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THE ASSAY OF ACIDIC POLYSACCHARIDES IN SOLUTION WITH POLY(HEXAMETHYLENEBIGUANIDINIUM CHLORIDE)

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ABSTRACT

The poly(hexamethylenebiguanidinium chloride) [$PHMBH^+Cl^-$] assay method, developed originally for the determination of sodium alginate in solution (0.01–0.5%), has been applied to a range of other acidic polysaccharides. Response in the assay is dependent upon the ratio of acidic groups to monosaccharide, which can be altered for polysaccharides with differing anionic charge densities. When the charge density is low, as with highly esterified propylene glycol alginates, the response can be increased by complexation of the polymer with borate ions which increases the negative charge. The nature of the response allows the determination of the extent of esterification up to ~70% in propylene glycol alginates. The method is suitable for samples of low molecular weight and is therefore more reliable than other assays for acidic polysaccharides which involve a precipitation step. The simplicity, rapidity, and reliability of the method make it suitable for both carboxylated and sulphated polysaccharides.

INTRODUCTION

With the increasing use of plant and microbial acidic polysaccharides in food and industry, it is often necessary to assay these materials in solution. There is a lack of convenient methods for the routine analysis of plant extract liquors, fermentation broths, or even simple solutions of acidic polysaccharides. Gravimetry of material precipitated by ethanol may be interfered with by contamination with non-polysaccharide solids. Ethanol-precipitated carboxylated polysaccharides can be assayed[1] on the basis of the carbon dioxide evolved during acid hydrolysis, but this procedure is time-consuming and not readily adaptable to routine analysis.

Various colorimetric methods, based on the generation of furfural or its homologues, can be used to analyse polysaccharides, although the upper concentration that can be measured directly is usually limited to ~100 μg/mL.

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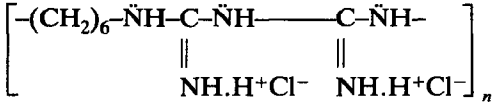
Thus, carbazole[2], naphthoresorcinol[3], phenol[4], and 3-hydroxybiphenyl[5] can be used in association with conc. sulphuric acid. Such methods measure total carbohydrate and do not distinguish between material of low and high molecular weight. Also, there is a variation of response with different sugars and the methods are unsuitable for such polysaccharides as alginate which vary in their carbohydrate composition (D-mannuronic acid/L-guluronic acid ratio). Also, high levels of protein or organic materials that char on contact with hot sulphuric acid or high levels of salt will interfere. The corrosive nature of these reagent mixtures is also preferably avoided for routine analysis.

Recently, we reported[6] the determination of alginates in solution using poly(hexamethylenebiguanidinium chloride) (**1**, $PHMBH^+Cl^-$) by measuring the u.v. absorption of residual $PHMBH^+Cl^-$ in the supernatant solution after precipitation of the polysaccharide. The method is simple, quick, and convenient for the determination of alginate in the range 0.1–5.0 mg/mL. The method has been applied[7,8] to determine the alginate content of industrial liquors extracted from brown seaweed. We now give details of other applications; a preliminary communication of some of this work has been reported[9].



1 (n = 4–6)

EXPERIMENTAL

Materials. — Samples of various sodium alginates, propylene glycol alginate esters, and kappa-carrageenan were obtained from Alginate Industries Ltd. (now Kelco/AIL International Ltd.). A sample of Keltrol (food-grade xanthan) was provided by Kelco Co. Inc. Samples were dried at 61° *in vacuo* over P_2O_5 for 24 h and used on a dry-weight basis. The poly(hexamethylenebiguanidinium chloride) [Vantocil 1B] was obtained from ICI Plc, Pollution Control Division (Hyde, Gt. Britain), as a 20% solution.

Assays. — *(a) Standard.* To duplicate aliquots (5 mL) of solutions of polysaccharide (1–5 mg/mL) were added, with continuous stirring, aliquots (10 mL) of 0.3% $PHMBH^+Cl^-$ in aqueous 1% sodium acetate. Smaller volumes can be used, provided the volume ratio of polysaccharide and $PHMBH^+Cl^-$ solution remains 1:2. Each solution was then stirred for a further 5 min, the polysaccharide–$PHMBH^+$ precipitate was collected by centrifugation (3,000 r.p.m., 5 min), the supernatant solution was diluted 100-fold, and the u.v. absorption at 235 nm was determined with a Pye Unicam SP6-550 spectrophotometer. Standard calibration curves are shown in Fig. 1. Modified responses and applications to alginates of low molecular weight are shown in Figs. 2 and 3 respectively.

(b) In the presence of borate. To aliquots (5 mL) of aqueous solutions of propylene glycol alginate ester (1–5 mg/mL) was added aqueous 2.5% sodium tetraborate (0.5 mL) followed by $PHMBH^+Cl^-$ solution (10 mL). The procedure in (*a*) was then followed. A comparison of the responses of propylene glycol alginate esters to the assay under standard and borate conditions is illustrated in Fig. 4 with appropriate corrections for differences in volume.

Application of the standard procedure to aqueous 0.4% solutions of propylene glycol alginates having various extents of esterification gave the results shown in Fig. 5. Sodium alginate was employed as the control.

DISCUSSION

The $PHMBH^+Cl^-$ assay has several advantages[6] for the quantitative determination of alginate. In particular, the response is easily reproducible and insensitive to variation of salt concentration, pH, and temperature within defined limits. The method is accurate and gives results in good agreement[6] with those obtained using the neutral equivalent procedure[10]. Also, the response is independent of the hexuronic acid composition of the alginate. The standard calibration curve covers the range 1–5 mg/mL, but this can be easily adjusted to 0.1–0.5 mg/mL. The method is based on the formation of an insoluble complex between $PHMBH^+Cl^-$ and the acidic polysaccharide, as is that[11] using Alcian Blue. However, the latter method required several hours for the formation of the insoluble complex, which contrasts with the instantaneous precipitation in the $PHMBH^+Cl^-$ method. Further, the concentration range for the Alcian Blue procedure[11] is limited (0–0.1 mg/mL), unless an additional dilution step is included.

It was expected that acidic polysaccharides having various charge densities per repeating unit would show different responses to the assay, and some results are shown in Fig. 1. Sodium alginate and chondroitin 4/6-sulphate, which have ratios of 1, gave similar responses. These polysaccharides have a relatively large number of acidic groups per molecule and thus show the largest response in the assay. Since a carboxylated polysaccharide (sodium alginate) and a sulphated/carboxylated polysaccharide (chondroitin 4/6-sulphate) can give similar responses, it is charge density and not the type of acidic group which is important. There is some difference in the responses of the two polysaccharides, but the purity of the chondroitin 4/6-sulphate was unknown.

Xanthan, which has the lowest ratio (0.3) of acidic groups per sugar molecule, gave the lowest response in the assay and the red-seaweed polysaccharide kappa-carrageenan, which has an intermediate ratio, gave an intermediate response. However, the range of responses given by kappa-carrageenan and xanthan is not wide enough to allow determinations as accurate as those with sodium alginate and chondroitin 4/6-sulphate. This problem can be overcome by altering the quantity of $PHMBH^+Cl^-$ used in the assay. Fig. 2 shows how the response of kappa-carrageenan having a lower ratio of acidic groups per sugar molecule can be suitably modified.

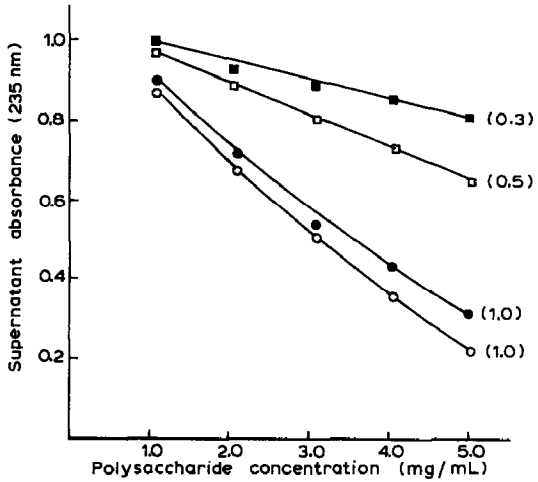


Fig. 1. Response of various acidic polysaccharides (5–25 mg; 1–5 mg/mL) to the standard $PHMBH^+Cl^-$ assay (see Experimental): xanthan (■), kappa-carrageenan (□), chondroitin 4/6-sulphate (●), and sodium alginate (○). The values shown in parenthesis are the acidic group/sugar molecule ratios.

The quantitative precipitation of polysaccharides of low molecular weight by alcohol or divalent metal ions is often difficult to achieve. A comparison of a sodium alginate of low molecular weight with a standard sample indicates that the molecular size of the polysaccharide is not important in determining the response to the assay (Fig. 3). There was a large difference in the molecular weights of the two samples since the solution of the standard sodium alginate was forty times as viscous as that of the low-molecular-weight sodium alginate. These results suggest

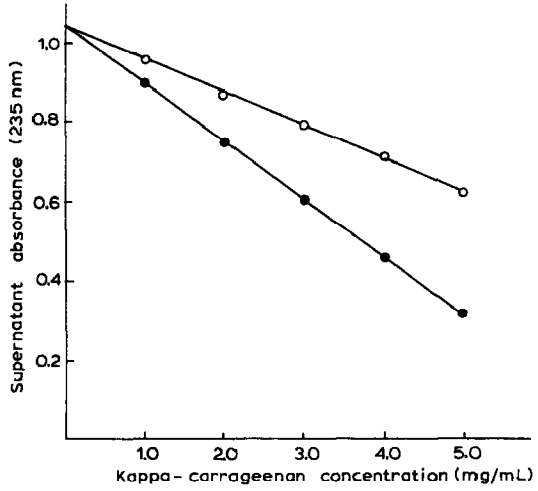


Fig. 2. Modification of the response by kappa-carrageenan (5–25 mg; 1–5 mg/mL) in the $PHMBH^+Cl^-$ assay on decreasing the amount of $PHMBH^+Cl^-$ added from 30 (○) to 15 mg (●).

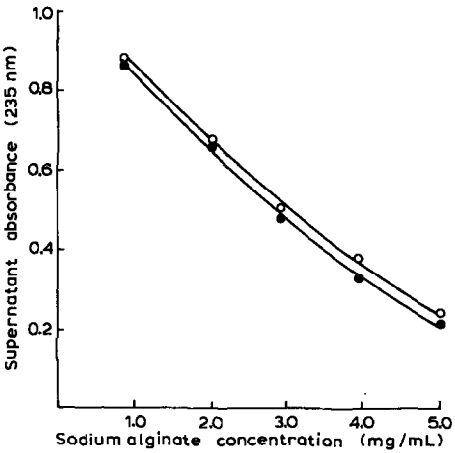


Fig. 3. The response of sodium alginate (5–25 mg; 1–5 mg/mL) in the standard $PHMBH^+Cl^-$ assay for standard (○) and low-molecular-weight (●) samples.

that cross-linking occurs in the precipitation of polysaccharides with $PHMBH^+Cl^-$, with large insoluble particles being formed from the smaller soluble ones.

Based on the foregoing results, the alginate contents of several samples of low-molecular-weight material were analysed. The results are included in Table I, together with those obtained by the neutral equivalent assay and dry-weight content. Although the results are parallel, the $PHMBH^+Cl^-$ method places the sodium alginate of the samples 7–12% closer to the dry-matter content than the neutral equivalent method. The low-molecular-weight sodium alginates analysed were pure samples which had been thermally degraded during their production cycle. Normally, samples of this type would be expected to contain some non-alginate material, but not 7–12%. It is likely that the results obtained by the $PHMBH^+Cl^-$ method are the more reliable; the quantitative precipitation of low-

TABLE I

COMPARISON OF THE $PHMBH^+Cl^-$ AND NEUTRAL EQUIVALENT METHODS FOR THE DETERMINATION OF THE SODIUM ALGINATE CONTENT OF LOW-MOLECULAR-WEIGHT SAMPLES OF ALGINATE

Sample	*Dry-matter content (%)*[a]	*Na alginate content (%)*	
		$PHMBH^+Cl^-$[b]	*N.e.a.*[c]
1	89.3	86.3	76.0
2	87.0	88.0	76.3
3	90.9	88.2	77.8
4	90.2	90.1	78.5
5	89.8	86.3	79.2

[a]Samples were dried for 4 h at 105°. [b]A sample of purified low-molecular-weight sodium alginate was used in the calibration of the assay. [c]Neutral equivalent analysis.

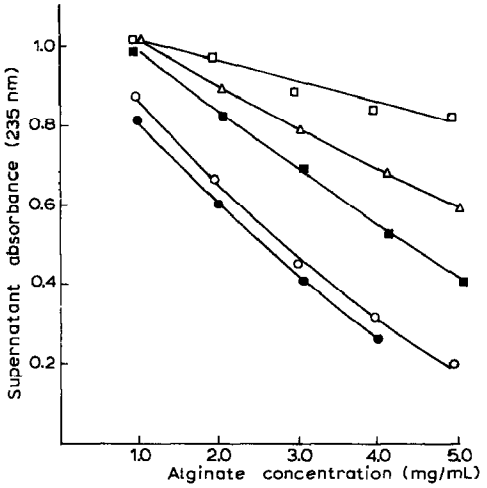


Fig. 4. Comparison of the response of Manucol E/RE (△), Manucol E/KM3 (□, ■), and sodium alginate (○, ●) to the $PHMBH^+Cl^-$ assay under standard conditions (unshaded points) and borate conditions (shaded points).

molecular-weight alginates during the initial stage of the neutral equivalent assay is considered to be suspect.

The influence of the charge density per repeating unit on the response in the $PHMBH^+Cl^-$ assay is illustrated further in the application to propylene glycol alginate esters, two commercial samples [Manucol E/KM3 (60% esterification) and E/RE (80–85% esterification)] of which were tested. The addition of $PHMBH^+Cl^-$ to Manucol E/RE produced slight turbidity, but did not give a precipitate. However, Manucol E/KM3 did give a precipitate, and a comparison of response with that of a standard sodium alginate is shown in Fig. 4. Clearly, the blocking of the acidic groups with propylene glycol disrupted the cross-linking complexation between the high esterified alginate sample and $PHMBH^+Cl^-$.

The negative charge on a polysaccharide can be increased by the formation of a borate complex and this phenomenon has been used to precipitate neutral polysaccharides with quaternary ammonium salts[12]. Since the L-guluronic acid and D-mannuronic acid residues present in alginate contain vicinal *cis*-hydroxyl groups, the formation of borate complexes would be expected. In the presence of borate, Manucol E/RE gave a precipitate with $PHMBH^+Cl^-$. The responses of Manucol E/RE, Manucol E/KM3, and a standard sodium alginate in the $PHMBH^+Cl^-$ assay in the presence of borate is shown in Fig. 4. Thus, the standard sodium alginate sample gave the largest response, Manucol E/KM3 (40% of carboxyl groups unsubstituted) gave an intermediate response, and Manucol E/RE (15–20% of carboxyl groups unsubstituted) gave the lowest response. All the responses were higher than those obtained under the standard conditions (Fig. 4). Indeed, the highest concentration of the standard sodium alginate could not be precipitated using the borate conditions.

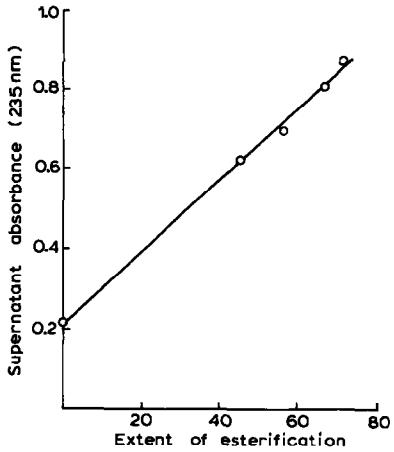


Fig. 5. Correlation between extent of esterification and response of propylene glycol alginates (0.4%) in the $PHMBH^+Cl^-$ assay. Sodium alginate (no esterification) was employed as the control.

The $PHMBH^+Cl^-$ assay was applied to determine the extents of esterification of the members of a series of propylene glycol alginates. The alginate esters were purified materials with an *Ascophyllum nodosum* origin and it was found that samples with up to 70% esterification could be precipitated by $PHMBH^+Cl^-$. The linear relationship between the response in the assay and the extent of esterification is illustrated in Fig. 5. The addition of borate to the assay procedure produced an erratic correlation, particularly with the highly esterified alginate samples.

The $PHMBH^+Cl^-$ method is simple, accurate, quick, and well suited for routine analysis. The biocidal properties of $PHMBH^+Cl^-$ avoids any need for the preparation of fresh solutions. The supernatant solution remaining after precipitation of each polysaccharide sample must be free from any turbidity before measurements of u.v. absorption are made. Turbidity[6] in the supernatant solution could reflect an excessively high concentration of salt or indicate that the concentration of the polysaccharide is outside the calibration range.

REFERENCES

1 I. R. SIDDIQUI AND G. MORRIS, *Carbohydr. Res.*, 69 (1979) 330–332.
2 T. BITTER AND H. M. MUIR, *Anal. Biochem.*, 4 (1962) 330–334.
3 W. WAGNER, *Anal. Chim. Acta*, 29 (1963) 227–239.
4 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
5 N. BLUMENKRANTZ AND G. ASBOE-HANSEN, *Anal. Biochem.*, 54 (1973) 484–489.
6 J. F. KENNEDY AND I. J. BRADSHAW, *Br. Polym. J.*, 16 (1984) 95–101.
7 J. F. KENNEDY AND I. J. BRADSHAW, *Carbohydr. Polym.*, in press.
8 J. F. KENNEDY AND I. J. BRADSHAW, unpublished results.
9 J. F. KENNEDY, S. A. BARKER, AND I. J. BRADSHAW, *Biochem. Soc. Trans.*, 10 (1982) 136–137.
10 W. B. ACHWAL, *J. Text. Assoc.*, 42(2) (1981) 61–64.
11 J. RAMUS, *J. Phycol.*, 13(4) (1977) 345–348.
12 S. A. BARKER, M. STACEY, AND G. ZWEIFEL, *Chem. Ind. (London)*, (1957) 330.