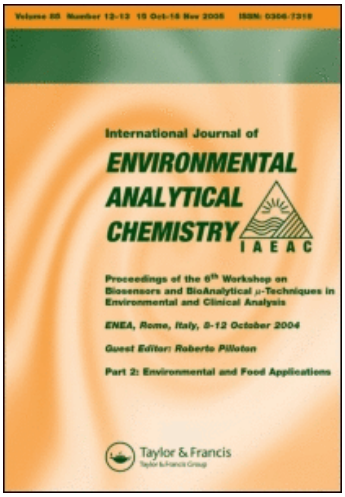

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Quantification of polysaccharides in water using capillary electrophoresis

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Two hydrolysis techniques were tested to optimize hydrolysis conditions for natural lake polysaccharides (PS). Modified sulphuric acid hydrolysis was found to be more efficient than 0.1 M HCl hydrolysis as assessed by a spectrophotometric method commonly known as TPTZ assay (2,4,6-tripyridyl-s-triazine). Capillary electrophoresis coupled with laser induced fluorescence (CE–LIF) was standardized to determine hydrolyzed saccharides in water. Two types of capillaries were tested for the CE technique. Fused silica capillary was found good for quantitative determination of different saccharides ($r^2 = 0.998$, $P < 0.001$). Excellent resolution of monosaccharides was achieved using a gel capillary (CGE), but correlation coefficients ($r^2 < 0.87$, $P < 0.05$) were not as strong as in the case of silica capillary.

CE–LIF and TPTZ assay were applied to quantify the vertical polysaccharide profile of the Lake Geneva (0–309 m). Maximum concentration of PS ($1.02 \pm 0.03\,mg\,L^{-1}$) was observed at surface. The concentration gradually decreased, with a minimum concentration of PS at 290 m ($0.27 \pm 0.04\,mg\,L^{-1}$). A fairly good correlation ($r^2 = 0.83$, $N = 9$, $P < 0.001$) between the total organic carbon (TOC) and APTS saccharide adduct measured by fused silica capillary was observed. The vertical profile of the lake as determined by CGE indicated glucose and fructose as dominant sugars with a minor contribution of xylose. The present study indicates the potential of CGE for qualitative as well as quantitative analysis of hydrolyzed saccharides in water samples.

Keywords: Polysaccharides; Glucose; Dextran; Hydrolysis; Capillary electrophoresis; Laser induced fluorescence; Total organic carbon; Capillary gel electrophoresis

1. Introduction

Carbohydrates are produced as neutral or acidic compounds by all living species, from micro-organisms to higher animal cells and it is not surprising to detect them in the environment (soils, waters) as a consequence of exocellular release (extracellular polysaccharides, EPS) or degradation processes [1]. In the aquatic environment, carbohydrates exist both as free monomers and as combined sugars such as oligo

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and polysaccharides. Polysaccharides are important in natural waters because they may destabilize inorganic colloids via flocculation [2], induce the formation of biofilms [3], aggregates [4] and complex trace metals [5–7]. The behavior of polysaccharides depends on their macromolecular structure, varying from totally linear (cellulose) to strongly branched compounds (e.g. spherocolloide like amylopectin or gum arabic). Due to minimal steric hindrances, the former have the tendency to form water insoluble thick fibers, while the latter are usually highly hydrated (hydrocolloids) and form gels that can contain up to 99.5% water [8]. Furthermore, the hydroxyl groups of polysaccharides tend to interact with the hydrated surfaces of dispersed mineral particles (oxyhydroxides) and with hydrophilic organic material by hydrogen bonding to form biofilms on submerged surfaces [9, 10]. Polysaccharides are also used in water treatment plants for the removal of undesirable substances by cross linking, micro gel formation and flocculation.

Due to the number of environmental processes in which polysaccharides are involved, development of analytical methods for their determination is an increasingly important research area. However, despite extensive efforts, carbohydrate analysis still presents a major challenge, especially for complex natural samples. Several problems have thus far slowed the development of analytical techniques to quantify the polysaccharides. First, the concentration of dissolved sugars is very low in natural waters. Second, the extraction of carbohydrates from complex sample matrices is a tedious job. Third, the separation of many closely related carbohydrate compounds poses a problem. Fourth, the carbohydrate compounds generally do not possess chromophores which absorb appreciably at UV–VIS wavelengths and therefore are not readily detected by conventional detectors. Most techniques to determine carbohydrates in environmental samples require a preliminary chemical hydrolytic procedure to convert polysaccharides into monosaccharides or smaller molecules prior to detection. The results obtained from the hydrolysis techniques themselves are highly variable, depending on the nature of the polysaccharides.

Capillary electrophoresis (CE) is a powerful technique that should in principal allow the simultaneous identification and quantification of carbohydrate of similar structure. CE uses only small amounts of samples and does not require purification prior to analysis. However, CE does not provide information on the polysaccharide structure. Secondly, since many carbohydrates carry no electrical charge and have a large number of hydroxyl groups, the electrophoretic procedures often need to be adapted. Keeping in view all these artifacts, the following studies were planned:

(a) Optimization of hydrolysis conditions for natural lake polysaccharides.
(b) Development of an analytical technique that gives a good separation of closely related monosaccharides, required for the identification of the polysaccharides.
(c) Application of the method to natural water samples.

2. Experimental

Analytical grade standards (>99% purity), were obtained either from Fluka or Sigma: D(+)-glucose, D(+)-mannose, D(+)-galactose D(+)-glucuronic acid, γ-lactone, D(+) xylose, sucrose, fructose, B-lactose, xanthan gum, cellulose (fibrous, medium), polygalacturonic acid (sodium polypectate 85–90%), α-Fe_2O_3,

2,4,6-Tripyridyl-s-triazine (98% sigma), potassium ferricyanide, ferric chloride (anhydrous), 1-aminopyrine-3,6,8-trisulfonic acid (APTS), sodium cyano borohydride. Fulvic acid, humic acid and peat humic acid were purchased from the International Humic Substances Society (IHSS). All dextran standards used (9.9 kD, 40 kD, 464 kD) were produced by *Leuconostoc mesenteroides*.

2.1 *Sample collection and preparation*

Water samples were collected from the lake Geneva in March 2001 at site SHL2 (figure 1) from 0, 5, 10, 20, 50, 100, 200, 290, 309 m depth. Lake Geneva covers an area of 7975 km^2 with an approximate volume of 89 km^3. The depth of lake varies at various points with an average depth of 153 m [11]. The maximum depth of the lake 309.7 m, is at point SHL2. Water samples were centrifuged at 4000 rpm ($3700 \times g$) for one hour (Heraeus Sepatech, Omnifug 2.0 RS) to remove suspended particles prior to storage at $-20°C$. The frozen samples were lyophilized and the powder obtained was stored in acid-washed polypropylene tubes at $-20°C$. The powder was pulverized with a clean glass rod to obtain a homogeneous sample for analysis. Demineralized water used for analysis was further purified through a Millipore water purification system (Milli-Q plus 185) equipped with a UV lamp. The purified water had a resistivity of $>18\,M\Omega$ and a total organic carbon content of $<5\,\mu g\,C\,L^{-1}$. All glassware and polymer bottles used in analysis were soaked for 24 h in methanol saturated with NaOH, followed by 24 h in each of 10% HNO_3 and 10% HCl. Finally, all labware were rinsed with copious amounts of Milli-Q water to completely remove any remaining cleaning agents.

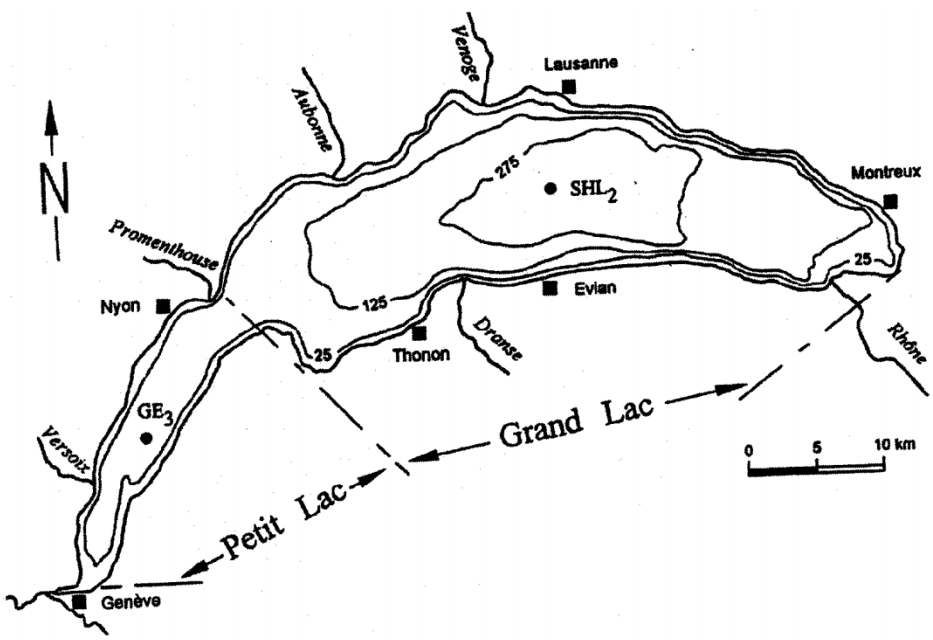

Figure 1. Sample collection site (SHL2) of lake Geneva.

In order to standardize the hydrolysis conditions for natural lake polysaccharides, two method of hydrolysis were evaluated, one using HCl and the other using H_2SO_4.

2.2 *HCl hydrolysis*

For hydrolysis with HCl, 0.0 to 1.0 mL of 1000 mg L^{-1} of 40 kD dextran was pipetted into teflon bombs in triplicate prior to the addition of 0.4 mL of 1.0 M HCl. The samples were diluted appropriately to 0.1 M HCl. Hydrolysis was carried out at 100°C for 24 h and the cool hydrolysate was neutralized to pH 8–9 by 1 M NaOH [12].

2.3 *Modified H_2SO_4 hydrolysis*

For the hydrolysis with sulfuric acid, 0.0 to 1.0 mL of 1000 mg L^{-1} of 40 kD dextran solution was pipetted into polypropylene tubes in triplicate. The concentrated H_2SO_4 and tubes containing the solutions were placed in an ice bath for half an hour. Two milliliters of cold concentrated H_2SO_4 were slowly added along the walls of test tubes to avoid caramelization of the carbohydrates. The samples were diluted appropriately with Milli-Q water to achieve a final acid concentration of 12 M. In this manner, prehydrolysis was performed for two hours at room temperature with 12 M H_2SO_4. From these solutions, 0.5 mL was sampled in teflon bombs, diluted 10 times with Milli-Q water and hydrolysed for 3 h at 100°C. The cool hydrolysate was neutralized by adding 2 M NaOH to pH 8–9 [13].

2.4 *Efficiency of hydrolysis*

The efficiency of the hydrolysis was evaluated using a spectrophotometric reaction. Reduced polysaccharides were subjected to oxidation at an alkaline pH in which Fe(III) was reduced to Fe(II). The reduced iron was then allowed to react with reagent 2,4,6-tripyridyl-s-triazine (TPTZ) to give a strongly colored $Fe(TPTZ)_2^{2+}$ complex [14]. A standard curve was prepared by measuring absorbance at 595 nm for 0, 2, 4, 6 and 10 mg L^{-1} of glucose solution. The stock solution was prepared from D-glucose dried under vacuum for 24 h.

2.5 *Derivatization with APTS*

The derivatization procedure for the monosaccharides was adapted from the method described by O'Shea [15]. Briefly, 0, 2, 4, 6 and 10 μL of a 500 μM glucose solution was mixed with 5 μL of 0.02 M APTS in 15% acetic acid and 5 μL of freshly prepared 1 M aqueous sodium cyano borohydride in a 500 μL Eppendorf micro centrifuge tube. The volume was made up to 20 μL with Milli-Q water. The reaction tube was centrifuged and incubated at 37°C, stirring at 70 rpm for 15 h. The samples were diluted appropriately with Milli-Q water for analysis. After dilution, samples were stored at −80°C.

2.6 *Measurement of fluorescence spectra*

Excitation and emission spectra of the APTS and its sugar derivatives (50 nM) were measured using a Jasco FP-750 Spectrofluorometer.

2.7 *Capillary electrophoresis*

Capillary electrophoretic separations were performed on a P/ACE Model 2100 coupled with an Argon Ion Laser using a laser intensity of 30 mW.

2.7.1 Silica capillary. A capillary column, 50 cm in length with an internal diameter of 100 μm, was assembled in the P/ACE cartridge equipped with an ellipsoidal mirror to collect fluorescence. The separation was performed at 26 kV. The instrumental setup was configured so that sample introduction took place at the anode and sample detection at the cathode. The buffer employed was 10 mM $Na_2CO_3/NaHCO_3$ adjusted to pH 10.5. The capillary was washed with Milli-Q water for 2 min, with 0.1 M NaOH for 1 min and with the running buffer for 1 min prior to sample injection. The sample was injected using a 10 s pressure injection (0.5 psi) and separation was carried out for 10 min. The buffer solution was changed after a maximum of 5–6 runs to avoid any pH changes, which might have occurred during electrophoresis.

2.7.2 Gel capillary. An e-cap N-CHO neutral capillary and carbohydrate separation gel buffer was also purchased from Beckman Coulter, Switzerland. The capillary was 50 cm in length with a 50 μm inner diameter. The sample was injected at the cathode for 10 s and separation was carried out for 15 min at 15 kV with detection at the anode. Data were analyzed with the statistical package origin.

3. Results and discussions

3.1 *Comparison between H_2SO_4 and HCl hydrolysis*

The calibration curve for D-glucose in Milli-Q-water indicated that relationship between absorbance of the TPTZ derivative and concentration of glucose was linear ($r^2 = 0.998$) and the results were reproducible ($P < 0.001$). In order to choose an appropriate acid hydrolysis condition, the 0.1 M HCl hydrolysis [12] was compared to the two step H_2SO_4 hydrolysis method [13]. Standard curves indicated no significant difference in the absorbance of unhydrolyzed ($r^2 = 0.999$) and 0.1 M HCl hydrolyzed ($r^2 = 0.998$) glucose solutions over a concentration range of 0 to 10 mg L^{-1}, but absorbance was significantly decreased ($P < 0.0001$, $N = 10$) following H_2SO_4 hydrolysis (table 1) as could be judged also from the 1.3 ± 0.2 times decrease in slope. It has been reported earlier [13] that sulfuric acid hydrolysis appreciably reduced the absorbance of standard carbohydrates monomers but no attempts have been made so far to improve it.

The HCl method was found least effective for the hydrolysis of commercially available 40 kD dextran (table 1), while the H_2SO_4 method appeared to be more effective in hydrolyzing higher molecular weight polysaccharides. Nonetheless, the absorbance signal obtained due to the reaction of monomers with TPTZ was less than the theoretical value obtained for the depolymerization of 40 kD dextran. (Note that the molar mass of a glucose unit is 180 while the D-glucosyl units each have a molar mass of 162, i.e. 10 mg L^{-1} glucose have 5.5×10^{-5} glucose units while 10 mg L^{-1} of 40 kD dextran should have 6.17×10^{-5} glucose equivalent.) During preliminary experiments, a slight coloration was observed during the 12 M H_2SO_4

Table 1. Comparison of hydrolysis of monsaccharide (glucose) and polysaccharide (40 kD and 464 kD dextran) with 0.1 M HCl and 1.2 M H_2SO_4 methods and reproducibility of results ($N = 10$).

	Glucose (mg L^{-1})		40 kD dextran (mg L^{-1})		464 kD dextran (mg L^{-1})	
	Slope	Intercept	Slope	Intercept	Slope	Intercept
Aqueous*						
Rep. 1	0.0917 ± 0.001	0.0139	–	–	–	–
Rep. 2	0.0949 ± 0.001	0.0018	–	–	–	–
HCl						
Rep.1	0.1063 ± 0.005	0.0041	0.0505 ± 0.0006	0.0013	–	–
Rep.2	0.0951 ± 0.002	0.0049	–	–	–	–
H_2SO_4						
Rep. 1	0.0697 ± 0.002	0.0058	0.0736 ± 0.0008	0.0027	0.0713 ± 0.0013	0.0091
Rep.2	0.0721 ± 0.001	0.0073	–	–	0.0688 ± 0.0019	0.0064

Rep. = replicate. (*Results for standard glucose curve are also given for comparison).

pre hydrolysis treatment which was hypothesized to be due to the caramelization of carbohydrates in the concentrated acid. Therefore the original method of Pakulski and Benner [13] was slightly modified. A chilled concentrated sulfuric acid was added to a chilled solution of saccharides in an ice bath to obtain the 12 M solution of H_2SO_4 for pre hydrolysis. After the addition of acid, pre hydrolysis was performed at room temperature. The method greatly improved the glucose calibration curve. The absorbance of both hydrolyzed ($r^2 = 0.998$) and unhydrolyzed ($r^2 = 0.999$) monomeric standard D-glucose indicated that it could be used as an acceptable standard for a wide variety of carbohydrates present in natural water samples. To compare the absorbance yields of the mono saccharides with those obtained from the corresponding polysaccharides, 9.9 kD, 40 kD and 464 kD dextran were hydrolyzed under similar conditions and results were presented in terms of glucose equivalents (figure 2). The data were in fair agreement with the theoretical values. Borch and Kirchmann [16] also noted during Sub arctic Pacific water samples analysis that the two step H_2SO_4 method gave lower concentrations, probably because of destruction of the liberated monosaccharides with more concentrated acid. Keeping in view all these artifacts, it was necessary to standardize the hydrolysis conditions with model polysaccharide.

3.2 *Effect of time on the hydrolysis of commercial and natural samples*

In the present study, the absorbance versus hydrolysis time was examined for solutions containing 10 mg L^{-1} of 464 kD dextran in order to optimize the hydrolysis conditions (figure 3a). The data indicated that maximum absorbance could be achieved after a 120 min pre hydrolysis time followed by a 180 min hydrolysis at 100°C with 1.2 M H_2SO_4. Similarly the hydrolysis time was varied while keeping the pre hydrolysis time constant (120 min). In that case, hydrolysis for 120 min was sufficient to generate a maximum absorbance and continued hydrolysis for four hours did not increase the absorbance significantly. The absorbance value at the plateau was in reasonable agreement with the theoretical absorbance for a dextran completely hydrolyzed into glucose. Hydrolysis conditions were also optimized for lake water samples (figure 3b). A plateau was achieved after 180 min of hydrolysis instead of 120 min as was the case for the 464 kD dextran. There was a significant ($P < 0.0001$, $N = 4$) increase

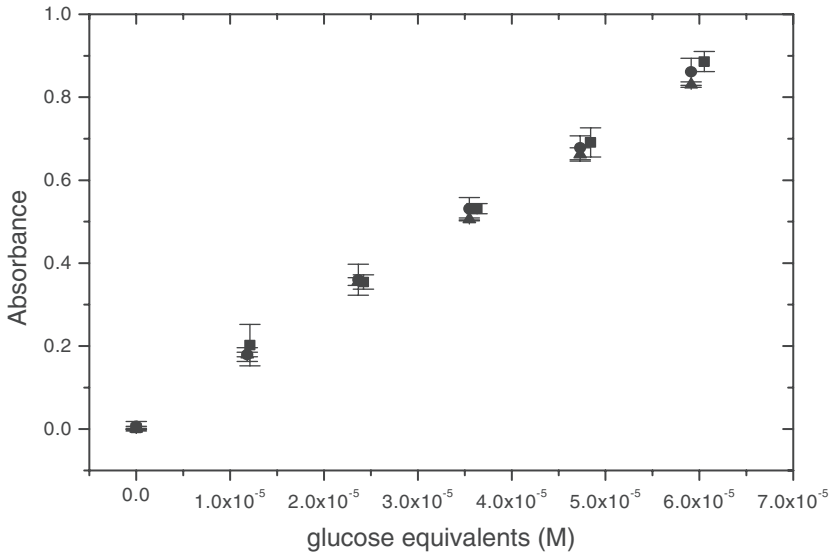

Figure 2. Monomers (glucose equivalents) of various polysaccharides obtained after H_2SO_4 hydrolysis: 9.9 kD (▲), 40 kD (●), and 464 kD (■) dextrans.

in absorbance after 120 min of hydrolysis at 100°C. A higher yield of hexoses, pentoses and fructose in water and sediments from the Black Sea and North Sea has been observed previously [17] using a 12 M H_2SO_4 pretreatment method. The higher yield was attributed to swelling of polysaccharides in the strong acid and partial cleavage of glycosidic bonds which greatly enhanced the effectiveness of subsequent hydrolysis step. The pretreatment technique was found especially advantageous when terrigenous plant debris, e.g. cellulose was present in the sample.

In order to understand the behavior of natural samples, other standards such as xanthan, cellulose and poly galacturonic acid, which may have similar characteristics to polysaccharides that are found in natural waters, were used to check the efficiency of the sulfuric acid hydrolysis. The absorbance of the cellulose standards in the concentration range of 1 to 10 mg L^{-1} were similar to the absorbance of an equal concentration of hydrolyzed glucose indicating that nearly complete hydrolysis had occurred (figure 4). These results were in agreement with other studies that showed that sulfuric acid pretreatment and hydrolysis was more effective in hydrolyzing structural poly saccharides [13]. In case of poly galacturonic acid, a significantly lower ($P < 0.0001$, $N = 10$, Student t-test) absorbance was observed as compared to the glucose standard. It had been reported that polymers containing uronic acids resisted acid hydrolysis because the carboxylic acid moiety stabilized the glycosidic linkage [18–20]. Therefore, the hydrolysis of the glycosidic linkages of uronic acid residues in polysaccharides could be considered to be the major limitation to the accurate sugar analysis of these polysaccharides. The calibration curve for xanthan in the concentration range 1 to 10 mg L^{-1} had a significantly higher ($P < 0.001$, $N = 10$) absorbance than polygalacturonic acid but significantly lower values were observed in comparison to the glucose standard. Lower values for xanthan in comparison to dextran have been reported using 0.1 M HCl hydrolysis [21] in which case the reduction was attributed to the presence of acetate, pyruvate, mannose and galacturonic acid

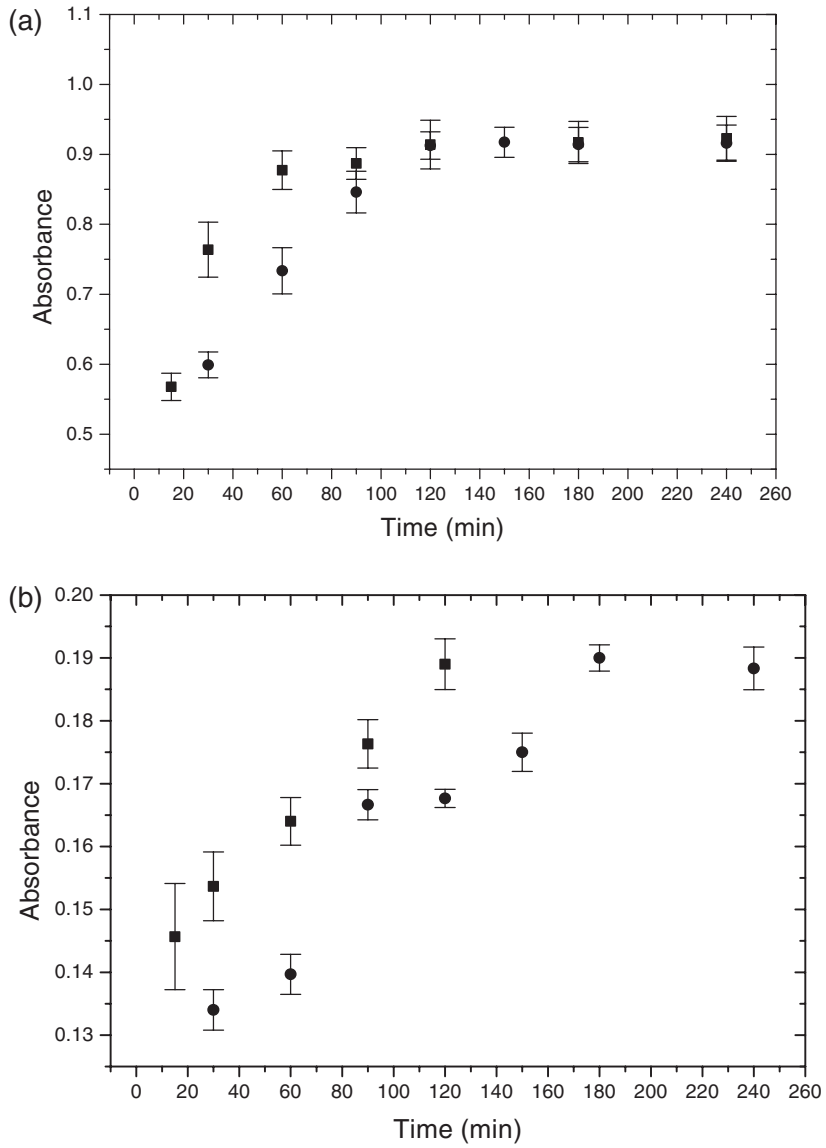

Figure 3. (a) Hydrolysis of the 464 kD dextran as a function of pre-hydrolysis (■) and hydrolysis time (●), (b) Effect of pre hydrolysis (■) and hydrolysis (●) time on lake samples.

in the polysaccharide. Non-reducing sugars, sugar alcohols, some amino acids, dicarboxylic acids and aldehydes after hydrolysis with 0.1 M HCl did not yield significant absorbance by TPTZ method [14].

3.3 *Potential interferences*

To study the possible interferences of other naturally occurring colloidal substances with the spectrophotometric method, the interaction of a fulvic acid (FA), a peat

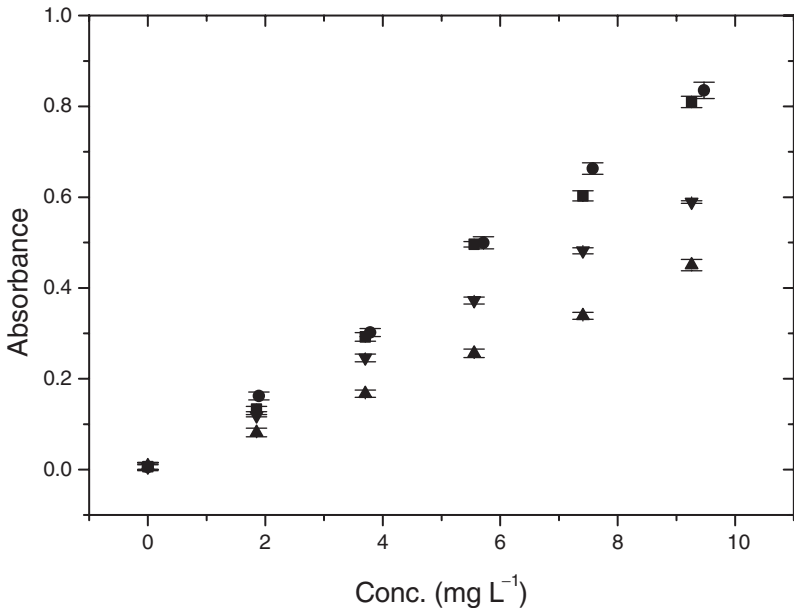

Figure 4. Comparison of absorbance of other polysaccharides with glucose (hydrolysed) standard as a function of concentration, cellulose (■), glucose (●), polygalacturonic acid (▲), xanthan gum (▼).

humic acid (PHA) and a Fe_2O_3 colloid were examined with respect to the hydrolysis of a 464 kD dextran. The inclusion of Fe in the study was based on its ubiquity, its likely interaction with organic material and its potential ability to interfere with the TPTZ assay. Humic substances were included in the study because it has been established by ^{1}H and ^{13}C NMR spectroscopic studies of natural humic substances [22, 23] and ^{13}C-enriched methylated derivatives of humic substances [24] that much of the carbon in humic substances is aliphatic and that many of the aliphatic carbons are singly bound to oxygen implying the presence of sugars or closely related structural moieties. In this case, the data indicated a significant ($P < 0.05$, $N = 14$, ANOVA) increase in absorbance due to the presence of both the FA and PHA. Note that the intercept (0.59 ± 0.003) corresponded to the absorbance due to $5\,mg\,L^{-1}$ of dextran (figure 5). The comparison in the increase of absorbance signal due to FA and PHA indicated that both might contribute saccharides either during the acid hydrolysis or directly forming a complex with TPTZ without cleavage of saccharide entities in the complex. Fe_2O_3 also contributed significantly to the absorbance of 464 kD dextran ($P < 0.05$, $N = 14$, ANOVA) in the concentration range of 1.75 to $7.01\,mg\,L^{-1}$ but less than for FA. It is certainly possible that most carbohydrates are recycled in the biosphere by the action of micro organisms. Therefore, the probability that humic substances contain sugar as structural components is quite high, considering the very high carbohydrate content of the biomass from which they are derived. Total dissolved sugar contents correlated well to humic carbon, estimated by the absorbance measured at 420 nm and pH 10 in the Williamson River, Oregon [25]. Correlation coefficients of 0.89, 0.80, 0.88 and 0.97 over the four month period were observed implying that dissolved sugars were likely associated with humic substances. Nonetheless, elemental and ^{13}C NMR spectra of water samples collected from two mesotrophic lakes indicated that

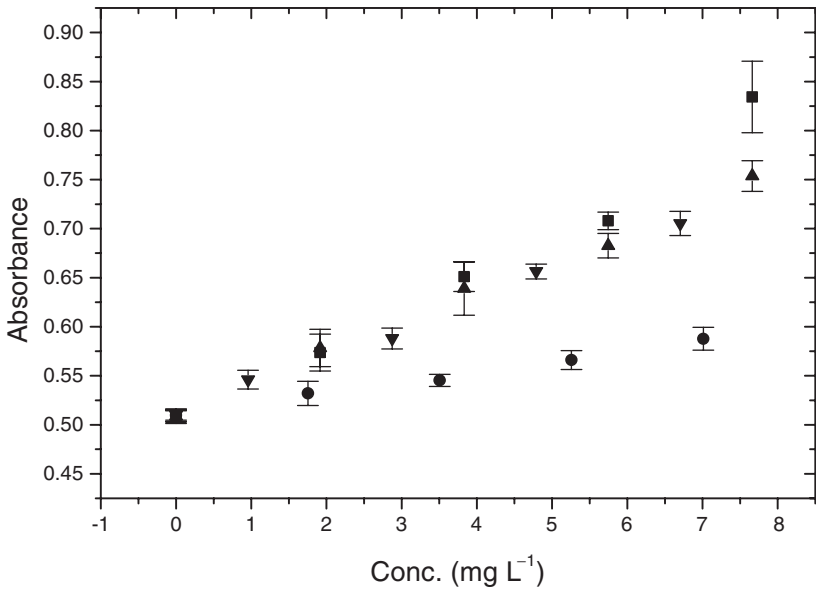

Figure 5. Comparison of the increase in absorbance as a function of concentration, FA (■), hematite (●), PHA (▲), hematite and FA (▼).

polysaccharides make up $< 15\%$ of the total carbon in fulvic acids and humic acids [26]. Another spectrophotometric procedure (phenol sulfuric acid assay) has also been shown to overestimate the total sugar contents in sea water mainly because of its reaction with humus and some organics other than sugars [27, 28]. The significant increase in the absorbance of dextran as a function of the hematite concentration might be due to the reduction of already existing iron at alkaline pH when the reagents were heated in a boiling water bath for TPTZ assay. It was reported that Fe(II) may interfere with TPTZ assay if present in significant amounts [14]. A significant decrease ($r^2 = 0.996$, $P < 0.05$) in the absorbance signal was observed due to increase in FA contents (0.957–6.71 mg L^{-1}) when both the dextran (5 mg L^{-1}) and hematite concentration (12.69 mg L^{-1}) were kept constant. This suggested a potential interaction of hematite with FA. It has been observed by using contrast tuning transmission electron microscopy (CT-TEM) technique that mineral micro particles (clays, SiO_2, FeOOH) interact strongly with the extra cellular polysaccharide networks in natural waters [29]. A cationic bridging mechanism was suggested in which multivalent metal cations complex with organic functional groups (e.g. COO^-) of microbial extra cellular polysaccharides [30, 31].

4. Capillary electrophoresis

4.1 *Reductive amination of sugars with APTS*

The primary amine of the fluorescent tag (APTS) and the C-1 aldehyde of the reducing sugar react to form a Schiff base, which can be reduced to the mixed aryl/aliphatic

secondary amine by sodium cyanoborohydride. An equilibrium between the hemiacetal and the aldehydic form of the sugar occurs spontaneously when the sugar is in solution [32, 33]. We adapted the derivatization with APTS because it imparts both electric charge and fluorescence to the saccharides. The three sulphonic acid groups in the APTS moiety are negatively charged over a wide pH range and the concurrently high electrophoretic mobility of the derivatized carbohydrates facilitates their rapid separation. The APTS derivatives can be detected both with UV and laser induced fluorescence detection [34, 35].

4.2 *Excitation and emission spectra*

The excitation and emission spectra were obtained from a diluted reaction mixture (50 nM) of APTS and APTS-saccharide adduct. The maximum excitation wavelength λ_{max} of APTS in milli-Q water was 463 nm with a nominal excitation intensity whereas the λ_{max} (422 nm) of APTS adduct with glucose, galacturonic acid and lactose had excitation intensities of 438, 392 and 442 respectively (figure 6a). The emission maxima λ_{max} of the APTS and APTS sugar derivatives were 514 and 501 respectively (figure 6b). The emission intensities of glucose, glucuronic acid and lactose were 312, 270 and 315 at excitation wavelength of 480 nm. The emission intensity of APTS (100 Arbitrary Units) was much lower than APTS-sugar derivatives at this wavelength. In the original method of O'Shea [15], 0.2M APTS was used and dilutions were carried out with an electrophoresis sample buffer consisting of 6 M urea in 0.04 M boric acid and 0.04 M tris (hydroxy methyl) amino methane (pH 8.6), whereas in the present study 0.02 M APTS was used and dilutions were carried out with Milli-Q water. These modifications required the standardization of complete experimental procedure.

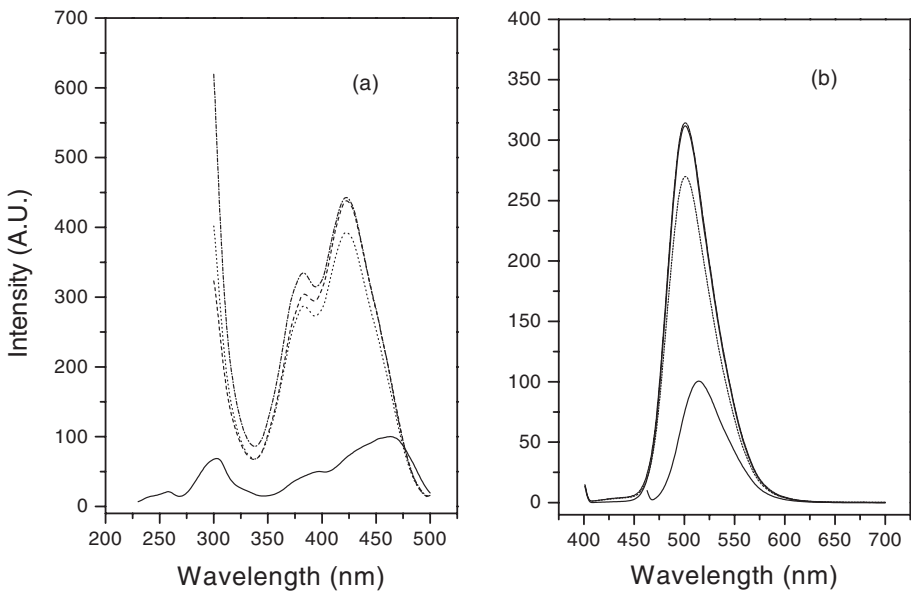

Figure 6. UV/Visible excitation (a) and emission (b) spectra of APTS (—) and its adduct with glucose (– –), galacturonic acid (– – –), lactose (– · – ·), AU = arbitrary units.

4.3 *Labeling efficiency*

For the precise determination of carbohydrates, the labeling and detection system must both be quantitative. In our study, APTS content in the reaction mixture was varied, keeping the glucose concentration constant (50 μM). The choice of this concentration was based on the observation that the maximum amount of carbohydrates never exceeded 50 μM in lakes samples, as determined by the TPTZ assay. The reaction mixture was diluted 100 times before analysis. There were no differences observed in the labeling efficiency of APTS over the concentration range of 2 to 15 mM (figure 7a). In the original method of O'Shea, oligosaccharides with degree of

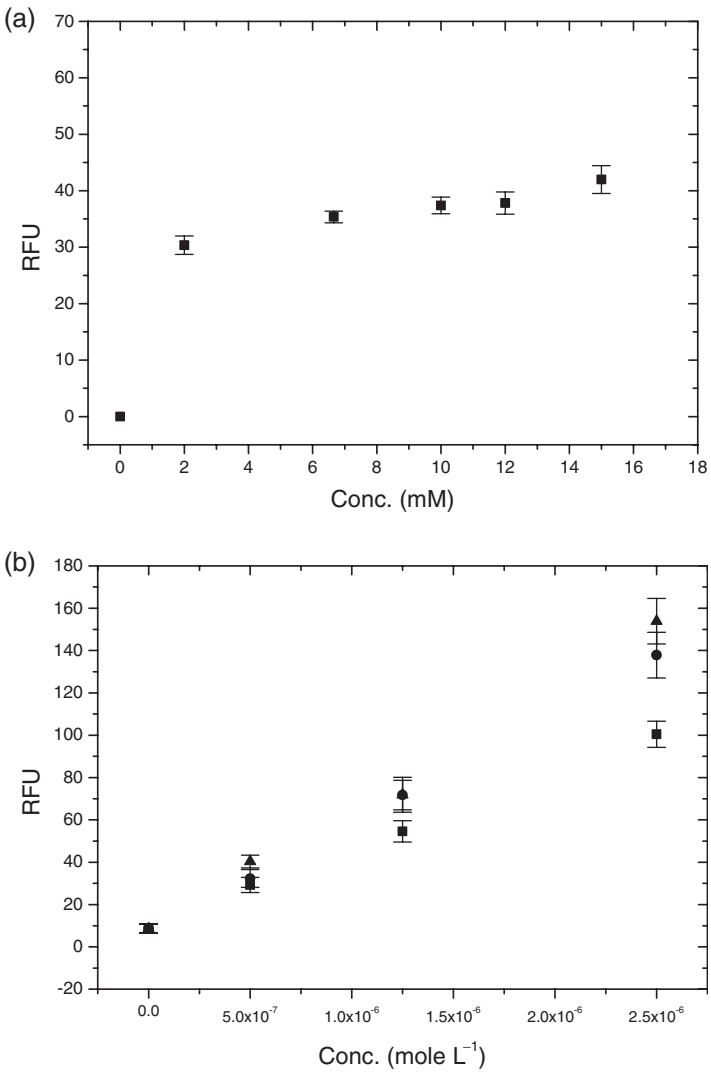

Figure 7. (a) Effect of the APTS concentration in the reaction mixture on the labeling efficiency (37°C, 15 h incubation), (b) Laser induced fluorescence detection linearity of APTS-sugar concentration, glucose (■), fructose (●), xylose (▲).

polymerization between 3 and 135 were labeled with 0.2 M APTS which may have required an excess fluorophore in the reaction mixture but in case of sterically unencumbered monosaccharides, the labeling efficiency of reductive amination seemed to be quantitive with 0.02 M APTS.

Based on these observations we used 5 mM APTS for labeling in subsequent experiments. The concentration of glucose was also varied from 50 μM to 0.25 mM in the reaction mixture in the presence of 5 mM APTS to check the labeling efficiency of the reaction. The samples were diluted 100 times before the analysis, the fluorescence signal increased linearly ($r^2 = 0.987$) with an increase in glucose concentration from 0.5 to 2.5 μM (figure 7). Using ^{14}C labeled saccharides it has been shown that the labeling efficiency is >95% for glucose-ANTS [36] and >97% for APTS-N-acetylglucosamine [37].

4.4 *Labeling selectivity*

One of the most important characteristics of carbohydrates is the labeling selectivity, meaning that under identical derivatization conditions different carbohydrate structures may yield different labeling efficiencies. This possibility of selective labeling led us to investigate the labeling reaction of various monosaccharides with APTS. The CE-LIF signals were linearly proportional to the amounts of sugar derivatized with correlation coefficients (r^2) greater than 0.97 (table 2). A linear increase in the LIF signal intensity for fucose, galactose, glucose, xylose and N-acetyl-glucosamine with APTS has already been reported in the picomole range [37]. In this study, a highly significant increase in fluorescence intensity was observed for mannose ($P = 0.0017$), galactose ($P = 0.003$), glucuronic acid ($P = 0.0056$) and xylose ($P = 0.003$, student t-test) as compared to glucose. The increased labeling efficiency might be due to the excess of aldehyde formation as compared to the hemiacetal form of the reducing sugar when the saccharides were dissolved in water [37]. Indeed differences in derivatization yield among the monosaccharides derivatized at equimolar amounts in a mixture have been reported for a reductive amination with 2-aminopyridine (mannose > galactose > L-fucose > N-acetyl-D-galactosamine > N-acetyl-D-glucosamin) [38].

40 kD dextran and the disaccharides lactose and sucrose were also derivatized with APTS following hydrolysis with sulfuric acid. There were no significant differences observed in the APTS labeling efficiency when the results were compared in terms of glucose equivalents for the 40 kD dextran (table 2). A significant increase in the labeling

Table 2. Observed slopes and statistical analysis ($N = 4$, student t-test) of APTS derivatives of mono, di- and polysaccharides by CE-LIF.

	(Slope ± error) × 10^{-7} (M)	Intercept	r^2	P
Glucose	3.479 ± 0.28	7.771	0.987	0.00645
Fructose	3.480 ± 0.10	8.313	0.991	0.00422
Xylose	5.728 ± 0.35	9.265	0.992	0.00375
Glucuronic acid	6.408 ± 0.62	32.403	0.981	0.00938
Mannose	12.72 ± 0.69	32.043	0.965	0.01733
Galactose	8.764 ± 0.30	7.718	0.989	0.00513
Dextran 40 kD	4.254 ± 0.38	2.552	0.976	0.0015
Lactose	3.819 ± 0.34	3.564	0.975	0.0016
Sucrose	1.808 ± 0.05	5.425	0.997	<0.0001

efficiency was observed for hydrolyzed lactose ($P = 0.009$, $N = 6$) with respect to the unhydrolyzed glucose whereas sucrose showed a highly significant ($P = 0.0001$, $N = 6$) decrease. The increase in labeling efficiency of the lactose after hydrolysis might be due to the fact that a ring opening and Schiff base formation are facilitated as both these steps are acid catalyzed [39]. Derivatization in the presence of organic acids of higher strengths than acetic acid produced substantially higher yields of APTS-sugar adducts [34]. This effect was most pronounced for N-acetylamino sugars. Optimum yields were obtained using citric acid (pK_a 3.1).

4.5 *Characterization of the APTS-saccharide complex*

The separation efficiency of the APTS-saccharide complex was verified using equimolar concentrations (0.125 μM) of monosaccharide standards. In case of the separation performed in the silica capillary, no peak resolution was observed, rather, one sharp peak was found in the electropherograms of various mixtures. The observed fluorescence was in reasonable agreement with the theoretical values calculated by summing contributions from each individual component (table 3). The poor resolution of the monosaccharides in the silica capillary might be due to an increase in electro-osmotic flow, because a higher electrolyte pH produces an increase in the ionization of silanol groups on the surface of fused-silica capillary. A better separation by CE-LIF of monomers liberated from glycoproteins and oligosaccharides, labeled with APTS and separated in 25 mM borate buffer at pH 10 has already been reported [40]. The increased separation was attributed to the interaction of cis diol hydroxyl groups in the carbohydrates with the borate ions.

4.6 *Interaction of humic substances*

It was shown by the TPTZ assay of reducing sugars that humic substances could increase the absorbance signal likely due to the contribution of some saccharide content during the sulfuric acid hydrolysis. In order to verify this effect, 24.7 μg L^{-1} of 40 kD dextran was added to 28.8 μg L^{-1} of fulvic acid, 29.9 μg L^{-1} of humic acid and 30.5 μg L^{-1} of peat humic acid. These concentrations were present in the APTS reaction mixture and the fluorescence was measured after 200 times dilution of the mixtures with milli-Q water. No increase in the APTS-saccharide content (peak 1) was observed in the presence of fulvic and peat humic acid but a slight increase in the sacchride peak was detected in the presence of humic acid (figure 8). It was quite obvious from the

Table 3. The difference in observed and theoretical fluorescence signal.

	Observed (RFU)	Theoretical (RFU)	Difference
Glu. + Fru.	64.293	59.56	+4.73
Glu. + Gl. Acid	91.274	101.68	−10.41
Glu. + Man.	161.951	140.77	+21.18
Glu. + Xyl.	89.616	74.29	+15.32
Glu. + Glt.	104.26	91.72	+12.54
Mixture*	265.179	268.69	−3.51

Glu. = glucose; Fru. = fructose; Gl. Acid = glucuronic acid; Man. = mannose; Xyl. = xylose; Glt. = Galactose, *Mixture = mixture of five monomers, RFU= relative fluorescence unit.

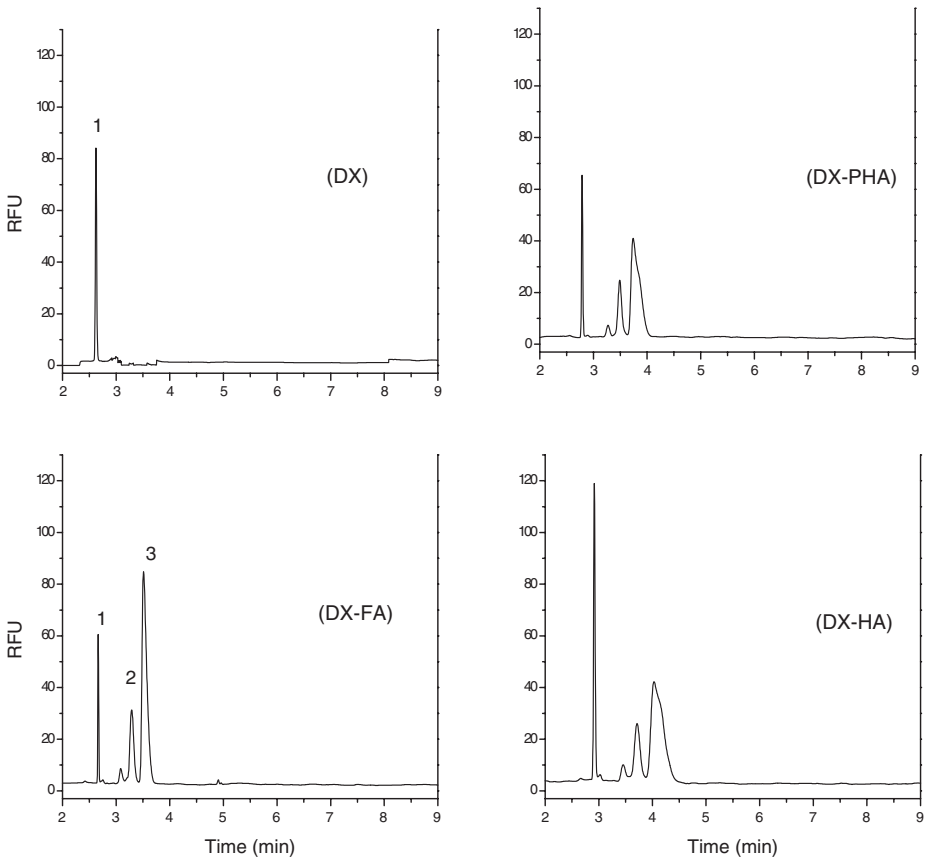

Figure 8. Effect of humic substances on the fluorescence spectra of hydrolysed 40 kD dextran using silica capillary. Separation potential 26 kV, detection at cathode, 10 mM carbonate buffer. 1 = APTS-saccharide peak, peak 2 & 3 = humic peaks; Dx = dextran, FA = fulvic acid, PHA = peat humic acid, HA = humic acid, RFU = relative fluorescence unit.

electropherograms of fulvic, humic and peat humic acids in the presence of 40 kD dextran that they did not interfere with APTS-saccharide peak at this concentration. These results indicated the possibility to use silica capillary for total saccharide analysis without any interference from humic substances. Although we observed a significant increase in saccharide content during the TPTZ assay, it has been reported previously [41] that another spectrophotometric method (phenol sulfuric acid assay) overestimated the total sugar contents in sea water mainly because of its reaction with humus and some organics other than sugars. These electropherograms also demonstrated the stability of relative migration times.

4.7 *Capillary gel-electrophoresis*

Following another ten times dilution (i.e., 1000 times dilution of the original mixture) the above mixtures were used to monitor the separation efficiency of the carbohydrate-APTS adduct using the e-cap N-CHO neutral capillary with carbohydrate separation

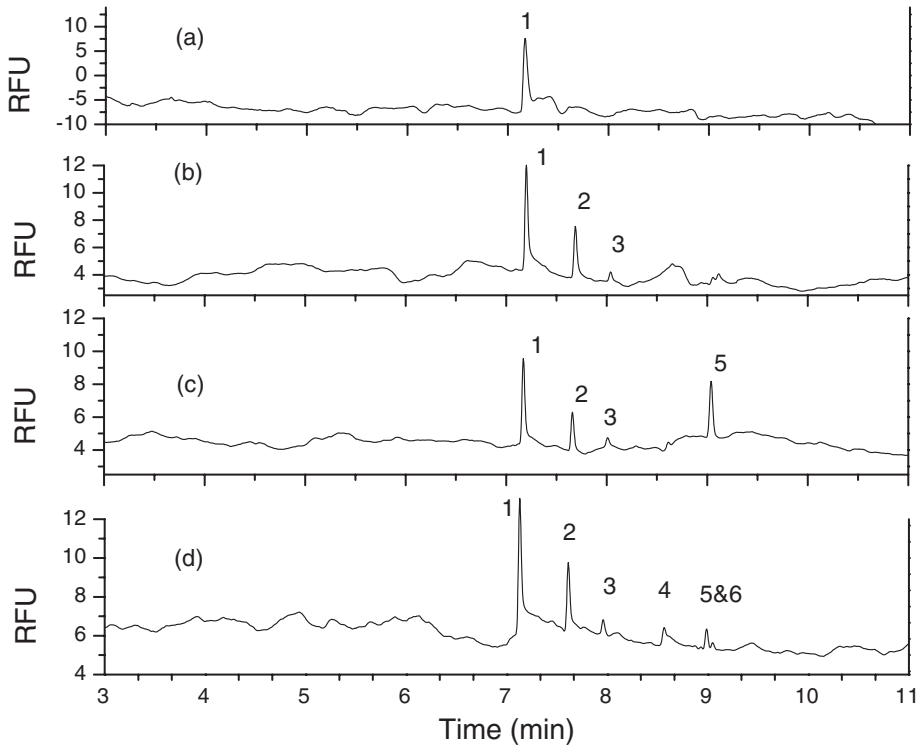

Figure 9. Capillary Gel Electrophoresis (CGE) separation of the APTS-labeled monosaccharides at 15 kV/ 8.653 μA (a) APTS blank, (b) mixture of glucose and fructose, (c) mixture of glucose, fructose, mannose (d) mixture of glucose, fructose, xylose, mannose, galactose. Peak identification, 1 = APTS, 2 = glucose, 3 = fructose, 4 = xylose, 5 & 6 = mannose and galactose.

gel buffer (pH = 4.90) and detection at the anode. Figure 9 depicts capillary gel electrophoresis (CGE) separations of various mixtures of monosaccharides. It was quite apparent that higher separation efficiency could be achieved by CGE using these separation conditions. The better resolution in the gel-packed capillary might be due to the negligible electro-osmotic flow such that the APTS-sugar adducts could have migrated to the anode solely by electrophoresis. The high separation efficiency observed in polyacrylamide gel-filled capillaries has also been attributed to a sieving effect associated with the use of the highly concentrated cross-linked polyacrylamide gel in the capillary [42, 43]. The high column efficiency was due not only to the anticonvective effect of the gel, but also to the small amounts of sample introduced (at the attomole level). Three positional isomers of the mannose-7 and mannose-8 oligosaccharides were observed during a CGE separation of the APTS-labeled high mannose type oligosaccharides released from bovine ribonuclease using 25 mM acetate buffer [44].

Another major advantage of the gel filled capillary was the reproducibility of the migration times which could be judged easily from the electropherograms of glucose and mannose. These two saccharides were selected due to their large difference in migration times. The essence of the elecropherograms in figure 10 was that the relative migration time was constant and the increase in concentration of the APTS-saccharide adduct (200×, 500×, 1000× dilution of the same reaction mixture) did not affect the

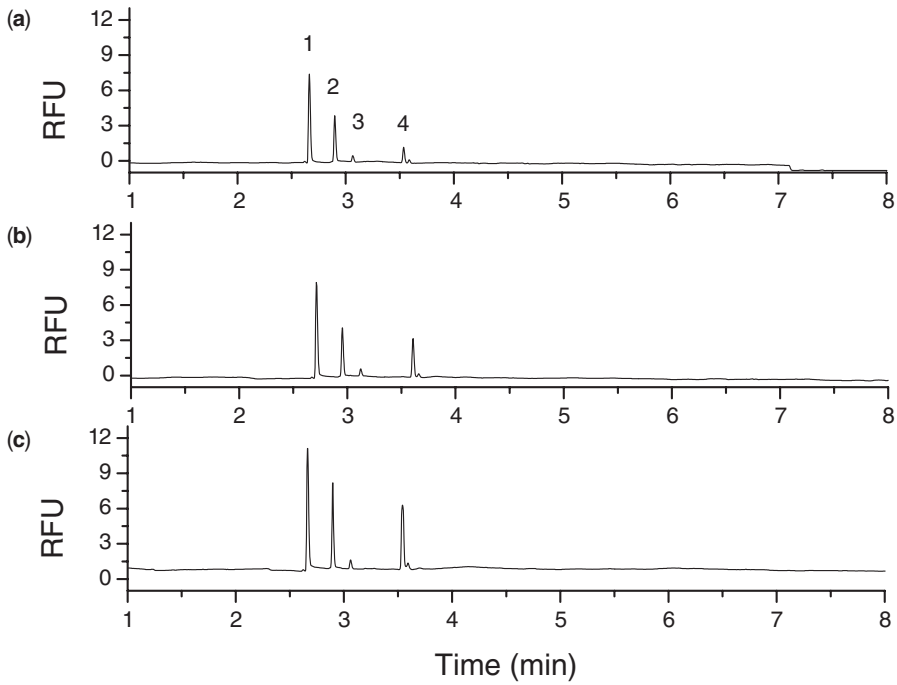

Figure 10. Reproducibility of the migration times in the CGE. Separation potential 26 kV. (a) 1000× dilution, (b). 500× dilution, (c) 300× dilution. Peak identification: 1 = APTS, 2 = glucose, 3 = impurity, 4 = mannose.

migration times. The coefficient of variation of migration time (C.V. = S.D./mean) for twelve replicates of the APTS-glucose samples was less than 2%. All of the experimental data suggested that gel-filled capillaries provided the best possible separation of labeled oligosaccharides and delivered reproducible good migration times. Twenty replicates of glucose, fructose and mannose in the concentration range of 0.5–2.5 nmole showed correlation coefficients (r^2) of 0.731, 0.704 and 0.685 respectively. These correlations were not as significant as obtained with silica capillary. This apparent discrepancy might be due to the fact that we standardized the derivatization and dilution steps using silica capillary that required more sample (180 nL injection volume) in comparison to silica gel cap (11 nL); implying the necessity to standardize the experimental setup using gel capillary. However, the present study indicated the potential of CGE for qualitative as well as quantitative analysis of hydrolyzed saccharides. Keeping in mind, that extremely small amount of sample is required for analysis in CGE, derivatization and dilution steps needs to be explored further.

5. Application to natural water samples

5.1 *Profile of mono and polysaccharides*

The concentration of mono- and polysaccharides in lake water samples were measured using the spectrophotometric method and capillary electrophoresis coupled with

Table 4. Concentration profile of monosaccharides (MS) and polysaccharides (PS-TPTZ) measured by TPTZ assay, CE-LIF method (PS-CE), total organic carbon (TOC) and total saccharides (TS*). Total saccharides were calculated by combining fluorescent signals of polysaccharide and saccharide peaks of humic substances as determined by CE-LIF method.

Depth (m)	MS ($mg\,L^{-1}$)	PS-TPTZ ($mg\,L^{-1}$)	PS-CE ($mg\,L^{-1}$)	TS* ($mg\,L^{-1}$)	TOC ($mg\,c\,L^{-1}$)
0	0.24 ± 0.01	1.31 ± 0.06	1.02 ± 0.06	1.63 ± 0.05	1.19 ± 0.02
5	0.17 ± 0.02	0.91 ± 0.02	0.88 ± 0.05	1.41 ± 0.05	1.01 ± 0.03
10	0.19 ± 0.02	0.72 ± 0.03	0.62 ± 0.04	1.25 ± 0.05	0.92 ± 0.02
20	0.15 ± 0.01	0.58 ± 0.01	0.45 ± 0.03	0.96 ± 0.06	0.88 ± 0.03
50	0.13 ± 0.02	0.36 ± 0.02	0.27 ± 0.02	0.48 ± 0.02	0.69 ± 0.03
100	0.13 ± 0.01	0.36 ± 0.05	0.30 ± 0.06	0.59 ± 0.04	0.67 ± 0.01
200	0.15 ± 0.02	0.42 ± 0.04	0.36 ± 0.03	0.53 ± 0.05	0.96 ± 0.04
290	0.12 ± 0.01	0.36 ± 0.06	0.29 ± 0.04	0.65 ± 0.06	0.72 ± 0.05
309	0.13 ± 0.01	0.45 ± 0.02	0.37 ± 0.03	1.041 ± 0.04	0.86 ± 0.02

the laser induced fluorescence detection (CE-LIF) system. Both concentration profiles (table 4) indicated the presence of maximum saccharide content at the surface and a gradual decrease with depth. The higher concentration of carbohydrates at surface could be due to the release of dissolved carbohydrates from phytoplankton [16]. Myklestad [45] reported that the production of dissolved carbohydrates by phytoplankton and consumption by bacterioplankton varies over time, so that the measured concentration could be the net result of production and consumption processes, both of which vary in space and time. This observation could likely explain the decrease in the carbohydrate concentrations in waters below the euphotic zone and the small variation in deep waters for Lake Geneva as well.

At the bottom of the lake (309 m), an increase in polysaccharide content ($\cong$30%) was observed. This could be due to the bacterial hydrolysis of organic matter that contributes to the dissolved carbohydrate pools. Using fluorescently labeled polysaccharides extremely rapid rates of hydrolysis in surface sediments have been reported [46]. In addition, since carbohydrates comprise an important fraction of total organic matter in sediments and since they tend to accumulate at the surface of the bottom sediments, the concentration at lake bottom is often slightly higher due to the re suspension of sugars [47, 48].

Monosaccharides contents measured by the TPTZ assay and TOC contents of the hydrolysed samples followed a similar pattern (table 4). The rapid decline of sugar concentrations with depth suggested that dissolved sugar was a major bioreactive component of the dissolved organic carbon pool. Monosaccharide concentrations were greatest at the surface ($0.237\,mg\,L^{-1}$) and decreased to $0.120\,mg\,L^{-1}$. High concentrations of free monosaccharides at the surface were assumed to be due to significant autotrophic activity [49].

5.2 *Relationship of saccharides with total organic carbon (TOC)*

A good correlation ($r^2 = 0.88$, $N = 9$, $P < 0.005$) of TOC with polysaccharides measured by the TPTZ assay and moderately good correlation ($r^2 = 0.83$, $N = 9$, $P < 0.001$) between the TOC and APTS saccharide adduct measured by CE-LIF was observed. The correlation improved significantly ($r^2 = 0.928$, $N = 9$, $P < 0.001$) when total fluorescence of all the peaks (humics and saccharides) was plotted against TOC. It could be hypothesized that humic substances might be contributing significantly to these values.

Our results were in agreement with previously published findings [25] evidencing that the total saccharide content correlated well with TOC, except for sugar carbon that accounted for an average of 8.0% of the total carbon. In the samples of lake Geneva the carbohydrates carbon comprised 20–40% of total organic carbon as measured by TPTZ assay and 15–35% by CE-LIF method. Burney *et al*. [50] reported that the percentage of polysaccharides contributing to DOC ranged from 69% along the shore to 37% in the mid ocean samples which is also much higher than values reported by other investigators [51, 52].

5.3 *Molecular composition of neutral sugars*

Capillary gel electrophoresis enabled us to examine the molecular composition of the carbohydrate pool. Electropherograms depicted that glucose and fructose were most abundant sugars with a minor contribution of xylose in surface and deep waters (figure 11). The concentration of glucose and fructose decreased with depth to 200 m and again a slightly higher concentration of glucose was observed in samples from the lake bottom (309 m). Our findings were in agreement with one of the monosaccharide spectrum of North Sea, Baltic Sea and Black Sea waters. Most sea water samples were characterized by a dominance of glucose and fructose [53]. The presence of fructose was attributed to both biotic and abiotic processes (epimerization of glucose to fructose). The samples from 50–100 m depths contained approximately half of the glucose concentration in comparison to the surface samples. Sugar concentrations of particulate and high molecular weight dissolved material could be highly variable in time and space and tend to resemble the composition of plankton on a composite basis [54]. On the other hand, some investigators reported the presence of glucose, galactose, mannose, xylose, fructose, fucose and galacturonic acid etc. in marine waters and particulate matter [44, 55, 56] and in lakes and marsh waters [25].

6. Conclusion

A modified hydrolysis of polysaccharide standards was found to be more efficient than hydrochloric acid hydrolysis. The hydrolysis efficiency was verified with a TPTZ assay. For the characterization of carbohydrates by capillary electrophoresis, the monosaccharides were labeled with APTS. The labeling efficiency and selectivity was verified using various model mono, di-, and polysaccharides. In order to mimic the natural water conditions, potential interferences due to fulvic, humic and peat humic acids were evaluated. Two types of capillaries were tested. Silica capillary was found to have poor resolution but well related to the concentrations of saccharide in the medium. Quantitative determination was only possible for the total carbohydrates contents because the mixture of various monosaccharides gave one peak. We did not observe any significant interference of humic substances during separation and detection. In case of a gel capillary, excellent resolution of monosaccharides was achieved; however the fluorescent signal of monosaccharide mixture was moderately correlated with their concentration.

Modified sulfuric acid hydrolysis method coupled with CE-LIF technique were applied to lake Geneva samples, collected during March 2001, to demonstrate its applicability to natural water samples. Maximum concentration of mono and

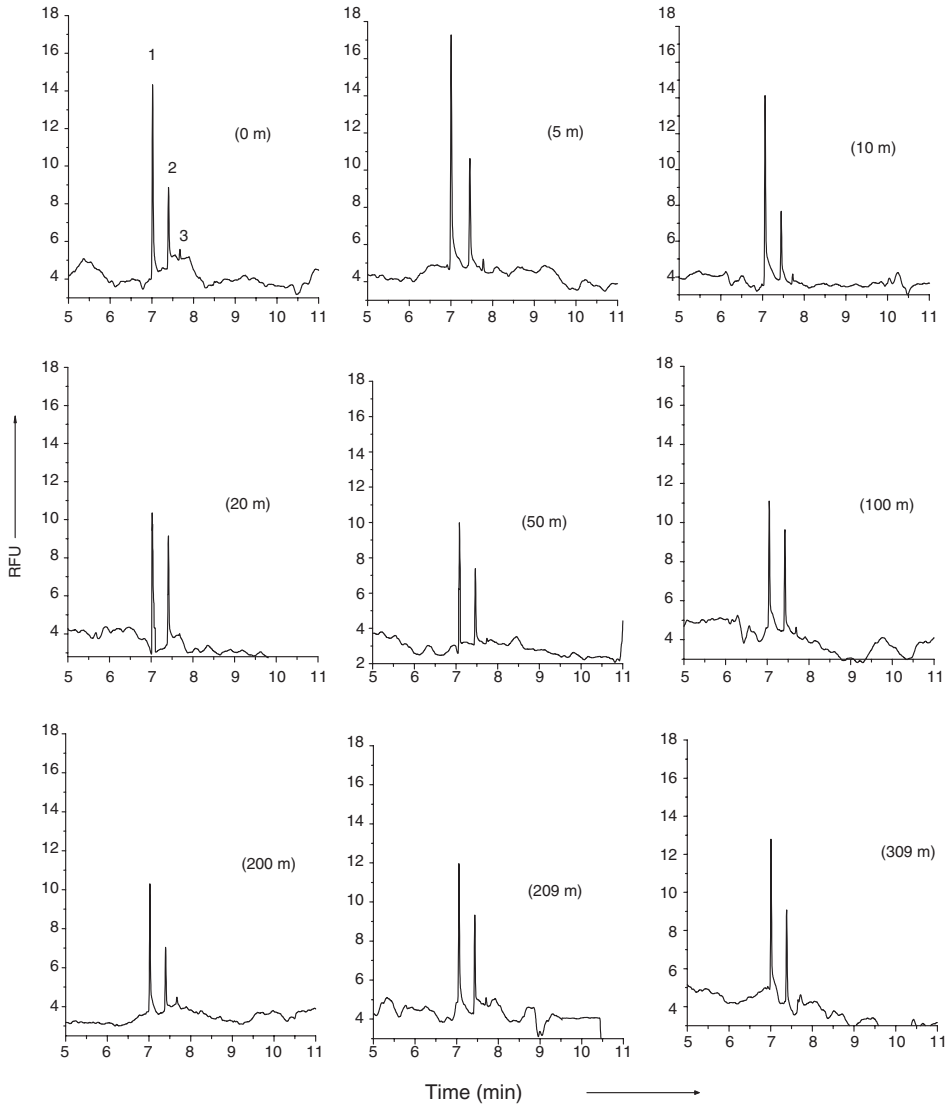

Figure 11. Depth profile of Geneva lake water samples with gel-cap in reverse polarity mode using an applied potential of 15 kV. Peak identification: 1 = glucose, 2 = fructose, 3 = xylose.

polysaccharides were observed at the lake surface with gradual decrease down to 200 m. The maximum concentration of saccharides at the surface could be due to phytoplanktonic activity in the euphotic zone. The concentration of saccharides was slightly higher at the bottom of lake that could be due to the remineralization of the organic matter by anoxic bacteria in sediments. The depth profile obtained by using gel-capillary electrophoresis indicated the dominance of glucose in water with an important contribution of fructose and minor contribution of xylose. The major advantage of CE-LIF technique was the small amount of sample required. However, further work needs to be done

to optimize the conditions for gel capillary electrophoresis so that with minimum amount of sample both quantitative and qualitative information can be achieved.

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