ELSEVIER

Journal of Chromatography A, 763 (1997) 271–277

JOURNAL OF CHROMATOGRAPHY A

# Analysis of monosaccharide composition by capillary electrophoresis

András Guttman*

*Beckman Instruments Inc., Fullerton CA 92634, USA*

**Abstract**

The monosaccharide composition analysis described in this paper employs capillary electrophoretic separation of sugar monomers liberated from glycoproteins or oligosaccharides, by high temperature acidic hydrolysis. Trifluoroacetic acid was used for sialo- and neutral-sugar hydrolysis, and hydrochloric acid was used for amino-sugar hydrolysis. The neutral- and amino-sugars in the hydrolyzates were then labeled with a charged fluorophore, 8-aminopyrene-1,3,6-trisulfonate, while sialic acids were labeled with 9-aminoacridone. The stoichiometry of labeling was such that only one fluorophore molecule was attached to each monosaccharide molecule. The labeled monosaccharides were then separated by high-performance capillary electrophoresis with laser induced fluorescence detection. The acidic hydrolysis and fluorophore labeling conditions described in this paper are suitable for monosaccharide composition analysis of a wide variety of complex carbohydrates from glycoprotein and/or oligosaccharide samples using capillary electrophoresis.

*Keywords:* Derivatization, electrophoresis; Monosaccharides; Carbohydrates; Glycoproteins; Oligosaccharides

## 1. Introduction

Carbohydrates play a key role in the process of life [1]. Besides their undoubted importance in nutrition, they also make up the polymeric backbone of DNA, they are part of many vital enzyme systems and are responsible for cell recognition [2]. Therefore, carbohydrate analysis is getting more and more important in modern bioanalytical chemistry [3]. The various low and high cost analytical methods used for carbohydrate analysis are mass spectrometry (MS) [4], $^{1}$H-nuclear magnetic resonance spectroscopy (NMR) [5], high-performance anion-exchange chromatography with pulsed amperometry detection (HPAEC–PAD) [6,7] and polyacrylamide gel electrophoresis (PAGE) [8,9]. One of the most important parts of the analysis of complex carbohydrates is the determination of their monosaccharide composition, which has been conventionally done by HPAEC–PAD and PAGE methods [6–9]. Recently, capillary electrophoresis (CE) has also emerged as a powerful separation tool in monosaccharide analysis [10–21].

Since most monosaccharides lack charge and UV absorbing and/or fluorophore groups, pre-separation derivatization is usually necessary in order to aid their detection in electrophoresis separations [22]. A large variety of charged and uncharged, UV active and fluorophore derivatization reagents have been suggested in the literature for this purpose, such as 2-aminopyridine; 4-aminobenzoic acid; 4-aminobenzoate; 4-aminobenzonitrile; 8-aminonaphthalene-1,3,6-trisulfonate (ANTS); 1-phenyl-3-methyl-2-pyrazolin-5-one (PMP); 8-aminopyrene-1,3,6-trisulfonate (APTS), 9-aminoacridone (AMAC), etc.

*Corresponding author. Present address: Genetic BioSystems Inc., 10171 Pacific Mesa Boulevard, Suite 307, San Diego, CA 92121-4326, USA.


*PII* S0021-9673(96)00750-9

[8–24]. Additionally, the kinetics of the derivatization reaction varies for the different sugar monomers with any given tag. Therefore, a derivatization reagent that is good for one sugar, might not be as good for another [24]. One of the usual ways for derivatization of reducing sugars, is the so called reductive amination technique [25]. Using the charged fluorophore of 8-aminopyrene-1,3,6-trisulfonate (APTS), Chen and Evangelista obtained successful CE based separation of up to ten different APTS labeled neutral- and amino-sugars [12].

It is well known, polyhydroxy compounds, such as carbohydrates, form anionic complexes with borate [26]. In CE at higher separation pH (>9), resolution among various sugars can indeed be enhanced using borate based buffer systems [10–12]. Interestingly, in borate mediated CE analysis of monosaccharides, increased separation efficiency has been observed with elevated temperature. Hofstetter-Kuhn et al. [27] showed a significant increase in resolution between glucose and xylose at 60°C, compared to no separation at 20°C. Monosaccharides are typically very hydrophilic, and therefore are hardly soluble in surfactant based micellar systems. Labeling them by a hydrophobic tag makes possible their separation by micellar electrokinetic chromatography (MECC). Eight monosaccharides, derivatized with 9-aminoacridone (AMAC), were separated by Greenaway et al. [18] in the presence of sodium taurodeoxycholate in borate buffer. Pre-separation labeling with PMP also resulted in a nice separation of a mixture of monosaccharides by SDS mediated MECC [23]. Employing an extremely high pH buffer system (pH=13), Zare and coworkers developed a relatively sensitive copper microelectrode based amperometric detection method for determination of unlabeled carbohydrates [28].

Oligosaccharides degrade at different rates to their constituent monosaccharide units when subjected to acid hydrolysis. Therefore, different digestion reactions are suggested to determine the amount of particular types of monosaccharides, such as sialic acids, neutral- or amino-sugars. Three different types of hydrolysis reactions are usually recommended for digestion of glycoproteins and intact carbohydrates, using 0.1 *M* trifluoroacetic acid (TFA) at 80°C for 30–60 min for sialic acids, and 2 *M* TFA/4–5 h for neutral-sugars, or 4 *M* HCl/3–5 h for amino-sugars, both at 100°C [9].

The method suggested in this paper provides monosaccharide composition information which can be used for predicting the nature of the glycans of interest, the presence or absence of sialic acids, and the extent of oligosaccharide processing, as well as evaluating changes in monosaccharide composition.

## 2. Materials and methods

### 2.1. Chemicals

Monosaccharides, N-acetylgalactosamine (GalNAc), N-acetylglucasamine (GlcNAc), mannose (Man), glucose (Glc), fucose (Fuc), galactose (Gal), and the composition control, N-acetyllactosamine (GlcNAc-Gal) were purchased from Sigma (St. Louis, MO, USA). The trisodium 8-aminopyrene-1,3,6-trisulfonate (APTS) was from Beckman Instruments (Fullerton, CA, USA), the 9-aminoacridone (AMAC) was from Glyco (Novato, CA, USA). The sialic acid standard (Neu5Ac) and all other chemicals were from Aldrich (Milwaukee, WI, USA). The APTS and AMAC-derivatized sugar samples were analyzed directly after derivatization or stored at −20°C. All buffer solutions were filtered through a 0.45 μm pore size filter and carefully vacuum degassed at 100 mbar.

### 2.2. Apparatus

CE separations were performed on a P/ACE 5000 CE system (Beckman Instruments) with the anode on the injection side and the cathode on the detection side (i.e., normal polarity). The separations were monitored on column with a Beckman laser induced fluorescence (LIF) detection system using a 4 mW argon-ion laser with the excitation wavelength of 488 nm using a 520 nm emission wavelength notch filter. A bare fused-silica capillary column [37 cm (30 cm effective length)×25 μm I.D.] was used for the separations, filled with 25 m*M* lithium tetraborate buffer (pH 10.0) as running buffer. The column was activated before each run by flushing for 1 min with 0.1 *M* NaOH, followed by a 1 min flush with water and 2 min equilibration rinse with the running buffer. The samples were injected by the pressure injection

mode of the system, typically for 5–10 s at 3.45 kPa. The temperature of the capillary in the P/ACE instrument was controlled at 20±0.1°C. The electropherograms were acquired and stored on an IBM 486/66 computer and evaluated by using the Caesar 4.1 software package (Analytical Devices, Alameda, CA, USA).

### 2.3. Procedures

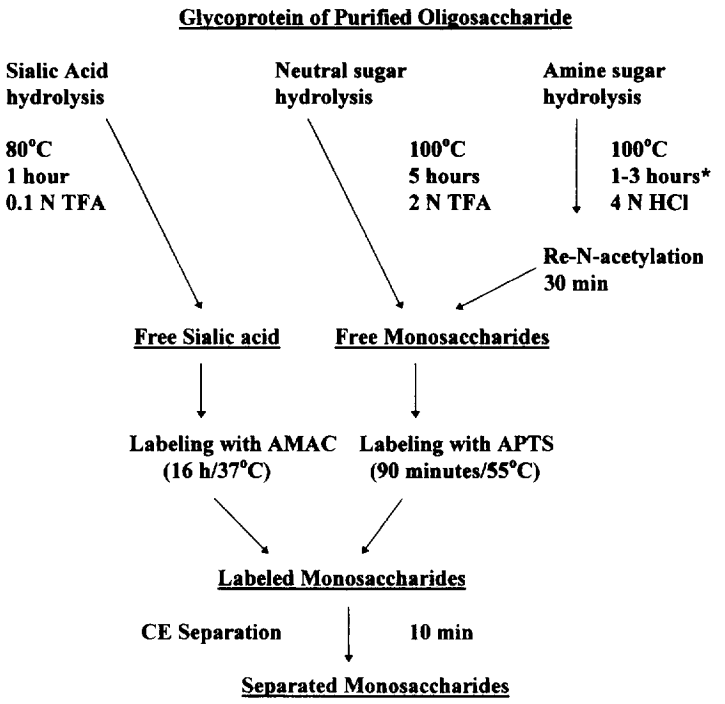
10 μg bovine fetuin was used for each hydrolysis reaction (sialic acid-, amino- and neutral-sugar hydrolysis) dissolved in 50 μl CE grade water in a 0.5 ml snap top tube with cap lock. For sialic acid hydrolysis 50 μl 0.2 *M* TFA was added, and the reaction mixture were incubated for 60 min at 80°C. For neutral-sugar hydrolysis 50 μl 4 *M* TFA, and for amino-sugar hydrolysis 50 μl 8 *M* HCl was added, and the reaction mixtures were incubated at 100°C for 5 h and 3 h, respectively. For the amino-sugar hydrolysis of intact oligosaccharides (e.g., N-acetyllactosamine) 1 h/100°C hydrolysis was used. Consistent oligosaccharide hydrolysis was best achieved using a sand heat block with the reaction tubes completely buried to prevent condensation. Please note that condensation of the reaction mixture at the top of the tubes may cause variable hydrolysis results. After hydrolysis, each reaction mixture was kept cool for 30 min at −20°C, then dried in a centrifugal vacuum evaporator, and the dried pellet was used in the derivatization reaction. Hydrolyzed amino-sugars were re-N-acetylated by re-suspending the pellet in 10% acetic acid anhydride and incubating at 0°C for 15 min.

With the exception of the sialic acids, the dried sugars were labeled by the addition of 2 μl 0.2 *M* APTS in 25% citric acid and 2 μl 1 *M* $NaBH_3CN$ in tetrahydrofuran (THF). The reaction mixtures were incubated at 55°C for 1.5 h for complete labeling (derivatization efficiency was >97% [17]). Derivatization of the sialic acids were accomplished by the addition of 3 μl 0.2 *M* AMAC in 15% acetic acid and 3 μl 1 *M* $NaBH_3CN$ in dimethyl sulfoxide (DMSO), and the reaction was incubated at 37°C overnight. The labeling reactions were stopped by the addition of 46 μl CE grade water, and samples of 5 μl were diluted to 200 μl by water before injection.

## 3. Results and discussion

Due to differences in the acid stability of various sugars, different hydrolysis conditions are recommended for the analysis of sialo-, neutral- and amino-sugars originating from isolated glycoproteins or oligosaccharides. As Fig. 1 shows, sialic acid hydrolysis is accomplished at 80°C for 1 h in 0.1 *M* TFA. The neutral-sugars are hydrolyzed from the glycoproteins by 2 *M* TFA at 100°C for 5 h. For amino-sugar hydrolysis a final concentration of 4 *M* HCl is used at 100°C for 3 h in the case of glycoproteins, and for 1 h in the case of intact oligosaccharides. Please note, that after the amino-sugar hydrolysis, a re-N-acetylation step is necessary to reformulate the N-acetylated amino-sugars of GlcNAc and GalNAc. If sufficient starting material is available, it is also recommended that a time course is performed for each hydrolysis, so conditions may be modified for optimal recovery of monosaccharides from specific glycoprotein samples. Also, due to possible large differences in the amounts of the different monosaccharides released from glycoproteins or isolated oligosaccharides, the volumes for sample preparations may need to be varied to optimize the loading of a particular sample.

After the hydrolysis of neutral- and amino-sugars, the hydrolyzates were labeled with the charged fluorophore of 8-aminopyrene-1,3,6-trisulfonate (APTS) (Fig. 2a). However, the sialic acids (Neu5Ac, etc.) were labeled with 8-aminoacridone (AMAC), a dye which is not ionized at the separation pH of 10. This change in derivatization agent was necessary in this instance, because the electrophoretic mobilities of the APTS labeled sialic acids were very close to the electroosmotic flow (EOF) at pH 10, thus the migration time of the APTS labeled sialic acids were found to be inconveniently long (data is not shown). The labeling reaction of a sialic acid with AMAC is shown in Fig. 2b. The fluorescently labeled monosaccharides were then separated by CE using LIF detection. An EOF marker (AMAC, which is, as mentioned earlier, not charged at the separation pH of 10) and an internal standard (APTS, which is fully charged at the separation pH of 10) were both coinjected with each sample in order to achieve higher precision in peak identification. The following equation developed by Suzuki and Honda [29] was applied for the high precision calculation of




* *3 hours for glycoproteins, 1 hour for isolated oligosaccharides.*

Fig. 1. Procedure.

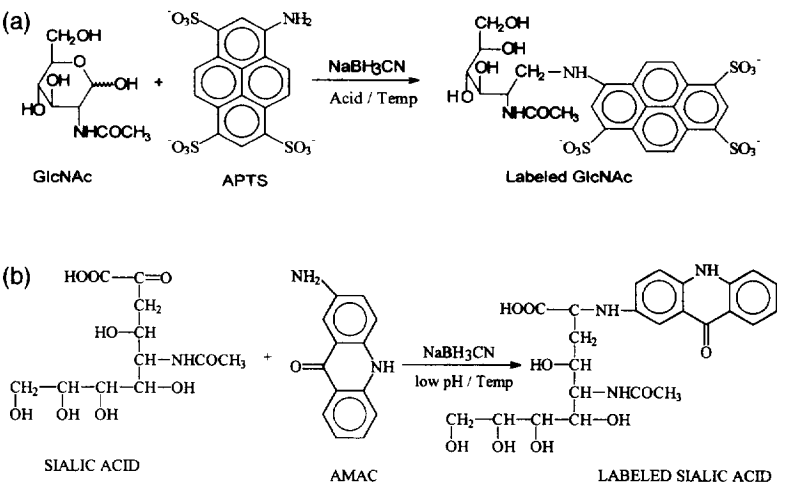



Fig. 2. Labeling reaction of N-acetylglucosamine (GlcNAc) with 8-aminopyrene-1,3,6-trisulfonate (APTS) (a) and N-acetyl-neuraminic acid (Neu5Ac) with 9-aminoacridone (AMAC) (b).

relative migration times of the individual monosaccharides:

$$R_{MT} = \frac{t_{APTS}}{t} \cdot \frac{t - t_{AMAC}}{t_{APTS} - t_{AMAC}} \quad (1)$$

where $R_{MT}$ is the relative migration time of a solute with a migration time $t$; $t_{APTS}$ is the migration time of the internal standard, APTS and $t_{AMAC}$ is the migration time of the EOF marker, AMAC. A typical separation of a monosaccharide composition test mixture is depicted in Fig. 3. The bracketing peaks of 1 and 9 correspond to the EOF standard (AMAC) and the internal standard (APTS), respectively. Peaks 2 to 8 were identified by coinjection with authentic individual monosaccharide standards, and proved to be AMAC labeled sialic acid (Neu5Ac)(2) and APTS labeled N-acetylgalactosamine (GalNAc)(3), N-acetylglucosamine (GlcNAc)(4), mannose (Man)(5), glucose (Glc)(6), fucose (Fuc)(7) and galactose (Gal)(8). The small peak between the AMAC (1) and the AMAC labeled sialic acid (2) peaks, and the right hand small shoulder of peak 2 are probably impurities from the sialic acid. The minor peak migrating between peaks 8 and 9 is an impurity of the APTS. Using Eq. (1), the relative migration time reproducibility of all monosaccharides in the test-mixture had less than 0.2% R.S.D. over the course of 100 injections.

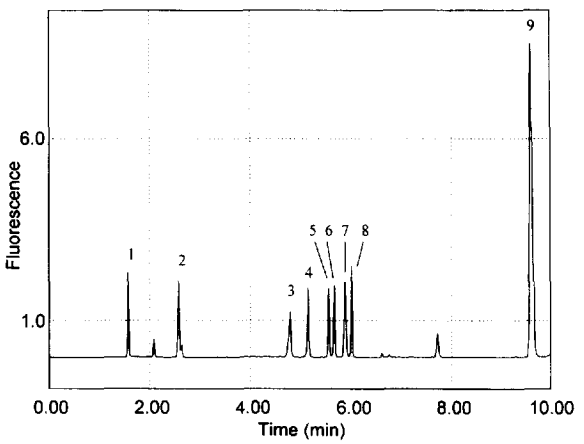



Fig. 3. Typical CE separation of the monosaccharide composition test mixture. Peak assignment: 1 = AMAC (9-aminoacridone, EOF marker) 2 = Neu5Ac-AMAC; 3 = GalNAc-APTS; 4 = GlcNAc-APTS; 5 = Man-APTS; 6 = Glc-APTS; 7 = Fuc-APTS; 8 = Gal-APTS; 9 = APTS (8-aminopyrene-1,3,6-trisulfonate, internal standard). Conditions: 30 cm (37 cm total) fused-silica capillary (25 μm I.D.), 25 m*M* borate buffer, pH 10.0, $E = 750$ V/cm, $i = 19$ μA, temperature: 20°C.

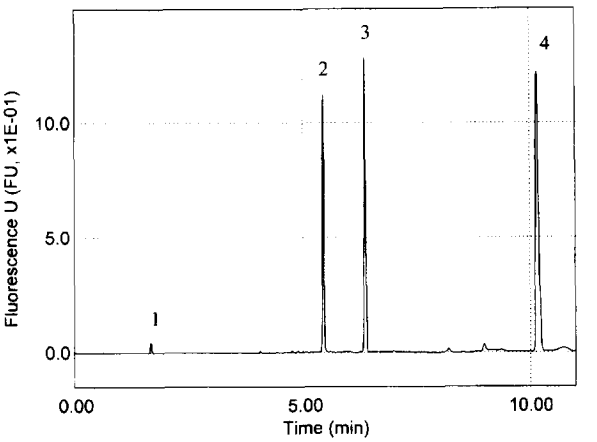



Fig. 4. CE separation of the APTS labeled monosaccharides from N-acetyllactosamine after amino-sugar hydrolysis and re-N-acetylation. Peak assignment: 1 = AMAC; 2 = GlcNAc-APTS; 3 = Gal-APTS; 4 = APTS. Conditions are the same as in Fig. 3.

Fig. 4 depicts the monosaccharide composition of N-acetyllactosamine after amino-sugar hydrolysis. Since the severe acid hydrolysis with 4 *M* HCl for 1 h/100°C practically removes all acetyl groups from the N-acetylglucosamine units, a re-N-acetylation step was necessary before the labeling step. It is important to note that, albeit, the amino-sugars, such as glucosamine and galactosamine can also be derivatized by APTS, their actual labeling efficiency is quite low (>50%). Therefore, to obtain the highest possible precision in monosaccharide composition analysis, the re-N-acetylation step for the amino-sugars is highly recommended. Similar to the test mixture separation the EOF marker (AMAC)(1) and the internal standard (APTS)(4) were both co-injected along with the hydrolyzed and labeled sugars. Peak 2 corresponds the GlcNAc (re-N acetylated glucosamine), peak 3 is galactose, and peak 4 is the APTS. The disaccharide test compound of N-acetyllactosamine contains GlcNAc and Gal in a 1:1 ratio, but the ratio of peaks 2 and 3 in Fig. 4 is slightly different. This discrepancy is probably due to some loss during the re-N-acetylation reaction of glucosamine (~10%), therefore assessment of the re-N-acetylation reaction efficiency is always highly recommended.

The CE separation of the APTS labeled products

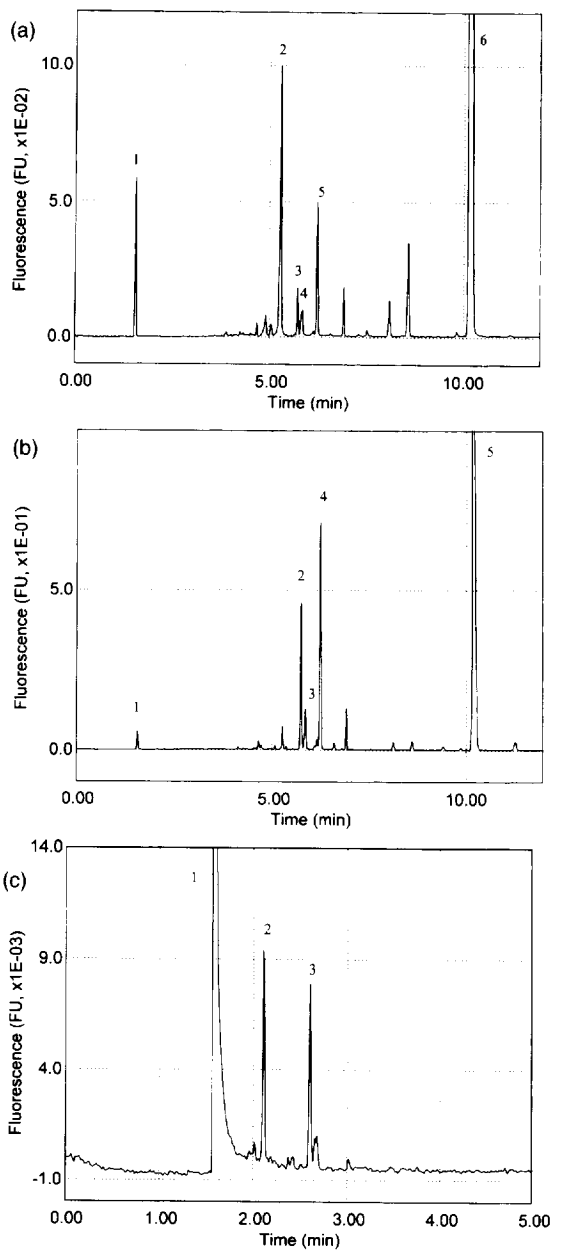



Fig. 5. (a) CE separation of the amino-sugar hydrolysis reaction products of bovine fetuin after re-N-acetylation. Peak assignment: 1 = AMAC; 2 = GlcNAc-APTS; 3 = Man-APTS; 4 = Glc-APTS; 5 = Gal-APTS; 6 = APTS. (b) CE separation of the neutral hydrolysis reaction products of bovine fetuin. Peak assignment: 1 = AMAC; 2 = Man-APTS; 3 = Glc-APTS; 4 = Gal-APTS; 5 = APTS. (c) CE separation of the sialic acid hydrolysis reaction products of bovine fetuin. Peak assignment: 1 = AMAC; 2 = unknown; 3 = Neu5Ac-AMAC. Separation conditions are the same as in Fig. 3.

of the amino-sugar hydrolysis (Fig. 1) of fetuin is shown in Fig. 5a. The monosaccharides corresponding to the numbered peaks 2–5 were identified by their relative migration times. Besides the EOF marker, AMAC (1) and internal standard, APTS (6) peaks 2–5 correspond to N-acetylglucosamine (2), mannose (3), glucose (4) and galactose (5), respectively. The small peaks before peak 2 were identified by coinjection as N-acetylgalactosamine and galactosamine, both probably originated from the *O*-linked glycans of the fetuin. The three large peaks between peaks 5 and 6 are probably neither labeled neutral-, nor amino-sugar species originating from fetuin, and are subject to further analysis. Please note, as Fig. 3 shows, galactose is always the last migrating sugar in the mixture of sialo- neutral- and amino-sugars, therefore anything migrating after Gal, is irrelevant for monosaccharide composition analysis point of view. Fig. 5b depicts the separation of the APTS labeled neutral sugars of fetuin after neutral-sugar hydrolysis (Fig. 1). Here, similar to Fig. 5a, the group of small peaks preceding peak 2 are probably the APTS labeled GalNAc, glucosamine and galactosamine. Peaks 2, 3 and 4 were identified as mannose, glucose and galactose, respectively. The unknown peaks between 4 and 5 are the similar origin (from the fetuin) as discussed under Fig. 5a. Fig. 5c depicts the CE trace of the AMAC labeled sialic acids of fetuin after the sialic acid hydrolysis. Here, besides the large peak of the unreacted AMAC(1), two major peaks can be observed. Peak 3 was identified as Neu5Ac by coinjection with the authentic individual standard. The identity of Peak 2 is still under investigation, but is probably one of the isoforms of sialic acids.

## 4. Conclusions

This paper has demonstrated the high separation power of CE in conjunction with various acidic hydrolysis methods for monosaccharide composition analysis of glycoproteins and intact oligosaccharides. The mixture of the released monosaccharides was labeled with the appropriate fluorescent tag of AMAC for sialic acids, or with APTS for neutral- and amino-sugars. Separation of the fluorophore labeled monosaccharides has been accomplished by

CE. The resulting peak pattern represented the monosaccharide composition of the starting material, and was compared to the monosaccharide composition standard trace to identify the individual monosaccharides in the sample using double internal standard method. Different hydrolysis conditions were optimized for sialo-, neutral- and amino-sugar analysis, and the particular hydrolysis that gives the greatest recovery of the monosaccharides should be used. It is also important to note that there is always some variability in the optimal conditions for acid hydrolysis of glycoproteins/oligoasaccharides, therefore taking two or more time-points is always recommended. Note that the appearance of glucose (Fig. 5A and B) does not necessarily mean that the glycoprotein/oligosaccharide sample contains glucose, its presence can be a result of a simple contamination by dust from cellulose wipes, etc. The presence of GalNAc in the hydrolyzed monosaccharide mixture always suggests the presence of O-linked oligosaccharides in the glycoprotein/oligosaccharide samples.

## Acknowledgments

The author gratefully acknowledges Dr. Nelson Cooke for his support. The stimulating discussions with Dr. Albert F-T. Chen are also greatly appreciated.

## References

[1] A.L. Lehninger, Principles of Biochemistry, Worth. Publ., New York, 1987.
[2] H.S. El Khadem, Carbohydrate Chemistry, Academic Press, San Diego, CA, 1988.
[3] Z. El Rassi, Carbohydrate Analysis, Elsevier, Amsterdam, 1995.
[4] R.A. Laine, Methods Enzymol., 193 (1990) 539.
[5] R. Barker, H. Nunez, P. Rosevear and A.S. Serianni, Methods Enzymol., 83 (1990) 58.
[6] M.R. Harty and R.R. Townsend, Proc. Natl. Acad. Sci. U.S.A., 85 (1988) 3289.
[7] Y.C. Lee, Anal. Biochem., 189 (1990) 151.
[8] P. Jackson, Biochem. J., 270 (1990) 705.
[9] C.M. Starr, R.I. Masada, C. Hauge, E. Skop and J.C. Klock, J. Chromatogr. A, 720 (1996) 295.
[10] Z. El Rassi, Adv. Chromatogr., 34 (1994) 177.
[11] M. Stefansson and M. Novotny, J. Am. Chem. Soc., 115 (1993) 11573.
[12] FT.A. Chen and R.A. Evangelista, Anal. Biochem., 230 (1995) 273.
[13] R.J. Linhardt, Methods Enzymol., 230 (1994) 265.
[14] P. Oefner and C. Chiesa, Glycobiology, 4 (1994) 397.
[15] J. Liu, O. Shirota, D. Wiesler and M. Novotny, Proc. Natl. Acad. Sci. U.S.A., 88 (1991) 2302.
[16] J.Y. Zhao, P. Dietrich, Y. Zhang, O. Hindsgaul and N.J. Dovichi, J. Chromatogr. B, 657 (1994) 307.
[17] A. Guttman, R.A. Evangelista, FT.A. Chen and N. Cooke, Anal. Biochem, 233 (1996) 234.
[18] M. Greenaway, G.N. Okafo, P. Camilleri and D. Dhanak, J. Chem. Soc. Chem. Commun., (1994) 1691.
[19] S. Honda, K. Yamamoto, S. Suzuki, M. Ueda and K. Kakehi, J. Chromatogr., 588 (1991) 327.
[20] H. Schwaiger, P.J. Oefner, C. Huber, E. Grill and G.K. Bonn, Electrophoresis, 15 (1994) 941.
[21] Y. Menchef and A. El Rassi, Electrophoresis, 15 (1994) 627.
[22] P. Jackson, Anal. Biochem., 216 (1994) 243.
[23] C. Chiesa, P. Oefner, L.R. Zieske and R.A. O'Neil, J. Cap. Electrophor., 2 (1995) 175.
[24] A. Paulus and A. Klockow, J. Chromatogr. A, 720 (1996) 353.
[25] S. Hase, S. Hara and Y. Matsushima, J. Biochem., 85 (1979) 217.
[26] A.B. Foster, Adv. Carbohydr., 12 (1957) 81.
[27] S. Hofstetter-Kuhn, A. Paulus, E. Gassmann and H.M. Widmer, Anal. Chem., 63 (1991) 1541.
[28] L.A. Colon, R. Dadoo and R.N. Zare, Anal. Chem., 65 (1993) 476.
[29] S. Suzuki and S. Honda, Trends Anal. Chem., 14 (1995) 279.