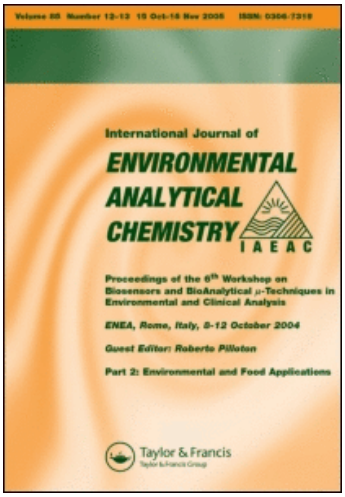

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The application of the MBTH method for carbohydrate determination in freshwaters revisited

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There is a lack of reliable and easy-to-use methods for the quantitative determination of carbohydrates in natural waters. Among the existing methods, the 3-methyl-2-benzothiazolinone hydrochloride (MBTH) method has been widely used to determine total dissolved carbohydrates in seawater. Its application to freshwaters has, so far, been less frequent. The objective of this study is to critically examine the application of the MBTH method to the analysis of carbohydrates in freshwater samples in order to understand what the method really measures. Following a comprehensive revision of the literature, the method has first been applied to representative model compounds and then to a variety of freshwaters of contrasting trophic characteristics. By simultaneously determining the total dissolved organic carbon and humic-type compounds in the same samples, it has been possible to show that sometimes a significant part of the organic carbon remains undetected. This seems to indicate that a substantial amount of carbohydrate present in some natural waters is probably not 'seen' by the MBTH method.

Keywords: Carbohydrates; Polysaccharides; MBTH; Natural organic matter; Freshwaters; Lakes; Rivers

1. Introduction

Carbohydrates are among the most abundant organic molecules in the biosphere. They play important roles in both cellular metabolism and recognition and as structural polymers. As well as being important cellular components, carbohydrates represent a major proportion of non-living organic matter in terrestrial and aquatic environments.

Carbohydrates are products of phytoplankton photosynthesis and are released by exudation, cell lysis, and microbial degradation. Along with amino acids and lipids, they are the main components of organic matter released by phytoplankton [1, 2]. Excretion from zooplankton is a further source of dissolved carbohydrates [3, 4].

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Low-molecular-weight carbohydrates, such as monosaccharides, are released directly from phytoplankton into the surrounding water or from polymeric carbohydrates during hydrolysis by microbial enzymes. Glucose is the most abundant carbohydrate, both as a monomer [5–8] and in polysaccharides such as glycans and cellulose [9, 10].

Molecular characterization of carbohydrates has identified the presence of neutral sugars (normal, deoxy, and methylated aldoses) [7, 8, 11–14], amino-sugars [11, 14, 15] and acidic sugars [16–21] in natural waters. Of these classes of carbohydrates, neutral sugars have been studied extensively and provide information on the origin of natural organic matter (NOM) and its diagenetic state. Considerably less is known about the abundance and distribution of other types of sugars because of the lack of suitable methods for analysis.

For years, the study of carbohydrates in natural waters has received much less attention than the study of other types of organic matter such as humic substances. The situation is changing, mainly as a result of the close attention paid to NOM in oceans, where a significant portion of NOM is composed of carbohydrates [5, 13, 19, 20, 22–37]. In effect, carbohydrates constitute up to 35% of the dissolved organic carbon in most seawaters [29], and in Antarctic phytoplankton blooms, dissolved carbohydrates may account for up to 50% of the semi-labile DOC pool [8]. Although studied much less frequently than carbohydrates in seawater, carbohydrates also constitute a large component (1–30%) of the dissolved organic matter in freshwater environments [21, 38–46].

There is no well-established, widely accepted method for carbohydrate determination in natural waters. Carbohydrate analysis is particularly difficult. More than 100 different sugar species and sugar derivatives are present as monomeric constituents in polysaccharides, and their complexity is heightened by the different linkage possibilities (including branching linkages) and the variable molecular weight (up to millions of Daltons) within a single family of polysacharides species. Furthermore, large variations in the secondary structure and molecular associations are possible. Carbohydrates are also extremely water-soluble and they lack light-absorbing chromophores, making direct detection difficult unless chemical derivatization is performed prior to analysis. Carbohydrates also exhibit multiple charge states at natural pH, including neutral sugars, the positively charged amino sugars, and the negatively charged uronic acids, which makes the isolation of carbohydrates as a single chemical class difficult.

Existing analytical methods can be classified in two groups: chromatographic and colorimetric methods. Chromatographic methods have been used for a long time [47–49]. They have been applied in freshwaters [39, 41–43, 50–53], sediments [5, 17, 54–56], and seawater [7, 13, 15, 16, 57–60]. Both gas chromatography [17, 39, 41, 43, 54–58, 61, 62] and liquid chromatography [5, 51, 60] have been used. Gas chromatography (GC), in conjunction with a flame ionization detector (FID), makes it possible to identify individual molecules and, with mass spectrometry (MS) [55, 58], to confirm their identity. However, GC involves time-consuming derivatization procedures that can limit the number of samples processed and contribute to sample loss. High-performance anion-exchange chromatography (HPAEC), coupled with pulsed amperometric detection (HPAEC-PAD or HPLC-PAD), has been widely used [7, 8, 11, 13, 15, 16, 50, 51, 53, 56, 59, 63, 64]. Unfortunately, desalting of the samples is necessary prior to anion-exchange chromatography and often causes the loss of a large percentage of charged sugars [8, 13]. The main advantage of the chromatographic

methods is that they allow the qualitative and quantitative identification of individual sugars. However, as mentioned, they are not without their limitations. Carbohydrate measurements in seawater taken using colorimetric methods are also known to show higher concentrations of reducing sugars than those observed using separation methods such as HPLC-PAD [13, 21, 53], for which no clear explanation exists. Hung and co-workers [21] suggested that it is due to the fact that a large fraction of the carbohydrate-type substances have not yet been characterized at the molecular level by chromatographic methods.

Sugar concentrations in natural waters are most often obtained colorimetrically and are usually reported as total sugar concentration (e.g., in glucose equivalents). The first colorimetric methods – the *N*-ethylcarbazol method [65, 66], the anthrone method [67–69], the L-tryptophan-sulphuric method [22, 70–72] and the phenol-sulphuric method [55, 60, 73–77] – are rarely used nowadays. Currently, the method that is most frequently applied is the MBTH (3-methyl-2-benzothiazolinone hydrochloride) method. This was originally developed to determine aldehydes [78] but has been adapted to the quantification of reducing sugars. The formation of a coloured product in the MBTH method involves a series of reactions: borohydride reduction of aldehyde to alcohol, periodate oxidation of the diol to formaldehyde, and nucleophilic attack of the electrophilic carbonyl in formaldehyde by MBTH. Under acid and oxidizing conditions, this compound reacts with a second molecule of MBTH to form a highly coloured final product. The MBTH method has been extensively applied to the determination of carbohydrates in seawaters [7, 23, 24, 26–30, 33, 34, 36, 79–91]. It has been applied much less frequently to freshwater systems [44, 46, 84, 92–97]. An alternative to the popular MBTH method is the TPTZ (2,4,6-tripirydyl-1,3,5,-triazine) method. This method is based upon the oxidation of free reducing sugars with ferricyanide [32, 98]. Environmental applications are recent. The method has been applied to the study of estuaries [19, 99], seawater [20, 35–37, 90, 100–102] and freshwaters [21, 103]. A comparison of the results obtained by both the TPTZ and the MBTH methods in seawater samples showed them to be very similar [90]. However, these authors suggested that the TPTZ method may suffer from interference if carbohydrates are particle-bound.

Polysaccharide or combined sugar concentrations are usually determined by measuring monosaccharides liberated after acid hydrolysis. It is therefore important that the hydrolysis step ensures the maximum formation of monosaccharides, while limiting their degradation. In spite of the number of studies devoted to this process, hydrolysis has remained the bottleneck for both chromatographic and colorimetric methods. As mentioned above, polysaccharides vary not only because of the different monosacharides present, but also because of the different linkage possibilities. Not all polysaccharides hydrolyse equally. For instance, uronic acid-containing polysaccharides have glycosidic bonds that are more resistant to hydrolysis due to the protective effect of the carboxyl groups [104, 105]. In contrast, drastic conditions of hydrolysis can either produce unexpected degradation products [22] or lead to the degradation of the monosaccharides [43, 50, 51, 53], both of which will cause the carbohydrates present in the analysed sample to be underestimated. A relatively wide range of hydrolysis methods and conditions have been tested and applied so far, mostly to seawaters (table 1). Some of the methods have been the subject of comparison exercises [13, 33, 43, 46, 53, 84, 90, 103]. As yet, no clear conclusions can be drawn from the work carried out, apart from the fact that no hydrolysis scheme successfully

Table 1. Some examples of polysaccharide acidic hydrolysis conditions that have been applied to surface waters.

Acid	Acid concentration ($mol\,L^{-1}$)	Hydrolysis length (h)	T(°C)	System	Comments	Reference
HCl						
	0.09	20	100	Seawater	*Reference article* Addition of a hydrolysis step to the MBTH method proposed by Johnson and Sieburth [79]	[23]
	0.09	20	100	Seawater and estuarine waters		[86, 91]
	0.09	20 or 1	100 or 150	Seawater		[32]
	0.09	1	150	Estuarine and river waters		[19, 21]
	0.1	20	95	Seawater		[85]
	0.1	20	100	Seawater		[35, 81, 106]
	0.16	20	100	Particulate material in seawater		[28]
	0.24	12	100	Streamwater	Comparison of the efficiency of hydrolysis conditions (0.02 to 1.3 $mol\,L^{-1}$ HCl; reaction times: 3, 12 h): relatively mild hydrolysis conditions give the best recovery; hydrolysis of uronic acids not assessed	[53]
	0.5	1	100	Estuarine waters	Different conditions tested: 0.1, 0.5, 1.0, 3.0 and 6 $mol\,L^{-1}$ HCl; temperatures (100, 125°C); times (1–2 h)	[43]
	0.5	4	100	Freshwater reservoir		[44]

	Different concentrations tested	3	100	Seawater	*Reference article* Optimal HCl concentrations: 1 mol L^{-1} for sugars and 0.5 mol L^{-1} for uronic acids	[5]
	1	7	100	Lake water		[68]
	1	15	100	Freshwater reservoir		[95]
	1	20	100	Estuarine and river waters		[96]
	1.5	4	100	Lake water		[50]
	1.8	3.5	100	Seawater	*Reference articles*	[25, 107]
	1.8	3.5	100	Estuarine and river waters; seawater during a diatom bloom		[34, 92]
	3	1	100	River waters		[39]
	3	5	100	Seawater		[15]
	6	24	110	Sea surface microlayer		[82]
H_2SO_4						
	0.5	10, 20, 40, 60, 150 min	100	Surface microlayer seawater particulate samples		[60]
	0.85	4–6	100	Seawater	*Reference article*	[59]
	0.85	24	100	Seawater		[8]
	Pretreatment: dried samples + 1 mL 12 mol L^{-1} H_2SO_4	2	Room	Seawater (dried samples)	*Reference articles* H_2SO_4 with pretreatment, more effective than dilute HCl for hydrolysis of structural polysaccharides (i.e., chitin and cellulose)	[30, 83]
	Hydrolysis: + 9 mL water, thus giving 1.2 mol L^{-1}	3	100			

(*Continued*)

Table 1. Continued.

Acid	Acid concentration (mol L^{-1})	Hydrolysis length (h)	T(°C)	System	Comments	Reference
	1.2 Pretreatment as in: Pakulski and Benner [83]	3 ?	? ?	River water		[41]
	1.2 Pretreatment as in: Pakulski and Benner [83]	3 15 min ultrasonic bath	100	Seawater	After pretreatment, samples were allowed to sit for 1 h 45 min	[7]
	1.2 Pretreatment as in: Pakulski and Benner [83]	3 2	100 25	Lake water		[93]
	1.2 Pretreatment as in: Pakulski and Benner [83]	3 2	100 Room	Estuarine waters		[87]
Trifluoroacetic acid				River water	From: [108]	[94]
Comparisons						
HCl H_2SO_4	0.09 0.85	20 4	100 100	Seawater	H_2SO_4 hydrolysis gives higher results than hydrolysis by HCl; raising HCl concentration reduces the yield substantially	[84]
HCl H_2SO_4 H_2SO_4	0.09, 0.25 0.85 1.2 Pretreatment as in: Pakulski and Benner [83]	24 24 3 2	100 100 100 Room	Seawater	No observable differences in hydrolysis recoveries in natural waters; the 24-h hydrolysis was less efficient in hydrolyzing commercially available polysaccharides compared with the 12 mol L^{-1} H_2SO_4 pretreatment method	[13]

HCl	0.09	20	100	Seawater	Parallel application of different hydrolysis conditions allows the distinction of two categories of compounds: polysaccharides and hydrolysis-resistant polysaccharides	[33]
H_2SO_4	1.2	3	100			
	Pretreatment as in: Pakulski and Benner [83]	2	28			
HCl	0.1	?	100	Eutrophic lake waters	Comparison of the efficiency of hydrolysis conditions: no technique 100% satisfactory	[103]
HCl	1	?	100			
H_2SO_4	1.2	3	100			
	Pretreatment as in: Pakulski and Benner [83]	2	25			
HCl	0.09	1	100	Seawater	Comparison of the efficiency of hydrolysis conditions: no significant difference	[90]
H_2SO_4	0.85	1	100			
HCl	0.09	24	100	Lake water	Different hydrolysis times tested (HCl: 3 to 38 mol L^{-1}; H_2SO_4: 0.5 to 6 mol L^{-1}); definition of HCl-hydrolyzable and HCl-resistant polysaccharides	[46]
H_2SO_4	2	2	100			
	Pre-treatment: 6 mol L^{-1} H_2SO_4	2	Room			

combines complete hydrolysis and complete monosaccharide preservation. Moreover, since the results obtained seem to be highly dependent on hydrolysis conditions, the fact that such a variety of hydrolysis methods has been used makes it difficult to compare the results obtained by different authors.

The aim of our study was to examine the application of the MBTH method to the determination of carbohydrates in freshwaters in order to better understand the meaning of the results obtained. We focused on identifying the problematic aspects of the method by applying it to model compounds and to natural freshwater systems of contrasting characteristics.

2. Experimental

2.1 *Reagents*

All standard and sample solutions were prepared with 18 MΩ cm Milli-Q water. All glassware and polyethylene bottles were cleaned with nitric acid (10%) and 0.5 mol L^{-1} sodium hydroxyde, and rinsed with Milli-Q water. Glass bottles for DOC were precombusted (3 h at 550°C).

All chemicals used were of analytical reagent grade except the mineral acid (HCl), which was of Suprapur grade. D-Glucose was from Merck. D-Fructose, D(+)-galactose, D(+)-cellobiose, D-galactosamine hydrochloride, D(+)-galacturonic acid, and poly-galacturonic acid were from Sigma-Aldrich. Alginic acid sodium salt from brown algae, xanthan gum from *Xanthomonas campestris*, D-raffinose pentahydrate, dextran from *Leuconostoc* ssp., bovine serum albumin and reduced L-glutathione were from Fluka. Humic and fulvic acids were from IHSS: Suwannee River humic acid standard II, 2S101H (52.6% C (w/w)) and Suwannee River fulvic acid standard, 1S101F (52.4% C (w/w)).

2.2 *Procedure*

Lake Brienz water samples were collected at different depths by using a membrane pump attached to a CTD probe. Water samples from other lakes were collected directly from the shore. River waters were sampled directly in bottles. Water samples for MBTH analysis were collected in polyethylene bottles and acidified at pH 2 with 10 mol L^{-1} of HCl. Samples for DOC determination were collected in precombusted glass bottles and acidified at pH 2 with HCl Suprapur. Immediately after collection, both samples were filtered through precombusted (3 h at 550°C) 1.2 μm size glass-fibre filters (Whatman GF/F filters) by vacuum filtration.

Dissolved organic matter (DOC) was determined by a high-temperature combustion method using a TOC 5000-A Shimadzu analyser (Shimadzu Co. Ltd). Milli-Q water was used as blank (0.00 mg C L^{-1} with an SD less than 0.005). Calibration was performed with a 1000 mg C L^{-1} TOC standard solution (CertiPUR, Merck). Standard solutions as well as blanks were acidified at pH 2 with HCl Suprapur.

Humic-type compounds were measured by an electrochemical method based on the adsorptive stripping voltammetry response of the complex formed by refractory organic matter in the presence of trace amounts of molybdenum(VI) [109]. This method is particularly well suited to the quantitative determination of low concentrations of humic-type compounds in freshwaters.

The total dissolved carbohydrates were analysed by a modified Pakulski and Benner method [83]. Fifty millilitres of acidified water samples were freeze-dried. The dried samples were then treated with 1 mL of 12 mol L^{-1} H_2SO_4 for 2 h at room temperature. After this, the samples were diluted down to a 2.4 mol L^{-1} H_2SO_4 concentration using 4 mL Milli-Q water, briefly sonicated (5–10 s), transferred into a Teflon bomb, and hydrolysed for 3 h at 100°C. After 3 h, the samples were removed, cooled, and 13 mL of 2 mol L^{-1} NaOH added. Next, monosaccharides were reduced to alditols by the addition of 250 μL of 10% (w/w) potassium borohydride (KBH_4) for 4 h in the dark at room temperature. After 4 h, the samples were acidified with 2 mL of 2 mol L^{-1} HCl. The samples were left overnight at 4°C. The following day, triplicate 1 mL aliquots of the solutions were oxidized to formaldehyde by the addition of 0.1 mL of 0.025 mol L^{-1} periodic acid for 15 min in the dark at room temperature. The oxidation reaction was terminated by the addition of 0.1 mL of 0.25 mol L^{-1} sodium metaarsenite to the samples, which were allowed to incubate for an additional 15 min in the dark. For blanks, 0.2 mL of a mixture of periodic acid and metaarsenite (1 : 1 v/v) was added and left for 15 min in the dark. Both the samples and blanks were acidified with 0.2 mL of 2 mol L^{-1} HCl. Then, 0.2 mL of freshly prepared MBTH solution (276 mg of MBTH per 10 mL of 0.1 mol L^{-1} HCl) was added. The tubes were incubated for 10 min at 100°C. Once cooled, 0.2 mL of ferric chloride solution (5 g per 100 mL of Milli-Q water, filtered through a 0.45 μm filter) was added to the tubes. A blue–green colour developed after incubating the solutions for 30 min in the dark at room temperature. After colour development, 2 mL of a solution of acetone and Milli-Q water (1 : 2 v/v) was added and the absorbance at 635 nm recorded with a Helios γ (Thermo Electron Corporation) spectrophotometer using a 1 cm quartz cell and the acetone-Milli-Q water solution as a blank.

The samples were analysed in triplicate. Absorbance of the blank was subtracted before calculating the monosaccharide content in the sample by means of a standard curve. Except where stated otherwise, calibration curves were prepared by using D-glucose.

The carbohydrate concentrations obtained from the glucose calibration curves were converted to organic carbon concentrations: (1 mg of glucose = 0.4 mg C) assuming that all monomers were hexoses. The detection limit, C_L, for glucose was calculated as: $C_L = kS_B/b$ [110] where S_B is the standard deviation of the absorptivity for blanks measured at 635 nm, b is the slope of the calibration plot, and k is a constant equal to 3 as recommended by IUPAC [110]. The standard deviation for five blank curves was 0.01, leading to a detection limit of 43 μg C L^{-1}.

3. Results

3.1 *Study of model compounds*

3.1.1 Monosaccharides. We determined the extinction coefficient of some monosaccharides with and without hydrolysis. The MBTH method estimates the number of terminal alcohol groups and terminal groups that can be converted to alcohol groups by reduction with KBH_4. In unhydrolysed samples, the analysis will include both monomeric sugars and free ends of sugars that are attached to other molecules. The results are shown in table 2. The monoccharides studied were: three abundant hexoses,

Table 2. Millimolar extinction coefficients at 635 nm for several compounds reacted with MBTH (for each compound, measurements were made for a carbon concentration of 2.5 mg C L^{-1}).

Compound			Unhydrolysed		12 mol L^{-1} H_2SO_4 hydrolysis		
			Millimolar extinction (mmol $L^{-1}cm^{-1}$)[a]	% Response unhydrolysed glucose	Millimolar extinction (mmol/L/cm)[a]	% Response hydrolysed glucose	% Response unhydrolysed glucose
Hexoses		D-Glucose	9.46 ± 0.01	100	6.05 ± 0.01	100	64
		D-Fructose	9.41 ± 0.04	99	3.74 ± 0.05	62	40
		D-Galactose	9.46 ± 0.05	100	6.34 ± 0.29	105	67
Polyoses		D-Cellobiose			5.57 ± 0.10	92	
		D-Raffinose			2.01 ± 0.08	33	
		Dextran			1.53 ± 0.12	25	
Amino sugars		D-Galactosamine	6.34 ± 0.20	67	6.67 ± 0.34	110	70
Uronic acids	Mono	D-Galacturonic acid	7.97 ± 0.09	84	2.69 ± 0.04	44	28
	Poly	Polygalacturonic acid			2.01 ± 0.08	33	
		Alginic acid			2.16 ± 0.05	36	
		Xanthan gum			3.41 ± 0.24	56	

[a] Errors are given as 1 SD, $n = 3$.

glucose, fructose, and galactose; one amino sugar, galactosamine; and one uronic acid, galacturonic acid. The MBTH response of a large amount of monosaccharides has been widely tested in the past (i.e., [79, 83, 46]). Our aim was not to repeat these studies but rather to test a few compounds representative of the various types present in natural waters. The results obtained for unhydrolysed samples confirmed that no significant differences exist among the MBTH absorptivities of the three hexoses [79, 83] but that the MBTH absorbances of some monosaccharides, such as galactosamine and galacturonic acid, are appreciably lower than the absorbance of glucose. Hydrolysis resulted in a general decrease in the measured absorbances. This decrease was particularly apparent for fructose and galacturonic acid. It is known that drastic conditions of hydrolysis lead to the degradation of some monosaccharides. Fructose has been reported to be destroyed [50, 51], even when mild hydrolysis conditions, such as $0.25\,mol\,L^{-1}$ HCl, are used [53, 103]. The results obtained for monosaccharides show that in samples rich in uronic acids, for example, regardless of the efficiency of the hydrolysis of polysaccharides (see next section), the use of glucose as standard will lead to a significant underestimation of their concentration.

3.1.2 Polysaccharides. The MBTH response of synthetic and natural polysaccarides has also been tested [23, 46, 83], but choosing model compounds representative of the polysaccharides present in natural waters remains a difficult task. On the one hand, as mentioned in section 1, polysaccharides are extremely heterogeneous, so no single standard or set of standards can adequately represent all freshwater polysaccharides. On the other hand, since there is no 'absolute' method for carbohydrate determination, the nature of all the polysaccharides present in natural waters is currently unknown. This makes any real validation of the existing methods impossible and the task of developing better methods difficult. In this study, we have tested several neutral polysaccharides: two glucose homopolymers – cellobiose and dextran –, raffinose (composed of repeating units of galactose, glucose, and fructose), two acidic carbohydrates – polygalacturonic acid and alginic acid – and a complex polysaccharide, xanthan gum. The use of an aminopolysugar, chitin, a polymer of *N*-acetylglucosamine that plays an important structural role in fungi, invertebrates, and algae, proved impossible because of solubility problems with the chitin that is commercially available (chitin from crab shells from Sigma).

With the exception of cellobiose, which is just $(Glu)_2$ with a $\beta(1\rightarrow4)$bond, the results obtained (table 2) show that all polysaccharides tested give a response that is significantly lower than expected. In the case of cellobiose, our results confirm those previously obtained by Pakulski and Benner [83], who also found a slight decrease in the response as compared with hydrolysed glucose, and of Borch and Kirchman [13], who showed that H_2SO_4 hydrolysis (24 h with $0.85\,mol\,L^{-1}$ H_2SO_4) was able to efficiently hydrolyse $\beta(1\rightarrow4)$ linkages in carbohydrate dimers. In other sugars, the deceptive response can be explained by the difficulty involved in breaking the glycosidic bonds. This is the case, for instance, with raffinose, whose unique glycosidic bonds cannot easily be broken down into their simple sugars. It is well known, for example, that raffinose cannot be absorbed by the small intestine and is often metabolized by bacteria in the large intestine to form unwanted gaseous by-products. The low response of acidic polysaccharides, polygalacturonic acid, and alginic acid (linear copolymers consisting mainly of residues of $\beta(1\rightarrow4)$-linked D-mannuronic acid

and $\alpha(1\rightarrow4)$-linked L-glucuronic acid) can be explained by the reported difficulty of hydrolysing acidic polysaccharides [32, 111]. As pointed out in section 1, uronic acid-containing polysaccharides have glycosidic bonds that are more resistant to hydrolysis due to the protective effect of the carboxyl groups. Alginic acid is considered to be a representative uronic acid of the extracellular polysaccharides produced by bacteria [112] and algae [113].

The low response of dextran is more difficult to explain. Dextran is a glucose homopolymer. It is flexible and globular, and has a relatively low molecular weight (40 kDa). Since it is a reserve polysaccharide, it is expected to be relatively biodegradable.

The relatively high response of the reserve polysaccharide xanthan gum is also surprising. Xanthan gum is a semi-rigid, fibrillar, high-molecular-weight (several thousand kilodaltons) polysaccharide. It has a fairly complex structure. It is an anionic polyelectrolyte with a $(1\rightarrow4)$-β-D-glucopyranose glucan (as cellulose) backbone with side chains of $(3\rightarrow1)$-α-linked D-mannopyranose-$(2\rightarrow1)$-β-D-glucuronic acid-$(4\rightarrow1)$-β-D-mannopyranose on alternating residues. Slightly less than half ($\sim$40%) of the terminal mannose residues are 4,6-pyruvated, and the inner mannose is mostly 6-acetylated. It has been suggested that its natural state is bimolecular antiparallel double helices. It may form a very stiff intramolecular double-stranded helical conformation by the annealing of the less stiff 'natural' denatured elongated single-stranded chains. The glucan backbone is protected by the side chains which lie alongside it, making it relatively stable to acids, alkalis, and enzymes. However, it seems to degrade more easily than raffinose in our hydrolysis conditions.

Irrespective of whether the low responses obtained for most of the compounds tested in this study or in previous studies can be justified on a case-by-case basis, the important point is that the MBTH method significantly underestimates the contents of most of the polysaccharides studied. Although, as explained above, it is impossible to prove the extent to which the compounds tested in this study are representative, there is no reason to think that the carbohydrates present in natural waters, if different, will provide a better response.

3.2 *Effect of the presence of other types of NOM in the samples*

We tested the effect of the presence of humic and fulvic compounds and of glutathion and BSA as protein models. Although it is well known that colorimetric techniques often suffer from potential non-selectivity, Burney and Sieburth [23] and Johnson and Sieburth [79] concluded in their seminal publications that little analytical interference from non-carbohydrate compounds in natural waters can be expected. However, Senior and Chevolot [92] observed interference from humic substances in their MBTH measurement of carbohydrates in an estuary. This was attributed to the existence of a continuum of sugars loosely associated with humics and to the presence of carbohydrate-like entities within them. A recent study showed that humic substances produced a systematic 10–20% error rate in the TPTZ method [102]. Hung *et al.* [21] results confirmed that humic substances provide a part of the TPTZ response.

The results are shown in table 3. Absorbances measured in $5\,\mathrm{mg\,C\,L^{-1}}$ Suwannee River humic and fulvic compound solutions are, respectively, 2.1 and 6.8% of the absorbance given by glucose. According to IHSS, the concentration of carbohydrate in their humic and fulvic compounds is 16.5 and 7.4% (w/w), respectively. This means

Table 3. Millimolar extinction coefficients at 635 nm for several potential interfering compounds.[a]

Compound		Millimolar extinction[b] (mmol C L^{-1} cm^{-1})	% glucose
Refractory organic matter	IHSS fulvic acid standard (1S101F)	0.412 ± 0.001	6.8
	IHSS humic acid standard (2S101H)	0.127 ± 0.007	2.1
Proteins	Glutathione	0.050 ± 0.009	0.8
	Bovine serum albumin	0.097 ± 0.008	1.7

[a] For each compound, millimolar extinctions coefficients were measured for a carbon concentration of 5 mg C L^{-1}.
[b] Errors are given as 1SD, $n = 3$.

Table 4. Concentration of 'dissolved'[a] carbohydrates (expressed as mg C L^{-1}) and % DOC[a] for different natural water samples.

System	Depth (m)	Sampling date	Type of system	Carbohydrates[b] (mg C L^{-1})	% DOC
Lake Brienz	1	21 February 2005	Ultra-oligotrophic perialpine lake	0.047 ± 0.005	14
	1	21 March 2005		0.431 ± 0.007	63
	1	20 July 2005		0.16 ± 0.02	38
	25	20 July 2005		0.07 ± 0.02	13
Lake Geneva	0.1	9 August 2005	Mesotrophic lake	0.65 ± 0.02	34
Lake Bret	0.1	26 July 2005	Eutrophic lake	1.54 ± 0.02	37
River Aare	0.1	23 July 2005	Alpine, modified glacial river, tributary of Lake Brienz	0.08 ± 0.01	36

[a] Defined by filtration at 1.2 µm.
[b] Errors are given as 1SD, $n = 3$.

that the presence of humic or fulvic substances does not lead to an overestimation of the carbohydrate concentration in the sample. Recently, Hayakawa [46] found that fulvic acid extracted with a XAD-7 resin from a Japanese river gave 4% of the glucose absorbance. The presence of the tested proteins (5 mg C L^{-1}) leads to an overestimation of the total carbohydrate of less than 2%. In natural waters, with a much lower protein content, the effect of the presence of proteins can be considered to be negligible.

3.3 *Application to natural water samples*

The method has been applied to the determination of polysaccharides in several freshwaters with contrasting physico-chemical characteristics and/or trophic status. The results are shown in table 4. Total DOC concentrations and humic-type compounds have been simultaneously determined in the samples. The chosen water bodies range from an ultra-oligotrophic perialpine lake, Lake Brienz, to a eutrophic lake, Lake Bret. Waters from a mesotrophic lake (Lake Geneva) and one river draining granitic and alpine areas (River Aare), have also been analysed. All systems are located in Switzerland. Their characteristics are given in table 4. Total DOC concentrations range from 0.22 mg C L^{-1} in the River Aare to 4.2 mg C L^{-1} in Lake Bret. MBTH-carbohydrate concentrations range from 0.047 mg C L^{-1} in Lake Brienz (February) to 1.5 mg C L^{-1} in Lake Bret. Interestingly, carbohydrates make up a similar percentage of DOC in the different systems in the same season (summer), about 36% on average, regardless of the trophic state of the system. However, the percentage changes considerably in a given system (Lake Brienz) through the seasons, i.e., MBTH-carbohydrates only represent 14% of total DOC in February, but 63% just a month

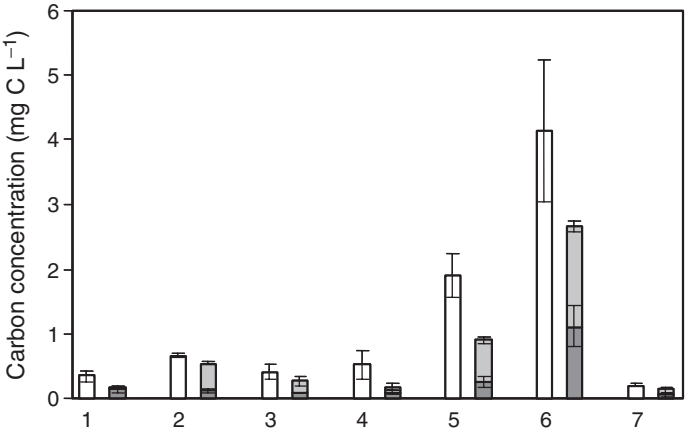

Figure 1. Concentrations of organic carbon (white bar), carbohydrates (light grey bar) and humic substances (dark grey bar) for several waters (filtered at 1.2 μm). 1: Lake Brienz 1 m (21 February 2005); 2: Lake Brienz 1 m (21 March 2005); 3: Lake Brienz 1 m (20 July 2005); 4: Lake Brienz 25 m (20 July 2005); 5: Lake Geneva (9 August 2005); 6: Lake Bret (26 July 2005); 7: Aare (23 July 2005). Error bars represent 3SD ($n = 3$).

later, in March, during the spring planktonic bloom. Significant differences are also observed at different depths, i.e., a small percentage of DOC is carbohydrate below the photic zone in July in the same lake.

In the past, it was difficult to ascertain whether the DOC undetected by the MBTH method was another type of NOM, such as humic-type compounds, or carbohydrates not 'seen' by the method. In this study, we have simultaneously analysed the samples for humic-type substances. The results are shown in figure 1. In some systems, the amount of 'unknown' DOC is relatively small and can be attributed to the presence of other types of NOM such as lipids and proteins. However, for some samples, such as Lake Brienz in July at 25 m, Lake Geneva, and Lake Bret, the percentage of non-classified DOC is high (66, 36, and 55%, respectively). A significant proportion of this undetected DOC is probably made up of carbohydrates that are either difficult to hydrolyse, or whose constituent monosaccharides give a lower MBTH-response than glucose. For example, for Lake Bret, if xanthan gum was used for the calibration instead of glucose, the concentration of carbohydrate plus humic-type compounds would represent 94% of total DOC, and if galacturonic acid was used, this would rise to 112%. 'Better' budgets are also obtained for Lake Geneva (74 and 91%, respectively) if these two carbohydrates are used as standards: thus, the results obtained might reflect distinct compositional differences between polysaccharides from the different systems or from the same system in different seasons. The 'best' standard would probably be a mixture of different carbohydrates that reflects the carbohydrates present in a given system. Such a standard is, for the moment, impossible to define. A possible future method could be to test different mixtures as standards in order to work out which were the main polysaccharides present by looking at how 'good' the budget calculations were.

The relevant data published for freshwater systems have been collected (table 5). Since chromatographic methods systematically give lower results than colorimetric methods [13, 21, 53], only the results obtained using colorimetric methods have been considered. The ratio of total carbohydrates to total organic matter is given

Table 5. 'Dissolved' carbohydrate concentrations in freshwater systems (published values using colorimetric methods).

System	Type	Date	Depth (m)	Concentration (as published)	Concentration ($mg\,C\,L^{-1}$)	% DOC	Method[a]	Reference
Lake Suwako, JP	Shallow, eutrophic	August 1978	Profile 0–5.5	0.92–14.25 mg L^{-1} [b]			Filtration: GF/C	[68]
Lake Nakanuma, JP	Small, eutrophic	May 1978	Profile 0–12	0.77–1.95 mg L^{-1} [b]			Hydrolysis: 1 mol L^{-1} HCl, 7 h, 100°C	
Lake Shirakomaike, JP	Dystrophic mountain lake	August 1978	Profile 0–6	0.77–1.01 mg L^{-1} [b]			Reagent: anthrone Standard: not given	
Elorn River, Bay of Brest, FR		February 1985–January 1986 (12 sampling campaigns)		230–860 µg C L^{-1}	0.23–0.86		Filtration: GF/C	[92]
							Hydrolysis: 1.8 mol L^{-1} HCl, 3.5 h, 100°C	
							Reagent: MBTH Standard: (glucose + manitol)/2	
Lake Constance, DE/CH	Mesotrophic lake	April–August 1992	Profile 0–50	1.7–5.5 µmol glucose L^{-1}	0.12–0.40		Filtration: 0.2 µm Nucleopore filters	[84]
							Hydrolysis: 0.85 mol L^{-1} H_2SO_4, 4 h, 100°C	
							Reagent: MBTH Standard: glucose	
Lobo Reservoir, BR		Aprril 1989–March 1990		0.26–0.74 mg C L^{-1} mean = 0.89, SD = 0.39 (n = 25)	0.26–0.74		Filtration: GF/C	[44]
							Hydrolysis: 0.5 mol L^{-1} HCl, 4 h, 100°C	
							Reagent: MBTH Standard: not given	
Lake Bret, CH	Eutrophic lake	April–July 1995 15 August 2005	3 Profile 0–14	0.5–1 mg $C\,L^{-1}$ [c] 0.3–1.2 mg $C\,L^{-1}$ [c]	0.5–1 0.3–1.2	15–22[d] 10–31[d]	Centrifugation (1 h, 3,700 g, < 1 µm)	[93]
							Hydrolysis: 1.2 mol L^{-1} H_2SO_4, 3 h, 100°C (after 12 mol L^{-1} H_2SO_4, 2 h, 25°C prehydrolysis)	
							Reagent: MBTH Standard: not given	

(*Continued*)

Table 5. Continued.

System	Type	Date	Depth (m)	Concentration (as published)	Concentration ($mg\,C\,L^{-1}$)	% DOC	Method[a]	Reference
Lena River, RU		July 1994	18 stations	190–470 μg C L^{-1}; mean = 299 μg C L^{-1}	0.19–0.47	1.2–2.5	Filtration: not given Hydrolysis: trifluoroacetic acid Reagent: MBTH Standard: glucose	[94]
Lena River, RU		July 1994		1.6 μmol glucose L^{-1}	0.12	18	Filtration: GF/F Reagent: L-tryptophan-sulphuric acid Standard: glucose	[72]
Clima Lake system, BR		20, 21 June 1995	9 sites	< DL (1.8)–4.3 $mg\,L^{-1}$ [b]			Filtration: GF/F Hydrolysis: not given Reagent: phenol-sulphuric acid Standard: glucose	[77]
Sep Reservoir, FR	Oligomesotrophic, recently flooded reservoir	April–August 1997 (17 sampling campaigns)		1.3–3.1 $mg\,L^{-1}$ [b]; mean = 2.0 $mg\,L^{-1}$ [b]			Filtration: 0.12 μm polycarbonate filters Hydrolysis: 1 $mol\,L^{-1}$ HCl, 15 h, 100°C Reagent: MBTH Standard: glucose	[95]
Sacramento River, CA, US		April–October 1996 (7 dates)		2.25–6.55 μmol glucose L^{-1}; mean = 3.23 μmol glucose L^{-1}	0.16–0.47 mean = 0.23	mean = 2.5	Filtration: GF/F Hydrolysis: 1 $mol\,L^{-1}$ HCl, 20 h, 100°C Reagent: MBTH Standard: glucose	[96]

Lake Biwa, JP	Mesotrophic lake	August 1998–February 1999	Profile 0–70 Profile 0–70	11.6–49.2 μmol C L^{-1} [c] 18.0–22.7 μmol C L^{-1} [c]	0.14–0.59 0.22–0.27	14–42 20–25	Filtration: GF/F Hydrolysis: 2 mol L^{-1} H_2SO_4, 2 h, 100°C (after swelling in 6 mol L^{-1} H_2SO_4 2 h room T) Reagent: MBTH Standard: glucose	[46]
Lake Crooked Lake Druzhby (Antarctic lakes)	Oligotrophic lakes (DOC < 3 mg L^{-1})	March 1999–February 2000 (7 dates)	60 40	0.1–0.7 μmol L^{-1} [c, d] ND–2.3 μmol L^{-1} [c, d]			Filtration: GF/F Hydrolysis: not given Reagent: MBTH Standard: not given	[97]
Trinity River, TX, US		2000–2001		35.6–155.4 μmol C L^{-1}	0.42–1.86	9–36	Filtration: GF/F Hydrolysis: 0.1 mol L^{-1} HCl 1 h 150°C Reagent: TPZ Standard: not given	[21]

[a] Pore size filters: GF/C = 0.7 μm, GF/F = 1.2 μm. Both are glass-fibre filters.
[b] It is not clear whether units are expressed as mg of C or mg of glucose.
[c] Scanned from published figures by using Un-Scan-It [114].
[d] It is not clear whether units are expressed as μmol of C or μmol of glucose.

wherever possible. Our data lie within the range of published values, but a meaningful comparison of data is difficult because of the variability of the filters, digestion procedures, and colorimetric method used. In some cases, even the exact meaning of the units used by the different authors is unclear.

4. Conclusions

The MBTH method is quite popular because it is sensitive without requiring expensive equipment such as GC and HPLC. Although the sensitivity and precision are good, the method involves numerous steps, including several chemical reactions, which makes the procedure laborious. However, the main weakness of the method is not that its application is cumbersome but that the measurement obtained is relative because the values are estimated in relation to one monosaccharide of general occurrence, usually glucose. The underlying hypotheses are that: (1) all monosaccharides that constitute natural carbohydrates give the same analytical signal with the MBTH reagent as glucose does and (2) that polysaccharides hydrolyse completely without any loss of monosaccharides. Our results indicate that both hypotheses are probably wrong: (1) model compounds systematically show a much lower MBTH reponse than expected; (2) the simultaneous determination of the so-called humic fraction and of the MBTH-carbohydrates in the same samples, with the estimation of the corresponding DOC mass balances, has allowed us to show that a significant part of the organic carbon remains undetected and that, as a consequence, a significant proportion of the carbohydrates present in some natural waters are probably not 'seen' by the method.

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