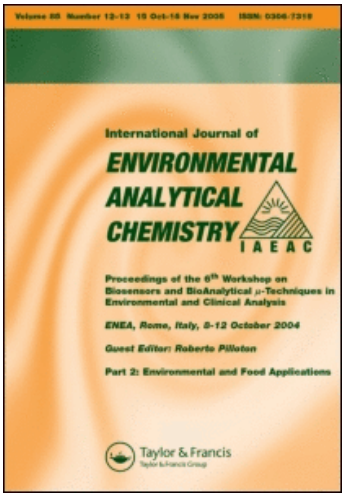

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OPTIMIZATION OF THE HYDROLYSIS OF FRESHWATER POLYSACCHARIDES

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Three techniques of acid hydrolysis are verified for their ability to efficiently hydrolyse freshwater polysaccharides. Polysaccharides isolated from two eutrophic lakes were hydrolysed rapidly as compared to model or marine polysaccharides. No single technique is 100% satisfactory for all polysaccharides (*i.e.* complete hydrolysis with no degradation). Hydrolysis by 0.1 M HCl appeared to give the best results while acceptable results were also obtained for 1.2 M H_2SO_4. The results suggest that it is prudent to hydrolyse freshwater polysaccharides under the least drastic conditions and for as short of a time as possible in order to minimize decomposition of the sample, (*esp.* uronic acids). Interferences due to humic acids did not appear to increase as a function of hydrolysis time.

Keywords: Polysaccharide; hydrolysis; dextran; xanthan; glucose; analytical determination

INTRODUCTION

Polysaccharides are quantitatively important in natural aquatic systems[1,2,3]. Furthermore, they have been shown to play important roles in natural waters by destabilizing inorganic colloids via flocculation[3,4], inducing the formation of biofilms and aggregates[5,6], or by binding trace elements[7]. Given their potential to affect such a wide variety of aquatic processes, it is essential to accurately quantify the aquatic polysaccharides, especially in freshwaters, where it is necessary to distinguish their contribution from that of the ubiquitous humic substances.

In natural waters, polysaccharides are mainly produced by a variety of microorganisms and are thus heterogeneous and polydisperse. For this reason, it is nearly impossible to quantify them without first breaking them down into smaller

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components such as monosaccharides, formaldehyde or CO_2. Several techniques are available for the quantification of degradation bi-products. For example, both spectrophotometric[8,9,10] and chromatographic[11] techniques have been used to quantify natural aquatic polysaccharides at environmental concentrations. Other techniques based upon capillary electrophoresis[12] or oxidation to CO_2[13] have also been developed for other uses and could eventually be applied to the analysis of carbohydrates in natural waters. In the majority of these techniques, the key step to the accurate measurement of the polysaccharides is their quantitative hydrolysis to monosaccharides. Therefore, it is essential to optimize the hydrolysis of the polysaccharides while limiting their degradation. This is especially important for the uronic acid containing polysaccharides, since in this case, the glycosidic bonds are more resistant to hydrolysis due to the protective effect of the carboxyl groups[14,15]. Although hydrolysis techniques have been examined and optimized for structural polysaccharides in the pulp and paper industry[16], and for marine polysaccharides[9], the techniques have not yet been examined critically with respect to problems specific to the determination of freshwater polysaccharides, *e.g.* presence of higher concentrations of inorganic particles or terrestrial humic substances.

The goal of this study was to verify three standard hydrolysis techniques which are commonly used for the hydrolysis and quantification of marine polysaccharides with respect to their applicability to freshwater polysaccharides.

EXPERIMENTAL

Freshwater polysaccharides are extremely heterogeneous[17]; no one standard or set of standards can represent all freshwater polysaccharides. In this study, we present detailed results for a model monosaccharide, D-glucose, and two model polysaccharides, dextran and xanthan. In addition, complementary experiments were performed on various carbohydrates such as galactose, galacturonic acid, polyglucuronic acid and schizophyllan. Dextran was employed because it is a glucose homopolymer, which allows direct comparison of its signal after hydrolysis with that of glucose. Xanthan was chosen because many of its properties are in direct contrast with dextran (*i.e.* xanthan is a semi-rigid, fibrillar, large molecular weight (several thousand kDa) polysaccharide while the dextran employed here is a flexible, globular, low molecular weight (40 kDa) polysaccharide). Furthermore, dextran is a reserve polysaccharide which should be relatively easily biodegradable, while xanthan is a structural polysaccharide which should be more resistant to enzymatic (and possibly acid) hydrolysis. In this paper, the sig-

nal obtained after hydrolysis of 4 mL of a 10 mg L^{-1} solution of polysaccharides was compared to the signal obtained for an identical concentration of glucose.

Natural samples were prepared in two different manners. For the freshwater samples, between 500 mL and 1 L of water was sampled from a eutrophic lake (Lake Bret or Lake Leman). Solutions were centrifuged (1 h, 3700 × g) to remove large particles and the supernatant was lyophilized. The separation of the marine polysaccharides has been described in detail elsewhere[1,18]. In summary, a 10 kD-0.2 μm colloidal fraction was isolated from the Middle Atlantic Bight by tangential flow ultrafiltration. The retentate, containing the colloidal polysaccharides was lyophilized and the sample frozen until use. Although the ultrafiltration is much more labor intensive, it allows the elimination of a large proportion of the marine salts and facilitates the pre-concentration of the polysaccharide fibrils which should not pass through the ultrafiltration membrane used here.

In these experiments, reducing monosaccharides were quantified by measuring the reduction of Fe^{3+} to Fe^{2+} using spectrophotometric analysis after reaction with 2,4,6-tripyridyl-s-triazine (TPTZ)[19]. In theory, the absorbance of $Fe(TPTZ)_2^{2+}$ complex is directly proportional to the concentration of the reducing sugars. In practice, we have found that the technique is not reproducible enough for the quantitative routine analysis of freshwater polysaccharides; small variations in temperature, light, reaction time, age of reactants, *etc.* can have large effects on the measured absorbances. On the other hand, for comparative work using identical samples under identical conditions, as is the case here, it is a much more routine technique than most other spectrophotometric or chromatographic techniques[9,11,20].

In the experiments presented here, three hydrolysis techniques were verified for their ability to hydrolyse model and natural aquatic polysaccharides completely, and without degradation. These treatments were selected based mainly upon the existing literature for the hydrolysis of marine polysaccharides[9,20,21,22]:

(i) 0.1 M HCl (100°C)

(ii) 1.0 M HCl (100°C)

(iii) 2 hours of pre-hydrolysis in 12 M H_2SO_4 (25 °C) followed by 3 hours in 1.2 M H_2SO_4 (100 °C)

Hydrolysis conditions were evaluated by comparing the absorbance of the TPTZ complex as a function of hydrolysis time (and conditions). To this end, teflon bombs containing the substrate to be analyzed were placed in a temperature controlled oven at different times, so that at the end of the experiment, all of the bombs could be removed and analyzed simultaneously (thus minimizing variability due to reactant quality). The bombs were cooled in a cold water bath and

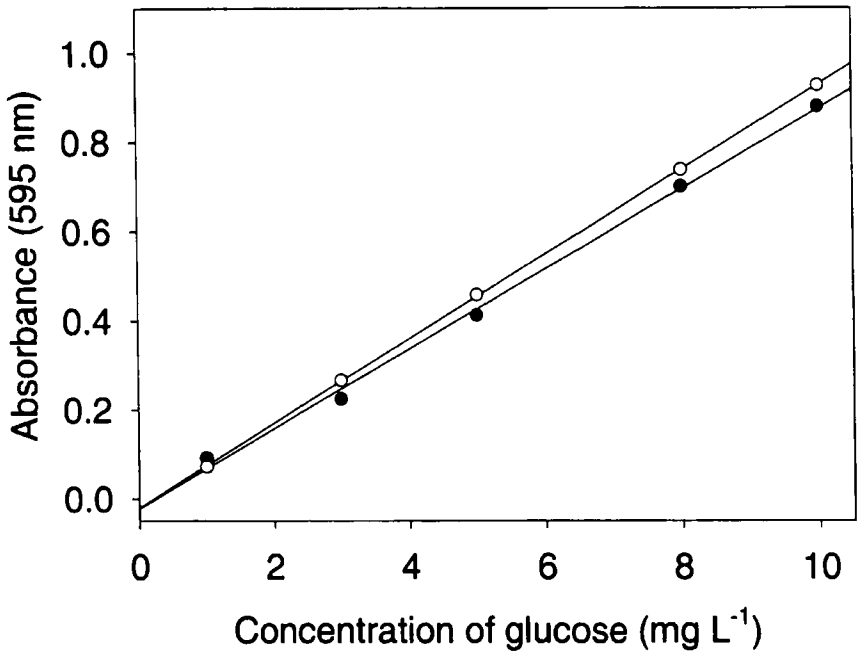

FIGURE 1 Calibration curves for glucose in the absence of an acid treatment. Absorbance of the $Fe(TPTZ)_2^{2+}$ complex formed subsequent to the oxidation of reducing monosaccharides (see text and reference 19 for further details)

the solutions were neutralized using 1 M NaOH. For the hydrolysis experiments presented below, each data point represents the mean and standard deviation of three replicate bombs.

Glassware and teflon digestion bombs were cleaned for trace organics by soaking in NaOH saturated methanol overnight, followed by a 24 hour soak in 5% HCl, extensive rinsing (5X) in double-distilled water followed by a Milli-Q water rinse (2X; TOC< 5 μg C L^{-1}).

RESULTS AND DISCUSSION

Standard solutions

In Figure 1, duplicate runs of the TPTZ calibration curves are shown as a function of the concentration of D-glucose. Although the reproducibility of the calibration curves given here is excellent (r^2 >0.99; slopes of 0.090 and 0.095), a

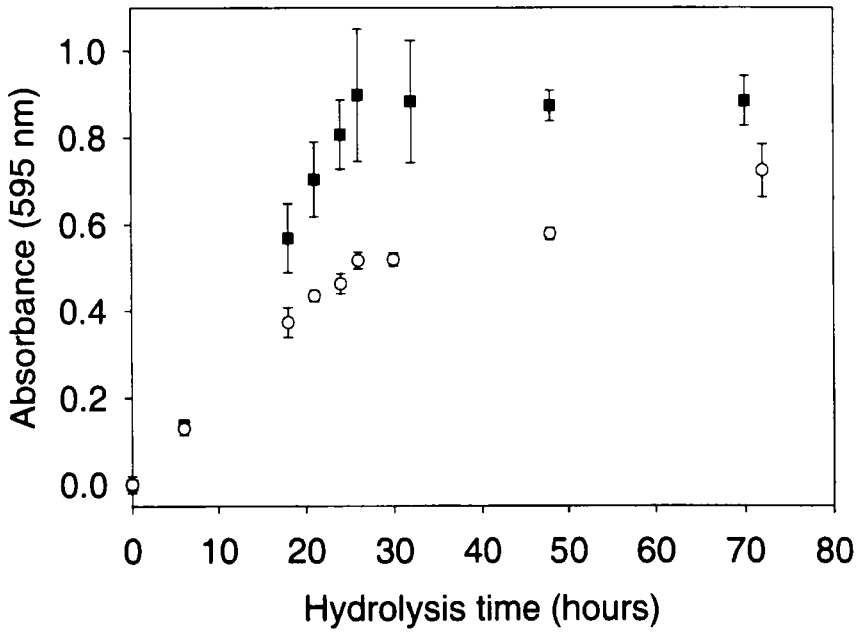

FIGURE 2 Absorbance of the $Fe(TPTZ)_2^{2+}$ complex as a function of hydrolysis time in 0.1 M HCl (100°C) for (■) dextran; (○) xanthan

variation of ±10% was more typical. In agreement with Myklestad *et al.*[19], our slopes obtained after reaction with other simple sugars (data not shown) were generally significantly lower than that obtained for glucose, decreasing by up to 30% for other monosaccharides and up to 60% for uronic acids.

In Figure 2, the hydrolysis of two model polysaccharides in 0.1 M HCl was followed over three days. Two types of information can be obtained from this format of data presentation: the time required to reach the plateau and the absorbance value of the plateau. For example, in this Figure, dextran absorbance attains a constant value (0.8–0.9) following 24 hours of hydrolysis. The absorbance value of the plateau is in reasonable agreement with the theoretical absorbance if the dextran is completely hydrolysed into glucose (recall that the molecular weight of a glucose unit is 180 while the D-glucosyl units each have a molar mass of 162). On the other hand, although a reduction in the absorbance of the plateau is expected for xanthan with respect to glucose due to the presence of acetate, pyruvate, mannose and glucuronic acid in the polysaccharide[19], the plateau for xanthan absorbance does not appear to be attained, even after 3 days of hydrolysis.

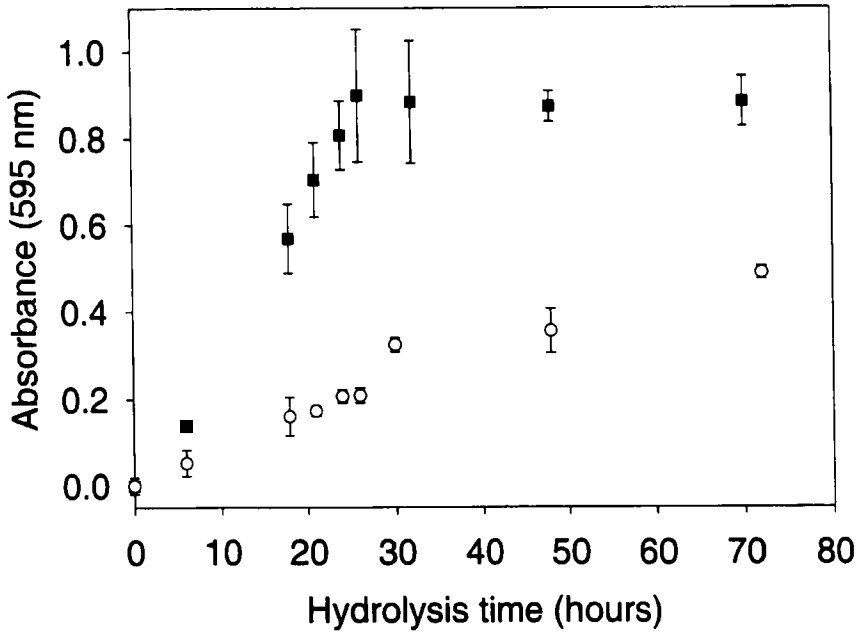

FIGURE 3 Absorbance of the $Fe(TPTZ)_2^{2+}$ complex as a function of hydrolysis time in 0.1 M HCl for dextran at (■) 100 °C; (○) 80 °C

Hydrolysis temperature was also an important parameter (Figure 3): a 20 °C reduction in temperature reduced the efficiency of the 0.1 M HCl hydrolysis of both dextran and xanthan by approximately 50% (data shown for dextran only). Furthermore, the concentration of HCl had an effect on the time required to reach the plateau. For 1.0 M HCl, only 4 hours was required to reach the absorbance plateau for dextran, >20 hours were required for the xanthan (Figure 4). In the case of dextran, the absorbance of the plateau did not appear to attain the theoretical absorbance based upon 100% glucose hydrolysis (Figures 1, 2), although these values may be within the precision errors of technique. For xanthan, maximum absorbance was observed after one day of hydrolysis but appeared to decrease for longer times. Such a decrease is only possible if part of the substrate is degraded. Therefore, experiments were performed to determine under what conditions the signal of the hydrolysed solution (corresponding to monosaccharides only) was likely to decrease. Glucose absorbance was constant over 24 hours for 0.1 M HCl (data not shown) but may have decreased slightly for 1.0 M HCl (Fig. 4A). On the other hand, the signal for galactose decreased significantly (ANOVA, $P<0.05$) and rapidly (>6 hours) during hydrolysis even for the 0.1 M HCl treatment (Figure 4A).

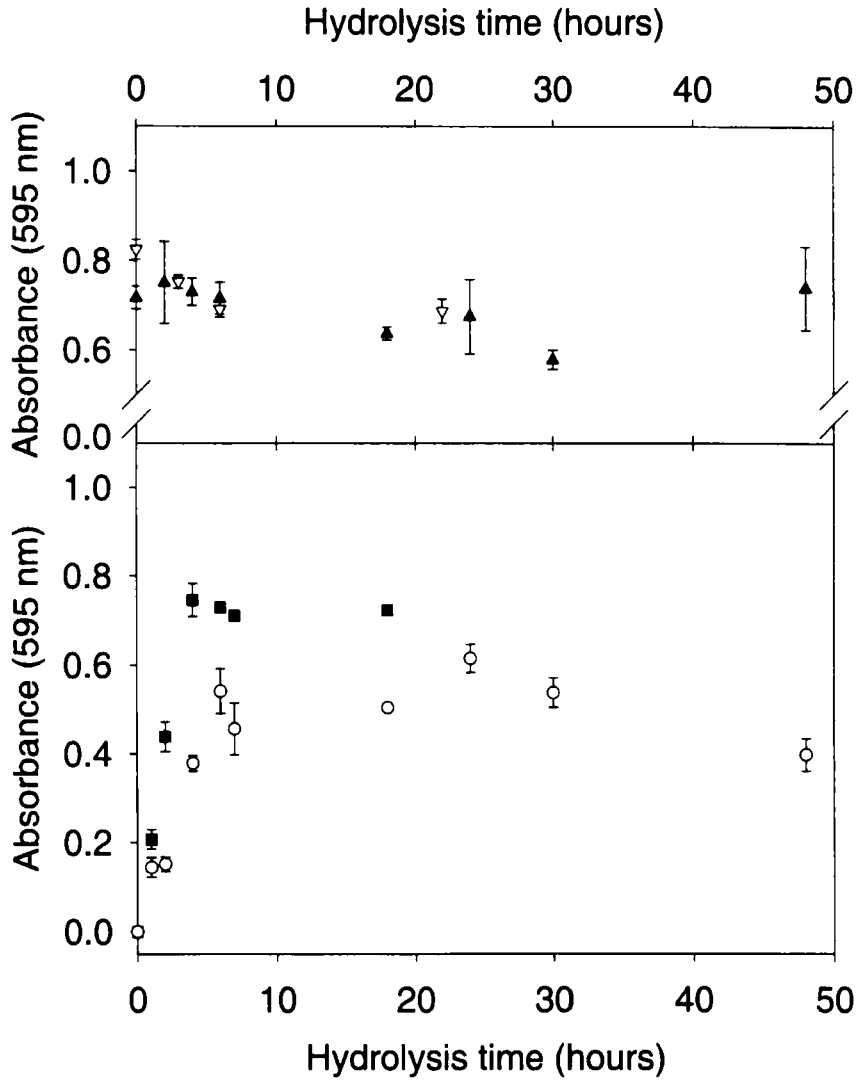

FIGURE 4 Absorbance of the $Fe(TPTZ)_2^{2+}$ complex as a function of hydrolysis time (at 100 °C) for glucose in 1.0 M HCl (▲), galactose in 0.1 M HCl (V); dextran in 1.0 M HC (■) and xanthan in 1.0 M HCl (○)

The sulfuric acid hydrolysis requires a 2 hour pretreatment at room temperature in 12 M H_2SO_4. Following this pretreatment, some hydrolysis is already apparent (non-zero intercept, Figure 5). As was observed for the HCl treatment, the efficiency of the hydrolysis depends to a large extent upon the temperature.

The slow initial increase in absorbance reflects the time required for the bombs to heat to 100 °C. While this slow initial heating may have also been present for the HCl treatment, it would have had a less obvious effect due to the much larger time scales employed (*i.e.* HCl hydrolysis was performed over a period of several days while H_2SO_4 hydrolysis was generally accomplished in a few hours). A maximum absorbance of approximately 0.8 was obtained after 3 hours of heating in agreement with the glucose standards prepared in the same manner. It is unclear whether the slight decrease in absorbance as compared to HCl hydrolysis is due to a degradation of the sugars, differences due to the acids (*e.g.* viscosity or absorptivity differences) or simply due to the reproducibility of the method. Nonetheless, in H_2SO_4, the absorbance of the xanthan plateau was only *ca.* 50% of that obtained for dextran (Figure 5) while in 0.1 M HCl the maximum absorbance of xanthan had attained 80% of the absorbance of the dextran. Furthermore, hydrolysis of the triple helical glucose homopolymer, schizophyllan, was not complete (absorbance 83% of glucose controls) even following 24 hours of hydrolysis in 1.2 M H_2SO_4 (data not shown).

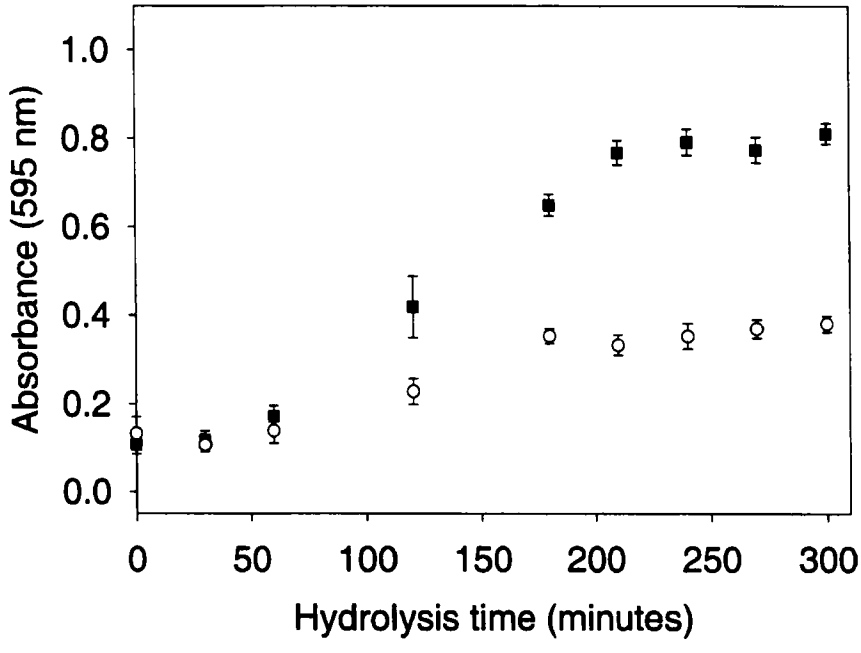

FIGURE 5 Absorbance of the $Fe(TPTZ)_2^{2+}$ complex as a function of hydrolysis time for 1.2 M H_2SO_4 at 100 °C following a 2 hour "pre-hydrolysis" in 12 M H_2SO_4 at room temperature. Results are presented for (■) dextran and (○) xanthan

Natural samples

Humic substances generally account for >50% of the organic matter in freshwaters[17,23], their concentration often exceeding that of the polysaccharides by a factor of 10. Humics have the potential to interfere with the analysis of the $Fe(TPTZ)_2^{2+}$complex by absorbing at 595 nm or by directly or indirectly reducing the Fe^{3+} (indirectly should their hydrolysis result in the production of compounds which can be oxidized). In the presence of 0.1 M HCl or 1.2 M H_2SO_4 (Figures 6A and 6C), absorbance was important but constant as a function of the hydrolysis time indicating that hydrolysis of the IHSS humics did not produce products which can reduce the Fe^{3+}. In Figure 6B, absorbance may have increased slightly due to the 1.0 M HCl. These results suggest that a blank measurement performed prior to hydrolysis should be sufficient to correct for the humics and reducing monosaccharides initially present in the sample (although it will be impossible to distinguish between these two groups of compounds using this technique). Absorbance of the natural samples generally increased in a similar manner to that observed for the standard solutions (Figure 7). On the other hand, the initial increase in absorbance was generally more rapid for the freshwater polysaccharides than was observed for the standard polysaccharides. For example, for the lake Bret polysaccharides, absorbance attained a plateau after 6 hours (0.1 M HCl), 1 hour (1.0 M HCl) or immediately following the initial 12 M H_2SO_4 hydrolysis (Figure 7). Similar results were obtained for polysaccharides obtained from the center of the much larger Lake Leman (data not shown). On the other hand, hydrolysis of the marine polysaccharides was substantially slower (Figure 8). Although the slow hydrolysis may have been due to a larger concentration of polysaccharides in the marine sample, it may also reflect a greater proportion of reserve polysaccharides in the lakes with respect to the structural polysaccharides in the less productive oceans. Further research is clearly merited in this direction.

Burney and Sieburth[9] have employed a 0.1 M HCl hydrolysis in concert with the MTBH spectrophotometric technique to determine total carbohydrates in marine samples. More recently, Pakulski and Benner[20] suggested that the 12 M H_2SO_4 hydrolysis was more appropriate for marine carbohydrates, giving values which were two to four fold greater than those determined after 0.1 M HCl hydrolysis. While our data support those of Burney and Sieburth in suggesting that the gentler 0.1 M HCl hydrolysis may be more appropriate for freshwater samples, the use of concentrated H_2SO_4 may be justified for marine samples or in other cases where highly recalcitrant polysaccharides predominate.

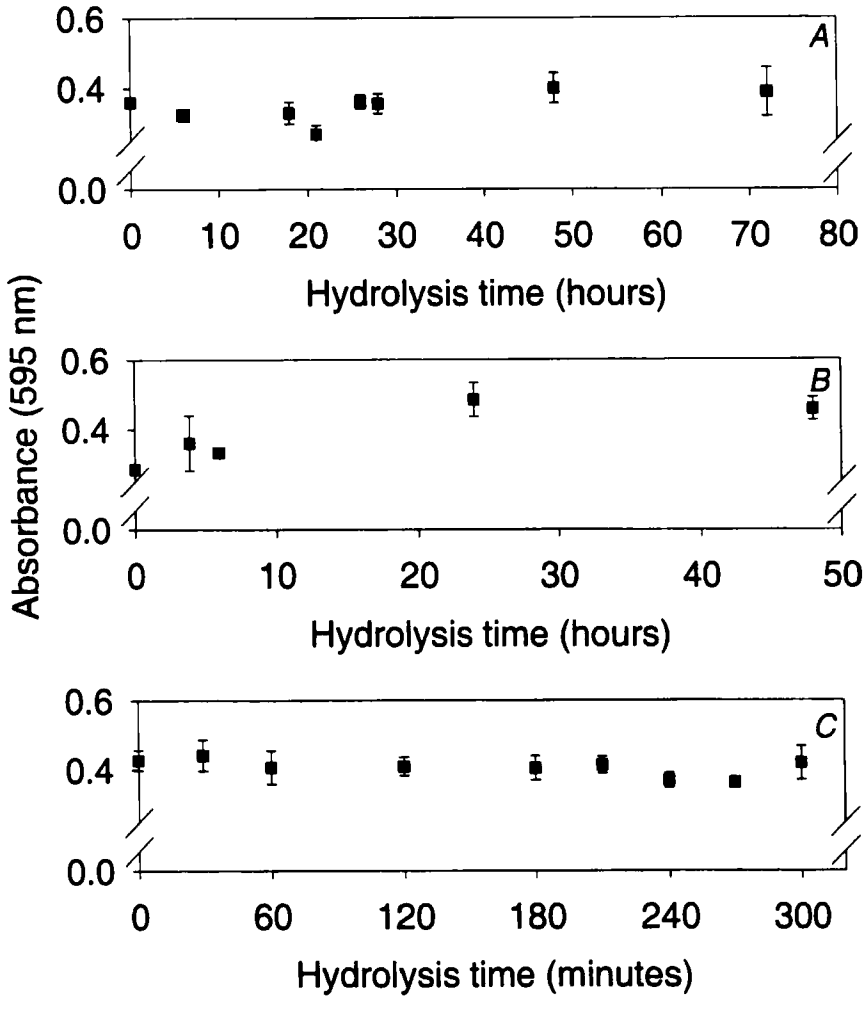

FIGURE 6 Absorbance at 595 nm as a function of hydrolysis time for 10 mg L^{-1} of a IHSS Suwannee River humic acid. Results are given for *A*. 0.1 M HCl; *B*. 1.0 M HCl and *C*. 1.2 M H_2SO_4

CONCLUSIONS

The data presented here indicated that two of the main techniques of acid hydrolysis used for marine samples are likely to be useful for the hydrolysis of freshwater polysaccharides. While the results using a fibrillar polysaccharide such as xanthan or schizophyllan suggested that long hydrolysis times are required to

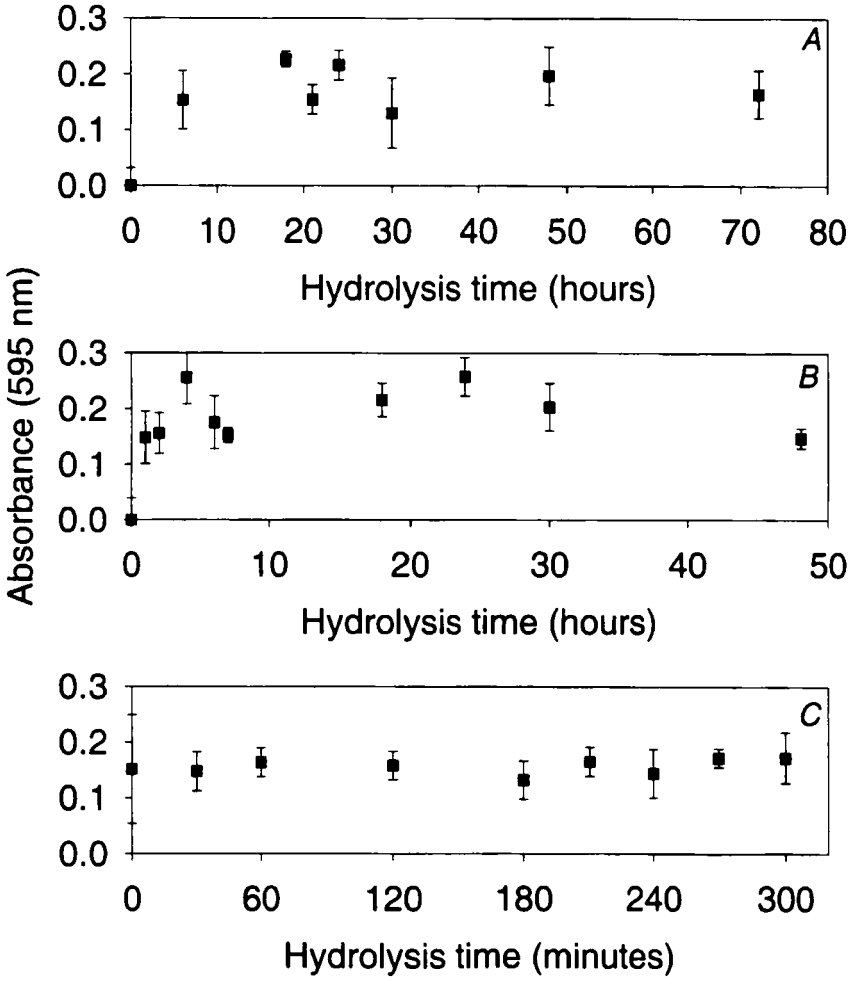

FIGURE 7 Absorbance of the $Fe(TPTZ)_2^{2+}$ complex as a function of hydrolysis time for a lyophilized freshwater mixture of natural organic matter isolated from a small eutrophic lake (Lake Bret). Hydrolysis conditions: *A*. 0.1 M HCl; *B*. 1.0 M HCl and *C*. 1.2 M H_2SO_4

ensure complete degradation of the polysaccharides, some evidence was presented suggesting that degradation of the sugars is occurring under the harshest conditions (*esp.* 1 M HCl). The data suggested that hydrolysis by 0.1 M HCl[9,19]may have resulted in a slightly higher efficiency than 1.2 M H_2SO_4[20] for the hydrolysis of mixed carboxylic acid containing polysaccharides. Nonetheless, both techniques gave similar results for lake polysaccharides, *i.e.* an

extremely rapid hydrolysis. These results suggest that it would be prudent to hydrolyse freshwater polysaccharides under the least drastic conditions and for as short of a time as possible in order to minimize decomposition of the sample, (*esp.* uronic acids). Other criteria such as the necessity to remove the acid following hydrolysis or the time available to perform the hydrolysis could be used to distinguish between the techniques. Interferences due to humic acids did not appear to increase as a function of hydrolysis time, suggesting that it should be possible to correct for their concentrations where necessary. Finally, temperature was shown to be an extremely important factor: a decrease of 20 °C significantly increased the time which was required to perform the hydrolysis. The results suggest that no technique will permit 100% recovery of the polysaccharides, especially in the presence of large proportions of structural polysaccharides or carboxylic acid containing polysaccharides. Because recovery is necessarily a compromise between cleavage of the glycosidic linkage and the decomposition of the liberated monosaccharides, it may be necessary to verify hydrolysis conditions for each sample type.

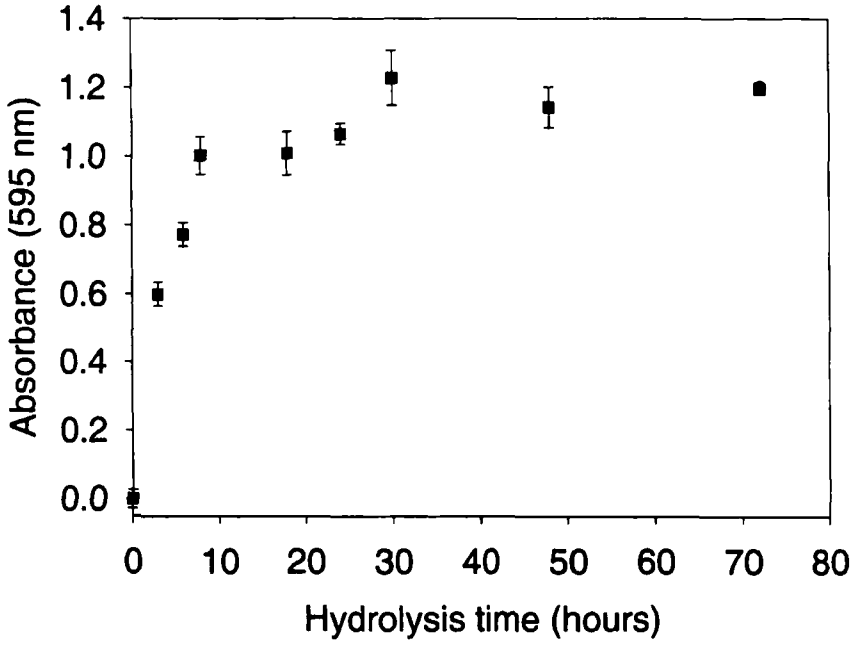

FIGURE 8 Absorbance of the $Fe(TPTZ)_2^{2+}$ complex as a function of hydrolysis time for marine fibrils collected by tangential flow ultrafiltration. Hydrolysis performed using 0.1 M HCl

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