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Carbohydrate analysis by methanolysis method and application to compositional analysis of transparent exopolymer particles

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ABSTRACT

Measurement of uronic acids (URAs) which are a group of acidic sugar, would be useful for the understanding of dynamics of bacterial extracellular polymeric substances (EPS) in marine environments. However, the URA analysis using traditional hydrolysis method which is used for neutral sugar analysis poses serious problems in URA that is unstable under hydrolysis. We developed the methanolysis method, which deploymerizes polysaccharides while retaining quantitative information. Our method was applied to coastal seawater, and the URAs distribution was compared with that of transparent exopolymer particles (TEP) which are acidic sugar containing particles. Since the relationship of URA with TEP was relatively weak, URA-containing polysaccharides present in bacterial EPS would not participate as a structural component of TEP.

Keywords: Uronic Acid; Transparent Exopolymer Particles; Methanolysis; Gas Chromatography Mass Spectrometry

1. INTRODUCTION

Quantitative and qualitative analyses of carbohydrates in seawater provide some information on dynamics of organic matter such as its origin and diagenetic processes [1-3], as well as its vertical transport and sedimentation [4-6]. Monosaccharides characterized in these studies were mainly neutral sugars (NSs), which comprise a major group of carbohydrates, but there are other minor sugar groups such as uronic acids (URAs). Although URA, a carboxylated acidic sugar, has minor contribution to total carbohydrates in most cases, it is known as an important component of bacterial extracellular polymeric substances (EPSs) (20% - 50% of total carbohydrate: [7,8]), and URA measurements are likely to reflect the dynamics of bacterial EPSs in a water column. EPSs readily adhere to each other as a result of their pronounced stickiness [9], and constitute amorphous aggregates such as transparent exopolymer particles (TEPs), which are defined as particles containing acidic sugar [10,11]. Since TEPs are relevant to sinking particle formation and the supply of food to filter feeders [11-14], URA measurements should allow us to better understand the contribution of bacterial EPSs to these processes.

Analysis of neutral sugars has been achieved by using chromatographic measurements after depolymerization under acid hydrolysis reaction [15]. On the other hand, application of acid hydrolysis method to URA analysis has some problems, because URA, once released after hydrolysis, forms lactones irreproducibly [16]. To overcome this, it is necessary to correct the recovery yield of URA after hydrolysis reaction, or to use other depolymerization method. In the present study, we apply the methanolysis method, which depolymerizes polysaccharides using methanolic HCl. High recovery yields for authentic standards and some plant materials were achieved by some previous researchers [17,18], but the suitability of the methanolysis method for marine environmental samples has yet to be examined.

In the present study, we modified a previous methanolysis method for the determination of URA in seawater samples [17,18]. In addition, we examined the monosaccharide composition of natural seawater sample in a coastal environment using the methanolysis method, and compared the distribution of URA in the water column

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with that of TEP to understand the contribution of URA to aggregate formation.

2. MATERIALS AND METHODS

2.1. Sample Collection

In 2007, seawater samples were collected from a coastal region at a station in Suo-Nada (30 m depth, 131,16E, 33,49N) during the fifth cruise of the TRV Toyoshio Maru of Hiroshima University in July, 2007. Seawater was collected from 1, 5, 10, 15, 20 and 25 m using a Rosette sampler fitted with Niskin bottles. The samples were filtered through precombusted (450˚C, 4 h) glass fiber filters (Whatman GF/F) and the filters were stored at −20˚C until analysis. In the present study, we carried out duplicate analyses for the samples at each depth.

2.2. Derivatization

The experimental scheme is a modification of the methods of Doco *et al*. (2001) and [19]. For analysis of particulate carbohydrates, chopped filter samples were placed in a 10 ml glass tube, and internal standard (*myo*-Inositol) was added. We putted the tube in a vacuum desiccator with phosphorous oxide (V) for 1 day to remove any water completely. After adding 2 ml 0.5 N methanolic HCl (mixture of 15 ml MeOH with 0.4 ml acetyl chloride) to the dried pellets, the tubes were sonicated for 15 min, sealed tightly and heated at 80˚C for 24 h (methanolysis reaction). After cooling to room temperature, the samples were centrifuged and the supernatant was transferred to another test tube. MeOH (1 ml) was added to the tube with chopped filter. The tube weresonicated for a few s, centrifuged, and resulting supernatant was combined with the previous one (this procedure was repeated 2×). After addition of 20 µl pyridine for neutralization of the supernatant, the samples were dried under an N_2 stream at 40˚C and stored in a vacuum desiccator with phosphorus oxide (V) for 3 days. Since the reagents for trimethylsilylation are readily decomposed by water, complete removal of water from the samples was checked.

TMSi-H [hexamethyldisilazane/trimethylchlorosilane/pyridine, 2/1/10 (v/v/v), GL Science] was added (0.2 ml) to the dried samples and the tubes were heated at 80˚C for 2 h for the conversion to TMS derivatives. After cooling to room temperature, the samples were dried under an N_2 stream at 40˚C and the dried pellets were dissolved in hexane and sonicated for a few s. The tubes were centrifuged and the supernatant was injected into a gas chromatograph/mass spectrometer within 24 h after trimethylsilylation at room temperature, because the derivatized sample becomes unstable a few days after the reaction. Standard materials for calibration also underwent methanolysis and trimethylsilyl reactions as well as the natural samples.

2.3. Analysis with Gas Chromatography/Mass Spectrometry

GC/MS (QP 2050, Shimadzu) used an HP-1 fused silica column (30 m × 0.25 mm i.d., 0.25 mm film thickness, Hewlett Packard) and the electron impact ionization (EI) mode. The detailed analytical condition were: inlet and interface temperature 250˚C, ion source temperature 200˚C: column oven temperature programme 50˚C (1 min), to 120˚C at 50˚C·min^{-1}, to 145˚C at 1˚C·min^{-1}, to 200˚C at 0.9˚C·min^{-1}, to 230˚C (held 10 min) at 10˚C·min^{-1} in order to separate eight NSs *i.e*. arabinose (Ara), ribose (Rib), rhamnose (Rha), fucose (Fuc), xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glc), and two URAs, galacturonic acid (Gal Ac) and glucuronic acid (Glc Ac) (**Table 1**). We confirmed the retention time by measuring authentic standards one by one; D-Arabinose (Wako), D-Ribose (Pfanstiehl Laboratories Inc), L-Rhamnose monohydrate (Wako), L-Fucose (Pfanstiehl Laboratory Inc), D-Xylose (Wako), D-Mannose (Wako), D-Galactose (Wako), D-Glucose (Wako), D-Galacturonic acid (Wako), and D-Glucuronic acid (Wako).

Table 1. Retention times of each monosaccharide.

Name of Sugar	Retention time (min)
Arabinose (1)	14.30 ± 0.021
Arabinose (2)	14.82 ± 0.022
Ribose (2)	15.00 ± 0.020
Ribose (1)	15.70 ± 0.021
Rhamnose (1)	16.01 ± 0.021
Rhamnose (2)	16.33 ± 0.019
Fucose (1)	17.22 ± 0.024
Fucose (2)	18.46 ± 0.023
Xylose (1)	19.78 ± 0.021
Xylose (2)	21.09 ± 0.027
Glucuronic acid (2)	24.88 ± 0.029
Mannose (1)	30.32 ± 0.029
Mannose (2)	32.20 ± 0.031
Galactose (1)	33.51 ± 0.035
Galacturonic acid (1)	34.64 ± 0.029
Galacturonic acid (2)	35.19 ± 0.032
Galactose (2)	36.06 ± 0.028
Glucose (1)	37.69 ± 0.038
Glucuronic acid (1)	39.06 ± 0.036
Glucose (2)	39.90 ± 0.036
Internal Standard (myo-Inositol)	57.74 ± 0.217

Peaks of two isomers were quantified in the present study, and order of peak intensity between the two isomers was indicated with number in parenthesis (higher peak: (1), lower peak: (2)).

Three fragment ions (m/z = 204 and 217 in 10 - 24.75 and 25.8 - 100 min, and m/z = 204 and 230 in 24.75 - 25.8 min) commonly found in the mass spectra of TMS derivatives of carbohydrates when EI is used were monitored in the selective ion monitoring (SIM) mode [19]. The methanolysis reaction theoretically generates 4 - 6 isomers from each monosaccharide, but it is difficult to quantify all of the isomers because some minor peaks were too small to quantify. Since the generation patterns of isomers would be constant if the condition (temperature and time) of methanolysis reaction does not change as described in latter section, we choose the highest and second highest peaks for quantification in most case. For galactose, the 1st and 3rd peak were quantified in the present study, because the 3rd highest peak of mannose coeluted with the 2nd highest one of galactose. The analytical errors (coefficients of variance between duplicate analyses) for each monosaccharide were 0.81% - 36%, and mostly the values were around 10% (**Table 2**).

2.4. Analyses of Particulate Organic Carbon and TEP

Particulate organic carbon (POC) concentration was determined using an elemental analyzer (FISONS EA 1108). For analysis of TEP concentration, 70 ml of seawater samples were filtered through polycarbonate filter (Millipore) with pore size of 0.4 μm, and immediately stained by prefiltered alcian-blue solution (0.2 μm, 0.02% alcian blue 8 GX in 0.06% acetic acid) which stains acidic sugar. The filters were transferred into 80% H_2SO_4 for 2 h, and the absorption at 787 nm was measured. The TEP concentration operationally defined as alcian blue-stained particles of size >0.4 μm [10,20], were measured colorimetrically according to the method of Passow and Alldredge [20]; the concentration was normalized with a gum xanthan equivalent per liter (GX equiv. l^{-1}).

3. RESULTS & DISCUSSION

When applying the methanolysis method to seawater samples, it is essential to take account of the presence of various organic compounds as concomitants, which cannot be completely separated from carbohydrates. Application of the EI/SIM mode in GC/MS enables us to minimize the possible effect from non-carbohydrate compounds which overlap with target compounds during the GC separation, since only the specific fragment ions for carbohydrates [19] were monitored in the present study, as described above. We have conducted preliminary measurements using GC-FID, which detects organic compounds non-selectively to estimate their concentrations. However, some of the monosaccharides cannot be quantified due to the overlap with concomitant peaks (data not shown). Thus, the application of selective detection in the GC/MS would be preferable for the analysis of the concentrations of carbohydrates using the methanolysis method.

Although 4 - 6 isomers theoretically occur from each monosaccharide, it is operationally difficult and laborious to detect all the isomers including minor ones. Since the generation patterns of isomers would be constant regardless of their initial form such as anomeric or ring size configurations of the carbohydrates before derivatization [19], we measured only the highest and second highest peaks in the present study. If such selection of major peak was appropriate for quantitative analysis, ratios of peak area of the second highest peak to the first one would be similar between authentic standards and environmental samples. Therefore, we also checked the isomeric ion composition (ratios of second highest peak against first one) between standard compounds used for calibration and natural sample. In conclusion, the isomer composition was similar between them (**Table 3**).

Concentrations of POC and TCHOs (sum of 8 NSs and 2 URAs) decreased from 24,000 - 26,000 and 3200 - 4000 nM C in surface layer (1 - 10 m) to 14,000 - 21,000 and 1300 - 2000 nM C in bottom layer (15 - 25 m) (**Figures 1(a)** and **(b)**). The proportions of TCHO to POC were 8.4% - 16%, and the value was higher in the surface layer (1 - 10 m: 13% - 16%, 15 - 25 m: 8.4% - 13%). These results for profiles of TCHO concentration and contribution of TCHO to POC were generally consistent

Table 2. Error ranges of duplicate samples.

Depth(m)	Ara	Rib	Rha	Fuc	Xyl	Glc Ac	Gal Ac	Man	Gal	Glc	TCHO
1	11.7	30.2	20.3	17.6	11.7	4.93	5.89	4.38	4.37	8.62	0.808
5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
10	4.08	21.2	5.51	4.10	3.75	6.15	4.46	1.99	1.07	0.697	5.40
15	5.01	16.8	8.74	7.00	10.5	10.1	9.34	16.5	0.909	11.0	11.3
20	6.55	1.02	13.9	11.6	5.51	12.1	17.9	24.6	10.9	1.58	13.1
25	16.0	5.82	36.2	25.6	18.7	3.24	3.77	10.1	0.810	2.73	11.4

Values are coefficient of variance between duplicate analysis. ND means not determined because single data was obtained for the sample at 5 m depth.

Table 3. Isomer ratios of each monosaccharide.

	Standard (n = 7)	Samples (n = 6)
Arabinose	60 ± 12	57 ± 2.2
Ribose	11 ± 2.1	17 ± 3.4
Rhamnose	7.6 ± 1.1	26 ± 2.4
Fucose	38 ± 2.5	50 ± 3.3
Xylose	49 ± 2.0	49 ± 1.5
Mannose	7.1 ± 1.0	7.3 ± 0.63
Galactose	34 ± 2.8	38 ± 2.6
Glucose	38 ± 1.0	39 ± 2.7
Galacturonic acid	42 ± 6.7	67 ± 9.8
Glucuronic acid	38 ± 1.2	39 ± 8.8

The values are peak area ratios of 2nd abundant isomer to 1st one for each monosaccharide. The column of standard indicates the values of authentic standard which is measured for calibration. Samples indicate the average values of among the samples from all the depths (1, 5, 10, 15, 20 and 25 m).

with those in previous findings [21-25]. The carbohydrate concentrations decreased more sharply with depth than bulk POC (**Figures 1(a)** and **(b)**), indicating that carbohydrates are relatively reactive components, as shown in other studies [1-3,26,27].

Concentrations of Gal Ac and Glc Ac in POM from surface seawater (1 - 10 m depth) were 22 - 30 and 29 - 40 nM C, respectively, slightly higher than those in the bottom layer (15 - 25 m; Gal Ac and Glc Ac, 16 - 26 and 14 - 25 nM C, respectively) (**Figure 1(c)**). Total URA (Gal Ac and Glc Ac concentrations) were 30 - 70 nM C, accounting for 0.19% - 0.29% and 1.5% - 2.5% of POC and particulate TCHO, respectively. In the previous studies, distributions of URA in POC have been investigated with the colorimetric method (1.4% - 4.5%, 4.7% - 23% [28,29]), being comparable with those in the present study. The trends in vertical profiles in the mol percentages of URA species in TCHO differed between Gal Ac and Glc Ac. Gal Ac in particulate TCHO accounted for 0.67% - 0.79% in the surface layer (1 - 10 m depth), relatively lower than that in the bottom layer (0.90% - 1.3%). On the other hand, the contribution of Glc Ac to particulate TCHO was 0.80% - 1.2%, with no distinctive vertical trend in Suo-Nada (**Figure 2(a)**).

The dynamics of NS components were also different among each component (**Figures 1(d)** and **(e)**). Glucose fractions in the particulate TCHO decreased with depth from 27% - 34% (1 - 10 m) to 20% - 23% (15 - 25 m) (**Figure 2(b)**), while other monosaccharides components were mostly constant or increased with depth (constant: Rha and Gal, increase: Ara, Rib, Fuc, Xyl and Man) (**Figures 2(b)** and **(c)**). Most of the glucose would originate from glucan, a carbohydrate reserved in phytoplankton [30]. Since glucan is considered as one of the most bio-labile components of OM derived from phytoplankton [3,31], it is conceivable that the glucan produced in surface layers by phytoplankton is rapidly decomposed during export to depth.

Transparent exopolymer particles (TEPs) are operationally defined as those above 0.4 μm when stained by alcian blue, a binding dye for acidic sugars, including URAs [10,11]. Since TEPs are likely to promote sinking particle formation and bacterial colonization in water columns, their biogeochemical and ecological importance in marine environments has been intensively studied [11,14,32]. In the present study, the depth profiles of TEPs showed that their concentrations ranged from 20 to 100 μg GX equiv. l^{-1} (**Figure 3**), and decreased from the surface with depth. The concentrations and depth profiles are comparable to those from previous studies of coastal

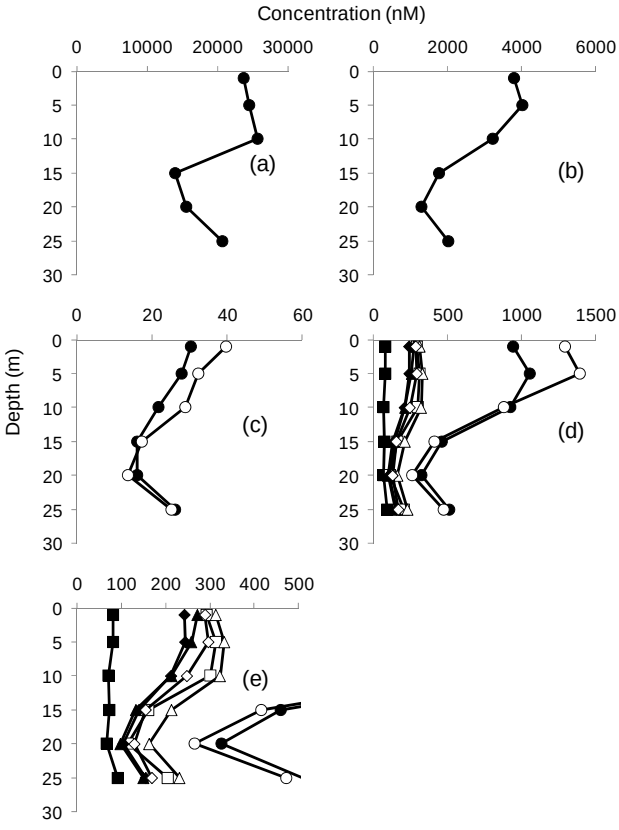

Figure 1. Vertical profiles of concentrations of POC, total carbohydrates, uronic acids (Gal Ac and Glc Ac) and neutral sugars. The ordinate and abscissa indicate depth (m) and concentrations (nM). The profiles of POC (a), total carbohydrates (b), each uronic acid (galacturonic acid (●) and glucuronic acid (○)) (c), each neutral sugar (arabinose (■), ribose (□), rhamnose (▲), fucose (△), xylose (◆), mannose (◇), galactose (●), and glucose (○)) (d and e) were shown. The abscissa of the fifth figure (e) is zoomed to show the minor components.

ecosystems (Monterey Bay and Santa Barbara Channel, 6 - 270 μg GX equiv. l^{-1}; [20]).

We had hypothesized that the concentrations of TEPs and particulate URAs have a close mutual relationship, given that the amounts of binding dye would be proportional to acidic sugar content [33]. However, the concentration of TEPs showed a relatively weak relationship with those of particulate URAs when compared with NSs in Suo-Nada (**Table 4**), even though URAs are one of the well-known acidic sugar groups. This suggests that the contribution of URAs to TEPs is a lesser one, making it essential to consider the contribution of other acidic sugar components such as sulfated carbohydrates. There have been just a few studies on the comparison between URA and sulfated carbohydrates so far [34,35], and they had found abundant formation of TEP, high stickness of aggregates, and higher contribution of sulfated carbohydrates compared with URA. Considering these finding together with our results, sulfated carbohydrate could be a major acidic sugar component in TEPContents of URA and sulphates in EPS would be variable among source organisms. Since it has been suggested that bacterial EPSs are relatively abundant in URA compared with phytoplanktonic EPSs [8], URA-containing polysaccharides present in bacterial EPS don't participate as structural components of TEP.

Table 4. Relationships of each monosaccharide and TEP concentrations.

	r^2	
Arabinose	0.0018	Not significant
Ribose	0.619	Not significant
Rhamnose	0.740	<0.05
Fucose	0.688	<0.05
Xylose	0.733	<0.05
Mannose	0.738	<0.05
Galactose	0.720	<0.05
Glucose	0.726	<0.05
Galacturonic acid	0.239	Not significant
Glucuronic acid	0.539	Not significant
TCHO	0.728	<0.05
POC	0.323	Not significant

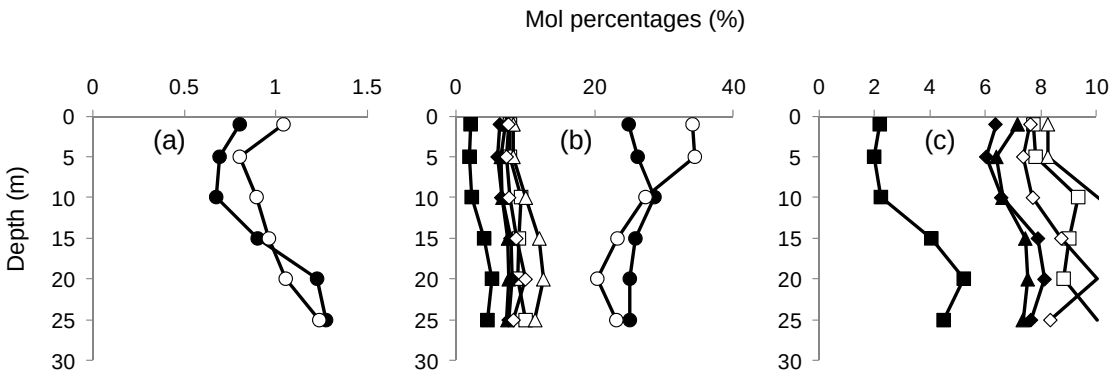

Figure 2. Vertical profiles of proportions of each monosaccharide. The ordinate and abscissa indicate depth (m) and mol percentages of each monosaccharide component. The profiles of each uronic acid (galacturonic acid (●) and glucuronic acid (○)) (a) and each neutral sugar (arabinose (■), ribose (□), rhamnose (▲), fucose (△), xylose (◆), mannose (◇), galactuose (●) and glucose (○)) (b and c). The abscissa of the third figure (c) is zoomed to show the minor components.

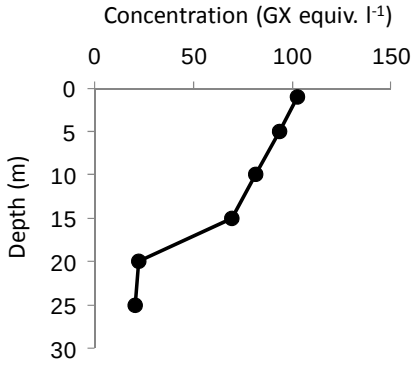

Figure 3. Vertical profile of the concentration of TEP. The ordinate and abscissa indicate depth (m) and concentrations (GX equiv. l^{-1}) of TEP.

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