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Sample Preparation for the Analysis of Complex Carbohydrates by Multicapillary Gel Electrophoresis with Light-Emitting Diode Induced Fluorescence Detection

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This paper evaluates various sample preparation methods for multicapillary gel electrophoresis based glycan analysis to support electrokinetic injection. First the removal of excess derivatization reagent is discussed. Although the Sephadex G10 filled multiscreen 96-well filter plate and Sephadex G10 filled pipet tips enabled increased analysis sensitivity, polyamide DPA-6S pipet tips worked particularly well. In this latter case an automated liquid handling system was used to increase purification throughput, necessary to feed the multicapillary electrophoresis unit. Problems associated with the high glucose content of such biological samples as normal human plasma were solved by applying ultrafiltration. Finally, a volatile buffer system was developed for exoglycosidase-based carbohydrate analysis.

There is a recently growing interest to understand the important multilateral biological functions of the carbohydrate moieties of glycoproteins. Of particular interest is the understanding of the role of glycoprotein microheterogeneity in biological recognition, receptor−ligand, or cell−cell interactions, in the modulation of immunogenicity and protein folding, as well as in bioactivity regulation.[1] Biopharmaceutical interest of recombinant glycoproteins demands high resolution and reproducible analysis methods of the glycosylation moieties. In the biotechnology industry, slight differences in the fermentation process of recombinant glycoproteins can lead to major glycosylation variations, possibly affecting the pharmacological outcome of the products.[2] Investigation of the human plasma glycoproteome, especially understanding glycosylation changes due to various diseases, is also of great interest, especially in biomarker discovery endeavors.[3]

Due to the high complexity of carbohydrate structures on glycoproteins, a variety of bioanalytical techniques should be usually applied for their characterization. These methods include enzyme assays, chromatography, mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR), polyacrylamide slab gel electrophoresis (PAGE), high-performance liquid chromatography techniques (HPLC) including anion-exchange chromatography with pulsed field amperometric detection (HPAEC-PAD), and capillary electrophoresis (CE).[1,4–9] Combination of some of these methods, such as LC−MS and CE−MS, offer encouraging opportunities in carbohydrate analysis.[10–11]

Most of the traditional methods for structural investigation of protein-bound sugar structures employ enzymatic or chemical cleavage of the carbohydrate moieties from the glycoproteins. As almost all carbohydrates lack chromophore and/or fluorophore moieties the use of high-resolution bioseparation techniques of reversed-phase HPLC and CE equipped with the usual UV or fluorescent detectors are not feasible without preseparation derivatization. In CE, the most broadly applied fluorophore tags are 8-amino-1,3,6-naphtalenetrisufonic acid (ANTS) and 8-amino-1,3,6-pyrenetrisulfonic acid (APTS),[12–15] both providing the necessary fluorescent characteristics and charge. Electric field mediated analysis of derivatized carbohydrates are then carried out either by traditional CE instrumentation,[16] capillary array electrophore-

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sis,[17] or in microfluidics format.[18] One of the problems with ANTS and APTS labeling is the very large molar access requirement of the labeling reagent in the derivatization reaction mixture. Apparently, the remaining unconjugated derivatization reagent makes the analysis of smaller (up to several sugar units) oligosaccharides problematic, due to their comigration with the overloaded labeling reagent peak. In addition, the large amount of highly charged labeling agent in the sample solution also represents a problem to CE systems with electrokinetic injection,[19] by biasing toward solute molecules with high electrophoretic mobilities, i.e., suppressing the intake of lower mobility sample components. Earlier attempts for postderivatization cleanup used Sephadex G10 packed 96-well filterplates to alleviate this problem.[20]

In this paper we introduce and compare several postderivatization labeling agent cleanup protocols to accommodate high-resolution capillary gel electrophoresis analysis of enzymatically released and APTS-labeled N-linked glycans. We also discuss options to remove the free monosaccharide content from human plasma in profiling applications and the introduction of volatile buffer systems for exoglycosidase digestion based carbohydrate analysis.

EXPERIMENTAL SECTION

Chemicals. Ribonuclease B (RNaseB), fetal bovine fetuin (FET), asialofetuin, α-1-acid-glycoprotein (AGP), and immunoglobulin G (IgG) (Sigma-Aldrich, St. Louis, MO) were reconstituted in HPLC grade water (Sigma-Aldrich) to 10 mg/mL final concentration. The pooled human plasma sample was from Dunn Labortechnik (Asbach, Germany). The APTS, acetic acid, $NaBH_3CN$ in tetrahydrofuran, acetonitrile, and trifluoroacetic acid were from Sigma-Aldrich. The maltooligosaccharide ladder was from Grain Processing Corporation (Muscatine, IA).

Oligosaccharide Sample Preparation. Glycoproteins (0.1 mg in 10 μL of water) were denatured at 100 °C for 10 min after the addition of 1 μL of denaturing buffer (Endoglycosidase buffer pack, New England BioLabs, Ipswich, MA). This step was followed by peptide-*N*-glycosidase F (PNGaseF, Sigma-Aldrich) digestion using 2 U of enzyme at 37 °C for 120 min in 25 μL of total reaction volume (2.5 μL of G7 and 2.5 μL of NP40 buffer solutions from the Endoglycosidase buffer pack of New England BioLabs). The deglycosylated proteins were then precipitated by the addition of three times volume of ice-cold ethanol (Sigma-Aldrich), followed by centrifugation at 11 000*g* for 10 min (Labofuge 400R, Heraeus, Osterode, Germany).

Labeling Procedure. The glycan-containing supernatants were dried in 0.2 mL microfuge vials in a centrifugal vacuum evaporator (Savant Instruments Inc., Farmingdale, NY) and labeled through reductive amination by the addition of 1 μL of 0.2 M APTS in 15% acetic acid and 1 μL of 1 M $NaBH_3CN$ in tetrahydrofuran.[15] The labeling reaction was incubated at 55 °C for 2 h or alternatively at 37 °C and overnight for sialic acid containing structures (FET and human plasma samples). The reaction was stopped by the addition of 100 μL of water to the reaction mixture. The oligosaccharide ladder standard was labeled the same way.

Desialylation Procedure. Removal of the sialic acid groups from FET was facilitated by neuraminidase (type VI from *Clostridium perfringens*, Sigma-Aldrich) in 0.1 M volatile carbonate, formate, or acetate buffers (pH 5.5, adjusted by NH_4OH), respectively. The exoglycosidase digestion reaction mixtures contained the enzymatically released FET glycans and 3.4 mU of neuraminidase. The reaction mixtures were incubated overnight at 37 °C and stopped by dilution with a 10× volume of ice-cold HPLC grade water. The samples were then dried in a centrifugal vacuum evaporator (Savant) to remove the volatile buffer constituents.

Sample Purification. *MultiScreen Filterplate.* MultiScreen filter plates (Millipore, Bedford, MA) were filled with 100 μL of Sephadex G10 beads (Sigma-Aldrich) and used for the purification of 200 μL of APTS reaction mixture. Filtration and size exclusion purification was carried out in a Hettich 30RF centrifuge (Tutlingen, Germany) at 2500 rpm for 15min.

G10 Pipet Tip. Excess APTS was removed by means of G10 bead filled 200 μL chromatographic pipet tips (PhyNexus Inc., San Jose, CA) using a semiautomated PhyNexus robot with multichannel pipettors. The PhyTips were filled with 160 μL of G10 resin, and 50% acetonitrile was used for conditioning and sample elution.

DPA-6S Pipet Tip. In this instance, 10 μL of DPA-6S normal phase polyamide resin containing PhyTips (PhyNexus) were used (1200 μL pipet tips with multichannel pipettor). The 100 μL sample was diluted with 900 μL of acetonitrile prior to the purification process. Acetonitrile (95%) was used in the washing and 20% acetonitrile in the elution process, respectively. Eight intake/expel cycles were applied followed by 24 washing cycles using four aliquots of 1 mL of 95% acetonitrile. The captured molecules were then eluted with 350 μL of 20% acetonitrile.

Reversed-Phase Pipet Tips. Five microliters of reversed-phase (C-18) chromatographic resin was filled in 200 μL pipet tips (PhyNexus) and applied to remove the glucose content of human plasma samples. The tips were conditioned with acetonitrile containing 1% trifluoroacetic acid (TFA). LC−MS grade water with 1% TFA was used in the washing steps, and 50% acetonitrile containing 1% TFA was used in the elution step, respectively.

Ultrafiltration. The low molecular mass components of the human plasma were removed by 3 or 10 kDa Microcon (Millipore) centrifugal filtration (Labofuge 400R centrifuge, Heraeus). The cellulose membrane filter devices were conditioned with 500 μL of 0.1 M NaOH solution, then washed with 500 μL of HPLC water. The plasma was first loaded onto the filter, followed by three washing steps with 150 μL of HPLC water. The glycoproteins were subsequently recovered by inverting the cartridge followed by centrifugation. The recovery was increased by washing the membrane four times with 10 μL of HPLC water.

Multicapillary Electrophoresis. The labeled and purified samples were diluted 10 times with HPLC grade water and analyzed on a CarbCE multicapillary electrophoresis system (eGene, Irvine, CA) using a 12-channel gel cartridge (GCK-CARB). The system was equipped with a blue light-emitting diode (LED; peak wavelength: 460−470 nm) excitation source. The fluorescent

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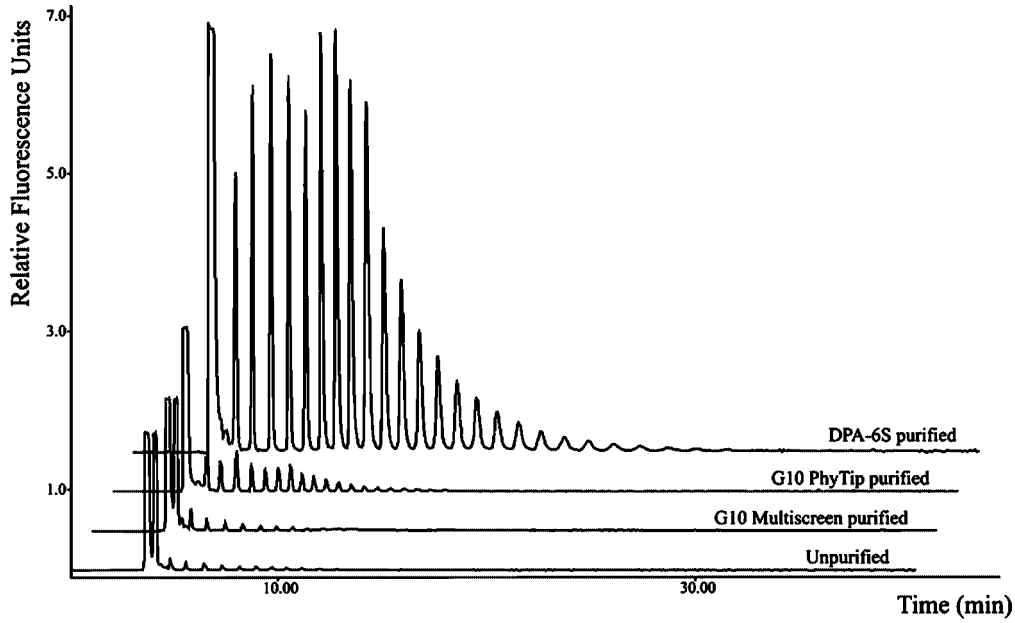

Figure 1. Comparison of unreacted derivatization reagent (APTS) removal methods from the carbohydrate derivatization mixture. Lower trace, unpurified maltooligosaccharide ladder; lower middle trace, Sephadex G10 multiscreen 96-well filter plate purified maltooligosaccharide ladder; upper middle trace, Sephadex G10 PhyTip purified maltooligosaccharide ladder; upper trace, DPA-6S normal phase PhyTip purified maltooligosaccharide ladder. Separation conditions: 10 cm long capillaries, 3 kV applied voltage at ambient temperature. Electrokinetic injection: 2 kV for 20 s. Detection: blue LED excitation (460−470 nm)/520 emission cutoff filter.

emission was detected by a photodiode using a 520 nm long-pass filter. Separations were performed in either 10 cm (12-capillary array) or 30 cm (4-capillary array) effective capillary lengths (20 μm i.d.). The capillaries were filled with CARB Separation Solution (eGene), and the samples were injected electrokinetically (2 kV for 20 s). Separations were carried out at 2−8 kV.

RESULTS AND DISCUSSION

Removal of Excess Derivatization Reagent Prior to Capillary Gel Electrophoresis. In capillary gel electrophoresis based carbohydrate analysis with electrokinetic injection, the large molar access of the derivatization agent in the sample may cause a biased injection problem. In addition, the unconjugated derivatization agent may make adequate analysis of lower unit number oligosaccharides problematic, due to their possible comigration with the large, overloaded labeling agent peak. Therefore, when pressure injection is not an option (i.e., with most manual systems and some commercial units with viscous separation gels), sample purification prior to injection is unavoidable in order to detect sample components in low concentration. To address this problem, the purification efficiency of three labeling reagent (APTS) depletion methods were evaluated and carefully optimized to attain the highest possible extent of APTS removal, while maintaining good sample recovery.

Figure 1 compares the electropherograms of the unpurified control oligosaccharide ladders (lower trace) with the G10 Multiscreen (lower middle trace), the G10 PhyTip (upper middle trace), and the DPA-6S PhyTip (upper trace) purified, i.e., APTS-depleted samples. As one can see, all three purification methods resulted in improved detectability over the unpurified sample, with the normal phase polyamide resin exhibiting the best performance in excess derivatization agent removal. Therefore, normal phase polyamide resin tips were used in subsequent APTS removal steps from PNGaseF-digested samples of IgG, RNaseB, FET, and AGP. Multicapillary gel electrophoresis separation of these four glycan samples was carried out in capillaries with 10 and 30 cm effective lengths using 12- and 4-channel cartridges, respectively. Figure 2 depicts the glycosylation patterns of the different glycoprotein samples. The upper trace in Figure 2A shows the separation of AGP glycans, featuring the characteristic large peak of highly sialylated structures at approximately 8 min. The distinctive two early migrating (~5 min) tri- and tetrasialylated structures (F1−F4) are clearly visible in the FET trace. The RNaseB trace nicely shows the well-known five major isoforms of its high mannose structures (M5−M9). The IgG trace shows the typical peak pattern of immunoglobulin glycans. To increase the resolving power of the separations, the effective capillary length was increased from 10 to 30 cm, in this instance using a 4-channel cartridge. The two separations shown in Figure 2B are good examples of the increased resolution. The upper trace in Figure 2B clearly depicts the doublet features of the two tetrasialylated (F1, F2) and two trisialylated glycans (F3, F4) of FET. The lower trace of Figure 2B shows adequate resolution of the individual positional isoforms of the mannose-7 (M7abc) and mannose-8 (M8abc) structures of the RNaseB glycans.

Elimination of the Monosaccharide Content from Human Plasma. Sample matrix related issues are usually minimal in the analysis of standard glycoproteins, as one can use appropriate solvents and reagents, which do not interfere with the derivatization and/or separation process. However, when biological samples are used, the sample matrix may certainly represent a problem. Indeed, we have faced some difficulties during profiling glycans released from human plasma samples because of their relatively high glucose content. As glucose gets derivatized along with the released glycan structures during the labeling reaction, it not only competes with the larger complex glycan structures

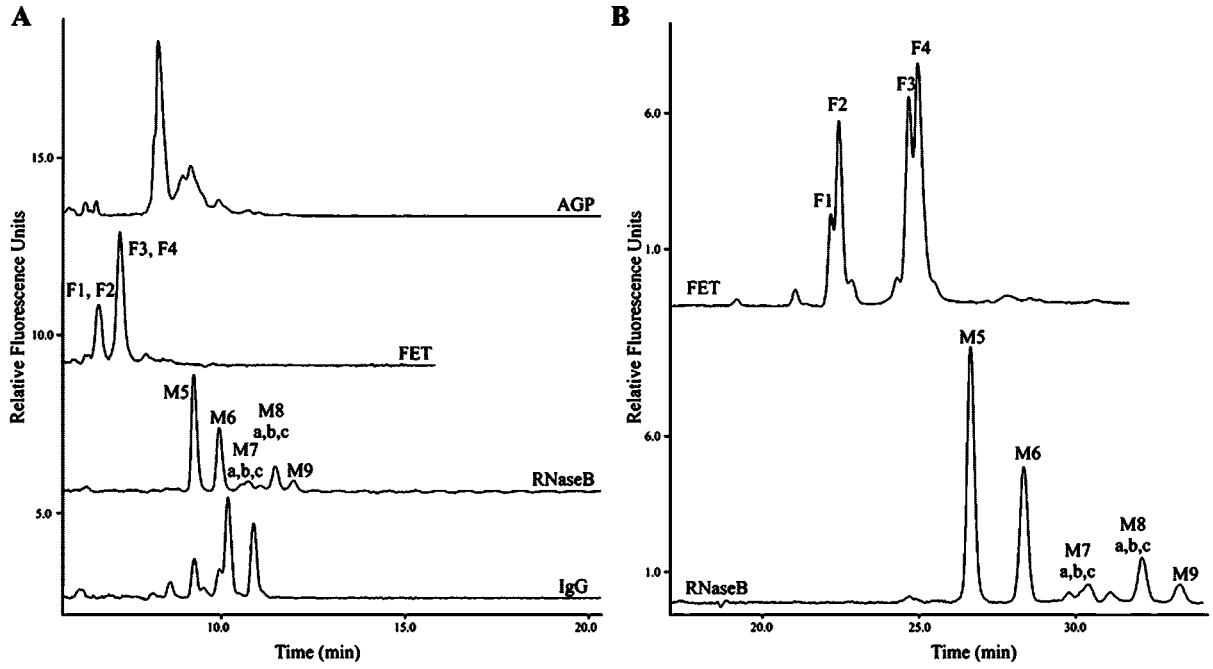

Figure 2. Capillary gel electrophoresis profiles of DPA-6S purified APTS-labeled glycans released from several glycoprotein standards. Samples: α-1-acid-glycoprotein (AGP), fetal bovine fetuin (FET), ribonuclease B (RNaseB), immunoglobulin G (IgG). Separation conditions: (A) effective separation length, 10 cm; applied voltage, 3 kV; (B) effective separation length, 30 cm; applied voltage, 8 kV. Other conditions were the same as in Figure 1.

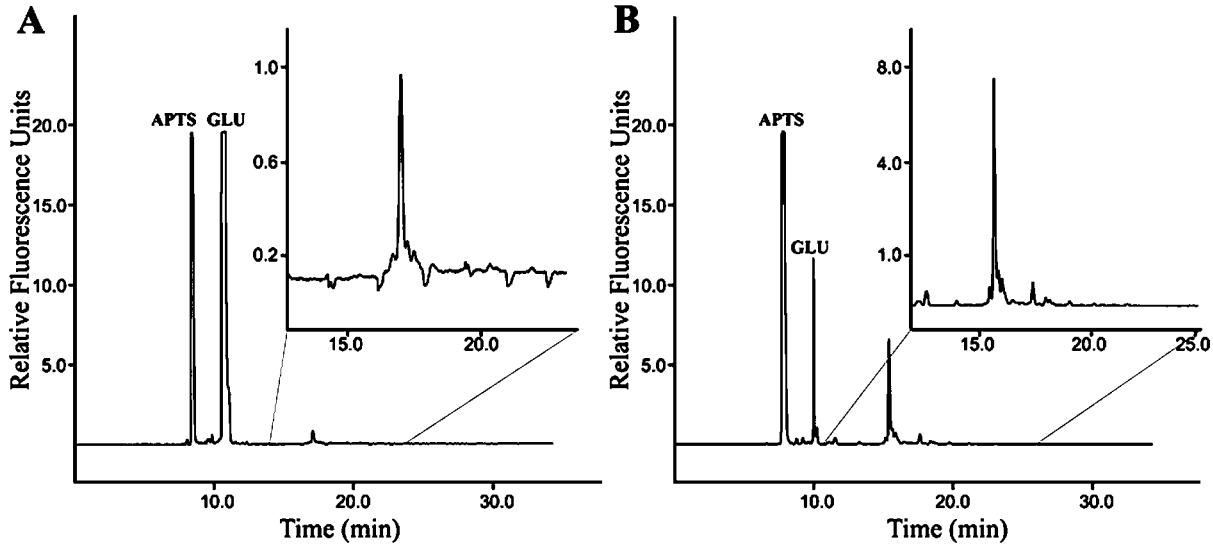

Figure 3. Ultrafiltration-mediated elimination of the monosaccharide content from human plasma samples analyzed by capillary gel electrophoresis. Panel A, normal human plasma; panel B, same sample after glucose removal by 3 kDa ultrafiltration. Separations conditions were the same as in Figure 2B.

of interest but also suppresses their entry into the separation capillary when electrokinetic injection is used. We investigated different methods to remove the blood sugar content prior to the enzymatic release of oligosaccharides from human plasma glycoproteins. Glucose removal was attempted by means of reversed-phase bead filled micropipet tips and ultrafiltration. Our results showed that ultrafiltration with either 3 or 10 kDa cutoff values provided significantly higher performance than that of reversed-phase bead filled pipet tips (data not shown). The only major difference between the 3 or 10 kDa cutoff ultrafiltration methods was the centrifugation time, which was significantly longer for the 3 kDa membranes. Figure 3 compares the capillary gel electrophoresis profiles of APTS-labeled N-linked glycans released from human plasma without glucose removal (Figure 3A) and after 3 kDa ultrafiltration (Figure 3B). Please note the improved signal intensity and signal-to-noise ratio in the insets of the panels before and after this sample preparation process. We consider this step to be necessary in order to see the fine distribution of human plasma glycan pool peaks, as their profile might possess valuable diagnostic information, such as altered glycosylation patterns.

Volatile Buffer System for Exoglycosidase Digestion. Determination of the composition and linkage information of carbohydrate structures released from glycoproteins of interest requires enzymatic analysis using specific exoglycosidases. For

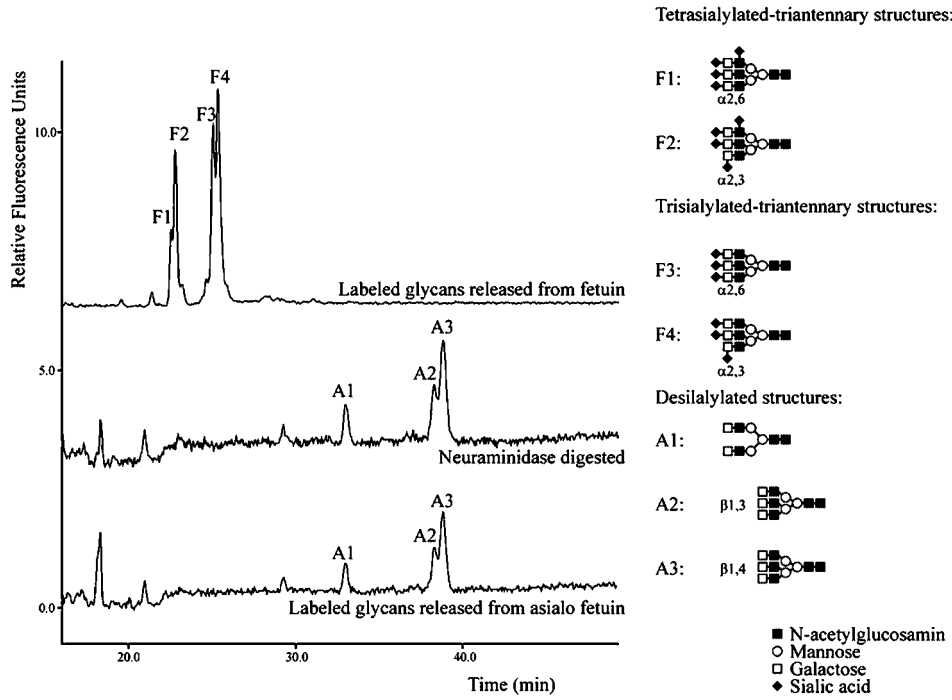

Figure 4. Capillary gel electrophoresis analysis of neuraminidase digestion products of fetal bovine fetuin (FET) glycans using volatile ammonium acetate reaction buffer. Upper trace, APTS-labeled fetuin glycans; middle trace, neuraminidase-digested APTS-labeled fetuin glycans; lower trace, APTS-labeled asialofetuin glycans (control). Symbols: ◆, sialic acid (Neu5Ac); □, galactose; ○, mannose; ■, *N*-acetylglucosamine (GlcNAc). Conditions: effective separation length, 30 cm; applied voltage, 8 kV. Other conditions were the same as in Figure 1.

example, carbohydrate sequencing can be done by using a digestion enzyme matrix, containing carefully chosen exoglycosidase mixtures.[21] Exoglycosidase-mediated analysis of carbohydrates can be applied to any CE-based methods, including multicapillary gel electrophoresis. However, when electrokinetic injection is the only option, the high ionic strength reaction buffer components should be accordingly modified or eliminated prior to injection. In this instance, ultrafiltration is not a good option, as some the labeled carbohydrate structures would be lost too. Therefore, we suggest the use of volatile reaction buffer systems that can be easily removed prior to CE analysis. Acetate-, formate-, and carbonate-based buffers were evaluated in 0.1 M concentration with their pH values adjusted by ammonium hydroxide to the optimal reaction pH of 5.5. To prove the applicability of this approach, neuraminidase enzyme was applied to release the sialic acid residues from FET glycans. After overnight incubation with neuraminidase at 37 °C, the volatile buffer components were removed in a centrifugal vacuum evaporator. In our hands, the ammonium acetate digestion buffer worked the best in the digestion and evaporation studies. The upper trace in Figure 4 depicts the relevant section of the separation trace of the undigested FET glycans, showing the two doublets of the tetra- and trisialylated structures (F1, F2 and F3, F4, respectively). The middle trace shows the results after neuraminidase digestion in 0.1 M ammonium acetate buffer (pH 5.5). As all sialic acid residues were dissociated at the separation pH of 4.5, their removal increased the mass to charge ratio of the solute molecules resulting in slower migration. In the middle trace, peak A3 represents the desialylated F1−F4 peaks, i.e., after the removal of all α2,3- and α2,6-linked Neu5Acs from the Man(α1,6) and Man(α1,3) side chains. A2 is the desialylated form of the Gal(β1,3)−GlcNAc middle antenna Man(α1,6) side-chain linkage isomer. On the basis of our earlier results we consider this peak to be originated from a mainly comigrated peak with F3.[22] A1 is assumed to be a digestion reaction byproduct with a Gal(β1,4) biantennary structure.[23] The lower trace in Figure 4 exhibits a control experiment with the injection of the glycan pool released from asialofetuin. Please note the excellent peak distribution similarity between our desialylation experiment and the asialofetuin trace.

CONCLUSION

Separation of complex carbohydrates by high-performance CE is a rapidly developing field. Applications in multicapillary formats enable high-throughput analyses of glycosylation changes in the extent and/or nature of oligosaccharide distribution (profiling) and exoglycosidase-mediated carbohydrate analysis (sequencing). As most sugars neither possess charged moieties nor chromophore/fluorophore groups, their electrophoresis-based analysis requires appropriate labeling protocols. APTS is one of the most frequently used labeling reagents in this field, supporting fluorescent detection with blue LED excitation. APTS also provides the required charged moiety for electrophoresis. However, the very large molar access of the labeling reagent in the derivatization reaction mixture may represent a problem during the injection and separation steps, i.e., besides the biased injection issue, the remaining large amount of unconjugated APTS makes adequate analysis of short

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oligosaccharides problematic, due to their possible comigration with the overloaded labeling reagent. In this paper we evaluated three sample preparation methods to remove the excess labeling reagent prior to CE. The Sephadex G10 bead filled multiscreen 96-well filter plate based method was compared to a semiautomated liquid handling system employing low-volume pipet tips with Sephadex G10 and polyamide DPA-6S stationary phases, respectively. All three methods were capable of APTS removal and enabled increased sensitivity analysis of the labeled carbohydrates of interest. The polyamide DPA-6S pipet tips showed the best performance. The important sample preparation issue of eliminating the glucose content from human plasma prior to glycan profiling was solved by ultrafiltration. Introduction of a volatile buffer system for exoglycosidase digestion mediated carbohydrate analysis enabled high-sensitivity detection of the digested reaction products using electrokinetic injection.

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