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Determination of marine monosaccharides by high-pH anion-exchange chromatography with pulsed amperometric detection

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Abstract

In this study, a method was developed for the determination of ten monosaccharides in marine particulate matter utilizing high-pH anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). Samples were analyzed using a Dionex CarboPac PA1 column with a flow-rate of 1 ml/min and addition of 380 m*M* NaOH post-column. The effect of NaOH concentration (between 0.5 and 50 m*M*) on the monosaccharide separations expressed by the capacity factor (k') was tested. The results showed that one isocratic elution was unfit to discriminate properly arabinose, fructose, fucose, galactose, glucosamine, glucose, mannose, ribose, rhamnose and xylose. Thus, two isocratic elutions, at 2.5 and 15 m*M* NaOH, were necessary to separate and quantify with significant selectivity (α) and resolution terms (R_s), respectively, glucose, mannose, xylose and fucose, rhamnose, arabinose, glucosamine, galactose, fructose and ribose. The method is linear for all sugars over the concentration range tested (25–250 ng per injection or 1–10 mg/g) expected in marine concentrated samples, and reproducibility was found to be satisfactory (1.7–4.8%), except for ribose (27%). Monosaccharide determinations from two kinds of marine matrix (hydrolyzates of surface sediment and of suspended particulate matter) are presented.

1. Introduction

In the marine environment, carbohydrates are commonly found as structural and storage compounds of marine plankton and terrestrial remains. The polysaccharide and, particularly, the monosaccharidic composition may be considered to be specific for each biological group. Like proteins and lipids, they constitute a large part of the marine organic matter with, respectively, 2–30% and about 10% of the organic carbon content in marine suspended particles and superficial sediments [1–4].

Organic particles originating from surface layers are degraded preferentially during settling through the water column [5–7]. Therefore, the monosaccharide spatial distribution may be used as a biogeochemical indicator of both the origin and the decomposition pathways of organic matter in the sea [8–12].

Several colorimetric and chromatographic methods are applied with relative success to determine carbohydrates in marine samples. Burney and Sieburth [13] have designed an improved colorimetric method to determine total

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carbohydrate concentrations in seawater. However, this method, which has been improved and is widely used [14–16], cannot distinguish between monosaccharides and any related substances, like humic compounds, with a terminal glycol function (CHOH–CH_2OH) [17].

Paper and thin-layer chromatography were the first chromatographic techniques used to separate individual sugars, but separation was limited to the number of recognized analytes [18]. The extremely polar, non-volatile and non-chromophoric properties of sugars caused difficulties in separation and detection steps. Chromatographic techniques require derivatizations to overcome these drawbacks. Numerous derivatization techniques have been applied with relative success to marine sample analysis, such as acetylation [19–21] and trimethylsilylation [22,23], in gas chromatography. Dansylhydrazine (DNS) reagent is widely used in high-performance liquid chromatography (HPLC) with reversed-phase separation and fluorimetric detection [4,24,25]. However, in both methods, the pretreatments for derivatization involve complex and time-consuming steps. High-pH anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) was recently developed for monosaccharide analysis in marine samples [26,27]. This method, which allows direct injection without derivatization steps, is more rapid and requires a shorter sample preparation.

Because carbohydrates are weak acids with pK_a values ranging from 12 to 14, they can be ionized in an alkaline eluent and separated by anion-exchange mechanisms. The electrochemical detector applies a repeated triple potential sequence to an electrode and measures the resulting current [28]. Non-chromophoric molecules are detected with excellent sensitivity [29]. In natural waters, the detection limits for mono- and disaccharides range from 2 to 10 n*M* or 0.4 to 0.8 pmol/injection [27].

The high complexity and diversity of the marine particulate organic matter lead to a large number of neutral sugars which are not easily discriminated. Mopper et al. [27] were the first to establish an extraction and separation protocol of sugars in seawater by HPAEC–PAD. However, the resolution with the used isocratic elution was not good enough to separate closely eluting sugars (galactose, glucose, xylose and mannose). In this paper, we discuss the selection of eluent conditions to achieve good separation and quantitation of ten main marine sugars by HPAEC–PAD. We examine then the validity of the selected method with two kinds of marine samples.

2. Materials and methods

2.1. Chemicals

Sugar standards were purchased from Sigma (St. Louis, MO, USA). NaOH and H_2SO_4 solutions were obtained from, respectively, a 50% low-carbonate NaOH solution (J.T. Baker, France) and a H_2SO_4 R.P. Normapur (min. 95%) (Prolabo, France) solution. Sugar standards, NaOH and H_2SO_4 solutions were prepared with fresh Milli-Q water (18.2 MΩ). Neutralization of hydrolyzed samples was performed with precombusted (450°C, 12 h) $CaCO_3$ powder (Merck, Germany).

2.2. Chromatographic system

The chromatographic system was purchased from Dionex (France) and consists of three modules:

–Three eluent reservoirs and an advanced gradient pump (AGP). Helium was used to pressurize the eluent reservoirs and to prevent dissolution of carbon dioxide by bubbling for 15 min prior to use. Otherwise carbonates would bind much more strongly on the columns and would induce reduced retention times. Tests of monosaccharide separations were performed with several isocratic gradients from solutions of Milli-Q water (eluent 1) and 50 m*M* NaOH (eluent 2). The system of the column was cleaned after each analysis with 300 m*M* NaOH for 5 min and weekly with 600 m*M* NaOH (eluent 3) for about 20 min.

–A basic high-pressure chromatography module.

This module contains a single hydraulic system which includes an injection valve, a column system and a detector cell. Samples were injected into the HPAEC–PAD system by a high-pressure injection valve with a 25-μl sample loop and eluted at a rate of 1 ml/min. Separations were performed with a CarboPac PA1 column (250 × 4 mm I.D.) and a CarboPac guard column (50 × 4 mm I.D.) (Dionex), which extends the CarboPac PA1 lifetime. A post-column delivery system of 380 m*M* NaOH with a flow-rate of 0.5 ml/min was added to the HPAEC–PAD system to increase the detector response and minimize the baseline drift. The detector cell contains a working gold electrode and a combination pH–Ag/AgCl reference electrode.

–A pulsed electrochemical detection module. Integrated and pulsed amperometric detection was performed with an adequate triple potential sequence (E_1, E_2, E_3) for carbohydrate analyses, supplied to the working gold electrode for durations T_1, T_2 and T_3 (Fig. 1) [30]. From the detection potential E_1 (+ 0.05 mV), which was found to be optimal by voltammetry of glucose,

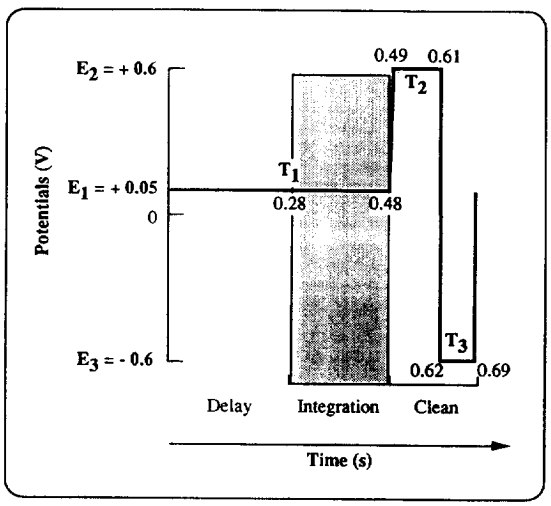

Fig. 1. Optimal setting for carbohydrate detection using a gold working electrode and an Ag–AgCl reference electrode. Carbohydrates are oxidized by the application of E_1 (+ 0.05 mV), a positive potential, and after a delay of 0.28 s the resulting current is integrated to give a chromatographic signal. The current generated is proportional to the carbohydrate concentration. E_2 (+ 0.06 mV) and E_3 (– 0.06 mV) are the positive and negative cleaning potentials.

a signal is measured by integrating the generated current for a fixed time (0.2 s). Anodic detection is followed by two cleaning pulses E_2 (+ 0.6 mV) and E_3 (– 0.6 mV), which regenerate the surface of the electrode [30].

A Kenitec 386DX-33 computer equipped with the Dionex AI-450 chromatography software pilotes the gradient pump, injection valve and detector and receives in real time data via a Dionex interface. This software is also used to calibrate and integrate the peaks.

3. Results and discussion

3.1. Separation optimization

Rocklin and Pohl [31] have described effects of NaOH on the retention behaviour of sugars and it was generally observed that selectivity changes as NaOH concentration is varied. Tests of separation were therefore achieved on a standard solution with 13 different NaOH concentrations (Fig. 2). Low NaOH concentrations are enough to ionize partly neutral sugars and to separate them. For every sugar retention time increased with decreasing NaOH concentration. This figure exhibits also separation problems such as overlaps between glucosamine, galactose, glucose and especially between the pairs rhamnose–arabinose and xylose–mannose. An elution order reversal is observed for xylose and mannose as the NaOH mobile-phase concentration varied, which is in agreement with the results obtained by Germain [26].

For high NaOH concentrations, we noted a large peak including galactose, glucose and mannose. For the low NaOH concentrations these components were well separated but rhamnose and arabinose shared the same peak. Intermediate concentrations can be divided into two ranges, 7.5–15 m*M* and 17.5–25 m*M* NaOH. The first range was characterized by the coelution of mannose and xylose; the second one discriminated them but with a low accuracy. The selectivity (α) and resolution (R_s) were estimated from chromatograms obtained with 17.5, 20 and 25 m*M* NaOH (Fig. 3). Although the

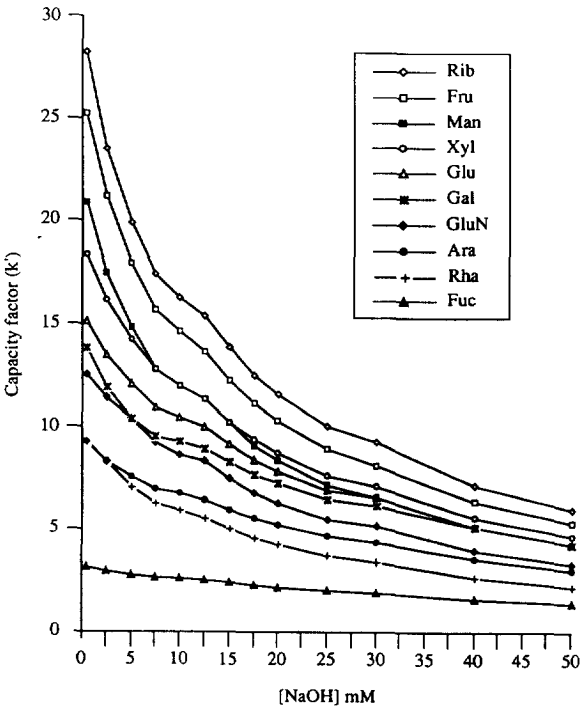

Fig. 2. Influence of the eluent NaOH concentration (0.5–50 mM) on the capacity factor (k') of ten sugars. Sugars are eluted under isocratic conditions. Abbreviations: Rib = ribose; Fru = fructose; Man = mannose; Xyl = xylose; Glu = glucose; Gal = galactose; GluN = Glucosamine; Ara = arabinose; Rha = rhamnose; Fuc = fucose.

three chromatograms contained 10 peaks, the α and R_s values were too low for a good integration, especially for glucose, mannose and xylose. Moreover, these values were optimal, i.e. they were obtained from standard solutions without contaminants such as anions.

This experiment showed that separation of all sugars was not possible with one isocratic elution. Addition of CH_3COONa to the NaOH eluent [32] did not improve the separation of rhamnose and arabinose and a gradient elution leading to fair separations of marine samples was difficult to obtain (data not shown). Therefore, we chose to determine monosaccharides with two isocratic elutions for obtaining accurate separations of the peaks with standard solutions as well as with marine samples. Isocratic elution with 2.5 and 15 mM NaOH was thus selected to quantitate, respectively, glucose, xylose, mannose and fucose, rhamnose, arabinose, glucosamine, galactose, fructose and ribose (Fig. 4). Excellent resolution of the integrated peaks was observed in both cases. Using a 2.5 mM NaOH isocratic elution, xylose and mannose are well separated with R_s and α values (Fig. 5) higher than those obtained with 17.5, 20 and 25 mM (Fig. 3) NaOH. The 15 mM NaOH isocratic elution represented the best compromise to integrate the remaining sugars with excellent separation parameters (Fig. 5). Although two elutions are time-consuming, this study has shown that they are necessary to determine all sugars contained in marine samples.

Elution time	NaOH 17.5 mM Rs	NaOH 17.5 mM α	NaOH 20 mM Rs	NaOH 20 mM α	NaOH 25 mM Rs	NaOH 25 mM α
Gal						
	1.04	1.09	1.08	1.08	1.13	1.07
Glu						
	1.27	1.08	1.09	1.07	0.77	1.04
Man						
	0.58	1.03	0.70	1.04	0.89	1.06
Xyl						

Fig. 3. Separation parameters (R_s: resolution parameter, α: selectivity factor) determined for closely eluting sugars with three NaOH concentrations (isocratic elutions). We consider peaks sufficiently resolved when $R_s \geq 1$ and $\alpha > 1$. Sugar abbreviations given in Fig 2.

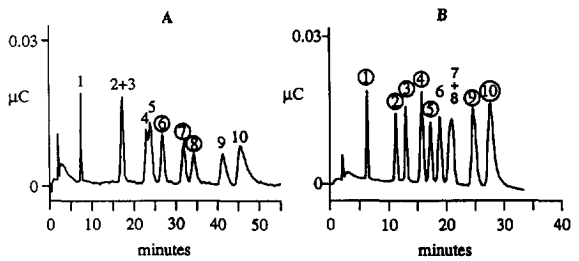

Fig. 4. Chromatograms of a standard solution of ten sugars obtained with two isocratic NaOH concentrations [(A) 2.5 mM and (B) 15 mM NaOH], selected to separate correctly every sugar. Encircled numbers correspond to the integrated peaks. Sugar solutions were injected onto the HPAEC–PAD system (injection volume: 25 μl; column: CarboPac PA1; flow-rate: 1 ml/min, addition of 380 mM NaOH post-column with a 0.5 ml/min flow-rate). Peaks: 1 = fucose; 2 = rhamnose; 3 = arabinose; 4 = glucosamine; 5 = galactose; 6 = glucose; 7 = xylose; 8 = mannose; 9 = fructose; 10 = ribose.

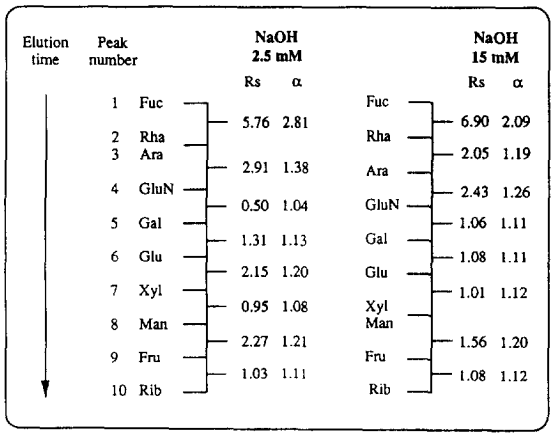

Fig. 5. Separation parameters (R_s and α) determined for all sugars with two selected NaOH (2.5 and 15 m*M*) concentrations as mobile phase. Sugar abbreviations given in Fig. 2.

3.2. Calibration and accuracy

Calibration was carried out by dissolving ten commercially available monosaccharides in 250 ml Milli-Q water. This standard solution was diluted to give a calibration range (25–250 ng/injection or 1–10 mg/g) with the same order of concentrations as those found in marine concentrated hydrolyzates. Calibration graphs and parameters of the resulting regression lines were obtained with 15 and 2.5 m*M* NaOH isocratic elutions (Table 1). The ten tested monosaccharides gave a linear response within the range studied, characterized by high correlation coefficients and *y*-intercepts near zero. Nevertheless, peak widths increased with elution time, so that the integration of late-eluting components such as fructose and ribose was less accurate. The large peak width of these two monosaccharides explains the reduced resolution. Higher concentration ranges for the calibration test would permit to correct this problem. Nevertheless the excellent fit ($R > 0.99$ for all sugars) demonstrated that this technique is suitable for quantitative determinations.

Reproducibility was tested by performing a series of six identical isocratic runs (15 and 2.5 m*M* NaOH) with a ten-component sugar standard solution (Table 2). The relative standard deviation (R.S.D.) of the retention time for all monosaccharides was found to be less than 3%. Moreover, the precision of this method, expressed as R.S.D. of individual monosaccharide areas, was satisfactory, despite a 27% value for ribose. For this component, a small variation of the integration parameters (start and end peak) induced a large variation of the peak area.

3.3. Application to marine particulate samples

Applications were performed on two kinds of matrices: marine suspended particles and surficial sediment collected, respectively, at 30 m depth off Banyuls (42°30′ N, 03°27′ E) and in the axis of the Grand-Rhône Canyon (1012 m)

Table 1
Linear regression parameters for calibration of ten main sugars expected in a marine environment

NaOH (m*M*)	Monosaccharides	Range (ng/inj.)	Slope ($\times 10^5$)	*y*-Intercept ($\times 10^4$)	Regression coefficient (*r*)
15	GluN	28–271	2.384	−2.213	0.9986
	Ara	25–248	1.992	−1.420	0.9997
	Gal	24–234	1.958	−2.522	0.9988
	Fuc	26–257	1.704	−1.098	0.9993
	Rib	45–438	1.679	−19.980	0.9992
	Rha	25–227	1.448	−0.118	0.9990
	Fru	47–459	1.060	−3.232	0.9993
2.5	Xyl	29–285	2.355	−7.614	0.9983
	Glu	28–285	2.294	−4.034	0.9991
	Man	25–248	1.876	−9.207	0.9974

Table 2
Relative standard deviation (R.S.D.) for sugar retention times and peak areas after repeated ($n = 6$) isocratic elutions (15 and 2.5 m*M* NaOH)

Eluent (mM NaOH)	Sugars	R.S.D. (%)	
		Time	Area
15	Fuc	0.9	1.7
	Rha	1.3	3.8
	Ara	1.1	3.8
	GluN	1.8	2.6
	Gal	1.4	2.9
	Fru	1.1	3.9
	Rib	1.2	27
2.5	Glu	1.5	1.7
	Xyl	1.2	2.1
	Man	2.2	4.8

(42°50′ N, 04°48′ E). Both stations are located on the Northwestern Mediterranean margin. Samples were collected with a Sea-Bird (WA, USA) carrousel water sampler fitted with twelve 8-l Niskin bottles for seawater recovery (suspended particles) and with a multi-tube correr for surficial sediment.

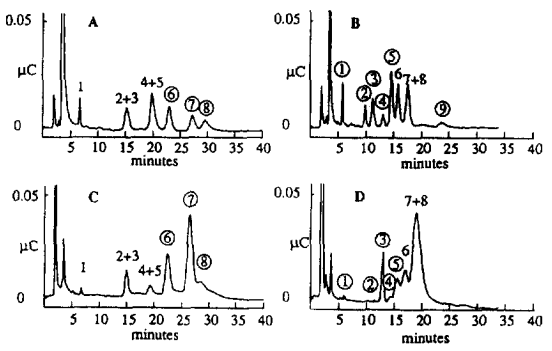

Fig. 6. HPAEC–PAD chromatograms of hydrolyzed marine surface sediment (A and B) and hydrolyzed marine suspended particulate matter (C and D). Chromatograms A and C are obtained with a 2.5 m*M* NaOH isocratic elution and B and D with 15 m*M* NaOH. Sampling stations are located on the Northwestern Mediterranean margin. Encircled numbers correspond to the integrated peaks. Analytical conditions given in Fig. 2.

Three litres of seawater were filtered through a precombusted (450°C for 12 h) Whatman GF/F filter. The filter and surficial sediment (about 50 mg) were stored in the dark at −20°C until lyophilization and hydrolyzed in 5 ml H_2SO_4 (1 *M*) at 90°C for 4 h. After cooling, acid-hydrolyzed samples were neutralized and sulphates precipitated by addition of $CaCO_3$ powder. Sonicated precipitates gave an opaque solution clarified by centrifuging at 3000 rpm for 5 min [27]. Amounts of 25 µl of the supernatants were injected directly onto the HPAEC–PAD system. Peaks were identified by comparing peak elution times and in doubtful cases by coinjection with standard monosaccharides.

Chromatograms are shown in Fig. 6. The presence of larger amounts of anions in the injected marine samples than in the standard solutions resulted in reduced retention times. A severe cleaning step between each injection is then performed to remove strongly retained anions. Nine monosaccharides, including fucose, rhamnose, arabinose, glucosamine, galactose, glucose, mannose, xylose and ribose have been identified and correctly separated by using 2.5 and 15 m*M* NaOH as eluent concentration. Comparison of chromatograms revealed a higher diversity of monosaccharides in sediment than in seawater samples, where fucose, rhamnose and fructose were not detected. The monosaccharide composition depends on the kind of sampled biological material and on its degradation state [5,6,33]. Concentrations of monosaccharides ranged between 0.12 (glucosamine) and 0.78 mg/g (galactose) and between 0.66 (fucose) and 28.13 mg/g (xylose) for the acid hydrolyzates of the surficial sediment and suspended particulate matter, respectively. Total monosaccharide concentration in the suspended particles (58.18 mg/g) was about ten times higher than in the surficial sediment (5.41 mg/g). The suspended particulate matter in the surface layers of the sea is more fresh, with living organisms; the settling material reaching the bottom corresponds to dead organisms which will be subject to biological, chemical and physical degradation processes [11,34–36].

4. Conclusion

This study has shown the presence of antagonistic pairs of monosaccharides (arabinose–rhamnose and mannose–xylose) and closely eluting peaks (galactose, glucose, mannose and xylose), and, therefore, the impossibility to determine accurately all sugars with one isocratic elution. Moreover, the high level of salt and the high complexity of the marine organic matter resulted in decreased resolution and unidentified peaks [27]. The use of two isocratic elutions (at 2.5 and 15 m*M* NaOH) consequently appeared necessary to separate and quantitate correctly the ten major monosaccharides expected in marine samples. Although this method requires two runs for each sample, it is quite simple, with a direct injection of hydrolyzates, rapid and particularly adapted to our multiple-sample study.

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