are separated from the native protein in less than 20 min on a ProPac WCX-10 weak cation-exchange column. This application note also compares the results of using a phosphate-based buffer to that of a 4-morpholineethanesulfonic acid (MES) -based buffer.

EQUIPMENT

Dionex UltiMate 3000 Titanium System consisting of:

SRD-3600 Solvent Rack with 6 Degasser Channels (P/N 5035.9230) and Eluent Organizer, including pressure regulator, and 2-L glass bottles for each pump

LPG 3400AB Quaternary Analytical Pump (P/N 5037.0015) or DGP-3600AB Dual Ternary Analytical Pump (P/N 5037.0014) for dual gradient capability

WPS-3000TBPL Biocompatible Analytical Autosampler (P/N 5823.0020)

TCC-3000 Column Compartment without Switching Valves (P/N 5722.0000) or TCC-3200B Column Compartment with 2 PEEK™ 10-port 2-position valves (P/N 5723.0025) for added productivity

VWD-3400 Variable Wavelength Detector (P/N 5074.0010) or PDA-3000 Photodiode Array Detector (P/N 5080.0020)

Biocompatible Analytical Flow Cell for VWD (P/N 6074.0200) or Biocompatible Analytical Flow Cell for PDA (P/N 6080.0220)

Chromeleon® Chromatography Data System

Helium; 4.5-grade, 99.995%, <5 ppm oxygen (Praxair)

Filter unit, 0.2 µm Nylon (Nalgene 90-mm Media-Plus, Nalge Nunc International, P/N 164-0020 or equivalent Nylon filter

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent; for degassing eluents)

0.3 mL polypropylene (Vial Kit, P/N 055428) injection vials with caps

Microcentrifuge tubes with detachable screw caps (polypropylene, 1.5 mL, Sarstedt, P/N 72.692.005; or equivalent)

REAGENTS AND STANDARDS

Deionized water, 18 MΩ-cm resistance or higher

MES hydrate, minimum 99.5% titration (Sigma-Aldrich; P/N M8250)

Sodium phosphate, dibasic (J.T. Baker; P/N 4062-01)

Sodium phosphate, monobasic monohydrate (J.T. Baker; P/N 3818-01)

Sodium chloride, crystal (J.T. Baker; P/N 4058-05)

Sodium hydroxide solution, 50% W/W (Thermo Fisher Scientific; P/N SS254)

Ammonium bicarbonate (Sigma-Aldrich; P/N A6141)

Ribonuclease A, Type III-A from bovine pancreas, minimum 85% (Sigma-Aldrich; P/N R5125)

CONDITIONS

Column:	ProPac® WCX-10 Analytical 4 × 250 mm (P/N 054993). Different columns were used with the phosphate-based and the MES-based buffers
Flow Rate:	1.00 mL/min
Inj. Volume:	10 µL (partial loop, no wash between re-injections)
Autosampler Temp:	5 ºC
Column Temp:	30 °C
Detection:	Absorbance, 280 nm (absorbance at 214 and 254 nm also collected)
Data Collection Rate:	1.0 Hz
Noise:	12-24 µAU
Typical System Operating Backpressure:	~ 130 bar (~1900 psi)
Mobile Phase:	MES-based buffers A: 20 mM MES, pH 5.7 B: 20 mM MES, 1.0 M sodium chloride, pH 5.7 Phosphate-based buffers A: 10 mM sodium phosphate, pH 6.0 B: 10 mM sodium phosphate, 1.0 M sodium chloride, pH 6.0
Gradient:	Linear, 4-70% B in 30 min (both buffer systems)

Method:

Time (min)	A(%)	B(%)	Comments
–10.00	96.0	4.0	Equilibration
0.00	96.0	4.0	Sample Injection
30.00	30.0	70.0	End Gradient
40.00	25.0	75.0	Column Regeneration
42.00	96.0	4.0	Re-equilibration

PREPARATION OF SOLUTIONS AND REAGENTS

Both sets of mobile phases used in this application note are prepared using concentrated reagent solutions. The MES buffers are prepared by diluting an MES concentrate solution (with or without added sodium chloride) with DI water and adjusting the resulting solution pH to 5.7 with concentrated sodium hydroxide. Slightly different volumes of sodium hydroxide may be required to produce 2 L volumes of mobile phases A and B. The sodium phosphate mobile phases are prepared by diluting appropriate quantities of monobasic and dibasic sodium phosphate concentrate solutions (with or without added sodium chloride) with DI water to yield pH 6.0. The following procedure is a recommended starting point for obtaining the desired mobile phases, but some deviation from this formula may be necessary after checking the pH. If the pH is not 6.0, then adjust the proportions of monobasic and dibasic concentrate solutions added. The combined total volume of phosphate concentrate solutions should remain at 100 mL to produce 2 L of 10 mM sodium phosphate mobile phases. Do not adjust the pH of the sodium phosphate mobile phases by adding NaOH or HCl.

Concentrated Solutions

All concentrated solutions are filtered through a 0.2 µm filter and stored at 5 ºC until needed.

400 mM MES

Dissolve 78.07 g MES in water to a final solution volume of 1.0 L.

2.00 M Sodium Chloride

Dissolve 233.76 g sodium chloride in water to a final solution volume of 2.0 L.

200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate ($Na_2HPO_4 \cdot H_20$) in water to a final solution volume of 1.0 L.

200 mM Sodium Phosphate, Monobasic

Dissolve 27.60 g monohydrate monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$) in water to a final solution volume of 1.0 L.

Mobile Phase Solutions

All mobile phases are filtered through a 0.2 µm filter under vacuum to remove particulates and to degas prior to their use. The mobile phases are blanketed under 34-55 kPa (5-8 psi) of helium headspace to reduce the growth of opportunistic microorganisms.

20 mM MES, pH 5.7 (A)

Combine 100 mL of 400 mM MES with 1900 mL DI water. Add 700 µL of 50% sodium hydroxide solution.

20 mM MES with 1.0 M Sodium Chloride, pH 5.7 (B)

Combine 100 mL of 400 mM MES, 1000 mL of 2.0 M sodium chloride, and 900 mL DI water. Add 700 µL of 50% sodium hydroxide solution.

10 mM Sodium Phosphate, pH 6.0 (A)

Combine 9.5 mL of 200 mM dibasic sodium phosphate, 90.5 mL of 200 mM monobasic sodium phosphate, and 1900 mL DI water.

10 mM Sodium Phosphate with 1.0 M Sodium Chloride, pH 6.0 (B)

Combine 40.0 mL of 200 mM dibasic sodium phosphate, 60.0 mL of 200 mM monobasic sodium phosphate, 1000 mL of 2.0 M sodium chloride, and 900 mL DI water.

Stock Standards

RNase A - 15 mg/mL solution in degassed, filtered DI water. Aliquot 400 µL volumes and store at –40 ºC until ready to use.

Ammonium bicarbonate – prepare a 10% w/v solution in filtered DI water. The measured pH of this solution is 8.0. Store at 5 ºC until ready to use.

Sample Preparation

Combine 334 µL of 15 mg/mL RNase A, 100 µL of 10% ammonium bicarbonate and 566 uL of DI water in a 1.5 mL microcentrifuge tube to make a 5 mg/mL RNase A solution in 1% ammonium bicarbonate. Incubate this solution at 37 ºC. Periodically withdraw 50 µL aliquots and freeze these aliquots. When ready to analyze the samples, thaw the aliquots, dilute five-fold with the appropriate Mobile Phase A (20 mM MES or 10 mM sodium phosphate), and place in the 0.3 mL autosampler vials. The final protein concentration is 1.0 mg/mL. This forced deamidation of RNase A follows the method described by Di Donato et al.[13] Controls consisting of 5 mg/mL RNase A and 1% ammonium bicarbonate were incubated with the samples and processed as described above.

RESULTS AND DISCUSSION

Separation

Figure 1 compares the chromatographic profiles for native RNase A run on the ProPac WCX-10 weak cation-exchange column using either MES- or phosphate-based buffers. The resolution between the two deamidation products (peaks 1 and 2 in Figure 1) is 1.37 using phosphate-based mobile phases and is 2.94 with the MES-based mobile phases.Visual inspection of the two chromatograms (see Figure 1A) shows more chromatographic detail with the MES mobile phases, increasing the ability of the system to separate more RNase A variants in a single chromatographic analysis. In contrast, Di Donato et al. reported the separation of the Asn[67] deamidation products of RNase A by cation-exchange chromatography followed by hydrophobic interaction chromatography to resolve the two deamidation variants.

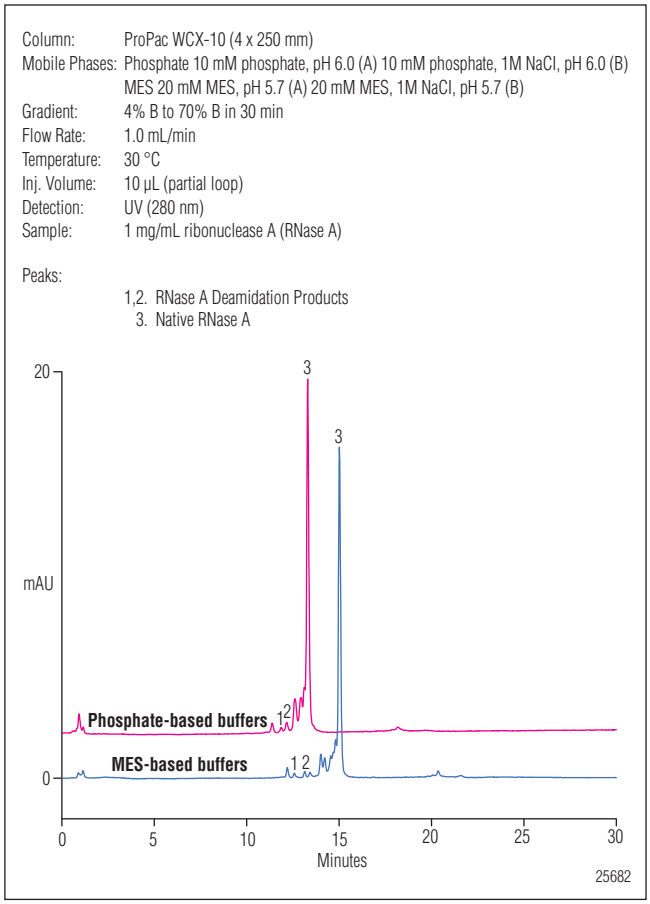

Figure 1: Full chromatogram. Comparison of the chromatography using a ProPac WCX-10 column with phosphate-based vs. MES-based mobile phases for ribonuclease A and its deamidation products.

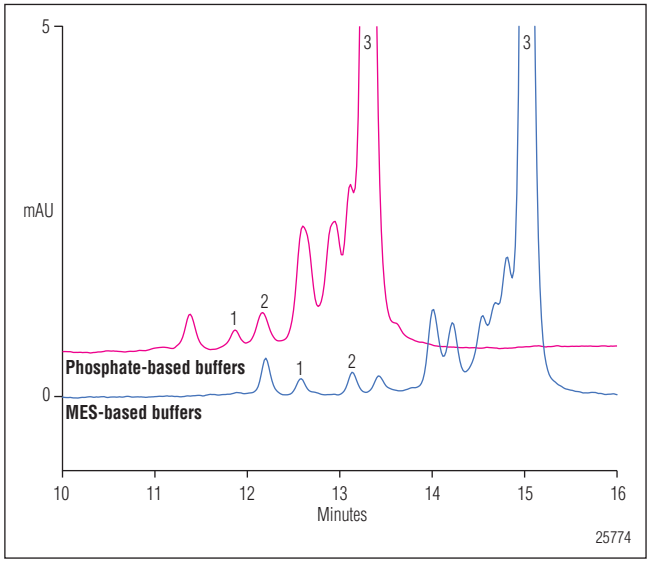

Figure 1A: 10–16 min region showing improved deamidation product resolution for MES-based vs. phosphate-based mobile phases.

Figure 2 displays the chromatographic profiles of samples taken during the 37 ºC incubation of RNase A in 1% ammonium bicarbonate. The deamidation peaks (12.5 and 13.1 min) increase in abundance while the native RNase A peak abundance (15.0 min) decreases. RNase A stability in aqueous solution at 37 ºC is demonstrated by observing little RNase A peak area change in the control (37 ºC incubation of RNase A in the absence of ammonium bicarbonate) over the 425 h incubation period (data not shown). This sample of untreated RNase A (Figure 1) shows additional peaks, including ones at the same retention times as the deamidation variants. It is likely that the untreated RNase A standard contains small amounts of deamidated RNase A.

Deamidation Monitoring

Insight into the deamidation kinetics can be gleaned by plotting the peak area of the deamidated forms of RNase A as a function of incubation time. Figure 3 shows the results of this plot (normalized to the RNase A peak area at time = 0 h) for both the full 425 h time course study (Figure 3A) and the initial 169 h (Figure 3B). Initially, the deamidation reaction followed first order kinetics.

CONCLUSION

The high efficiency of the ProPac WCX-10 column, coupled with an inert, titanium HPLC system, allows for the rapid analysis of protein deamidation variant forms. Using MES-based mobile phases improve peak resolution when compared to typical phosphate-based mobile phases.

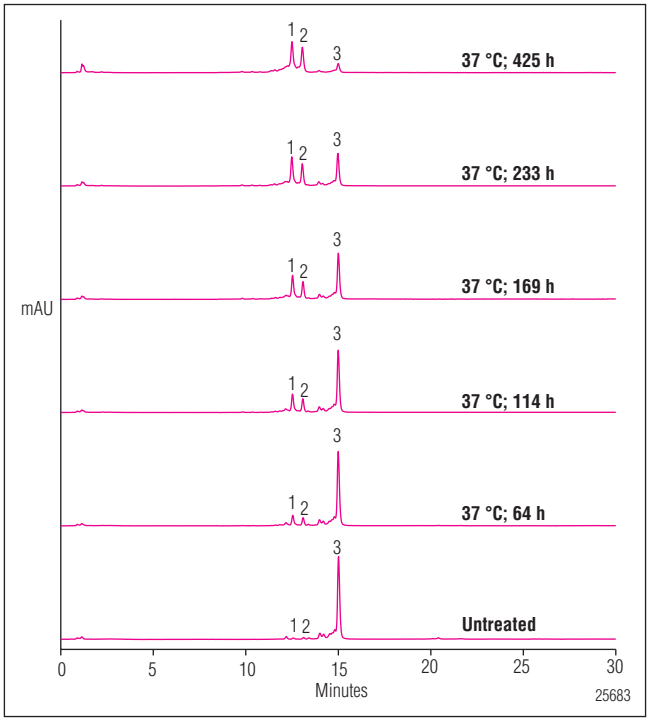

Figure 2: Use of MES-based mobile phases to separate ribonuclease A and its two deamidation products during the time course of forced deamidation. Ribonuclease A (5 mg/mL) was incubated in 1% ammonium bicarbonate at 37 ºC, aliquots diluted 5-fold with mobile phase A (see Figure 1 for conditions).

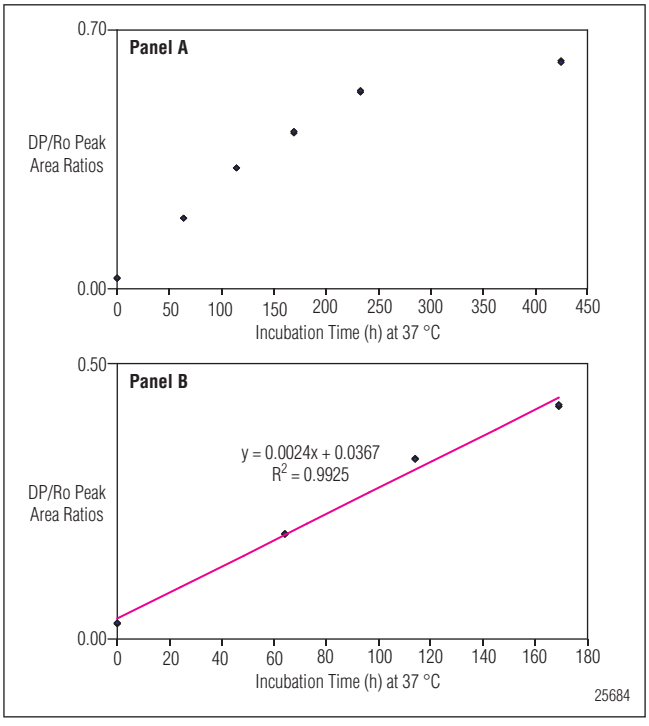

Figure 3: Formation of deamidation products (DP) of ribonuclease A as a function of time (Ro is the initial RNase A peak area at T=0 h). (A) Plot displayed of the full time course (425 h). (B) Plot displayed of the first 169 h. Duplicate injections for each time point.

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LIST OF SUPPLIERS

Gast Manufacturing Corp., 2550 Meadowbrook Road, Benton Harbor, MI 49022, U.S.A. Tel: 1- 269-926-6171, http://www.gastmfg.com

Mallinckrodt Baker, 222 Red School Lane, Phillipsburg NJ 08865, U.S.A. 1-800-582-2537, http://www.mallbaker.com

Nalge Nunc International, 75 Panorama Creek Drive, Rochester, NY 14625, U.S.A. Tel: 1-800-625-4327, http://www.nalgenunc.com

Praxair, 39 Old Ridgebury Road, Danbury, CT 06810-5113, U.S.A. Tel: 877-772-9247, http://www.praxair.com

Sarstedt AG & Co., Rommelsdorfer Straße, Postfach 1220, 51582 Nümbrecht, Germany Tel.: +49-2293-305-0, http://www.sarstedt.com

Sigma-Aldrich Chemical Company, P.O. Box 14508, St. Louis, MO 63178, U.S.A., Tel: 1-800-325-3010, http://www.sigma.sial.com

Thermo Fisher Scientific, 4500 Turnberry Drive, Hanover Park, IL 60133, U.S.A. Tel: 1-800-766-7000, http://www.fishersci.com

Thermo SCIENTIFIC

Application Note 129

Separation of Tryptophan and Methionine Oxidized Peptides from Their Unoxidized Forms

INTRODUCTION

Protein and peptide microheterogeneity can sometimes be attributed to oxidation of tryptophan (Trp) or methionine (Met) residues. Amino acid oxidation in proteins and peptides is a common post-translational modification. In vivo oxidation is caused by oxygen radicals and other biological factors (e.g., exposure to certain oxidizing drugs or other compounds). In vitro oxidation can be due to conditions encountered during purification or formulation. Oxidation can also occur during storage and from frequent freeze-thawing cycles. Protein chemists in process development and quality control are concerned with oxidation because it can adversely impact the activity and stability of biotherapeutics.[1]

In this Application Note, luteinizing hormone-releasing hormone (LH-RH), a peptide containing Trp, was forcibly oxidized with dimethyl sulfoxide/HCl.[2,3] α-Melanocyte stimulating hormone (α-MSH), a peptide containing Met was forcibly oxidized with hydrogen peroxide/ammonium bicarbonate.[3,4] The resulting peptides were separated using the ProPac™ WCX-10 weak cation exchange column.

EQUIPMENT

DX-500 BioLC® liquid chromatography system consisting of:

- GP50 gradient pump (PEEK)
- AD20 variable wavelength absorbance detector
- AS50 autosampler
- LC30 or LC25 chromatography oven or AS50 thermal compartment

PeakNet Chromatography Workstation

Dionex ProPac WCX-10 cation-exchange column, 250 × 4 mm i.d. (P/N 54993)

REAGENTS AND SAMPLES

Deionized water

Monobasic sodium phosphate, monohydrate, crystal (J.T. Baker, Inc.)

Dibasic sodium phosphate, anhydrous, powder (J.T. Baker, Inc.)

Sodium chloride, ACS grade (VWR Scientific)

Ammonium bicarbonate, HPLC grade (J.T. Baker, Inc.)

Ethylenediaminetetraacetic acid (EDTA), disodium, dihydrate (Sigma Chemical Co.)

Hydrogen peroxide, 30% (Sigma Chemical Co.)

Dimethyl sulfoxide (DMSO), ACS reagent (Sigma Chemical Co.)

Hydrochloric acid (HCl), 11–12 N, Ultrex II Ultrapure Reagent (J.T. Baker, Inc.)

Acetic acid, glacial, HPLC grade (J.T. Baker, Inc.)

Luteinizing hormone-releasing hormone (LH-RH), Human; 84% of dry weight is peptide, 97% of peptide contains the following sequence: p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Sigma Chemical Co.)

α-Melanocyte Stimulating Hormone (α-MSH), 78% of dry weight is peptide, 98% of peptide contains the following sequence: N-Acetyl-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (Sigma Chemical Co.)

CONDITIONS

Column:	Dionex ProPac WCX-10 cation-exchange column, 250 × 4 mm (P/N 54993)
Temperature:	30 °C
Flow Rate:	1 mL/min
Inj. Volume:	10 µL
Detection:	UV, 254 nm
Eluents:	A: 10 mM Sodium phosphate (pH 6.0)
	B: 10 mM Sodium phosphate with 500 mM Sodium chloride (pH 6.0)

Gradient Program:

Time (min)	A (%)	B (%)
0.0	98	2
30.0	84	16
30.1	98	2
45.0	98	2

Autosampler Cycle Time: 45 min

PREPARATION OF SOLUTIONS AND REAGENTS

Two eluents are used for the chromatography: 10 mM sodium phosphate (pH 6.0) and 10 mM sodium phosphate (pH 6.0) with 500 mM sodium chloride (NaCl). The sodium phosphate buffer system was prepared by diluting appropriate quantities of monobasic and dibasic sodium phosphate concentrate solutions with water to attain the desired pH 6.0. The relative amount of monobasic and dibasic sodium phosphate solution added differed between the eluents with and without sodium chloride because NaCl is not normally pH neutral. The following procedure is a recommended starting point for obtaining the desired eluents, but some deviation from this formula may be necessary to achieve the desired pH. If the pH is not 6.0, then adjust the proportions of monobasic and dibasic solutions added. The combined total volume should remain 100 mL to produce 10 mM sodium phosphate for 2 L of eluent.

1 M Sodium Chloride

Dissolve 58.45 g of sodium chloride in water and fill to a final volume of 1.0 L. Filter through a 0.22-µm filter.

200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate in 1.0 L of water. Filter through a 0.22-µm filter.

200 mM Sodium Phosphate, Monobasic

Dissolve 27.60 g monohydrate monobasic sodium phosphate in 1.0 L of water. Filter through a 0.22-µm filter.

10 mM Sodium Phosphate, pH 6.0

Combine 14 mL of 200 mM dibasic sodium phosphate, 86 mL of 200 mM monobasic sodium phosphate, and 1900 mL of water. If a 6.0 pH is not attained, adjust relative proportions of 200 mM dibasic sodium phosphate used, maintaining a total volume of 100 mL.

10 mM Sodium Phosphate with 500 mM Sodium Chloride, pH 6.0

Combine 35 mL of 200 mM dibasic sodium phosphate, 65 mL of 200 mM monobasic sodium phosphate, 1000 mL of 1 M sodium chloride, 900 mL of water. If pH 6.0 is not attained, adjust relative proportions of 200 mM mono- and dibasic sodium phosphate used, maintaining a total volume of 100 mL.

1 M Ammonium Bicarbonate, pH 8.8

Combine 7.91 g ammonium bicarbonate with 90 mL water. Adjust the pH to 8.8 with an 11–12 N HCl solution. Adjust to 100 mL with water, and filter through a 0.22-µm filter.

75 mM EDTA, pH 8.0

Combine 78.8 mg ethylenediaminetetraacetic acid (EDTA), disodium, dihydrate with 900 mL water. Adjust the pH to 8.0 with 0.05% (w/w NaOH). Adjust to a final volume of 1 L with water, and filter through a 0.22-µm filter.

400 mM Hydrogen Peroxide

Combine 0.455 mL hydrogen peroxide (30%, 8.79 M) with 9.55 mL water. Make a fresh solution the day of use.

SAMPLE PREPARATION

Oxidation of Tryptophan in LH-RH

Reconstitute a vial containing 5 mg of the LH-RH preparation (4.2 mg peptide content) with 243 µL glacial acetic acid, 32.7 µL of 11–12 N HCl, and 6.4 µL water. Non-oxidized buffer control is prepared by combining 41.7 µL glacial acetic acid, 5.6 µL 11–12 N HCl, and 1.1 µL water. Oxidized buffer control is prepared by combining 41.7 µL glacial acetic acid, 5.6 µL 11–12 N HCl, and 1.1 µL DMSO. Transfer two 48.4 µL aliquots of the LH-RH into two microinjection vials, add 1.1 µL

of DMSO to one of the vials for oxidation (oxy-LH-RH), and 1.1 µL of water to the other vial. Incubate all vials at room temperature for 15 min. No action is taken to stop oxidation. Add 94.7 µL of water to all vials. Dilute 100-fold by transferring 10 µL of each vial into 990 µL of water. Mix and inject 10 µL for analysis of the 0.05 mg/mL LH-RH and oxy-LH-RH, and the two buffer controls.

The peptides are detected at 254 nm. These peptides can also be detected at 210 nm and 280 nm. After Trp oxidation, LH-RH's response increases at 254 nm, and decreases at 280 nm. For this reason, it is not possible to obtain accurate mass balance of peptides based on peak area or height measurements without characterizing the response of the products.

Oxidation of Methionine in Melanocyte Stimulating Hormone (MSH)

Reconstitute 1 mg of the MSH preparation (~0.78 mg peptide) with 533 µL water to make 1.5 mg peptide/mL. Prepare 1 mg/mL untreated peptide control by combining 105 µL of the 1.5 mg peptide/mL with 15 µL of 1 M ammonium bicarbonate, 10 µL 75 mM EDTA, and 20 µL water. Prepare 1 mg/mL Met-oxidized peptide sample by combining 105 µL of 1.5 mg peptide/mL with 15 µL of 1 M ammonium bicarbonate, 10 µL of 75 mM EDTA, and 20 µL 400 mM hydrogen peroxide. Prepare a buffer control by combining 105 µL of water with 15 µL of 1 M ammonium bicarbonate, 10 µL of 75 mM EDTA, and 20 µL of 400 mM hydrogen peroxide. This control and all oxidized samples (oxy-MSH) had final concentrations of 0.1 M ammonium bicarbonate, 5 mM EDTA, and 53 mM hydrogen peroxide. Incubate all samples for 30 min in an icewater bath. No steps are taken to stop oxidation. After incubation, analyze 10 µL of the 1 mg/mL MSH, oxy-MSH, and the buffer control.

RESULTS AND DISCUSSION

Figure 1A shows the elution of the non-oxidized LH-RH at 16 min on the ProPac WCX-10 column. The two peaks eluting within the first 1.5 min are also seen in the buffer control (data not shown). After Trp oxidation, the peptide elutes earlier at 11–12 min (Figure 1B), completely resolved from the native peptide. A small peak at 16 min (peak 5) suggests that LH-RH is not completely oxidized. Two unidentified peaks are observed at 2 and 19 min (peaks 3 and 6).

Figure 2 shows MSH before (Panel A) and after oxidation (Panel B) with hydrogen peroxide. These are conditions that have been reported to oxidize methionine in Substance P.[3] MSH elutes at 23–24 min (peak 7) before oxidation, and at 16–17 min (peak 6) after oxidation. MSH contains a single Trp residue. Minor peaks are also observ-ed at 11, 12, and 13 min (peaks 3, 4, and 5, respectively) that could be Trp oxidation products.

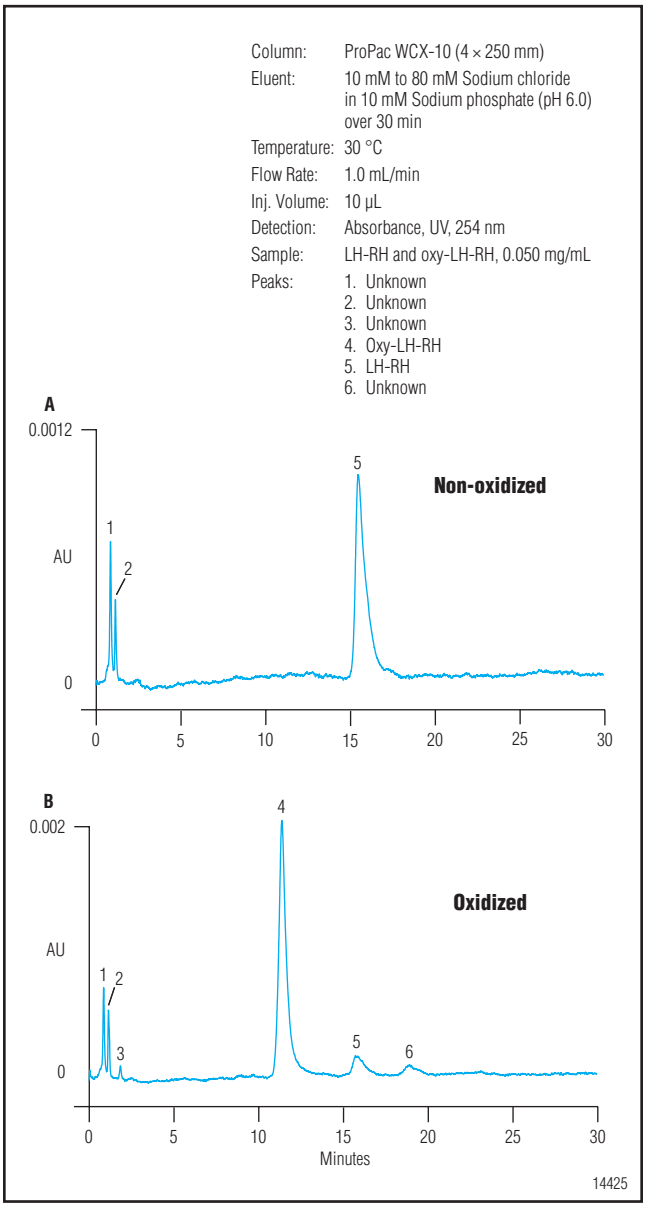

Figure 1. Separation of (A) LH-RH and (B) Oxy-LH-RH using the ProPac WXC-10 column with detection at 254 nm.

Experiments using the same Trp oxidation conditions described for LH-RH showed the evolution of peaks at these retention times (data not presented), but the interpretation of this experiment was complicated by an additional peak between 10–20 min in the peptide control (results not presented). Figure 2 shows that MSH and oxy-MSH are easily separated on the ProPac WCX-10 column.

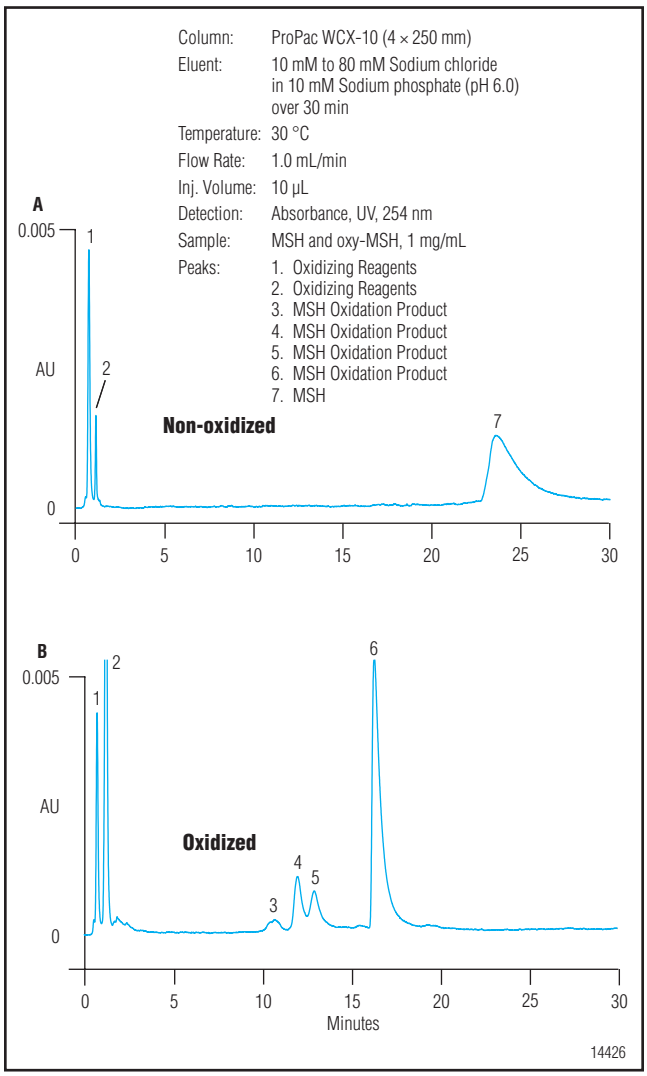

Figure 2. Separation of (A) MSH and (B) Oxy-MSH using the ProPac WCX-10 column with detection at 254 nm.

CONCLUSION

These separations demonstrate the resolving ability of the ProPac WCX-10 column to separate peptides with differences as small as the oxidation of a single amino acid. These separations use easily disposable aqueous eluents and are complimentary or an alternative to reversed-phase chromatography for characterizing peptides.

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4. Teh, L.-C. et al. *J. Biol. Chem.,* **1987**, *262,* 6472–6477.

LIST OF SUPPLIERS

Millipore Corporation, 80 Ashby Road, Bedford, MA 01730, USA. Tel: (800) 645-5476

J.T. Baker, Inc., 222 Red School Lane, Phillipsburg, NJ 08865, USA. Tel: (800) 582-2537

VWR Scientific, P.O. Box 172, 3745 Bayshore Blvd., Brisbane, CA 94005, USA. Tel: (800) 932-5000

Sigma Chemical Corporation, P.O. Box 14508, St. Louis, MO 63178, USA. Tel: (800) 325-3010

Thermo SCIENTIFIC

Application Note 214

Separation of Protein Phosphoisoforms Using Strong Anion-Exchange Chromatography

INTRODUCTION

Posttranslational modifications (PTMs) of proteins are considered one of the major determinants of an organism's complexity. Protein phosphorylation is one of the most studied PTMs because it provides a rapid, reversible means of regulating protein activity and numerous other cellular processes including cell differentiation, proliferation, and migration.[1] It is estimated that approximately thirty percent of all proteins in a cell are phosphorylated at any given time.[2] Changes in levels of phosphorylated isoforms often signal developmental or pathological disorders.[3] To better understand the role of phosphorylation in regulation, and for clinical diagnostics, it is important to differentiate phosphorylated forms of proteins from their dephosphorylated forms.

Conventional antibody-based phosphopeptide mapping methods used to assay for protein phosphorylation are laborious, difficult to control, and require the use of large amounts of 32P. Additionally, these methods can only be used when the protein of interest is relatively abundant in the tissue homogenate. Mass spectrometric analysis of protein phosphorylation has several inherent problems. Phosphopeptides are often missed during the mass spectrometric analysis due to (1) selective suppression of ionized phosphorylated proteins relative to large amounts of ionized unphosphorylated proteins, and (2) lower detection efficiencies of phosphopeptides compared to their unphosphorylated cognates.[4]

This application note demomstrates the use of the ProPac® SAX-10 column for separation of phosphorylated protein variants. This novel anion-exchange column consists of linear polymer chains grafted onto polymer beads coated with a hydrophilic surface. An inert liquid chromatography instrument is used to eliminate the possibility of complex formation between phosphorylated proteins and leachable transition metals (e.g., iron) often observed using stainless steel-based chromatography systems. The high efficiency of the strong anion-exchange resin allows the resolution of several phosphorylated isoforms using a simple gradient. Comparisons of chromatograms for selected model phosphoproteins with and without alkaline phosphatase treatment, which cleaves phosphate from the amino acid side chains, shows that peaks are resolved based on the extent of phosphorylation.

The phosphate content of the phosphorylated proteins is determined using ion chromatography with suppressed conductivity detection, as described in Application Note 210. Here, we present a simple and quick method combining anion-exchange chromatography and UV detection to analyze phosphorylated isoforms of different phosphoproteins: ovalbumin, synthetically phosphorylated BSA, and casein.

EQUIPMENT

ICS-3000 liquid chromatography system consisting of:

- ICS-3000 SP single pump (P/N 079820) or DP dual pump (P/N 061713)
- ICS-3000 VWD variable wavelength detector (P/N 064653) with PEEK™ flow cell, 10 mm, 11 µL (P/N 066346)
- ICS-3000 TC (P/N 064444) or DC (P/N 061767)
- AS Autosampler (P/N 056859)

Or

UltiMate® 3000 Titanium system consisting of:

- SRD-3600 Solvent Rack with 6 degasser channels (P/N 5035.9230) and Eluent Organizer, including pressure regulator, and 2 L glass bottles for each pump. Eluents were maintained under helium head space (5–8 psi)
- LPG 3400AB Quaternary Analytical Pump (P/N 5037.0015) or DGP-3600AB Dual Ternary Analytical Pump (P/N 5037.0014) for dual gradient capability
- WPS-3000TBPL Biocompatible Analytical Autosampler (P/N 5823.0020)
- TCC-3000 Column Compartment without switching valves (P/N 5722.0000) or TCC-3200B Column Compartment with 2 PEEK 10-port, 2-position valves (P/N 5723.0025) for added capability
- VWD-3400 Variable Wavelength Detector without flow cell (P/N 5074.0010) or PDA-3000 Photodiode Array Detector (P/N 5080.0020)
- Biocompatible Analytical Flow Cell for VWD (P/N 6074.0200) or Biocompatible Analytical Flow Cell for PDA (P/N 6080.0220)

Chromeleon® 6.8 Chromatography Management Software

Other Equipment

SpeedVac™ Evaporator System (ThermoQuest Savant E/C Division) consisting of :

- SpeedVac, Model SVC100
- Refrigerator Vapor Trap, Model RVT400
- Vacuum Gauge, Model VG-5
- Welch Duo-Seal Vacuum Pump, Model 1402 capable of pulling 0.2 Torr (200 µm Hg) vacuum

Microcentrifuge, Model 5415C (Eppendorf)

Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarstedt 72.692.005)

Filter unit, 0.2 µm nylon (Nalgene® Media-Plus with 90 mm filter, Nalgene Nunc International, P/N 164-0020) or equivalent nylon filter

Biodialyser™ microdialysis system with 500 dalton molecular weight cutoff (MWCO) cellulose acetate membranes (AmiKa, Inc., The Nest Group, P/N SSM0500)

Reacti-Therm III Heating Module with Reacti-Block™ H (Pierce Chemical Co., P/N 18940ZZ or equivalent)

Helium; 4.5-grade, 99.995%, <5 ppm oxygen (Praxiar Specialty Gases)

REAGENTS AND SAMPLES

Deionized water 18.2 (MΩ-cm)

Hydrochloric Acid, 36.5-38.0% (J.T.Baker, 9530-33)

Tris (Base), ACS Reagent (J.T. Baker, X171-7)

Sodium Chloride, ≥99.5% (Sigma-Aldrich, S7653)

Alkaline phosphatase, bovine intestinal mucosa, lyophilized, 35% protein (Sigma-Aldrich, P6772-2KU)

Ovalbumin from chicken egg white, Grade V, VI, >98%, lyophilized powder (Sigma-Aldrich, A5503, and A2512, 45 kDa), five lots

Bovine serum albumin (BSA), ≥96%, (Sigma-Aldrich, A6003, 69 kDa)

Micro BCA™ Protein Assay kit (Pierce Biotechnology, 23250)

Phospho-serine-BSA, 2 mg/mL in 10 mM phosphate buffer (pH 7.4), (Sigma-Aldrich, P3717, 45 kDa)

2-Mercaptoethanol, Molecular biology grade (Sigma-Aldrich, M6250)

Imidazole, Molecular biology grade (Sigma-Aldrich, I5513)

Urea, Molecular biology grade (Sigma-Aldrich, U5378)

Casein from bovine milk (Sigma-Aldrich, C8654)

Dephosphorylated casein from bovine milk (Sigma-Aldrich, C4032)

CONDITIONS

Chromatography Conditions for the Separation of Ovalbumin and BSA Isoforms

Columns: ProPac SAX-10 column, 4.0 × 250 mm, (P/N 054997)
ProPac SAX-10G column, 4.0 × 50 mm, (P/N 054998)
Flow Rate: 1.0 mL/min
Temperature: 25 °C
Inj. Volume: 10 µL
Detection: UV, 280 nm

Eluents and Gradient Program for Ovalbumin:

A: 20 mM Tris (pH 8.5)
B: 20 mM Tris, 500 mM Sodium chloride (pH 8.5)

Time (min)	A%	B%
0.0	100.0	0.0
15.0	87.5	12.5
17.0	76.0	24.0
19.0	76.0	24.0
19.1	100.0	0.0
25.0	100.0	0.0

Eluents and Gradient Program for BSA and Synthetically Phosphorylated BSA:

Eluents: A: 20 mM Tris (pH 8.5)
B: 20 mM Tris, 2 M Sodium chloride (pH 8.5)

Time (min)	A%	B%
0.0	100.0	0.0
2.0	100.0	0.0
30.0	10.0	90.0
39.0	10.0	90.0
39.1	100.0	0.0

Chromatography Conditions for Separation of Casein Isoforms

Columns: ProPac SAX-10 column, 4.0 × 250 mm, (P/N 054997)
ProPac SAX-10G column, 4.0 × 50 mm, (P/N 054998)
Flow Rate: 1.0 mL/min
Temperature: 25 °C
Inj. Volume: 75 µL
Detection: UV, 280 nm
Eluents: A: 4 M Urea, 0.1 M 2-mercaptoethanol, 10 mM imidazole, pH 7.0
B: 1 M Sodium chloride in 4 M urea, 0.1 M 2-mercaptoethanol, 10 mM imidazole, pH 7.0

Gradient Program:

Time (min)	A%	B%
–10.0	100.0	0.0
0.0	100.0	0.0
3.0	95.0	5.0
30.0	70.0	30.0
32.0	70.0	30.0
35.0	100.0	0.0

Phosphate Determination

Phosphate determinations were performed using an IonPac® Fast Anion IIIA analytical column (3 × 250 mm, P/N 062964) using 25 mM potassium hydroxide. Method details are described Application Note 210.[7]

PREPARATION OF SOLUTIONS AND REAGENTS

20 mM Tris (pH 8.5)

Dissolve 2.42 g of Tris in approximately 1700 mL of 18.2 MΩ-cm DI water. Adjust the pH to 8.5 using concentrated (36.5–38.0%) hydrochloric acid. Adjust the volume to 2 L with DI water and filter through a 0.2 µm filter.

20 mM Tris, 200 mM Sodium Chloride (pH 8.5)

Dissolve 2.42 g of Tris and 23.38 g of sodium chloride in approximately 1700 mL of 18.2 MΩ-cm DI water. Adjust the pH to 8.5 using concentrated (36.5–38.0%) hydrochloric acid. Adjust the volume to 2 L with DI water and filter through a 0.2 µm filter.

20 mM Tris, 2 M Sodium Chloride (pH 8.5)

Dissolve 2.42 g of Tris and 233.76 g of sodium chloride in approximately 1700 mL of 18.2 MΩ-cm DI water. Adjust the pH to 8.5 using concentrated (36.5–38.0%) hydrochloric acid. Adjust the volume to 2 L with DI water and filter through a 0.2 µm filter.

200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate (Na_2HPO_4) in water to a final solution volume of 1.0 L.

4 M Urea, 0.1 M 2-mercaptoethanol, 10 mM Imidazole, pH 7.0

Dissolve 240.24 g of urea in approximately 700 mL of water. Dissolve 0.6808 g imidazole in the urea solution. Add 90 µL of 2-mercaptoethanol and adjust the pH to 7.0 using concentrated (36.5–38.0%) hydrochloric acid. Adjust the volume to 1 L with DI water and filter through a 0.2 µm filter.

1 M Sodium Chloride in 4 M Urea, 0.1 M 2-Mercapto-ethanol, 10 mM Imidazole, pH 7.0

Dissolve 58.44 g sodium chloride in approximately 700 mL of water. Dissolve 240.24 g in the urea solution, and then dissolve 0.6808 g of imidazole to the urea solution. Add 90 µL of 2-mercaptoethanol and adjust the pH to 7.0 using concentrated (36.5–38.0%) hydrochloric acid. Adjust the volume to 1 L with DI water and filter through a 0.2 µm filter.

SAMPLE PREPARATION

5 mg/mL Ovalbumin Standard

Dissolve 20 mg ovalbumin into 4 mL DI water in a 20 mL scintillation vial. Gently swirl the solution until thoroughly mixed. Dispense 200 µL aliquots of the 5 mg/mL ovalbumin into 1.5 mL sterile micro-centrifuge tubes. Store the solutions at –20 °C or less.

5 mg/mL Bovine Serum Albumin (BSA) Standard

Dissolve 20 mg BSA into 4 mL DI water in a 20 mL scintillation vial. Gently swirl the solution until thoroughly mixed. Dispense 200 µL aliquots of the 5 mg/mL BSA into 1.5 mL sterile micro-centrifuge tubes. Store the solutions at –20 °C or less.

1 mg/mL Casein Standard

Dissolve 20 mg casein into 20 mL eluent A. Gently swirl the solution to mix. Dispense 200 µL aliquots of the 1 mg/mL casein into 1.5 mL sterile micro-centrifuge tubes. Store the solutions at –20 °C or less.

1 mg/mL Dephosphorylated Casein Standard

Dissolve 20 mg dephosphorylated casein into 20 mL eluent A. Gently swirl the solution to mix. Dispense 200 µL aliquots of the 1 mg/mL casein into 1.5 mL sterile micro-centrifuge tubes. Store the solutions at –20 °C or less.

Dephosphorylation of Protein with Alkaline Phosphatase

Digest 96 µg of ovalbumin using 19 units of alkaline phosphatase and 120 µL of 50 mM Tris buffer (pH 9). Bring the total volume to 300 µL using DI water. Incubate the mixture on a heat block at 37 °C for 5 h. Centrifuge the digested protein at 14,000 rpm for 5 min, and pipette the supernatant into 1.5 mL sample vials. Prepare, heat, and and centrifuge control samples of Tris buffer, alkaline phosphatase, and proteins in buffer in the same manner as the protein samples. See Application Note 210 for preparation of reagents and a detailed discussion of the assay. Determine total protein in all proteins according to the instructions on the Micro-BCA Protein Assay kit.

Microdialyze ovalbumin fractions overnight in water in order to remove salt, then dry in the SpeedVac. Because the fractions are eluted in high concentrations of salt, it is important to remove the salt to allow quantitation of the phosphate that is present at much lower concentrations. Resuspend the dried fractions in 50 mM Tris buffer prior to alkaline phosphatase digestion.

RESULTS AND DISCUSSION

The ProPac SAX-10 column was selected for the separations discussed in this application note because it is a high resolution column well suited for the separation of closely related anionic protein variants. The column has a nonporous core particle and a hydrophilic layer over the core to prevent unwanted secondary interactions. The strong anion-exchange moieties grafted onto the hydrophilic layer provide increased capacity, while still maintaining high resolution.

Ovalbumin is a 43 KDa glycoprotein present in avian egg white with two potential serine phosphorylation sites at positions 68 and 344.[5,6] Reported phosphate values for ovalbumin preparations vary from 1.75 to 1.89, indicating that both sites are phosphorylated. Figure 1 shows ovalbumin (magenta trace) and its dephosphorylated form after alkaline phosphatase treatment (blue trace). The figure shows that treatment with alkaline phosphatase results in earlier elution of peaks, which is consistent with a loss of negative charge. The early eluting peaks are

unaffected by alkaline phosphatase treatment. Ovalbumin has been reported to have four isoforms with varying phosphate content, one with zero, two with one and one with two phosphates.[6] The figure shows that treatment with alkaline phosphatase reduced the number of peaks from nine to one large and three small peaks. The first three peaks in the dephosphorylated sample correspond to the peaks in the native sample, while peak 4A corresponds to the initial shoulder of peak 4 in the native sample. This confirms that the separation of the ovalbumin sample is in part due to differences in the phosphorylation state of the protein backbone. Peak 2 appears to represent ovalbumin with no attached phosphate. Peaks 1 and 3 in the dephosphorylated sample may represent the known sequence microheterogeneity of ovalbumin. Peaks 2, 4, and 7 in the native preparation seem to represent varying phosphate contents, and all the minor peaks (1, 3, 5, and 6) represent different phosphorylation states of ovalbumin due to sequence microheterogeneity.

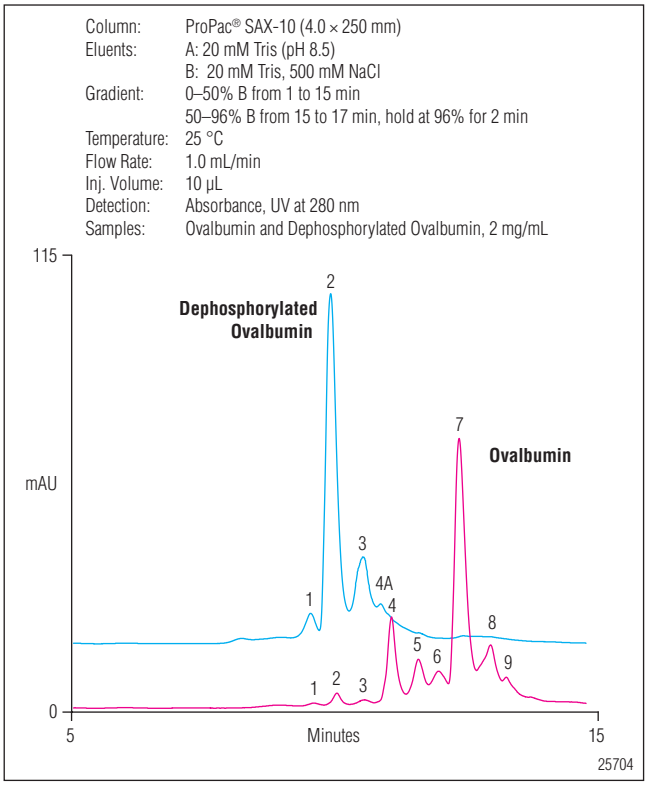

Figure 1. Separation of ovalbumin before and after alkaline phosphatase treatment (dephosphorylation) using the ProPac SAX-10 column

Figure 2 shows the separation of five lots of ovalbumin before and after dephosphorylation with alkaline phosphatase. The magenta trace shows the separation of native ovalbumin and the blue trace shows the separation of dephosphorylated ovalbumin. All the traces show a reduction of peaks after alkaline phosphatase treatment. Based on the chromatography in Figure 1 and reports of varying phosphate content, peak 1 was identified as an isoform with no phosphate, peak 2 as an isoform with one phosphate, and peak 3 as an isoform with two phosphates. Following alkaline phosphatase treatment, all the traces show the presence of only peak 1, an ovalbumin isoform with zero phosphates.

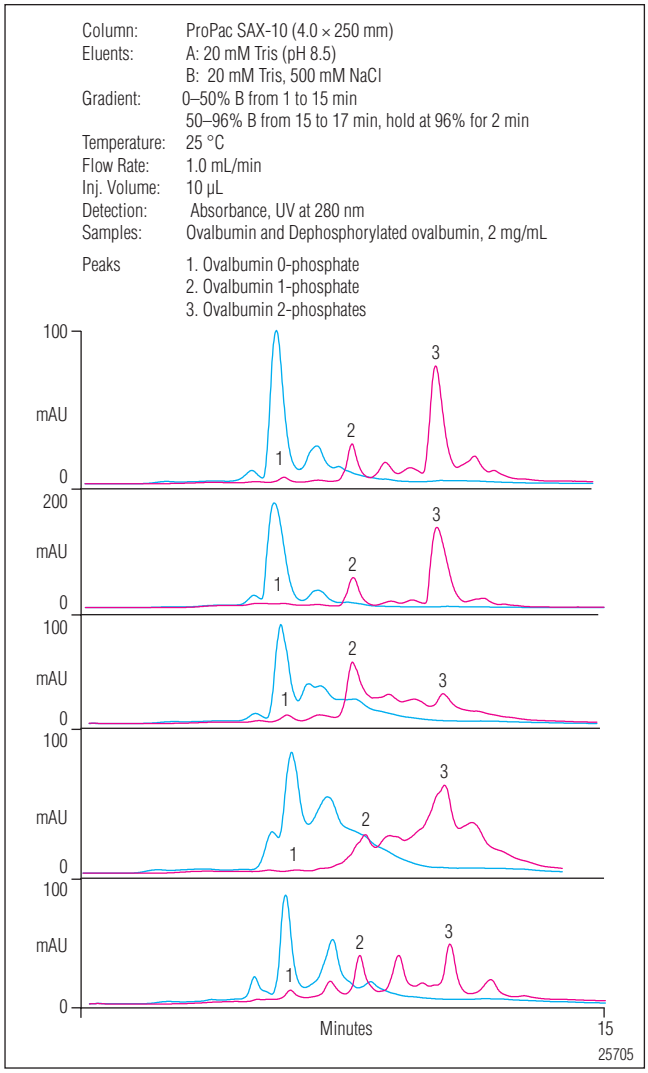

Figure 2. Separations of 5 lots of ovalbumin and their dephosphorylated forms using the ProPac SAX-10 column

Table 1 shows the ratios of the peak areas of peaks 2 and 3 to the total peak area prior to alkaline phosphatase treatment and the amount of phosphate removed after treatment, as measured by ion chromatography.[7] The amount of phosphate in each ovalbumin lot was predicted based on the sum of weighted peak area ratios of peaks 2 and 3. The predicted values for phosphate content were consistent with the IC determinations, suggesting that the assignment of peaks 2 and 3 as single and doubly phosphorylated isoforms of ovalbumin is correct.

Table 1. Phosphorylation Analysis of Five Commercial Ovalbumin Samples

Protein Grade and Lot	Ratio of peak 2 area to total peak area mAU*min	Ratio of peak 3 area to total peak area mAU*min	*Predicted Mole Ratio (Phosphate/ Protein)	Found Mole Ratio (Phosphate/ Protein)
Ovalbumin, grade V, lot 1	0.27	0.61	1.49	1.64
Ovalbumin, grade VI, lot 5	0.24	0.63	1.50	1.22
Ovalbumin, Grade V, lot 2	0.57	0.20	0.97	1.14
Ovalbumin, grade VI, lot 4	0.22	0.39	1.00	0.78
Ovalbumin, grade VI, lot 3	0.30	0.26	0.82	0.37

* Predicted mole ratio of phosphate to protein calculated based on the following formula: Predicted mole ratio of phosphate to protein = 2(ratio of peak 3) + ratio of peak 2

Fractions shown in Figure 3 were collected from one lot of ovalbumin, and the phosphate content of each was assayed as described in Application Note 210.[7] After dialysis the three collected fractions were treated with alkaline phosphatase and then analyzed for free phosphate. Table 2 presents the moles of phosphate measured per mole of protein. This data confirms the conclusion that peaks 2 and 3 are singly and doubly phosphorylated isoforms of ovalbumin.

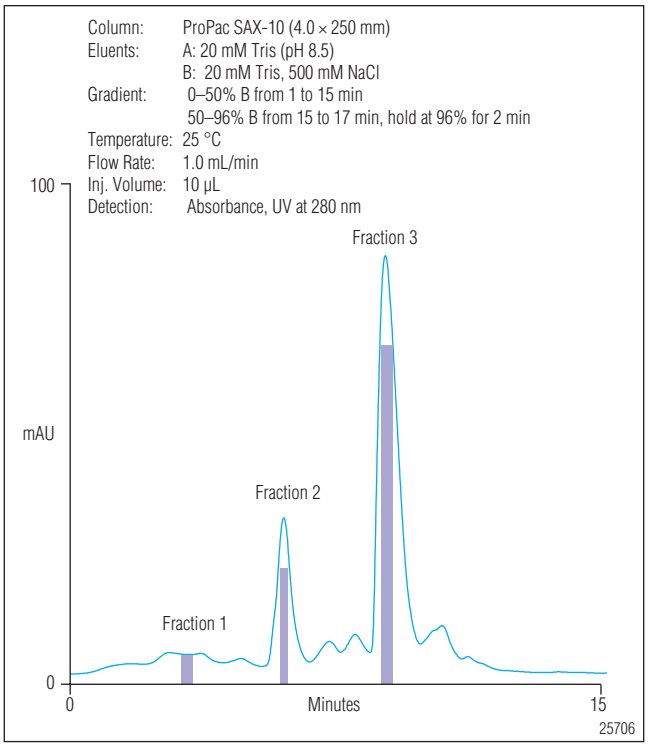

Figure 3. UV chromatogram showing the collection of fractions from a lot of ovalbumin separated on a ProPac SAX-10 column.

Table 2. Phosphate Content Of Ovalbumin Fractions Prepared By ProPac SAX-10 Separation

Sample	Moles of Phosphate/Mole of Protein
Fraction 1	0.00
Fraction 2	0.93
Fraction 3	1.97

Bovine serum albumin (BSA) is a 66 KDa single polypeptide chain consisting of 583 amino acid residues.[8] The pI of BSA is 5.1 with a charge of –17 at pH 7.4.[9] O-phospho-L-serine BSA is a commercially available synthetically phosphorylated form of bovine serum albumin. The ProPac SAX-10 column was selected for this separation based on pI of the protein. Figure 4 shows the separations of BSA and a commercially available synthetically phosphorylated form on a ProPac SAX-10 column. Phosphorylated BSA is retained longer on the column than BSA as would be predicted based on its increased negative charge.

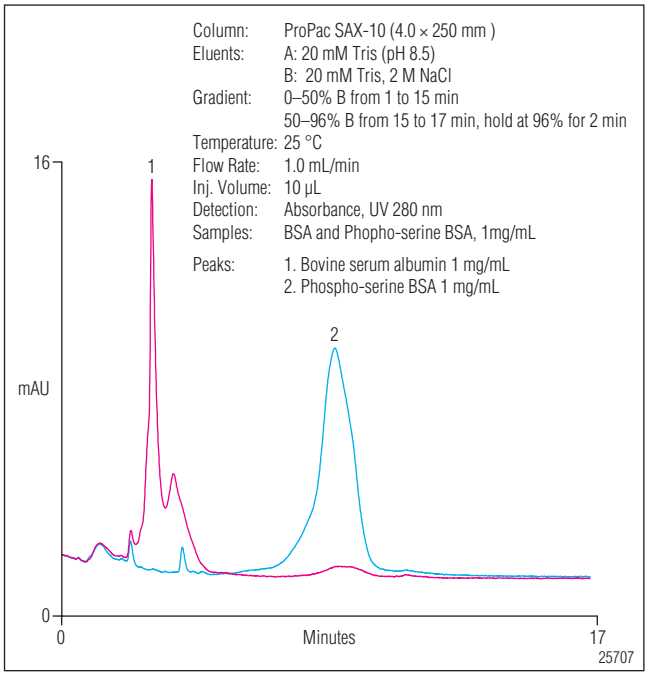

Figure 4. Separations of bovine serum albumin and its synthetically phosphorylated form using the ProPac SAX-10 column

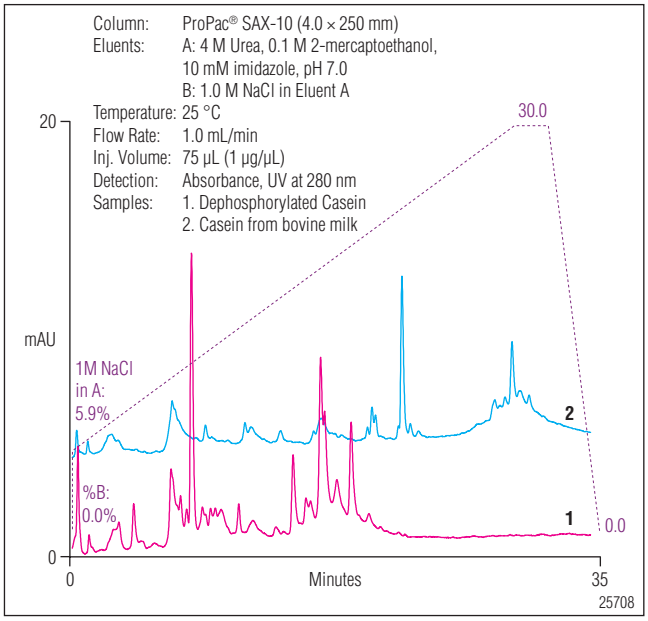

Figure 5. Separations of casein from bovine milk and commercially available dephosphorylated casein using the ProPac SAX-10 column

Caseins are a unique group of acid insoluble phosphoproteins that represent approximately eighty percent of the total protein in bovine milk.10 Caseins can be differentiated according to the homology of their primary structures and based on their relative electrophoretic mobility into the following families: αs1, αs2, β, and κ-caseins. The αs1 and αs2 subunits comprise 40% and 10% of casein in bovine milk and contain 8 and 11 phosphoserine residues respectively. The β and κ-subunits constitute 45% and 5% of casein in bovine milk and contain 5 and 1 phosphoserine residues respectively.11 Figure 5 shows the separations of casein and a commercially available dephosphorylated form of casein on a ProPac SAX-10 column. This figure reveals that casein is a complex sample with considerable heterogeneity and that some of its retention is due to phosphorylation. The majority of the peaks in the commercially available dephosphorylated form of casein show less retention than the majority of the peaks in the untreated casein preparation due to a net loss of negative charge.

SUMMARY

Phosphorylation of proteins confers variability in the protein's charge, resulting in charged isoforms. Grafted pellicular anion and cation exchangers are useful for resolution of charged protein isoforms.[12] In this application note we demonstrate a quick and simple method to separate phosphorylated isoforms from their dephosphorylated forms using the ProPac SAX-10, a high efficiency strong anion-exchange column. This column can separate proteins with differences as small as one or more phosphates on a single amino acid residue. Phosphoprotein analysis on an inert PEEK or titanium chromatography system ensures that there are no protein-transition metal complexes that reduce protein recovery, or transition metal fouling of the ProPac column.

PRECAUTIONS

Avoid contact and inhalation of any of these proteins. Material safety data sheets (MSDS) for these materials need to be reviewed prior to handling, use, and disposal.

SUPPLIERS

Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103, Tel: 800-521-8956, www.sigmaaldrich.com.

Mallinckrodt Baker, Inc., 222 Red School Lane, Phillipsburg NJ 08865, Tel: 908-859-2151, www.solvitcenter.com.

Nalge Nunc International, 75 Panorama Creek Drive, Rochester, NY 14625. U.S.A., Tel: 800-625-4327, www.nalgenunc.com.

Sarstedt INC, 1025 St. James Church Road, P.O. Box 468, Newton NC 28658-0468, Tel: 828-465-4000, www.sarstedt.com.

Pierce Biotechnology, Inc. P.O. Box 117, Rockford, IL 61105, Tel: 800-874-3723, www.piercenet.com.

Savant Instruments/E-C Apparatus (a division of Thermoquest), 100 Colin Drive, Holbrook, NY 11741-4306 USA, Tel: 800-634-8886, www.thermoquest.com.

The Nest Group, Inc., 45 Valley Road, Southborough, MA 01772-1323. Tel: 800-347-6378 www.nestgrp.com

Eppendorf, One Cantiague Road, PO Box 1019, Westbury, NY 11590, Tel: 800-645-3050, www.eppendorf.com

Praxair Specialty Gases and Equipment, 39 Old Ridgebury Road, Dansbury, CT 06810-5113 USA, Tel: 877-772-9247, www.praxair.com

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Complete sequence coverage

Protein therapeutics applications notebook

Thermo SCIENTIFIC

Application Note 99

Peptide Mapping of Ovalbumin Using Reversed-Phase High-Performance Liquid Chromatography and Prediction of Phosphopeptide Elution

INTRODUCTION

Protein variants arise from post-translational modifications, such as glycosylation, oxidation, and phosphorylation. Variants of proteins produced for medicinal purposes can occur during manufacturing, handling, and storage, and can impact the activity and stability of the biotherapeutic.[1] Protein phosphorylations at serine, threonine, and tyrosine residues are the most-studied and best-characterized of all post-translational modifications (PTMs).[2] Peptide mapping is used routinely to study PTMs and is capable of identifying a single PTM difference in a protein. Peptide mapping is commonly used in the biopharmaceutical industry to establish product identity by confirming the primary structure of a product.[3] The chicken egg white protein ovalbumin has a molecular weight of 44,281 Da and is 386 amino acid residues long. Ovalbumin has two serine phosphorylation sites (S69 and S345) thus making it a good model for the analysis of protein phosphorylation.[4]

This application note (AN) describes a method to reduce, alkylate, and enzymatically digest ovalbumin into peptides using trypsin. These peptides are separated by a reversed-phase HPLC method using an Acclaim® 300, C18 column. The Acclaim 300 column with its 300 Å pore size and 3 µm silica particle size is designed for the rapid analysis of peptide mixtures or proteins. The stable bonding of the Acclaim 300, C18 columns results in predictable reversed-phase separations with minimal secondary interactions and makes them compatible with LC/MS applications. Treating the ovalbumin tryptic digest with alkaline phosphatase removes phosphate from the phosphopeptides. Comparing tryptic maps with and without alkaline phosphatase treatment enables a tentative identification of the phosphopeptides. The Dionex UltiMate® 3000 HPLC system equipped with an Acclaim 300, C18 column is an excellent solution for routine peptide mapping applications.

EQUIPMENT

Dionex UltiMate 3000 system consisting of:

- SRD-3600 Solvent rack with six degasser channels (P/N 5035.9230)
- Eluent organizer, including pressure regulator (eluents were maintained under helium or nitrogen head space 5–8 psi)
- 2 L glass bottles for each pump
- DGP 3600M UltiMate 3000 quaternary low-pressure proportioning analytical pump system (P/N 5035.0050)
- WPS-3000T Well plate sampler (P/N 5823.0020)
- FLM-3100 Nano Flow Manager (P/N 5720.0010) or UltiMate 3000 column compartment (recommended module when using 2 and 3 mm i.d. columns, P/N 5722.0025)
- PDA-3000 Photodiode array detector (P/N 5080.9920)
- Biocompatible analytical flow cell for PDA (P/N 6080.0220)

SpeedVac® Evaporator System (Thermo Scientific Savant or equivalent) consisting of:

- SpeedVac Concentrator, Model SVC100
- Refrigerator vapor trap, Model RVT400
- Vacuum gauge, Model VG-5
- Welch DUOSEAL™ vacuum pump, Model 1402, capable of pulling 0.2 Torr (200 µm Hg) vacuum

Microcentrifuge, Model 5415C or equivalent

Microcentrifuge tubes, sterile with assembled screw cap, 1.5 mL (Sarstedt 72.692.005)

Filter unit, 0.2 µm nylon (Nalgene® Media-Plus with 90 mm filter, Nalgene Nunc International, P/N 164-0020) or equivalent nylon filter

Vacuum pump (Gast Manufacturing Corp., P/N DOA-P104-AA or equivalent)

0.3 mL Polypropylene (Vial Kit, P/N 055428) injection vials with caps

Reacti-Therm™ III heating module with Reacti-Block™ (Pierce Chemical Co., P/N 18940ZZ or equivalent)

Nitrogen 4.8 grade, 99.998%, <2 ppm oxygen (Praxair Specialty Gases)

SnakeSkin™ pleated dialysis tubing (Pierce, P/N 68100)

SnakeSkin dialysis tubing clips (Pierce, P/N 68011)

REAGENTS AND STANDARDS

Deionized water 18.2 (MΩ-cm)

Hydrochloric acid, ULTREX® II Ultrapure Reagent, 36.5–38.0% (J.T. Baker, 9530-33)

Tris (Base), ACS Reagent (tris(hydroxymethyl) aminomethane, J.T. Baker, X171-7)

Alkaline phosphatase, bovine intestinal mucosa, lyophilized, 35% protein, ≥2,000 DEA units/mg protein.* (Sigma-Aldrich, P6772-2KU)

Ovalbumin (albumin from chicken egg, Sigma-Aldrich, A5503, Grade V ≥98% pure by agarose gel electrophoresis, lyophilized powder)

Iodoacetamide (MP Biomedicals, P/N 100351)

DL-Dithiothreitol (Fluka, P/N 43815)

Trypsin, sequence grade, modified, lyophilized, 20 µg/vial (5 vials/kit), specific activity 17,000 U/mg. Kit includes 100 mM ammonium bicarbonate or trypsin resuspension buffer, 1 mL vial (Promega, P/N V5111)**

Ammonium bicarbonate (Sigma, A6141)

Trifluroacetic acid (TFA), sequanal grade for making 0.1% v/v TFA solutions (Thermo Scientific, P/N 28904)

Fetuin, fetal calf serum, lyophilized powder (Sigma-Aldrich, F2379, 250 mg, 48.4 KDa)

Micro BCA protein assay kit (Thermo Scientific, P/N 23235)

Acetonitrile, HPLC grade (Honeywell, P/N 015-4)

Sodium hydroxide, >98%, pellets, anhydrous (Sigma-Aldrich, P/NS8045)

Cytochrome C digest, 1.6 nmol/vial, lyophilized (Dionex, P/N 161089)

*One DEA unit will hydrolyze 1 µmole of 4-nitrophenyl phosphate per min at pH 9.8 and 37 °C.

**One unit is the amount of trypsin required to produce a change in absorbance (253 nm) of 0.001 per min at 30 °C with the substrate N-α-benzoyl-L-arginine ethyl ester (BAEE). The substrate is dissolved in 50 mM tris-HCl, 1mM $CaCl_2$ (pH 7.6), and the enzyme is diluted in 50 mM acetic acid.

CONDITIONS

Method

Columns	Acclaim 300, C18, 3 µm, Analytical, 2.1 × 150 mm, (P/N 060264) Acclaim 300, C18, 3 µm, Guard, 2 × 10 mm, (P/N 060395) Guard Kit (holder and coupler), (P/N 059526)
Eluents:	(A) 5% Acetonitrile, 0.1% TFA (B) 95% Acetonitrile, 0.1% TFA
Flow Rate:	0.2 mL/min
Temperature:	50 °C
Inj. Volume:	10 µL
Detection:	UV, 214 nm and 260 nm Minimum peak height setting for peak counts: 3.0 mAU (for ovalbumin and fetuin) and 1.0 mAU for cytochrome C

Gradient Method for Ovalbumin, Cytochrome C, and Fetuin

Time (min)	A%	B%
–33.0	95	5
0.0	95	5
45.0	20	80
60.0	20	80
60.1	0	100
65.0	0	100

PREPARATION OF SOLUTIONS AND REAGENTS

Eluents

5% Acetonitrile, 0.1% TFA

Add the contents of 1 ampule (1 mL) TFA to approximately 800 mL of 18.2 MΩ-cm DI water. Add 50 mL acetonitrile to the mix and make up the volume to 1000 mL with DI water.

95% Acetonitrile, 0.1% TFA

Add the contents of 1 ampule (1 mL) TFA to approximately 800 mL acetonitrile. Add 50 mL DI water to the mix and make up the volume to 1000 mL with acetonitrile.

As a precautionary note, TFA oxidizes over time and this may result in interfering chromatographic peaks. For best performance:

a. Use fresh TFA that is supplied in sealed ampules.
b. Prepare fresh TFA-containing eluents daily.
c. Store the eluents blanketed with inert gas during use on the system (helium or nitrogen).
d. Protect eluents from photoreactions by covering them with aluminum foil.
e. Though not used for this work, 0.08% TFA in mobile phase B will partially compensate for the rise in baseline observed when executing the gradient.

Sample Preparation Reagents

100 mM Ammonium Bicarbonate (pH 8.0–8.5)

Dissolve 1.58 g of ammonium bicarbonate in approximately 180 mL of 18.2 MΩ-cm DI water, bring the volume to 200 mL with DI water.

7 M Guanidine Hydrochloride Solution

Dissolve 3.34 g guanidine hydrochloride in 5 mL of 100 mM ammonium bicarbonate solution.

0.10 M HCl

Add 8.3 mL of concentrated HCl (11.65 M) to a volumetric flask containing 500 mL of DI water. Bring the volume to 1 L with DI water.

1 M Dithiothreitol (DTT) Solution

Dissolve 0.077 g DTT to 5 mL in 100 mM ammonium bicarbonate solution. This solution should be prepared just prior to use.

1 M Sodium Hydroxide Solution

Dissolve 0.200 g sodium hydroxide in 250 µL of 18.2 MΩ-cm DI water.

1 M Iodoacetic Acid

Dissolve 0.0465 g iodoacetic acid in 250 µL of 1 M sodium hydroxide solution. Warning: photosensitive, shield from light, and prepare fresh each day.

50 mM Ammonium Bicarbonate

Dissolve 7.91 g ammonium bicarbonate in approximately 1800 mL DI water and bring the volume to 2000 mL with DI water. Store at room temperature for up to 2 weeks.

Preparation of 50 mM Tris buffer (pH 9)

Dissolve 0.606 g of tris in 18.2 MΩ-cm DI water. Adjust to pH 9.0 (± 0.05) with 0.1 M HCl, if needed. Store at 4 ºC for up to 4 weeks.

Alkaline Phosphatase Working Solutions

To prepare 700 units/mL (0.7 units/µL) working solution of alkaline phosphatase, dissolve the entire contents of the 35% alkaline phosphatase, bovine intestinal mucosa bottle in 3 mL of 50 mM tris buffer. Swirl gently to dissolve. Dispense 200 µL aliquots into separate 1.5 mL micro-centrifuge tubes. Store the aliquots at –20 to –40 ºC for up to 6 months.

Preparation of Cytochrome C for System Qualification

Reconstitute the lyophilized cytochrome C (1.6 nmol/vial) in 200 µL of 5% acetonitrile, 0.1% TFA (eluent A) to obtain a concentration of 8 pmol/µL. Reconstitute the peptides by vortexing and wait for at least 10 min before use. Store all unused material at –20 °C for up to 2 weeks.

Procedure

Part I: System Qualification

1. Perform four replicate 10 µL injections of the cytochrome C tryptic digest solution for system qualification.
2. Confirm retention time RSD of ≤ 0.3% and peak area RSD ≤ 1.2% using a wavelength of 214 nm.
3. Confirm the presence of 21 ± 4 peaks, and the absence of any significant artifacts.

Part II: Reduction and Alkylation (Fetuin and Ovalbumin)

1. Weigh 5 mg of each protein (ovalbumin, and fetuin as a control, in triplicate) in a microcentrifuge tube and add 1 mL of the 7 M guanidine chloride solution. Gently mix to dissolve.
   a. Fetuin controls are used to demonstrate the reproducibility of the trypsin digestion method.
   b. A reagent control (without protein) is also prepared by adding 1 mL of 7 M guanidine chloride to a vial. The guanidine unfolds the protein by destabilizing both intra- and interchain electrostatic attractions.
2. Add 10 µL of 1 M DTT to each vial, followed by gentle mixing. Incubate for 60 min at 60 °C. Mix gently every 5 min. DTT reduces the protein's disulfide bonds.
3. Cool to room temperature.
4. Add 20 µL of 1 M iodoacetamide to the mixture. Gently mix and incubate in the dark for 30 min at room temperature. Iodoacetamide alkylates the cysteine and histidine residues and prevents proteins from assuming their native conformation, facilitating proteolytic digestion.
5. Add another 40 µL of 1 M DTT to each solution. Gently mix and incubate for 30 min at room temperature.
6. Dialyze (10 KD MW cutoff) each sample for 24 h at room temperature in 2 L of 50 mM ammonium bicarbonate while stirring with a magnetic stirrer. The dialysis exchanges the reduction, and alkylation buffer with ammonium bicarbonate is used for trypsin digestion in part III below.
7. After dialysis, a precipitate may form. Carefully measure the entire volume of the preparation, (including the precipitate) and transfer to a clean vial. Repeat the process for the reagent control. Calculate protein concentration based on the dialysis volume. If the protein concentration is unknown or questionable due to precipitation, determine the concentration using a Pierce micro BCA protein assay kit.[5]

Part III: Trypsin digest

Unmodified trypsin autoproteolyzes, generating fragments that can interfere with peptide mapping. The sequencing grade-modified trypsin, recommended for this method is altered by reductive methylation, making it resistant to proteolytic digestion.

1. The trypsin digest requires the following: reduced and alkylated protein, two vials of trypsin (20 µg/vial) and 100 mM ammonium bicarbonate (provided with the trypsin kit).
2. Prepare the trypsin by adding 25 µL of 100 mM ammonium bicarbonate to a lyophilized trypsin vial. Pipette 0.5–2 mg of each reduced and alkylated protein to a trypsin vial. Gently mix and transfer the contents to the second vial of trypsin. Digest fetuin in triplicate to evaluate reproducibility of the trypsin digestion method.

3. Based on the volume of protein solution added to the vial of trypsin, pipette an equivalent volume of the reagent control (from the reduction and alkylation) into a vial of trypsin. Transfer the contents of the first tube into a second vial of trypsin to prepare the reagent control.
4. Incubate the protein mixtures and the reagent control in a 37 °C water bath for 20 h. Freeze to stop the reaction.
5. Remove ammonium bicarbonate from the sample by placing frozen samples into a SpeedVac evaporator system. Add 100 µL of water to dissolve the dried solid.

Part IV: Evaluating the tryptic digestion using the fetuin tryptic digest control samples

1. Analyze three 10 µL injections of each fetuin tryptic digest control and the reagent control to evaluate the tryptic digestion.
2. Confirm the presence of a consistent peak count for all the three fetuin digests with no significant artifacts. Artifacts include extra peaks due to nonalkylated or nondigested protein or incompletely digested protein, nonspecific restriction peaks, and interfering contaminants from the digestion components.
3. Confirm a retention time RSD of ≤ 0.3% and a peak area RSD of ≤ 4.0% for replicate digests.

Part V: Alkaline phosphatase treatment

1. Alkaline phosphatase-treated tryptic digest: Pipette 96 µg of trypsin-digested ovalbumin or other peptide mixture to a vial containing 120 µL of 50 mM tris buffer (pH 9) and 107.6 µL of alkaline phosphatase (19 units). Add DI water to a total volume of 300 µL.
2. Untreated tryptic digest: Pipette 96 µg of trypsin-digested protein to a vial containing 227.6 µL of 50 mM tris buffer (pH 9). Add DI water to a total volume of 300 µL.
3. Reagent control with alkaline phosphatase: Pipette a volume of reagent control (equal to that used for the tryptic digest in steps 1 and 2) into a vial containing 120 µL of 50 mM tris buffer (pH 9.0) and 107.6 µL of alkaline phosphatase (19 units). Add DI water to a volume of 300 µL.
4. Reagent control without alkaline phosphatase: Pipette the same volume of reagent control used in step 3 into a vial containing 227.6 µL of 50 mM tris buffer (pH 9.0). Add DI water to make a volume of 300 µL.
5. Incubate the mixtures at 37 °C for 5 h. Freeze to stop the reaction. Store the mixtures at –20 °C for up to 6 months.

RESULTS AND DISCUSSION

Trypsin is an endopeptidase that specifically cleaves peptide bonds on the carboxyl side of lysine and arginine residues in proteins.[4] Peptides derived from the trypsin digestion of ovalbumin were separated by reversed-phase HPLC. High retention time repeatability is a requirement for peptide mapping. The predigested cytochrome C from Dionex was used to qualify the HPLC system. Figure 1 shows an overlay of four injections of the cytochrome C tryptic digest. Based on published amino acid sequence information, the tryptic digest of cytochrome C should result in 21 peptides that range in

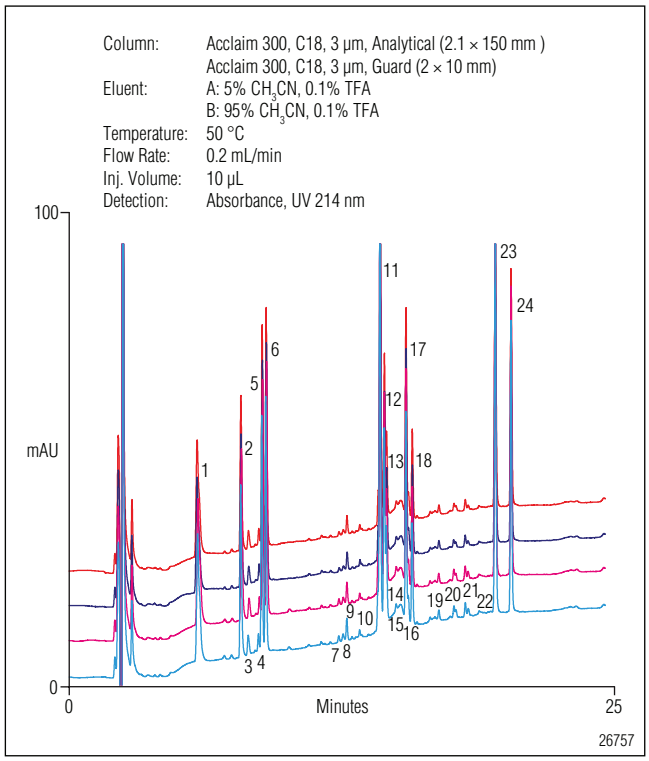

Figure 1. Overlay of four injections of the cytochrome C tryptic digest.

size from 1–37 amino acid residues. This is however, the theoretical peak count assuming no miscleavages have occured, and that all the shortest peptides retain on the C18 column. The tryptic digest of this protein using our method contained 24 peaks. The peak count for the predigested cytochrome C surpassed the theoretical number of tryptic peptides. This discrepancy in the peak counts can result from peak threshold values set too low during peak integration. The presence of small amounts of contaminating proteins in the protein preparation, miscleavages, and contaminants in test-tubes and other labware can also increase peak counts. Commercially available trypsin can have small amounts of contaminating proteases that cause nonspecific digestion of proteins resulting in extra peaks. Post-translational modifications can also increase peak counts.

Table 1. Retention Time and Peak Area Reproducibilities of Eight Arbitrarily Chosen Peaks for Four Replicate Injections of the Cytochrome C Tryptic Digest Measured at 214 nm

	Retention Time		Peak Area	
Peak #	**Mean (min)**	**RSD**	**Mean (mAU/min)**	**RSD**
1	5.99	0.30	5.750	0.22
5	8.36	0.28	0.430	0.6
11	12.90	0.02	0.350	0.45
14	14.70	0.04	2.250	1.1
18	15.70	0.01	3.160	1.08
21	17.20	0.04	0.170	0.69
25	19.90	0.04	7.290	1.09
26	20.60	0.03	5.460	0.55

*See Figure 1 for peaks analyzed

Table 1 shows the average retention times and RSDs for eight representative early and late eluting peaks from the cytochrome C peptide map. The retention time RSDs for these peaks were less than 0.3, which indicates reproducible gradient delivery. Peak area RSDs were less than 1.2, indicating repeatable sample injections.

The trypsin digestion method was qualified based on results for three separate tryptic digests of fetuin and determining the peak count reproducibility. Figure 2 shows an overlay of three fetuin digests and shows a consistent peak count of 36 peaks for each of the samples. The peak area RSDs for the three digests were less than 3.5, indicating the digestion procedure was reproducible. Based on published amino acid sequence information, the tryptic digest of fetuin should result in 25 peptides. The peak count for fetuin surpasses the theoretical number of tryptic peptides and is attributed to miscleaved peptides and to the fact that fetuin is a glycosylated protein. A glycosylated peptide has a different retention than a nonglycosylated peptide with the same amino acid sequence. The number of sugar residues also affects retention of the peptide, and this results in extra peaks in a tryptic map. A comparison of peptide maps before and after treatment with enzymes such as PNGase F can indicate which peaks are glycopeptides. Reference

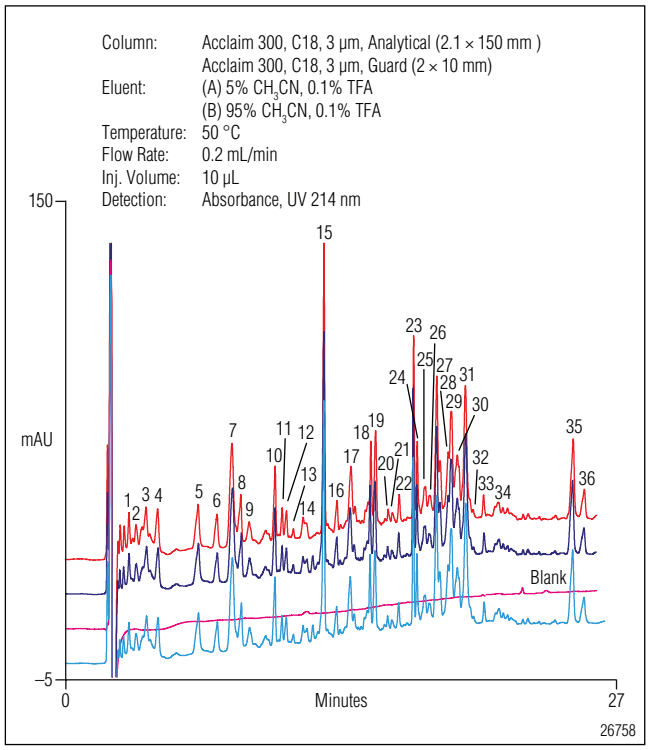

Figure 2. Overlay of three fetuin tryptic digests showing digestion and separation reproducibility.

7 describes the isolation and enzymatic carbohydrate removal of at least nine different glycopeptides peaks present in fetuin. The 36 peaks that we observed in the fetuin peptide map are therefore, well within the expected peak count range for this glycosylated protein.

Table 2 shows the expected 34 peptides and their amino acid sequences for an ovalbumin tryptic digest. These peptides range in length from 2–33 amino acids. Figure 3 shows separation of a tryptic digest of ovalbumin. The 35 peaks found surpass the theoretical number of 34 peaks, but within the range of 34–36 peaks possible when both phosphorylated and dephosphorylated peptide forms are considered.

Figure 4 shows a comparison of an ovalbumin peptide map with and without alkaline phosphatase treatment at 214 nm. The highlighted peaks 11 and 15 are believed to be the phosphorylated peptides EVVGS*AEAGV DAASVSEFFR and LPGFGDS*I EAQCGTSVNV HSSLR. When treated with alkaline phosphatase, the phosphates are removed from peptide peaks 11 and 15 resulting in a net loss of negative charge, therefore eluting later as peaks 11* and 15*. To evaluate the phosphopeptide identification and to suggest the elution order of the two phosphorylated peptides, the two separations were plotted at 260 nm, because both phosphopeptides contain phenylalanine.

Table 2. The Expected Tryptic Peptides of Ovalbumin

Amino-terminal…….	
MGSIGAASME FCFDVFK	DEDTQAMPFR
ELK	VTEQESKPVQ MMYQIGLFR
VHHANENIFY CPIAIMSALA MVYLGAK	V ASMASEK
DST R	MK
TQINK	I LELPFASGTM SMLVLLPDEV SGLEQLESII NF
VVR	LTEWTS SNVMEER
F DK	K**
LPGFGD S*I EAQCGTSVNV HSSLR	IK**
DILNQ ITKPNDVYSF SLASR	VYLPR
LYAEE R	MK
YPILPEYLQ CVK	MEE K
ELYR	YNLTSVLMA MGITDVFSSS ANLSGISSAE SLK
GGL EPINFQTAAD QAR	ISQAVHA AHAEINEAGR
ELINSWV ESQTNGIIR	EVVG S*AEAGV DAASVSEFFR
N VLQPSSVDSQ TAMVLVNAIV FK	ADHPFLFCIK
GLWEK	HIATNAVLFF GR
AFK	CVSP
	……Carboxy-terminal

S* Phosphorylated Serine
** This is an unlikely cleavage as trypsin is a poor exopeptidase

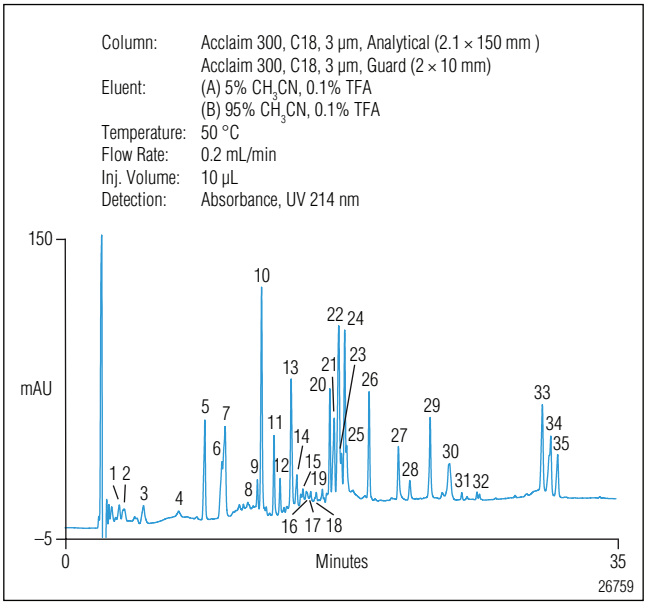

Figure 3. Tryptic map of ovalbumin.

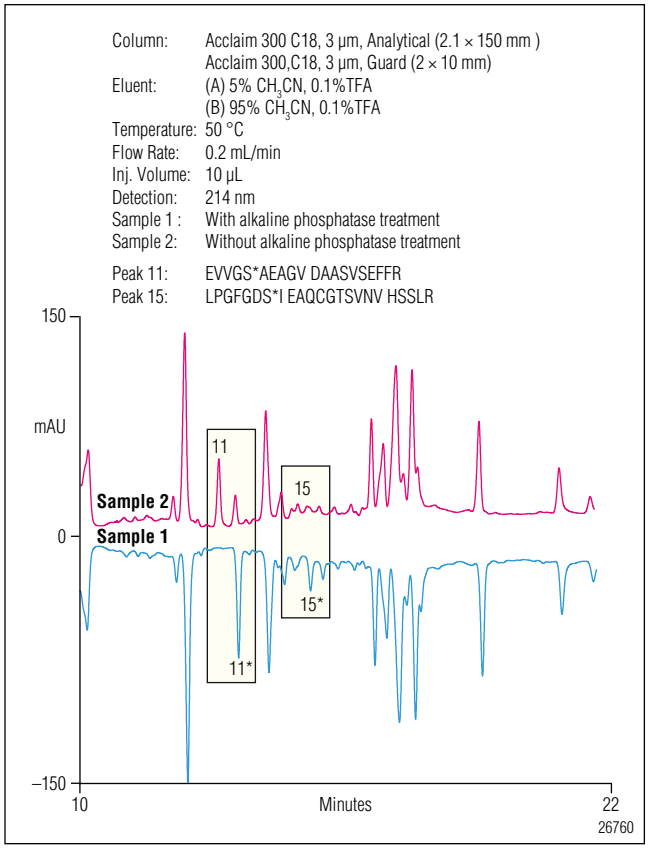

Figure 4. Ovalbumin peptide map with and without alkaline phosphatase digestion measured at 214 nm.

The phosphopeptide EVVGS*AEAGV DAASVSEFFR has two phenylalanine residues and should have a stronger absorbance at 260 nm compared to LPGFGDS*I EAQCGTSVNV HSSLR, that has only one phenylalanine. Figure 5 shows the data to be consistent with our hypothesis that peaks 11 and 15 are phosphorylated, and the absorbance intensities suggest they are EVVGS*AEAGV DAASVSEFFR and LPGFGDS*I EAQCGTSVNV HSSLR, respectively. The calculated average hydrophobicity (cal/mol)[6] for EVVGS*AEAGV DAASVSEFFR was 643 and for LPGFGDS*I EAQCGTSVNV HSSLR was 772 (ignoring the phosphate contribution in both peptides). The calculated hydrophobicity of these two peptides was consistent with their elution order, supporting their identification in the peptide map.

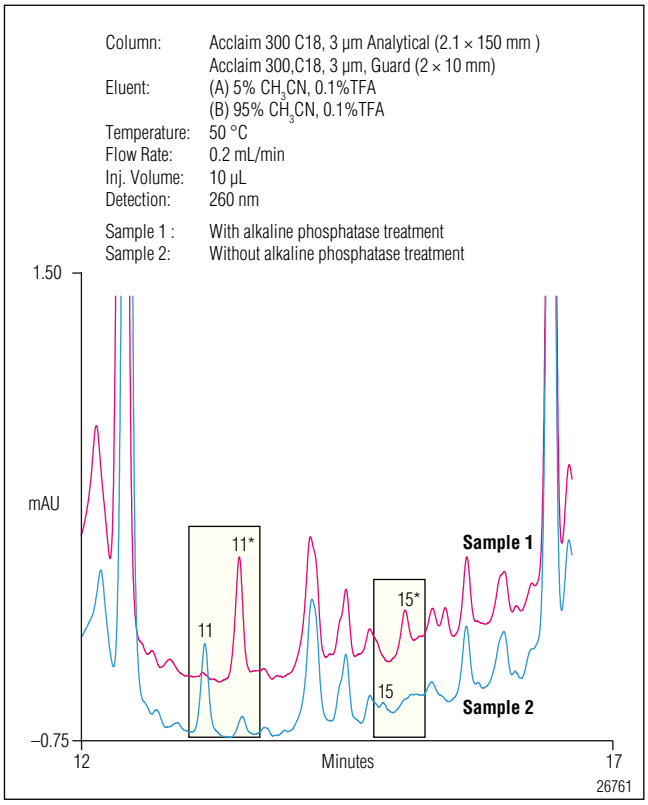

Figure 5. Ovalbumin peptide map with and without alkaline phosphatase digestion measured at 260 nm. The identification of the two phosphopeptide peaks is possible by comparing the relative peak heights at 214 and 260 nm wavelengths.

CONCLUSION

This application note demonstrates a quick and simple method to perform peptide mapping using the Acclaim 300 C18 reversed-phase column designed for high-resolution separation of proteins and peptides. This method also demonstrates the capability of the Acclaim 300 column in combination with the UltiMate 3000 chromatography system to separate and identify phosphopeptides in a peptide map.

PRECAUTIONS

Avoid contact and inhalation with any of the materials used. Material safety data sheets (MSDS) for these materials should be reviewed prior to handling, use, and disposal.

LIST OF SUPPLIERS

Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103, Tel: 800-521-8956, www.sigmaaldrich.com

Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, Tel: 608-274-4330, www.promega.com

Thermo Fisher Scientific (Pierce Biotechnology, Nalgene, Mallinckrodt Baker, J.T. Baker, and Savant Instruments), 308 Ridgefield Court, Asheville, North Carolina 28806-2210, Tel: 866-984-3766, www.thermo.com

Sarstedt Inc., 1025, St. James Church Road, P.O. Box 468, Newton NC 28658-0468, Tel: 828-465-4000, www.sarstedt.com

Honeywell International Inc., 101 Columbia Road, Morristown, NJ 07962, Tel: 973-455-2000, www51.honeywell.com

MP Biomedicals LLC, 29525 Fountain Pkwy, Solon, Ohio 44139, Tel: 800-854-0530, www.mpbio.com

Praxair Specialty Gases and Equipment, 39 Old Ridgebury Road, Dansbury, CT 06810-5113, Tel: 877-772-9247, www.praxair.com.

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Thermo SCIENTIFIC

Application Update 183

Separation of Peptides from Enzymatic Digestion on Different Acclaim Columns: A Comparative Study

INTRODUCTION

Separation of peptides—which can originate from protein enzymatic digestion or peptide synthesis—is of interest in modern biological research. High-resolution and high-peak capacity are the primary concerns in peptide mapping and general proteomics. In purification of synthetic peptides or separation of peptides with post-translational modifications (PTM), however, both column selectivity and efficiency are needed to discriminate minor structural differences.

While possible protein conformation changes in organic solvents (i.e., denaturation) limit reversed-phase chromatography for most protein separations,[1] this concern does not apply to peptides, making reversed-phase chromatography the first choice for peptide separations. To achieve better peptide separations, more information is needed on selectivity and resolution of different reversed-phase columns and how the separation will be affected by separation parameters such as pH, ionic strength, etc.

A protein digest—a mixture of peptides that differ in hydrophobicity, charge, and size—provides a good test sample to understand a column's separation character (i.e., selectivity). In proteomics applications, a reversed-phase-based peptides separation provides a necessary preliminary step before mass spectrometry (MS), especially for complicated protein mixture digests.[2]

Thermo Scientific Acclaim™ 300 C18 columns are designed to provide excellent resolution and capacity for peptide mapping,[3] synthetic peptide separations,[4] and small protein separations. The Acclaim RSLC 120 C18 columns are designed for fast separation without sacrifice in resolution. The Acclaim RSLC PolarAdvantage II (PA2) columns feature amide-embedded chemistry with selectivity complementary to RSLC C18 columns, especially for polar peptides. This specially designed column can work with 100% aqueous mobile phase and has a wide pH stability range from 1.5–10. Both features can be useful for separating peptides.

In the work shown here, a tryptic digest of myoglobin was separated on these three Acclaim columns. Resolution and selectivity of the three columns were compared and major peaks in the myoglobin digest were identified using MS data. The effect of pH on peptide retention time was evaluated with respect to the peptides' characteristics (e.g., hydrophobicity, charge). The Acclaim PA2 column's two prominent features—compatibility with 100% aqueous and high pH mobile phases—were demonstrated to be useful for separating peptides. All separations were performed on the Thermo Scientific Dionex UltiMate™ 3000 RSLC system with diode-array detection (DAD).

EQUIPMENT

UltiMate 3400 RSLC system including:

- LPG 3400RS Pump
- WPS 3000TRS Autosampler
- TCC-3000 Thermostatted Column Compartment
- DAD-3000RS Diode Array Detector

Thermo Scientific MSQ™ Plus Single Quadrupole Mass Detector with an electrospray ionization (ESI) source

Thermo Scientific Dionex Chromeleon™ 6.80, SR9 Chromatography Workstation

REAGENTS AND STANDARDS

Deionized (DI) water, Gradient A 10, Milli-Q®

Acetonitrile (CH_3CN) HPLC grade, (Cat.# AC6100-0040) Fisher Chemical

Formic acid, 98%, Fluka

Ammonium formate, 99%, Acros Organics

Myoglobin from equine heart, ≥ 90% (Sigma P/N M1882)

Trypsin from bovine pancreas, TPCK Treated (P/N T1426)

DL-Dithiothreitol, ≥ 99% (Fluka P/N 43819)

Iodoacetamide, ≥ 99% (Sigma P/N I6125)

CHROMATOGRAPHIC CONDITIONS

Analytical Columns:	Acclaim RSLC 120, C18, 2.2 µm, 2.1 × 100 mm (P/N 068982) Acclaim 300, C18, 3 µm, 2.1 × 150 mm (P/N 060264 Acclaim RSLC PolarAdvantage II (PA2), 2.2 µm, 2.1 × 100 mm (P/N 068990)
Mobile Phase:	(A) 2% acetonitrile in water (B) 2% water in acetonitrile (C) 10 mM formic acid (D) 10 mM ammonium formate
Flow Rate:	0.42 or 0.2 mL/min
Inj. Volume:	2 µL
Column Temp.:	30 °C
UV Detection:	Absorbance at 214 nm

Gradient for Figure 1 at pH 2.9: (Flow Rate as 0.2 mL/min)

Time (min)	A%	B%	C%	D%
0	78	2	19	1
55	30	50	19	1

Gradient for Figures 2, 3 at pH 2.9: (Flow Rate as 0.42 mL/min)

Time (min)	A%	B%	C%	D%
0	78	2	19	1
22	40	40	19	1
28	0	80	19	1

For gradients at other pH values in Figures 1–3, simply change the ratio C/D to 10/10 and 1/19. Mobile phase and gradient methods for Figures 4 and 5 were similar to those in Figures 2 and 3 with slight modifications as indicated in the figures.

MSQ-PLUS MASS DETECTOR CONDITIONS

Ionization Mode:	ESI
Operating Mode:	Positive Scan
Probe Temperature:	400 °C
Needle Voltage:	3.5 kV
Mass Range:	400~2000 amu
Scan Time:	0.5 sec
Cone Voltage:	50 V
Nebulizer Gas:	Nitrogen at 75 psi

PREPARATION OF SOLUTIONS

Ammonium Bicarbonate, 50 mM

Dissolve 0.395 g of ammonium bicarbonate in 90 mL DI water and bring the volume to 100 mL with DI water.

Urea Solution, 8 M

Dissolve 0.48 g of urea in 0.6 mL ammonium bicarbonate solution and bring the volume to 1 mL with ammonium bicarbonate solution.

Dithiothreitol (DTT) Solution, 0.5 M

Dissolve 7.7 mg DTT in 100 µL ammonium bicarbonate solution. Prepare this solution immediately before use.

Iodoacetamide Solution, 0.5 M

Dissolve 18.5 mg iodoacetamide in 100 µL ammonium bicarbonate solution. Prepare this solution immediately before use.

PROTEIN DIGESTION PROCEDURE

Reduction and Alkylation

1. Weigh 5 mg of myoglobin in a microcentrifuge tube and add 1 mL 8 M urea solution. Gently mix to dissolve.
2. Add 20 µL of 0.5 M DTT to the microcentrifuge tube, followed by gentle mixing. Incubate for 60 min at 56 °C.
3. Cool the sample to room temperature.
4. Add 40 µL of 0.5 M iodoacetamide to the mixture. Gently mix and incubate in the dark for 30 min at room temperature.
5. Add another 80 µL of 0.5 M DTT to the mixture. Gently mix and incubate for 30 min at room temperature.

Dialysis of Myoglobin Sample

Dialyze (3.5 kD molecular weight [MW] cut-off) the reduced and alkylated myoglobin sample against 2 L of 50 mM ammonium formate for 24 h at room temperature. Change the dialysis buffer once during the 24 h period.

Trypsin Digest

Dissolve 2 mg trypsin in 200 µL ammonium bicarbonate solution. Add 20 µL trypsin solution (1 µg/µL) to the protein mixture. Gently mix and incubate for 20 h at 37 °C. Boil the mixture for 15 min to stop the reaction.

RESULTS AND DISCUSSION

Identified Peptides with Predicted MW

Table 1 shows the expected 18 myoglobin peptides, with no miscleavage, after tryptic digestion; among them are the 14 peptides that were identified by MS. Note: Peptide fragmentation by tandem mass spectrometry is commonly used to identify peptides in peptide mapping experiments. Table 2 shows identified peptides in Figures 1–3.

The MSQ Plus Mass Detector does not have tandem MS capability; therefore, the authors used the intact peptides' mass comparison with experimental tryptic digestion data for identification. For this study with the focus on LC separation and only a single protein tryptic digest (myoglobin), this approach can be used with confidence.

Table 1. Expected and Detected Myoglobin Tryptic Peptides

No	Range	Mn	z	MC#	Sequence
1	[1–16]	1946.9	1	0	GLSDGEWQQVLNVWGK
2	[17–31]	1606.8	1	0	**VEADIAGHGQEVLIR**
3	[32–42]	1271.7	1	0	**LFTGHPETLEK**
4	[43–45]	409.2	1	0	**FDK**
5	[46–47]	294.2	1	0	**FK**
6	[48–50]	397.3	1	0	**HLK**
7	[51–56]	708.3	1	0	**TEAEMK**
8	[57–62]	662.3	1	0	ASEDLK
9	[63–63]	147.1	1	0	K
10	[64–77]	1378.8	1	0	**HGTVVLTALGGILK**
11	[78–78]	147.1	1	0	K
12	[79–79]	147.1	1	0	K
13	[80–96]	1854.0	1	0	GHHEAELKPLAQSHATK
14	[97–98]	284.2	1	0	**HK**
15	[99–102]	470.3	1	0	**IPIK**
16	[103–118]	1885.0	1	0	**YLEFISDAIIHVLHSK**
17	[119–133]	1502.7	1	0	**HPGDFGADAQGAMTK**
18	[134–139]	748.4	1	0	**ALELFR**
19	[140–145]	631.3	1	0	**NDIAAK**
20	[146–147]	310.2	1	0	**YK**
21	[148–153]	650.3	1	0	**ELGFQG**

Sequences with no miscleavage, noted in bold, were observed in the experiment. MC# stands for miscleavage number. Sequences (FDKFK, ASEDLKK, KK, HKIPIK, and KGHHEAELKPLAQSHATK) with a miscleavage were observed as well.

In addition to the correctly cleaved peptides, four peptides with one miscleavage were also observed by MS. Multiple properties affect a tryptic peptide's detectability by MS, as 50–90% sequence coverage has been reported in the literature.[5] Together with the coverage of the peptides with a miscleavage, approximately 90% coverage was achieved in this experiment.

Table 2. Identified Peptides in Figures 1–3				
Peak No.	**Expected *m/z***	**Observed *m/z***	**Range**	**Sequence**
2 (single charged)	470.3	470.3	99–102	IPIK
3 (single charged)	650.3	650.2	148–153	ELGFQG
5 (single, double charged)	684.4/342.7	684.4/342.7	43–47	FDKFK
7 (double charged)	1502.7/751.8	751.8	119–133	HPGDFGADAQGAMTK
9 (double charged)	735.5/368.2	368.2	97–102	HKIPIK
12 (double, triple charged)	1271.7/636.6/424.5	636.6/424.5	32–42	LFTGHPETLEK
13 (double, triple charged)	1606.8/803.9/536.3	803.9/536.3	17–31	VEADIAGHGQEVLIR
16 (single charged)	748.4	747.8	134–139	ALELFR
17 (triple charged)	1982.0/661.3	661.2	79–96	KGHHEAELKPLAQSHATK

Comparison of the Separation of Myoglobin Tryptic Peptides on Three Acclaim Columns at Different pH Values

As shown in Figure 1, the Acclaim 300 C18 column delivers a good separation of myoglobin tryptic peptides. When mobile phase pH changes from 5.6 to 2.9, peak width is slightly broadened, but the more acidic condition produces a better separation. For example, peaks 6, 8, and 3 are separated at pH 2.9 and they are not baseline resolved at pH 5.6. Peaks 15 and 16 are eluted as one peak at pH 5.6 and well separated at pH 2.9. Better separations and more sensitive MS signal at pH 2.9 confirm that acidic pHs (pH < 3) are preferred for reversed-phase high-performance liquid chromatography (RP-HPLC) peptide mapping.

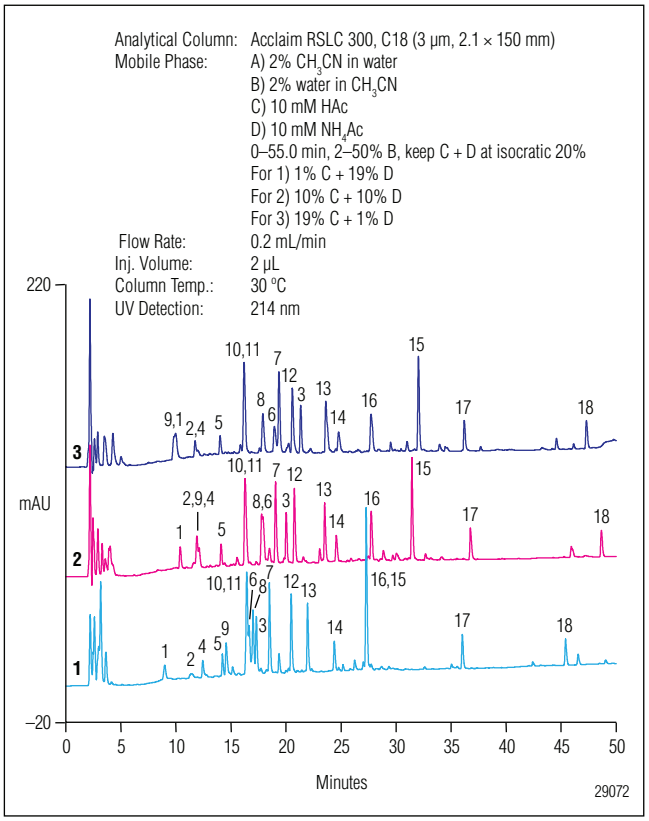

Figure 1. Peptide mapping of myoglobin tryptic digest on the Acclaim 300 C18 column at pH 5.6 (1), pH 3.7 (2), and pH 2.9 (3).

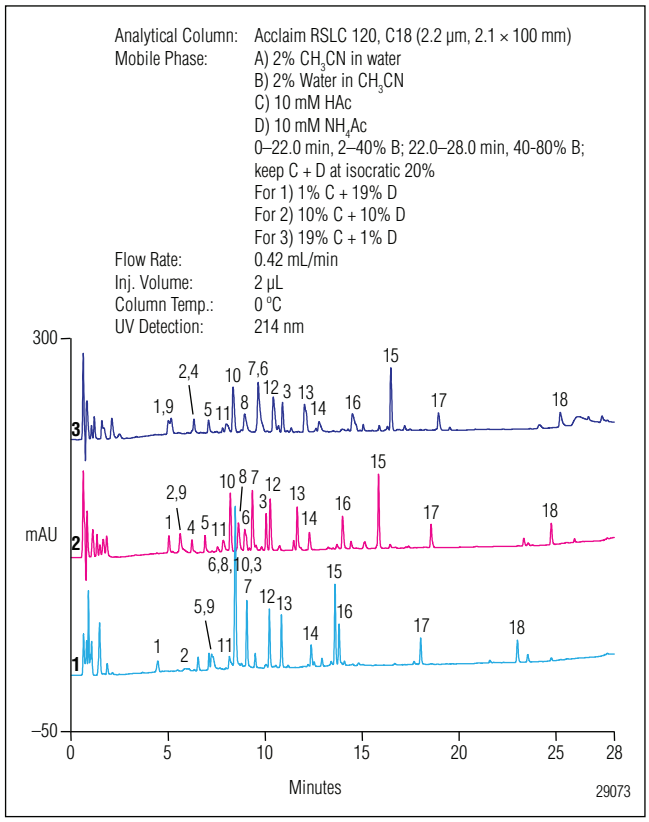

Figure 2 Peptide mapping of a myoglobin tryptic digest on the Acclaim RSLC C18 column at pH 5.6(1), pH 3.7 (2) and pH 2.9 (3).

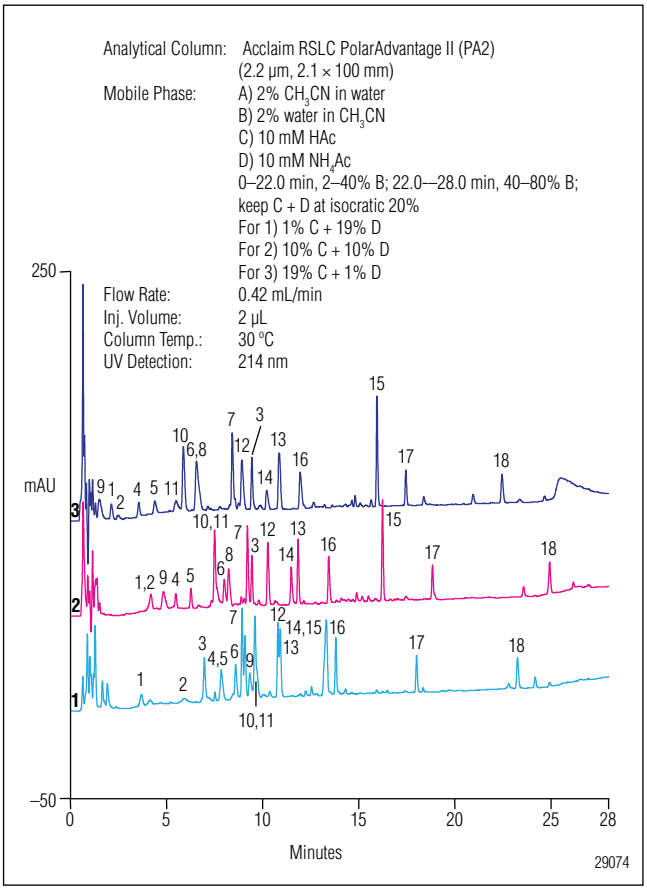

Figure 3. Peptide mapping of myoglobin on the Acclaim RSLC PA2 column at pH 5.6 (1), pH 3.7 (2) and pH 2.9 (3).

This does not appear to be the case with the Acclaim 120 C18 column. As Figure 2 shows, the Acclaim RSLC C18 column achieves very good resolution of the myoglobin peptides, especially at pH 3.7. Almost all peaks are baseline resolved at pH 3.7. At pH 2.9, three pairs of peaks (peaks 1 and 9, peaks 2 and 4, and peaks 7 and 6) coelute. At pH 5.6, peaks 6, 8, 10, and 3 coelute.

These data show that the pH of the mobile phase is important to retention time of peptides, and simply changing pH can be a good solution to resolve peaks that coelute under other conditions. Figure 3 shows the same pH experiment using the third column in this study, the Acclaim PA2 column in RSLC format. The best separations were achieved at pH 2.9 and 3.7, while pH 5.6 was not suitable for separation.

Effects of pH on Myoglobin Tryptic Peptides Elution Order

Most tryptic peptides maintain the same elution order on the Acclaim 300 C18 column with change in pH. Peaks 9 and 3 are exceptions. Peak 9 elutes earlier with the most acidic mobile phase. This peptide is identified as HKIPIK with pI approximately 10.6. Considering that the pKa of the histidine residue in the sequence is about 6.0, changing mobile phase pH from above 5.6 to below 2.9 adds one more positively charged residue to the peptide, thus reducing its hydrophobicity. This can explain the earlier elution of peak 9 at lower pH. This reduced affinity for the stationary phase is also observed with the Acclaim 120 C18 and PA2 columns. The most dramatic retention time change of peak 9 is observed on the Acclaim PA2 column. In contrast, peak 3 elutes later in the most acidic mobile phase. Peak 3 is identified as ELGFQG with a pI of 3.3. When mobile phase pH moves from near 5.6 to 3.3, the net charge of the peptide changes to near 0 from –1. The loss of charge increases the peptide's hydrophobicity and thus its retention.

Comparison of Myoglobin Tryptic Peptides Elution Order on Different Columns

The peptides eluted in very similar order on the Acclaim 300 C18 and 120 C18 columns, regardless of mobile phase pH. In contrast, there was a considerable difference in the peptide elution order at pH 5.6 when comparing the Acclaim C18 columns and the Acclaim PA2 column. This shows that the Acclaim PA2 column's embedded amide group provides a complementary selectivity to the Acclaim C18 column. As mobile phase acidifies, less difference was observed in the peptides' elution order between the Acclaim PA2 and C18 columns.

Advantages of the Acclaim PA2 Column: Compatibility with 100% Aqueous Mobile Phase

The stationary phase design of a hydrophilic group between the hydrophobic alkyl chain and silica surface enables the Acclaim PA2 column to remain wetted even in 100% aqueous mobile phase. While the conventional Acclaim C18 column will rapidly lose its selectivity due to dewetting in 100% aqueous mobile phase, the Acclaim PA2 column yields reproducible analyte retention time. A fraction of tryptic digested peptides, especially small peptides, are quite hydrophilic.[6] Even under 98% aqueous mobile phase, these peptides eluted with almost no retention. This results in poor chromatography and MS signal suppression.

As shown in Figure 4, peaks 1–10 eluted within 2 min using 98% aqueous mobile phase, a typical starting condition for peptide mapping. While some of this poor retention may be due to the small pore size of the Acclaim PA2 column, using 100% aqueous mobile phase separates peaks 4–10. In the myoglobin tryptic digest, these separated peptides constitute about 40% of all identified peptides. Additionally, 100% aqueous mobile phase provides some separation of peaks 16 and 17, which are unresolved in 98% aqueous mobile phase.

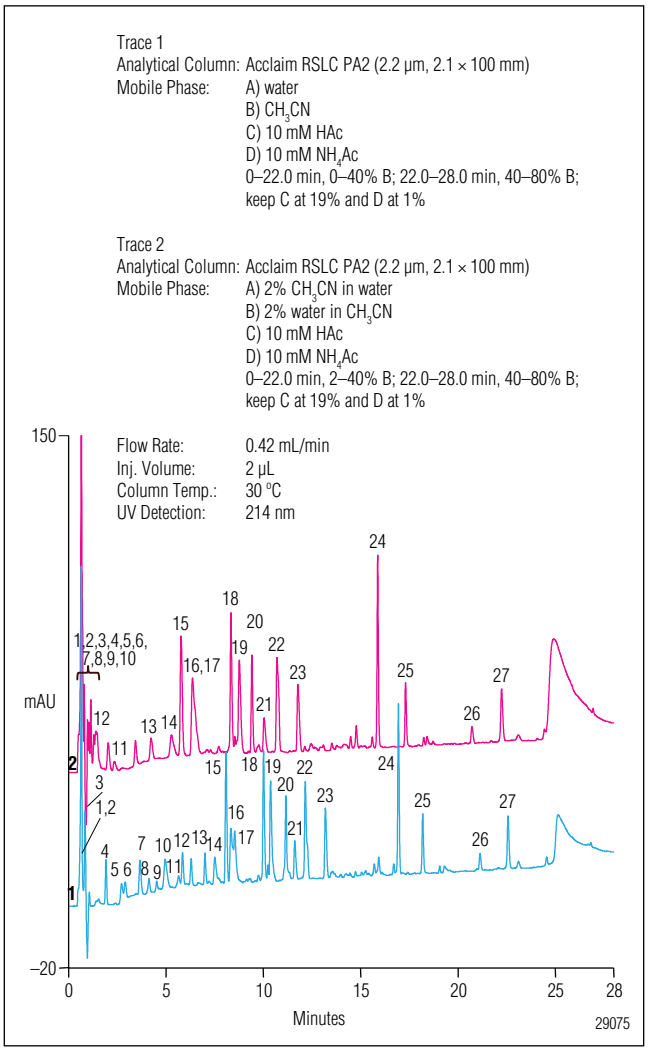

Figure 4. Peptide mapping of myoglobin on the Acclaim RSLC PA2 column with initial condition of mobile phase as (1) 100% water and (2) 2% CH_3CN in water.

Basic Mobile Phase on the Acclaim PA2 Column Provides Dramatically Different Selectivity

Routine peptide mapping involves separation of peptides by reversed-phase chromatography on a silica C18 column using an acidic mobile phase. Low pH usually provides high resolution, but separation with an alkaline mobile phase has been shown to be useful for peptides that are difficult to dissolve in acidic mobile phases and for some peptides that cannot be separated in acidic mobile phase.[7]

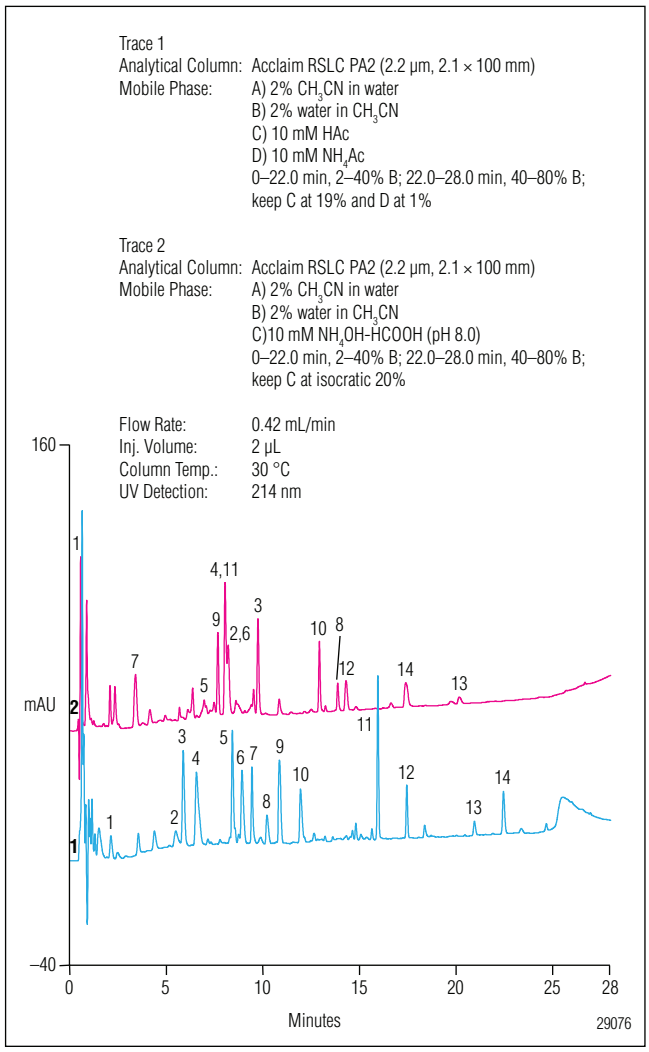

Figure 5. Peptide mapping of myoglobin on the Acclaim RSLC PA2 column at pH (1) 2.9, and (2) 8.0.

Conventional silica and most polar-embedded phases can not tolerate alkaline conditions. The Acclaim PA2 column is specially designed to withstand high pH conditions, and is highly stable in a wide pH range (1.5–10). Figure 5 shows peptide mapping on the Acclaim PA2 column at pHs 8.0 and pH 2.9. The elution order of the peptides is dramatically different. Although low pH separations can provide higher resolution, alkaline separation provides good resolution and complementary selectivity.

Peak 7, identified as peptide ELGFQG, is eluted much earlier at pH 8.0 than at pH 2.9. Due to its pI of 3.3, the peptide is fully charged at pH 8.0, whereas it is partially charged at pH 2.9. This explains the retention difference between the two pHs. Actually, most peaks elute earlier at pH 8.0 than at pH 2.9. This may be attributed to the embedded amide group in the Acclaim PA2 column. The amide will be partially charged under acidic conditions, which will contribute additional retention to polar analytes. The amide will lose its charged state at basic condition, which may decrease its retention of polar analytes.

Peak 10, peptide ALELFR, is an exception because it eluted earlier at pH 2.9 than at pH 8.0. With pI 7.0, peptide ALELFR can be almost noncharged at pH 8.0 and fully charged at pH 2.9. So the peptide is predicted to have longer retention time at pH 8.0.

CONCLUSION

This study showed the effect of pH on the separation of tryptic peptides on the Acclaim 300 C18, RSLC 120 C18, and RSLC PA2 columns. With the help of MS data, peptides were partially identified. Three types of columns achieved highest separation under different pHs. The elution of an individual peptide will be affected by charge status under different pHs. The compatibility with 100% aqueous mobile phase of the Acclaim PA2 column was shown to be advantageous for separating small polar peptides. The Acclaim PA2 column, with its featured hydrolytic stability under basic condition, demonstrated quite different selectivity with a basic mobile phase compared to routine acidic conditions. Both features are useful for peptide separations.

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Poster Note

UHPLC Peptide Mapping of Biopharmaceuticals

ANTIBODY STABILITY ANALYSIS

Peptide mapping is used in various stages of the development and production of biopharmaceutical proteins to ensure product integrity and stability. Stability of monoclonal antibody therapeutics is an important aspect of characterization programs. The most reliable information can only be obtained by long-term monitoring of the stability at the normal storage conditions throughout the shelf-life of a product. However, to obtain information of the most susceptible degradation pathways and the most vulnerable sites of the molecules, stability of molecules can be assessed under extreme conditions, such as elevated temperatures, exposure to chemicals, light, or any combination of these.

Typically, enzymatically-derived peptides are separated by reversed-phase ion-pairing with acidic conditions. Modifications to biopharmaceutical proteins may include truncation, oxidation, reduction, glycosylation, isomerization, deamidation, and clipping.

These changes in the product are revealed by changes in the retention times and by mass-spectrometric detection. In the case of monoclonal antibody analysis, this challenging task is further augmented by the presence of numerous other peptides. Shallow solvent gradients can be applied as a means to increase resolution but the resulting elongated analysis times are disadvantageous.

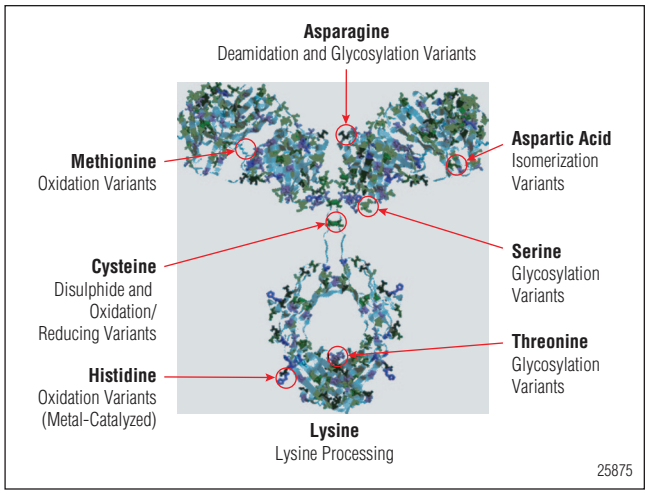

Figure 1. Challenges to characterize and analyze MAb: possible variations in a MAb.

Here we have applied U-HPLC column technology for peptide mapping of biopharmaceutical proteins. The effects of gradient LC conditions and column technology on peak capacity and analysis time were investigated. Parameter validation has been performed for stressed samples that represent relevant molar differences between native and modified peptides.

INSTRUMENTATION AND COLUMNS

HPLC experiments were carried out using an UltiMate® 3000 RSLC (Dionex, Germany) equipped with:

- SRD-3600 Membrane Degasser
- HPG-3400RS High Pressure Gradient Binary Rapid Separation Pump
- TCC-3000RS Thermostatted Column Compartment
- WPS-3000TRS Rapid Separation Well Plate Sampler
- VWD-3400RS Variable Wavelength Detector equipped with a 2.5 µL flow cell

Unless otherwise noted, Dionex Acclaim® C18 RSLC 100 × 2.1 mm I.D. columns were used, packed with particles sizes of 2.2, 3, and 5 µm, 120 Å.

All separations were carried out using acetonitrile as an organic modifier and trifluoroacetic acid as ion pair in a concentration of 0.05% v/v.

INFLUENCE OF PARTICLE SIZE ON TOTAL ANALYSIS TIME AND PEAK CAPACITY

Faster monoclonal antibody peptide mapping can be achieved by reducing the particle size. Reducing the particle size from 5 to 3, to2.2 µm, while maintaining the optimum linear velocity in the column for each particle size, results in increasing peak capacity (nc is inversely proportional to the square root of the plate height), and shorter analysis time (Figure 2). Average peak capacities of 290, 350, and 380 were found for 5, 3, and 2.2 µm particulate columns respectively.

The total analysis time was reduced by reducing the particle size from 5 to 2 µm, while maintaining the same linear velocity.

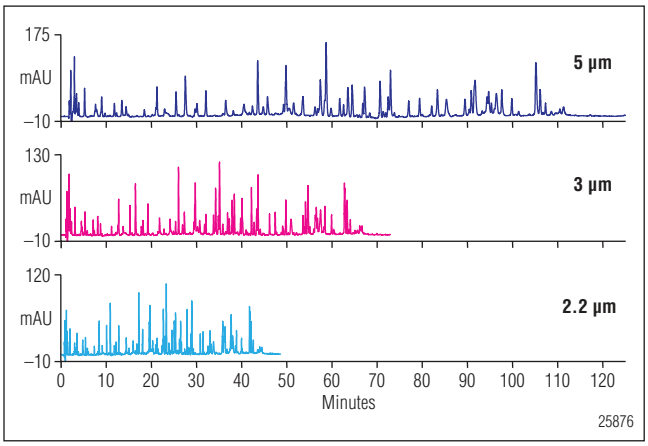

Figure 2. Separation of tryptic digest of monoclonal antibody on Acclaim C18 stationary phases with different particle sizes. Column dimensions: 2.1 × 100 mm.

Maintianing a constant flow rate while increasing the column length causes the total analysis time and the system backpressure to increase proportionally. Combining the effect of a smaller particle size stationary phase (with higher optimum velocity) and an increased column length, a considerable increase in efficiency can be expected. The tryptic digest of a MAb was separated on a 200 mm long column (2 × 100 mm) packed with 2.2 µm C18 particles (Figure 3). The calculated average peak capacity was found to be 570, significantly higher than for the 100 mm columns.

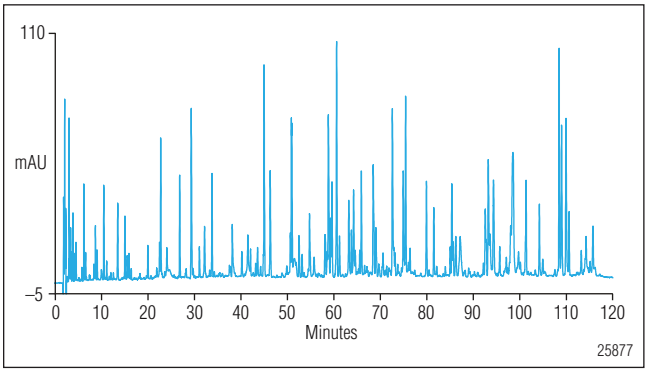

Figure 3. Separation of tryptic digest of monoclonal antibody on Acclaim C18 stationary phases with various particle sizes. Column dimensions: 2.1 × 200 mm and applying a 120 min gradient.

RETENTION TIME PRECISION

For the comparison of different batches of antibody, e.g., stability batches or stressed samples, it is important to have high retention time precision. This allows comparing the chromatograms of normal antibodies with modified antibodies in great detail and also evaluating the peptide mapping data visually. For MS detection, high chromatogram precision yields better comparability of different datasets. An example of six consecutive MAb peptide maps illustrating the high reproducibility of the UltiMate 3000 HPLC platform is shown in Figure 4. The retention time precision for most peptides was below 0.05% RSD.

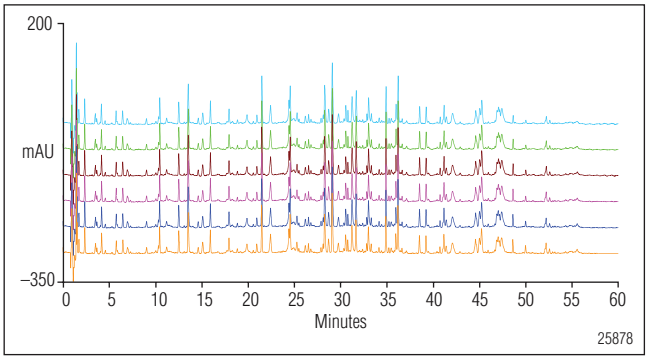

Figure 4. Repetitive peptide maps of a digested monoclonal antibody on Acclaim C18 2.2 µm.

IDENTIFICATION OF PROTEIN MODIFICATIONS

Susceptible degradation sites of therapeutic proteins are routinely assessed under accelerated conditions, such as exposure to chemicals or incubation at elevated temperature or a combination of both. In this study, deamidation of asparagine in the MAb (see Figure 5) was induced by thermally stressing a monoclonal IGg1 antibody protein at pH 7.4 at 37 °C for one week in PBS. (Deamidation protocol was adapted from Huang et al., *Anal. Chem.* **2005,** *77,* 1432–1439). The control antibody and the deamidated antibody were then subjected to tryptic digestion followed by RPLC-MS/MS analysis.

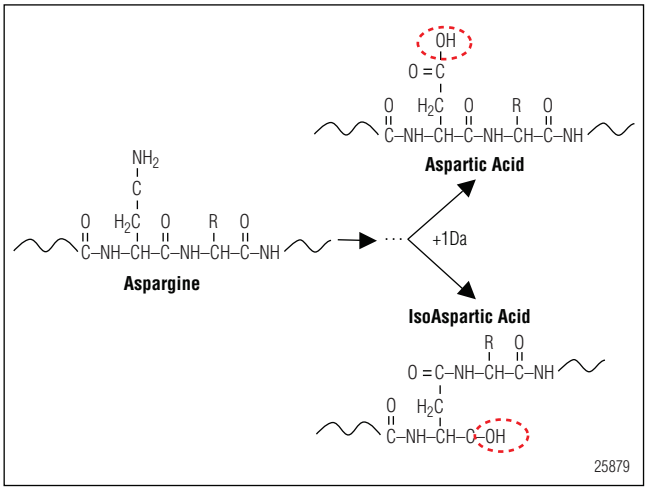

Figure 5. Deamidation of asparagine via a succinimide intermediate is a common degradation.

In this study, several deamidation sites were found, including one for a peptide related to the antibody light chain (SGTASVVCLLNNFYPR). This deamidation was expressed in the MS data by a shift of 1 Da and a retention time shift of 0.2 min at approximately 47 min in the Figure 6.

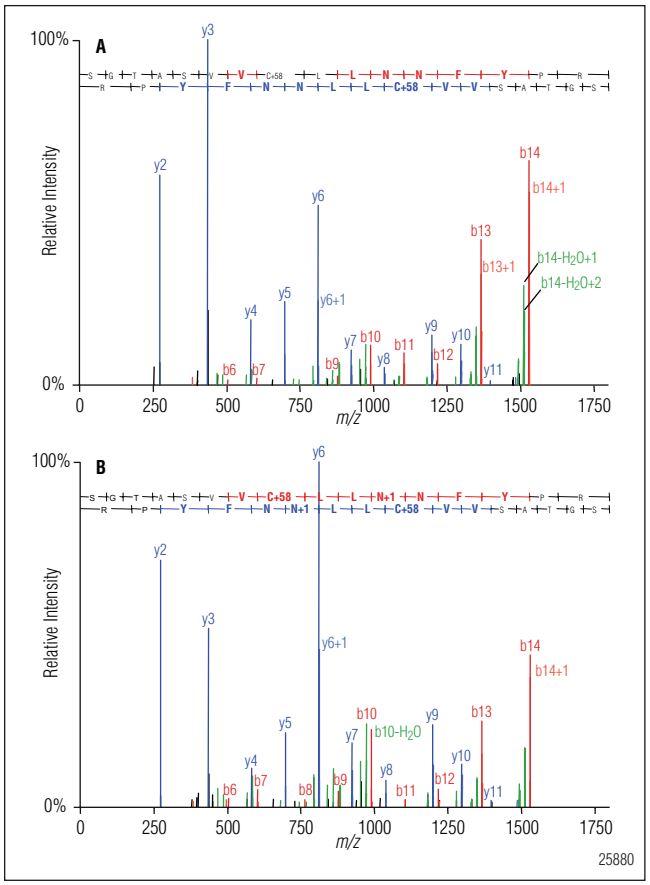

Figure 6. (A) MS/MS spectrum of a tryptic peptide in the antibody digest with sequence SGTASVVCLLNNFYPR (t_R = 47.09 min). (B) Corresponding peptide in the light chain of the stressed antibody (t_R = 46.88 min) featuring deamindation of asparagines and a shift in mass of 1 Da.

UV detection can be applied to quantify these protein modifications. As a result, to the high resolution small modifications can be resolved and eventually quantified. Deamidation was quantified to be 4% in the normal sample and 30% in the stressed antibody (Figure 7).

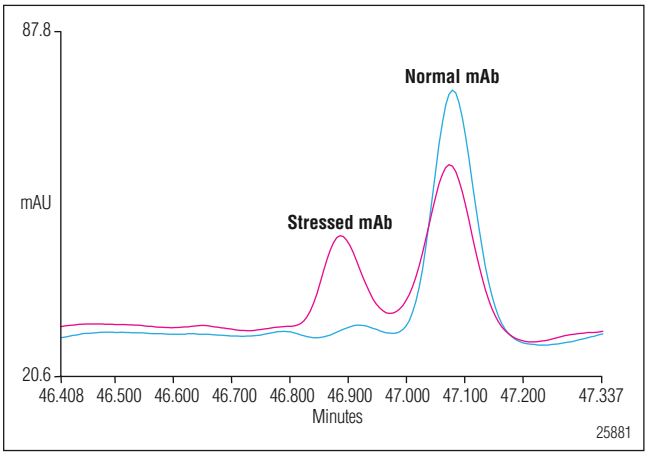

Figure 7. Overlayed chromatograms of the normal and the stressed antibody for one of the deamidated peptides.

CONCLUSIONS

- The Acclaim C18 2.2 µm stationary phase is ideally suited for peptide mapping, e.g. the analysis of biologics.
- High resolution peptide maps with peak capacities up to 570 can be obtained on 200 mm columns by applying shallow gradients.

Product focus:

Systems

Protein therapeutics applications notebook

Thermo SCIENTIFIC

Biocompatible LC Systems

Exceptional results and speed, resolution, versatility with Bio-LC separations

Thermo Fisher Scientific provides innovative and flexible solutions for biopharmaceutical LC analysis. The UltiMate 3000 Biocompatible Systems provide solutions for biochromatographic demands from micro to analytical range. System components are perfectly matched to meet the requirements for high-performance analysis as well as purification. The wide range of solvent options allows easy implementation of different gradient profiles, essential for method development. Additionally, these LC systems provide superior ease of use and are compatible with all Thermo Scientific MS systems, including our hybrid and Orbitrap instruments.

Benefits of our Bio-LC systems include:

- Superior chromatographic performance
- Industry-leading range of biocompatible pumps
- Titanium and PEEK™ flow-path for full biocompatibility
- Dual-gradient pump for true parallel, tandem, or multidimensional chromatography
- High-precision autoinjections from 0.1 to 250 µL (default) with ultralow carryover
- Sample fractionation and reinjection with the WPS-3000TBFC Autosampler with Integrated Fraction Collection and Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) software

The UltiMate 3000 biocompatible system ensures full biocompatibility, critical to integrity of proteins during separation, while delivering high day-to-day reproducibility and robust operation, even under harsh salt and pH conditions.

IC Systems

A complete range of IC solutions developed for flexibility, modularity, and ease of use

Thermo Fisher Scientific offers a premier IC product family that consist of an extensive set of fully inert, PEEK-based modules engineered for high performance, ease of use, and reliability. We offer a wide variety of autosamplers, injectors, pumps, thermostatted column compartments, and detectors with reliable, precise, and accurate operation. Whether you require a dedicated system or modularity for flexible applications, all components are integrated and single-point controlled through Chromeleon™ software or easy-to-use transistor-transistor logic (TTL) control. Additionally, these systems automatically remove mobile phase ions for effort-free transition to MS detection, and are fully compatible will all Thermo Scientific MS instruments.

Benefits of our IC systems include:

- Automated eluent generation for simplicity and reliability
- Single and dual isocratic and gradient pumps
- Autosamplers from basic to state-of-the-art automation and high-volume sample preparation
- Column and detector compartments that integrate thermal control with sample preparation and injection
- High-performance detectors, including conductivity, electrochemical, optical, and MS

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Orbitrap MS Instruments

Single-point control and automation for improved ease of use in LC/MS and IC/MS

Thermo Fisher Scientific provides advanced integrated IC/MS and LC/MS solutions which combine ease of use with modest price and space requirements. UltiMate 3000 System Wellness technology and automatic MS calibration allow continuous operation with minimal maintenance. The Thermo Scientific Dionex ICS instruments and Reagent-Free™ IC (RFIC™) systems automatically remove mobile phase ions for effort-free transition to MS detection. The Orbitrap™ family makes up some of the most powerful MS instruments on the market to date. With resolving powers and MS^n capabilities, these systems offer broad screening possibilities and confidence in identification and quantitation analysis.

Some of the benefits of our Orbitrap MS instruments include:

- High-confidence, high-resolution/accurate mass (HR/AM) intact mass analysis
- Resolving power >240,000 full width at half maximum (FWHM) on our most advanced systems
- Spectral multiplexing for enhanced duty cycle
- Self-cleaning ion sources for low-maintenance operation
- Chromeleon™ software for single-point method configuration, instrument control, and data management
- Compatible with all existing IC and LC methods

Our Orbitrap MS instruments are not only ideal for interfacing with our chromatography systems, they also provide confidence with HR/AM detection to deliver excellent performance and tremendous versatility.

Product focus:

Consumables

Protein therapeutics applications notebook

Thermo SCIENTIFIC

Protein and Peptide Columns

Exceptional separations and resolution with a wide variety of applications

Thermo Fisher Scientific provides a large portfolio of high-resolution, high-efficiency columns for separations of proteins and peptides. This bio-LC column line offers a complete selection of ion-exchange and reversed-phase columns for the analysis and purification of proteins and peptides. Stationary phases include polymer bead and monolith ion-exchange phases, and polymer and silica-based reversed-phase. Thermo Scientific also supports size-exclusion chromatography (SEC), and hydrophobic interaction chromatography (HIC) applications with specialty columns.

Benefits and columns within our bio-LC column line include:

- Thermo Scientific ProPac ion-exchange columns for protein, glycoprotein, and monoclonal antibody analysis
- Thermo Scientific MAbPac columns specifically designed for the high-resolution, high-efficiency analysis of monoclonal antibodies
- Thermo Scientific Acclaim PepMap reversed-phase columns for peptide analysis
- Thermo Scientific ProSwift and PepSwift monolith columns for fast high-resolution separation and purification of proteins and peptides
- Specialty columns for SEC and HIC separations

Within our bio-LC column family, the ProPac™ ion-exchange columns provide exceptionally high resolution and efficiency for separations of protein variants. These columns are capable of resolving isoforms that differ by as little as one charged residue, and are an excellent complement to our MAbPac™ columns, which are specifically designed for the separation of monoclonal antibodies. Applications with these columns include analysis of monoclonal antibodies as well as blood and proteins. Acclaim™ silica-based columns provide both traditional and capillary formats for peptide mapping and high-efficiency protein separations.

Carbohydrate Columns

Reliable, high-resolution separations optimized for various carbohydrate compounds

Thermo Fisher Scientific provides a wide range of carbohydrate separations, with each column optimized for a different class of compound. The Thermo Scientific Dionex CarboPac family of columns provides high-resolution separations of saccharides, and when combined with pulsed amperometric detection (PAD), provide high sensitivity without the need for derivatization. Dionex CarboPac™ columns support simple, reliable techniques to separate sugars. The Dionex CarboPac MA1 column provides high resolution of reduced sugars. The Dionex CarboPac PA10 and PA20 columns provide high-resolution separation of mono- and disacharides. The Dionex CarboPac PA100 and PA200 columns provide high resolution of oligosaccharides for analysis and mapping.

Compound analysis classifications within the Dionex CarboPac column line include:

- Sugar alcohols
- Mono- and disaccharides
- Poly- and oligosaccharides

The Dionex CarboPac family provides high-resolution separations of glycoprotein oligosaccharides and complex carbohydrates from dietary fiber, including fructans, maltodextrins, and amylopectins; monosaccharides, such as those obtained from glycoprotein hydrolysates or plant hydrolysates; and mono- and disaccharides.

Product focus:

Software

Protein therapeutics applications notebook

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Chromeleon 7 Chromatography Data System Software

The fastest way to get from samples to results

Discover Chromeleon software version 7, the chromatography software that streamlines your path from samples to results. Get rich, intelligent functionality and outstanding usability at the same time with Chromeleon software version 7—the Simply Intelligent™ chromatography software.

- Enjoy a modern, intuitive user interface designed around the principle of operational simplicity.
- Streamline laboratory processes and eliminate errors with eWorkflows, which enable anyone to perform a complete analysis perfectly with just a few clicks.
- Access your instruments, data, and eWorkflows instantly in the Chromeleon Console.
- Locate and collate results quickly and easily using powerful built-in database query features.
- Interpret multiple chromatograms at a glance using MiniPlots.
- Find everything you need to view, analyze, and report data in the Chromatography Studio.
- Accelerate analyses and learn more from your data through dynamic, interactive displays
- Deliver customized reports using the built-in Excel-compatible speadsheet.

Chromeleon software version 7 is a forward-looking solution to your long-term chromatography data needs. It is developed using the most modern software tools and technologies, and innovative features will continue to be added for many years to come.

The Cobra™ integration wizard uses an advanced mathematical algorithm to define peaks. This ensures that noise and shifting baselines are no longer a challenge in difficult chromatograms. When peaks are not fully resolved, the SmartPeaks™ integration assistant visually displays integration options. Once a treatment is selected, the appropriate parameters are automatically included in the processing method.

Chromeleon software version 7 ensures data integrity and reliability with a suite of compliance tools. Compliance tools provide sophisticated user management, protected database structures, and a detailed interactive audit trail and versioning system.

Column selection guides

Protein therapeutics applications notebook

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Thermo Scientific Biocolumns Selection Guide

Analyte	Mode of analysis	Recommended column
Proteins	Size Exclusion	BioBasic SEC
		MAbPac SEC-1
	Ion Exchange	BioBasic AX
		ProPac SCX-10, WCX-10, SAX-10, WAX-10
		MAbPac SCX-10
		ProSwift IEX
	Reversed Phase	BioBasic 18, 8, 4
		Acclaim 300 C18
		ProSwift RP
	Hydrophobic Interaction	ProPac HIC-10
	Affinity	ProPac IMAC-10
		ProSwift ConA-1S
Peptides	Proteomics	Acclaim PepMap
		PepSwift
		EASY-Column
		PicoFrit, IntegraFrit
		KAPPA
	Analytical	BioBasic 18, 8, 4
		Acclaim 300
	Preparative	BioBasic
Amino Acids (derivatized)	Ion Exchange	AminoPac PA10
	Reversed phase	Hypersil GOLD
Amino Acids (underivatized)	Ion Exchange	AminoPac PA10
	Reversed phase	Hypercarb
Nucleotides	Anion Exchange	BioBasic AX
	Polar retention	Hypercarb
Oligonucleotides	Ion Exchange	BioBasic AX
		DNAPac PA100, PA200
		DNASwift
Carbohydrates	Ligand Exchange	HyperREZ XP
	Ion Exchange	CarboPac
	HILIC	Acclaim HILIC
		Hypersil GOLD HILIC
		Syncronis HILIC
	Polar retention	Hypercarb

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Thermo Scientific Protein Columns Specifications

Polymeric Ion Exchange, Reversed Phase and Affinity Columns

Column	Phase	Target Applications	Base Matrix Material	Functional Groups	Capacity	Recommended Flow Rate	Solvent Compatibility	Maximum Backpressure	pH Range
MAbPac SCX-10	Strong Cation Exchange	High Resolution separation of monoclonal antibody variants	Highly crosslinked divinlybenzene 10µm nonporus particles	Sulfonic	30µg/mL	0.2-2.0 mL/min	50% acetonitrile	3000psi (21 MPa)	2.0-12
ProPac WCX-10	Weak Cation Exchange	High resolution separations of proteins and protein variants	Ethylvinylbenzene cross linked with 55% divinlybenzene 10µm nonporous particles	Carboxylate	6mg/mL lysozyme	0.2-2.0 mL/min	80% ACN, acetone. Incompatable with alcohols and MeOH	3000psi (21 MPa)	2.0-12
ProPac SCX-10	Strong Cation Exchange	High resolution separations of proteins and protein variants	Ethylvinylbenzene cross linked with 55% divinlybenzene 10µm nonporous particles	Sulfonate	3mg/mL lysozyme	0.2-2.0 mL/min	80% ACN, acetone, MeOH	3000psi (21 MPa)	2.0-12
ProPac SCX-20	Strong Cation Exchange	High Resolution separations of proteins and protein variants	Divinlybenzene 10µm nonporus particles	Sulfonic	30µg/mL	0.2-2.0 mL/min	50% acetonitrile	3000psi (21 MPa)	2.0-12
ProPac WAX-10	Weak Anion Exchange	High resolution separations of proteins and protein variants	Ethylvinylbenzene cross linked with 55% divinlybenzene 10µm nonporus particles	Tertiary amine	5mg/mL BSA	0.2-2.0 mL/min	80% ACN, acetone, MeOH,	3000psi (21 MPa)	2.0-12
ProPac SAX-10	Strong Anion Exchange	High resolution separations of proteins and protein variants	Ethylvinylbenzene cross linked with 55% divinlybenzene 10µm nonporous particles	Quaternary ammonium	15mg/mL BSA	0.2-2.0 mL/min	80% ACN, acetone, MeOH	3000psi (21 MPa)	2.0-12
ProSwift RP-1S	Reversed Phase	Fast protein analysis with high resolution	Monolith; polystyrene-divinylbenzene	Phenyl	5.5mg/mL Insulin	2.0-4 .0 mL/min	Most common organic solvents	2800psi (19.2 Mpa)	1.0-14
ProSwift RP-2H	Reversed Phase	Fast protein analysis with high resolution	Monolith; polystyrene-divinylbenzene	Phenyl	1.0mg/mL Lysozyme	1.0-10 mL/min	Most common organic solvents	2800psi (19.3 Mpa)	1.0-14
ProSwift RP-3U	Reversed Phase	Fast protein analysis with high resolution	Monolith; polystyrene-divinylbenzene	Phenyl	0.5mg/mL Lysozyme	1.0-16 mL/min	Most common organic solvents	2800psi (19.3 Mpa)	1.0-14
ProSwift RP-4H	Reversed Phase	Fast protein analysis with high resolution	Monolith; polystyrene-divinylbenzene	Phenyl	2.3mg/mL Lysozyme	0.1-0.3 mL/min	Most common organic Solvents	1500psi	1.0-14
ProSwift SAX-1S	Strong Anion Exchange	Fast protein analysis with high resolution	Monolith; polymethacrylate	Quaternary amine	18mg/mL BSA	0.5-1.5 (4.6mm)	Most common organic solvents	1000psi (4.6mm) 2000psi (1.0mm)	2.0-12
ProSwift SCX-1S	Strong Cation Exchange	Fast protein analysis with high resolution	Monolith; polymethacrylate	Sulfonic acid	30mg/mL Lysozyme	0.5-1.5 mL/min (4.6mm)	Most common organic solvents	1000psi (4.6mm)	2.0-12
ProSwift WAX-1S	Weak Anion Exchange	Fast protein analysis with high resolution	Monolith; polymethacrylate	Tertiary amine (DEAE)	18mg/mL BSA	0.5-1.5 mL/min (4.6mm)	Most common organic solvents	1000psi (4.6mm) 2000psi (1.0mm)	2.0-12
ProSwift WCX-1S	Weak Cation Exchange	Fast protein analysis with high resolution	Monolith; polymethacrylate	Carboxylic acid	23mg/mL Lysozyme	0.5-1.5mL/min (4.6mm), 0.05-0.20	Most common organic solvents	1000psi (4.6mm) 2000psi (1.0mm)	2.0-12
ProPac IMAC-10	Immobilized Metal Affinity	High resolution separation of certain metal-binding proteins and peptides	Polystyrene divinylbenzene 10µm nonporus particles	Poly (IDA) grafts	>60mg lysozyme/ mL gel (4x250mm)	1.0mL/min	EtOH, urea, NaCl, non-ionic detergents, glycerol, acetic acid, guanidine HCl	3000psi (21MPa)	2.0-12
ProSwift ConA-1S	Affinity	Concanavalin A binding glycans, glycopeptides and proteins	Monolith; polymethacrylate	Concanavalin A ligands	12-16mg of protein	0-1.0mL/min	Up to 10% methanol	2000psi	5.0-8

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Protein therapeutics applications notebook

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Select Peer-Reviewed Publications

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