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Carbohydrate Analysis of Bacterial Polysaccharides by High-pH Anion-Exchange Chromatography and Online Polarimetric Determination of Absolute Configuration

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A significant problem in structure determination of complex carbohydrates, especially for bacterial polysaccharides, is determination of the absolute configuration of the component monosaccharides. A number of analytical methods have been used for this purpose but, as a result of the wide variety of chemical properties of sugars found in complex polysaccharides, no single method is universally applicable. High-resolution gas chromatography of volatile derivatives with chiral reagents is the most widely used method. Optical activity, although direct and simple, lacks sensitivity generally requiring a large quantity of pure monosaccharide. We report a combination of high-performance anion-exchange chromatography (HPAEC) with combined electrochemical pulsed amperometric detection and in-line detection of optical rotation with an in-line laser polarimeter for analysis of a number of sugars found in complex polysaccharides. We show that application of the method for analysis of capsular polysaccharides of several gram-positive and gram-negative pathogenic bacteria provides useful information simultaneously on carbohydrate composition and the enantiomeric configuration of component sugars. © 2002 Elsevier Science (USA)

Key Words: **absolute configuration; optical rotation; carbohydrate; polysaccharide; sugar; HPLC.**

Absolute configuration of carbohydrates in biological samples is an essential feature since biosynthesis of different enantiomers must be completely distinct. Whereas the absolute configuration of most naturally occurring amino acids is L, that of carbohydrates is less straightforward. While the absolute configuration of the majority of sugars is D, there are many instances of L-sugars in glycoproteins and glycolipids. In bacterial polysaccharides, both enantiomers of a particular monosaccharide may be found, occasionally even within the same polysaccharide (1–3). Capsular polysaccharides and lipopolysaccharides are key virulence factors in bacterial infections in humans caused by, for instance, *Vibrio cholerae* (4), *Vibrio vulnificus* (5), and *Streptococcus pneumoniae* (6). Moreover, the difference between virulent and nonvirulent strains within one species is often associated with the structure and composition of the bacterial polysaccharide and structural investigations must include the chirality of each sugar residue.

Analysis of the absolute configuration (also referred to as enantiomeric configuration) of carbohydrates has been done in several different ways, the most common being gas–liquid chromatography. In this method, monosaccharides are derivatized into their (–)-2-butyl glycosides, trimethylsilylated, and separated on a capillary GLC[2] column yielding multiple peak patterns specific for the individual enantiomers (7). An adaptation of this technique using trimethylsilylated dithioacetals from (+ or –)-1-phenylethanethiol gives rise to only one enantiomer-specific peak and limits the number of necessary reference compounds (8). Unfortunately, both techniques need two derivatization steps. Chromatography columns with chiral stationary phases eliminate the treatment of a monosaccharide with a chiral reagent, but in practice, this method has

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[2] Abbreviations used: GLC, gas–liquid chromatography; CD, circular dichroism; OR, optical rotation; HPAEC-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; CPS, capsular polysaccharides; TFA, trifluoroacetic acid; PS, polysaccharides.

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only rarely been used for carbohydrates since it requires authentic samples of both the D- and the L-sugar (9, 10). Hořejší *et al.* have described a technique based on inhibition of binding of a series of lectins by (underivatized) monosaccharides (11). The relatively small range of available lectins limits this technique to common monosaccharides and probably explains its rare use. More commonly used approaches to determine the absolute configuration without derivatization are NMR spectroscopy and X-ray crystallography. Both methods have the ability to study the chirality of individual residues in the native poly- or oligosaccharides, even though amounts over 1 mg are required for the analysis. In NMR spectroscopy, comparison of ^{13}C chemical shifts with those of known compounds, NOE correlations, and ^{1}H coupling constants have been used to establish the absolute configuration of some of the sugar residues (12, 13). Although 2D-NMR has proven to be a powerful technique to determine the identity, sequence, linkage position, and anomeric configuration of constituting monosaccharides of complex carbohydrates, there is no general and completely reliable methodology to prove the enantiomeric configuration. The information obtained is relative stereochemistry, so additional chirality data are still needed. X-ray crystallography on the other hand provides absolute stereochemistry and has been used for some carbohydrates (14, 15) but its use is restricted to those carbohydrates that form crystals.

Techniques based on optical activity, circular dichroism (CD) and optical rotation (OR), rely on a chiral relationship between chromophores of the molecule. Although carbohydrates have few natural chromophores suitable for CD spectroscopy, they may be derivatized with, for instance, *O*-benzoyl groups (16) with the resulting analysis sensitive in the submicrogram range (17). OR, in use since the 19th century, is a simple way of determining the absolute configuration provided that the native compound is pure and available in sufficient amounts. Using a polarimeter (OR detector) linked to liquid chromatography assures that the first condition is met and simplifies the analysis (18). In the present study, we have combined in-line laser OR detection with an established method for monosaccharide analysis (19, 20), high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). As we describe in this paper, an OR detector in-line with an HPAEC column and a PAD detector has enabled us to perform monosaccharide analysis on the complex polysaccharides of six pathogenic bacteria while simultaneously resolving the absolute configuration of the constituent neutral monosaccharides.

MATERIALS AND METHODS

Materials

Monosaccharides (D(+)- and L(–)-fucose, D(+)- and L(–)-galactose, D(+)-galactosamine, D(+)-glucose, D(+)-glucosamine, D(+)- and L(–)-mannose, L(–)-rhamnose, and D(–)- and L(+)-ribose) were purchased from Sigma-Aldrich (St. Louis, MO). Purified capsular polysaccharides (CPS) of *V. cholerae* O139 (4) and *V. vulnificus* strains BO62316 (21), 6353 (22), and MO6-24 (5) were provided by Dr. J. Adegeye in the laboratory of Dr. J. G. Morris Jr. (University of Maryland School of Medicine, Baltimore, MD). Cell wall PS of *Streptococcus mitis* J22 (23) and CPS of *S. pneumoniae* type 12F (24) were provided by Dr. J. O. Cisar (National Institute of Dental Research, Bethesda, MD) and Dr. C. Abeygunawardana (Merck Research Laboratories, West Point, PA), respectively.

Methods

Polysaccharide hydrolysis. One milligram of CPS of each *V. vulnificus* strains BO62316, 6353, and MO6-24 was dissolved in 1 ml 4 M HCl in water. Samples of 1 mg PS of *V. cholerae* O139, *S. mitis* J22, and *S. pneumoniae* type 12F were dissolved in 1 ml 2 M trifluoroacetic acid (TFA; Eastman, Rochester, NY) in water. After hydrolysis at 100°C for 8 h in a heating block, the samples were cooled to room temperature and the acid was removed by evaporation with nitrogen gas. The residue was taken up in 200 μl water, filtered with a 0.45-μm Millex-HV filter (Millipore, Bedford, MA), and 25 μl was used for each HPAEC injection. A second hydrolysis in the case of *V. cholerae* O139 used 1 mg CPS in 1 ml 1 M TFA in water and was heated at 100°C for 2 h prior to evaporation.

Chromatography. The system used for HPAEC of the fucose samples was a chromatography system DX-500 (Dionex, Sunnyvale, CA) consisting of a gradient pump GP40 and electrochemical detector ED40 utilizing a CarboPac PA1 (4 × 250 mm) pellicular anion-exchange column equipped with a CarboPac guard column (Dionex). The injection valve was fitted with a 25-μl injection loop and samples were introduced to the valve via an autosampler AS50 (Dionex). The optical rotation detector (laser wavelength, 670 nm) Advanced Laser Polarimeter (PDR-Chiral, Lake Park, FL) was placed in-line following the gradient pump, before the electrochemical detector. The fucose samples were eluted isocratically using 18 mM NaOH at a flow rate of 1 ml/min, prepared from a dilution of 50% NaOH solution (J. T. Baker, Phillipsburg, NJ) with high-purity water. Data were collected and processed using PeakNet 5.1 (Dionex) and Origin 6.0 (Microcal, Northampton, MA) software. The HPAEC system for the other samples consisted of a gradient pump BioLC

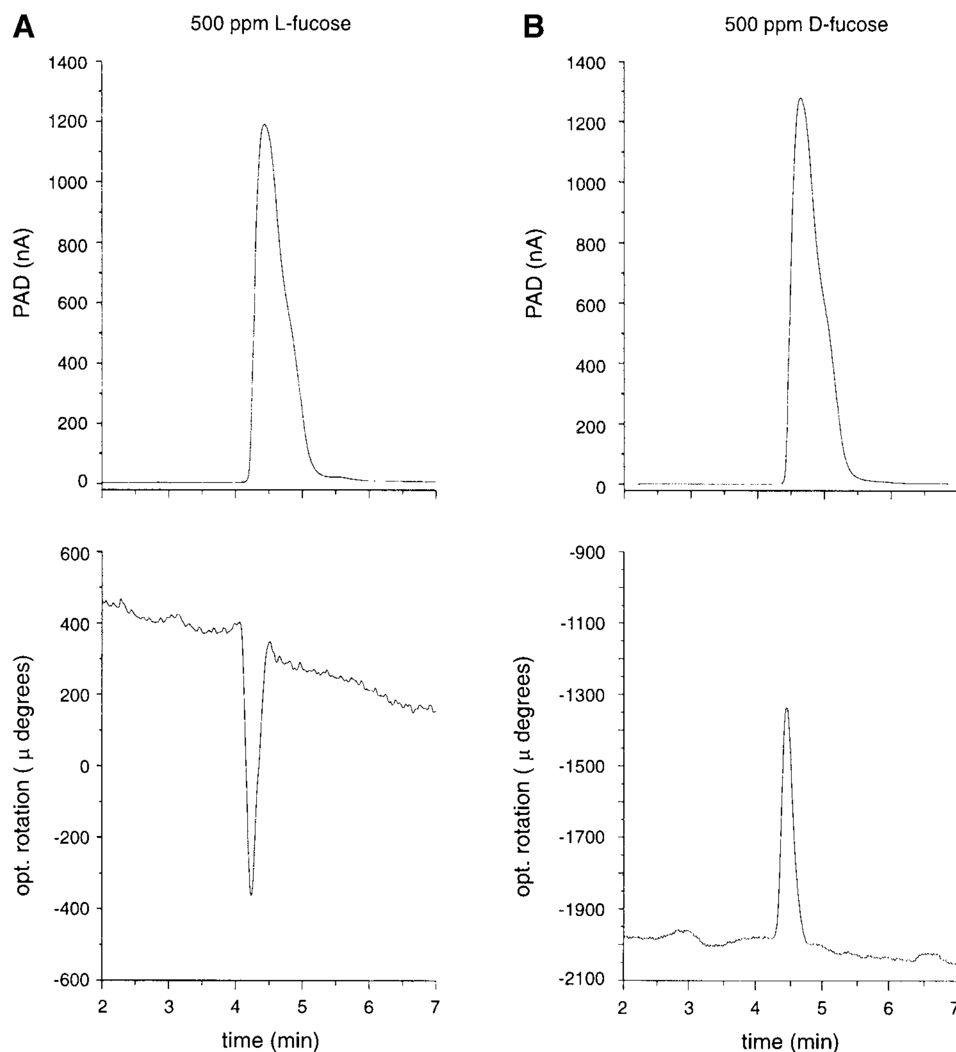

FIG. 1. HPAEC profile of 500 ppm (A) L-fucose and (B) D-fucose. The bottom panel shows the corresponding optical rotation profile.

(Dionex), an autosampler SP8880 (Spectro-Physics), and a CarboPac PA1 (4 × 250 mm) column equipped with a CarboPac guard column. The effluent (0.9 ml/min flow rate at room temperature) was monitored with an Advanced Laser Polarimeter (PDR-Chiral) and a PAD (Dionex). The analyses were done isocratically with 16 mM NaOH, prepared by suitable dilution of 50% NaOH solution with high-purity water. Data collection and handling were done with the PeakNet 4.3 (Dionex) software.

RESULTS

Polarimetric, or optical rotation (OR), detection was implemented in the monosaccharide analysis to simultaneously obtain the absolute configuration and the identity of the constituting monosaccharides. First, water was injected as a blank resulting in no peaks other than the injection peak. Next, samples of 500 ppm (4.56 $\mu g/\mu l$ or 2.78 mM) D(+)- and L(–)-fucose were injected giving identical PAD responses (Figs. 1A and 1B, top). The signals from the OR detector are comparable in size, but opposite in sign (Figs. 1A and 1B, bottom). The PAD peak has a delayed retention time which results from the setup with the pulsed amperometric detector placed after the polarimetric detector. This order is chosen based on the higher sensitivity of electrochemical detection. This sensitivity ratio between the two detection methods, which depends on the specific response of a compound in both PAD and OR, is approximately 10^3 in the case of fucose and differs for each monosaccharide. The PAD peak is broader and has, unlike the OR peak, a shoulder, which is the likely consequence of PAD cell overloading. The limit of detection of OR detection, at three

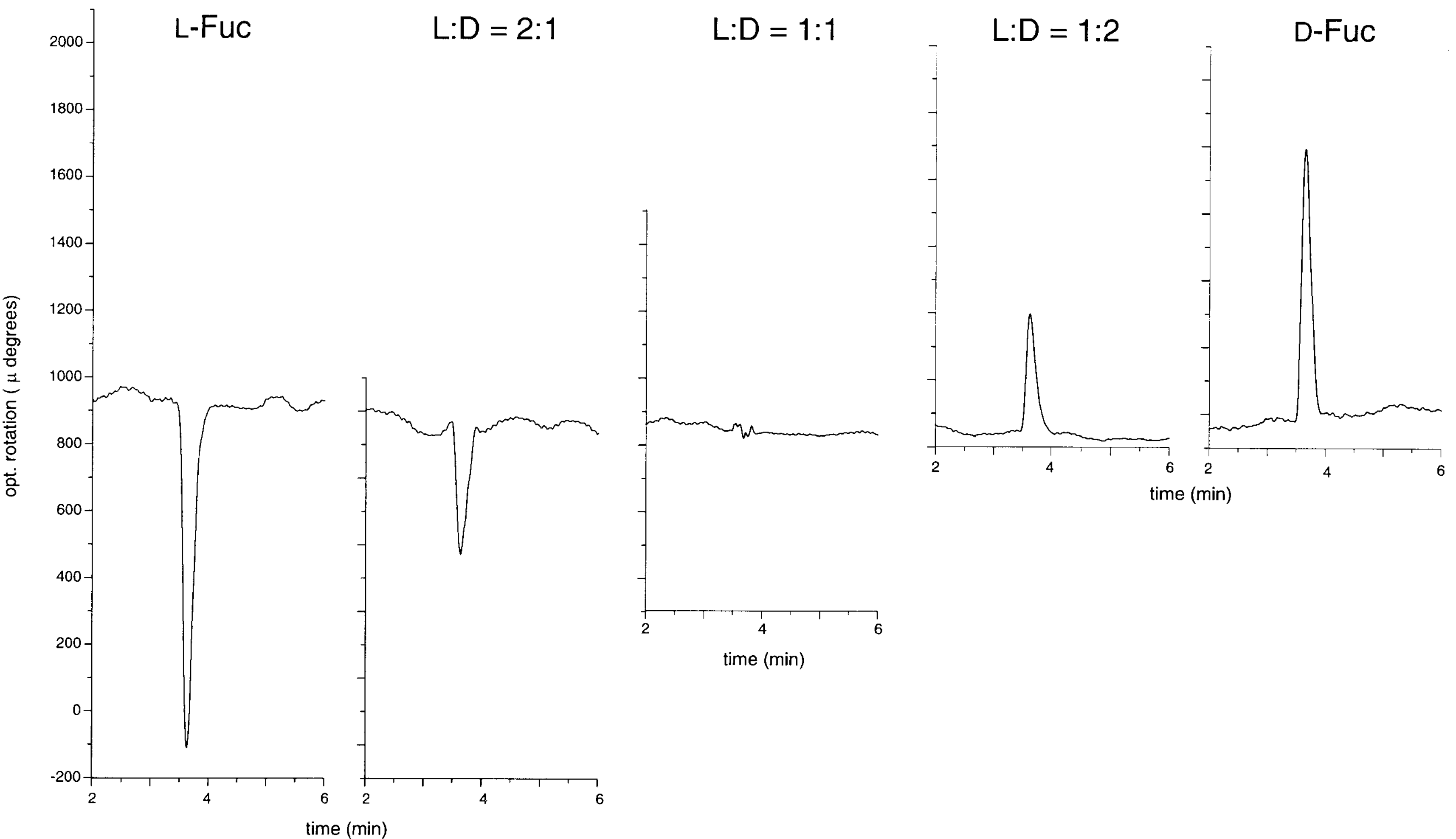

FIG. 2. Elution profiles of fucose (125 μg) mixtures composed of different ratios of the L- and D-isomer as detected by optical rotation.

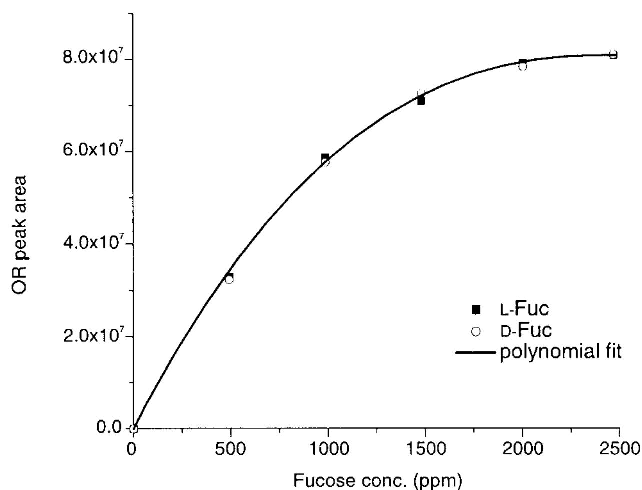

FIG. 3. Relation between the concentration (in ppm) of L- and D-fucose, respectively, and the response of the optical rotation detector, expressed in peak area. Negative peak area of L-fucose is represented as positive peak area to facilitate the comparison of the two isomers. The third-order polynomial fit was calculated with Microcal Origin 6.0.

times the noise, is also residue-specific and is 7.5 μg for fucose in Fig. 1. Yeung *et al.* (18) have reported an optical activity detection limit for fucose of 0.5 μg. This was obtained, however, in a considerably different setup and is therefore not suitable for accurate comparison.

Approximately 125 μg of fucose mixtures (of equal concentrations) composed of different ratios of the D- and L-isomer were applied to HPAEC and monitored by polarimetry to test for the effect of enantiomeric mixtures. Such a situation could arise in the analysis of a polysaccharide containing both enantiomers of a given sugar. The OR profiles of these mixtures (Fig. 2) show peaks with size and sign consistent with the isomeric ratios.

In order to test the relationship between the OR response and the concentration of the analyte, 25-μl samples of up to 2500 ppm of both D- and L-fucose were injected. Figure 3 shows the relationship between integrated OR peak area and fucose concentration. Up to 1000 ppm, corresponding with 228 μg carbohydrate injected, that relationship is approximately linear, whereas beyond this concentration the response levels

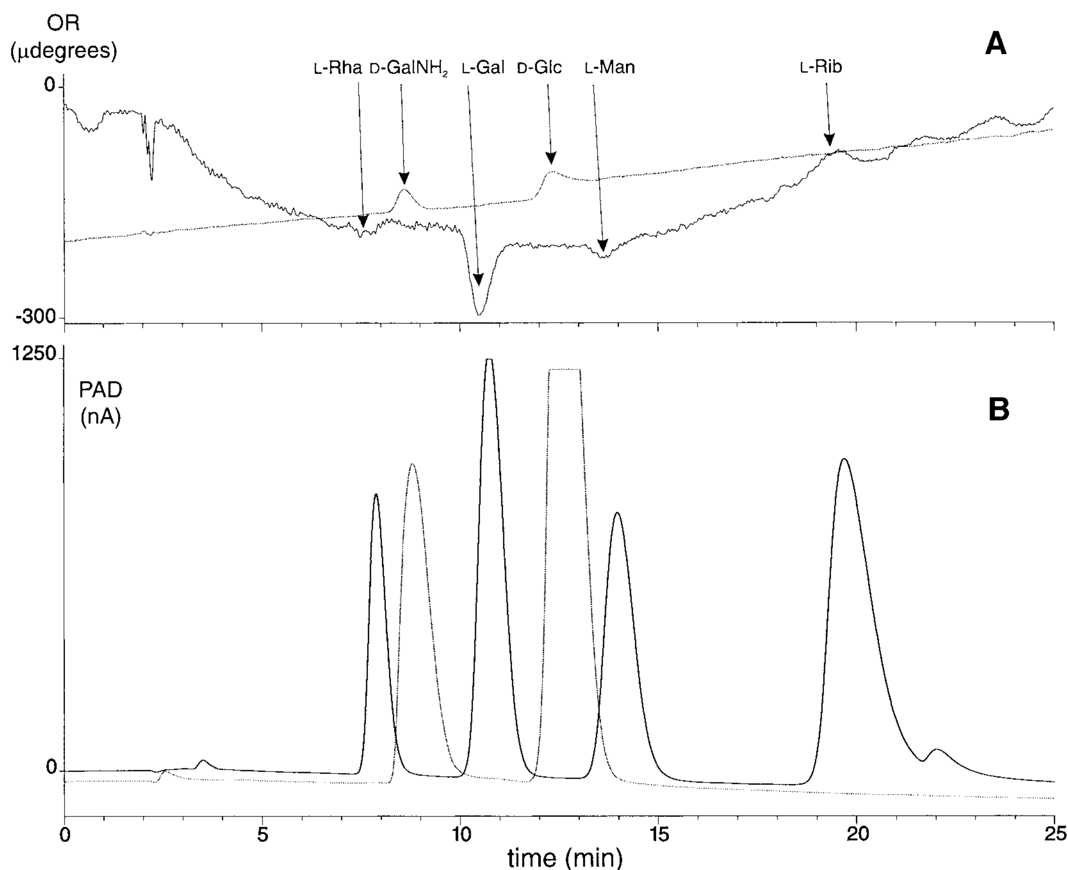

FIG. 4. Superimposed HPAEC profiles, as monitored by (A) optical rotation and (B) pulsed amperometric detection, of a mixture of D-sugars (dotted line; galactosamine and glucose) and a mixture of L-sugars (solid line; rhamnose, galactose, mannose and ribose).

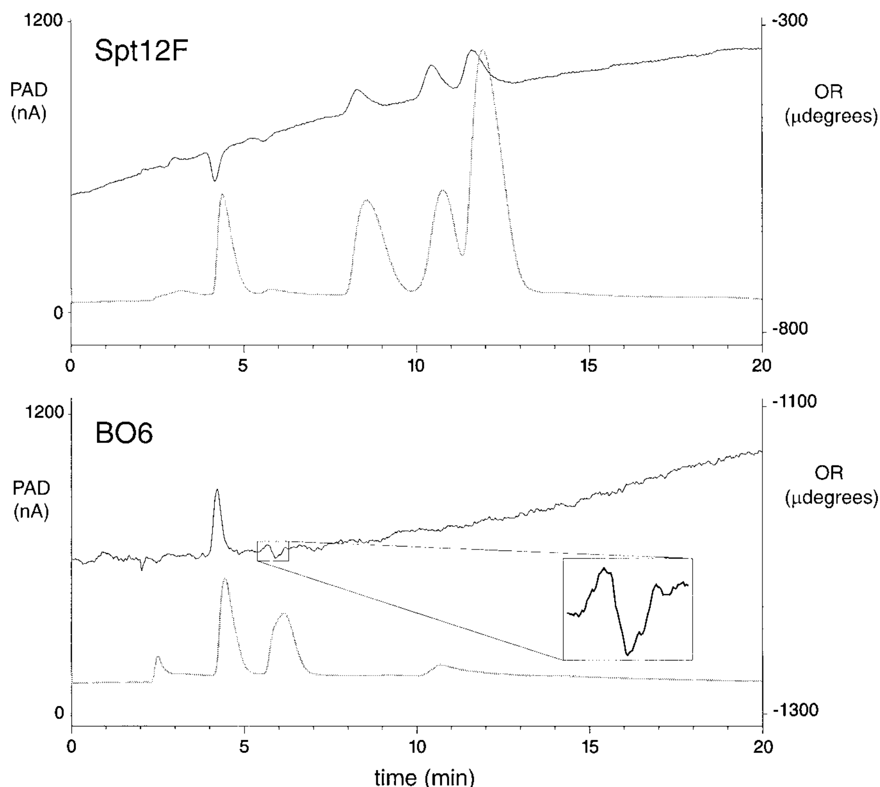

FIG. 5. HPAEC profiles of the constituent neutral monosaccharides of *S. pneumoniae* type 12F CPS (Spt12F, top) and *V. vulnificus* strain BO62316 CPS (BO6, bottom), as monitored by optical rotation (OR, solid line) and pulsed amperometric detection (PAD, dotted line).

off. Figure 3, which uses OR peak area rather than peak height, eliminates the influence of chromatographic peak broadening. The nonlinearity at high concentration could result from the instrument design or from interaction between solute molecules which can lead to nonlinear optical rotations, even for less concentrated solutions (25, 26).

The OR-PAD detector combination was also used with prepared mixtures of sugars. In Fig. 4, the separation of a mixture of L-rhamnose, L-galactose, L-mannose, and L-ribose as well as a mixture of D-galactosamine and D-glucose is shown. The size of the OR signal depends on the stereochemistry of the monosaccharide. Whereas compounds like galactose and galactosamine give sizeable OR responses, those of rhamnose and mannose are small as can be predicted based on the compound's specific rotation, $[\alpha]_D$, at the sodium D line (Table 1). Such a prediction can be made for sugars, because the polarimeter measures at 670 nm, which is not far from the sodium D line wavelength (589.3 nm) on a plateau of the optical rotatory dispersion curve, far away from chromophore adsorption bands (33). As the signal for L-ribose shows, peak broadening as a result of a late elution reduces the height of the OR signal. It is therefore advisable to run late-eluting sugars with a low $[\alpha]_D$ value at slightly higher NaOH concentrations. Even though small specific rotations make the analysis more difficult for these poorly responding carbohydrates, they can also validate the identity of the monosaccharide.

The HPAEC-OR-PAD system was used to analyze a number of bacterial polysaccharides (PS) containing several uncommon monosaccharides whose absolute configuration has been previously determined through established procedures. Each PS was analyzed multiple times and the results are summarized in Table 2. The CPS of *S. pneumoniae* type 12F (Spt12F) among other sugars contains L-fucosamine (24), while the CPS of *V. vulnificus* strain BO62316 (BO6) has one residue of D-fucosamine in its repeating unit (22, 34). In both CPSs, the fucosamine is found as an *N*-acetyl derivative, but that group is lost upon acid hydrolysis. The HPAEC chromatograms of the acid hydrolysates are shown in Fig. 5. The fucosamine peak elutes around 4.4 min and is clearly present in both Spt12F and BO6. The OR response for fucosamine, which is substantial as could be expected from its specific rotation (Table 1), clearly points out the difference in optical rotation between Spt12F (–) and BO6 (+). The other neutral constituent monosaccharides of Spt12F are, in order of

TABLE 1

Specific Rotations of Monosaccharides

Monosaccharide	D/L	+/−	$[\alpha]_D$	Ref.
Abequose[a]	D	−	−5°	(27)
Fucosamine	D	+	93.0°	(1)
	L	−	−95.0°	(1)
Fucose	D	+	76.0°	(28)
	L	−	−75.6°	(28)
Galactosamine	D	+	93°	(28)
Galactose	D	+	80.2°	(28)
	L	−	−80.0°	(29)
Glucose	D	+	47.9°	(28)
	L	−	−53°	(30)
Glucosamine	D	+	47.5°	(28)
Mannose	D	+	14.2°	(28)
	L	−	−14.5°	(30)
Quinovosamine[b]	D	+	53.0°	(1)
Rhamnosamine	D	−	−23.0°	(28)
	L	+	26.0°	(31)
Rhamnose	L	+	8.9°	(28)
Ribose	D	−	−25°	(28)
	L	+	23°	(32)

[a] Abequose, 3,6-dideoxy-D-*xylo*-hexose, which is the D-isomer of colitose.
[b] Quinovosamine, 2-amino-2,6-dideoxy-D-glucose.

elution: D-galactosamine, D-galactose, and D-glucose. All three show positive optical rotations consistent with reported absolute configurations (24). Noteworthy in the case of BO6 are the largely overlapping peaks for quinovosamine and rhamnosamine (Fig. 5). Reddy *et al.* (21) have been successful in partly separating the two components using a slightly lower NaOH concentration for elution establishing that rhamnosamine elutes just before quinovosamine. With the larger sample amounts required for OR detection, the chromatographic resolution is slightly degraded and the OR profile of Fig. 5 shows a small negative peak of about 5 μdeg of which the front end goes slightly up. Such a small signal is consistent with the overlap of L-rhamnosamine and L-quinovosamine, whose specific rotations are 26° and −53° (Table 1), respectively, and which would result in a small, net negative peak with a slightly positive leading edge.

Other quinovosamine-containing CPSs of *V. vulnificus* (strains 6353 and MO6-24) were also analyzed, and their previously reported absolute configurations (22, 35) were confirmed by our analyses (Table 2). MO6 has only one strong negative signal (−97 μdeg) at 6.1 min, which is clear evidence that all three quinovosamine residues are indeed L-quinovosamine (35). Strain 6353 yields two well-separated positive signals at 5.73 and 7.97 min, representing D-quinovosamine and D-galactosamine (22), respectively. As can be predicted from specific rotation values for the two residues (Table 1), the signal for galactosamine is considerably larger than that for quinovosamine (19 and 6 μdeg, respectively).

TABLE 2

Optical Rotations of Neutral Constituent Monosaccharides of Bacterial Polysaccharides

PS	Monosaccharide[a] [no. of residues]	Retention time (min)	Opt. rotation[b] (μdeg)	Absolute configuration D/L (obs.)	Absolute configuration D/L (lit.)
Spt12F	Fucosamine [1]	4.37	−31	L	L
	Galactosamine [1]	8.57	21	D	D
	Galactose [1]	10.77	30	D	D
	Glucose [2]	11.90	37	D	D
BO6	Fucosamine [1]	4.37	25	D	D
	Rhamnosamine [1]	5.9[c]	+[c]	L[c]	L
	Quinovosamine [1]	5.9[c]	−[c]	L[c]	L
MO6	Quinovosamine [3]	6.10	−97	L	L
6353	Quinovosamine [1]	5.73	6	D	D
	Galactosamine [1]	7.97	19	D	D
O139[d]	Colitose [2]	4.20	20	L	L
	Quinovosamine [1]	6.13	5[d]	D	D
	Glucosamine [1]	10.8	13	D	D
	Galactose [1]	11.3	20	D	D
J22	Rhamnose [1]	7.80	nd[e]	?	L
	Galactosamine [2]	8.53	24	D	D
	Galactose [2]	11.03	51	D	D
	Glucose [1]	12.20	14	D	D

[a] For systematic names, see Table 1.
[b] ORD peak height.
[c] Overlapping signals, see Fig. 5 and text.
[d] O139 values are derived from 1 M TFA incubation, except for quinovosamine.
[e] Not determined.

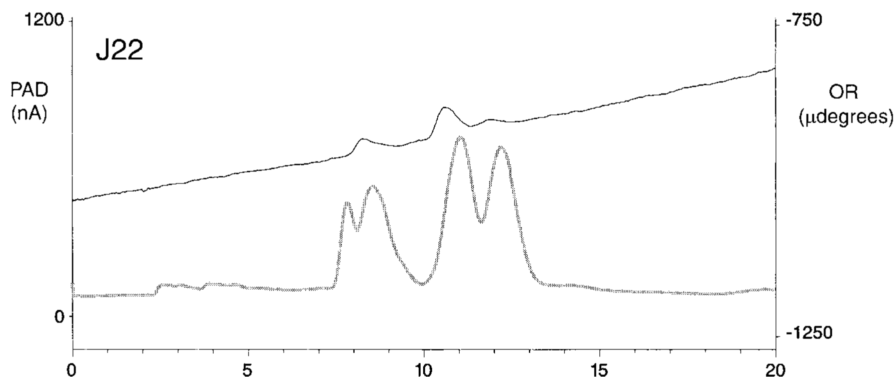

FIG. 6. HPAEC profile of the constituent neutral monosaccharides of *S. mitis* J22 cell wall PS, as monitored by optical rotation (OR, solid line) and pulsed amperometric detection (PAD, dotted line).

Colitose, a constituent monosaccharide in *V. cholerae* O139 CPS (4, 36), is relatively acid-labile and is, therefore, underestimated in a standard hydrolysis using 2 M TFA for 8 h. A second hydrolysis (1 M TFA for 2 h) was used to get a better response (both OR and PAD) for colitose, even though the aminosugars give a lower response (4). Data for quinovosamine (5 μdeg at 6.13 min) are derived from the standard hydrolysis for that reason and show that O139 contains its D-isomer. The second hydrolysis shows that the peak at 4.20 min was positive and consequently represented L-colitose, because the D-isomer is referred to as abequose and has a negative specific rotation (27). The retention times for galactose and glucosamine are normally very close (within 0.5 min) and are partly overlapping in this analysis due to the relatively high amount of material (125 μg) loaded on the CarboPac PA1 column. OR values for these two residues are thus approximate values: 13 μdeg at 10.8 min (D-glucosamine) and 20 μdeg at 11.3 min (D-galactose).

Finally, the cell wall PS of *S. mitis* J22 (17, 23) was subjected to hydrolysis and analysis by HPAEC-OR-PAD. As Fig. 6 and Table 2 show, the determination of absolute configuration is straightforward for D-galactosamine (8.53 min), D-galactose (11.03 min), and D-glucose (12.20 min), but this is not the case for rhamnose for which the OR response is small. Because its signal overlaps with the much more pronounced positive signal for D-galactosamine, it is difficult to determine whether the rhamnose signal is indeed positive as is to be expected for its L-isomer.

DISCUSSION

This first application of HPAEC-OR-PAD for carbohydrate analysis clearly shows how information can be easily obtained about both the monosaccharide composition and the absolute configuration of the residues in one analysis without chemical derivatization. The primary technical limitation is the small signal-to-noise of the OR requiring relatively large sample amounts (10–50 μg per residue per injection). While this is generally not a problem for analysis of bacterial, plant, or invertebrate polysaccharides which are available in adequate quantities, larger sample amounts than used here would exceed the capacity of the pellicular PA-1 column leading to degraded resolution. In standard analyses, the low sensitivity of OR only poses a problem for monosaccharides with low specific rotations ($<15°$). Even then, an early elution (i.e., a narrow peak) or more than one residue per repeating unit can still provide the absolute configuration, as is shown for L-colitose in O139 CPS. Improvement in the OR sensitivity can be obtained measuring at lower temperature, as follows from the temperature dependence of optical rotation and using shorter wavelength light. The use of other lasers could improve the OR sensitivity (18, 25, 26).

A common problem in analysis of complex carbohydrates results from the wide variations in sensitivity of different linkages and of free sugars to degradation by acid. This fact is illustrated by the CPS of *V. cholerae* O139 which contains the highly acid-labile 3,6-dideoxysugar, colitose, as well as uronic acid and aminosugars whose glycosidic linkages are very stable to acid hydrolysis. We have resolved this problem with multiple analyses using different hydrolytic conditions.

The simultaneous use of electrochemical and optical rotation detectors permits quantitative evaluation of the OR signal allowing detection of mixtures of enantiomeric sugars, a situation which could arise for a polysaccharide containing both D- and L-residues of a single monosaccharide (3). Quantitative OR estimates could also be useful for identification of sugars as well as in detecting situations of chromatographic overlap (Fig. 5).

Most of these polysaccharides contain, in addition to the monosaccharides discussed in this paper, acidic sugars such as uronic acids which were not detected under the chromatographic conditions used. Testing of this method with stronger elution conditions will be the subject of future investigation.

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