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THE QUANTITATIVE ANALYSIS OF URONIC ACID POLYMERS BY INFRARED SPECTROSCOPY

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ABSTRACT

I.r. absorption bands associated with the functional groups of carboxylic acid derivatives are useful for the analysis of alginates and pectins. The ester, amide, and uronate contents of pectins and the uronate content of alginates were determined, respectively, from the ester-carbonyl stretching band (1740 cm^{-1}), the amide I band (1650 cm^{-1}), and the carboxylate antisymmetric stretching band (1607 cm^{-1}) obtained from the spectra of solutions in D_2O–phosphate buffer. The results are accurate to within ±2–4%, are self consistent, and agree well with the few reliable results that are available. The method should be applicable for the determination of carboxylic acid derivatives in other polysaccharides.

INTRODUCTION

The Zeisel[1–3], modified carbazole[4,5], orcinol[6], and ashing–titration–atomic absorption procedures, although necessary for reference purposes, are not always the most suitable or the quickest methods for determining the uronate contents of pectins and alginates. Recently, uronate contents of pectins have been measured from the i.r. absorption bands of the ester, amide, and carboxylate groups. Sub-milligram amounts of pectin samples have been examined as KBr or KCl discs[7,8]. We now report on the use of i.r. spectra for measuring uronate contents of polysaccharides in solution in D_2O.

The i.r. disc method has an advantage in sample size over the D_2O method, which needs 10–50 mg of pectin, but the latter method does not require the drying of samples to decrease the H_2O absorption. In addition, D_2O solutions are more easily handled quantitatively and the extinction values are independent of the crystalline character of the solid. Whereas the disc method only gives the relative amounts of ester, amide, and carboxylate, the D_2O method also gives the absolute amounts together with the total uronate content.

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METHODS

Carboxylate and ester content of pectins. — Calibration lines for the acid–salt content and the ester content, obtained from dialysed samples of sodium pectate and fully methyl-esterified pectin prepared from the same pectin, allowed determination of the ratio of the methyl ester content compared to the sum of the ester and carboxylate contents in an unknown, unamidated sample. Because pectins may contain neutral sugar units in addition to galacturonic acid, it was necessary to correlate the i.r. absorbances with the standard analytical determinations of uronate and moisture, in order to obtain absolute values for the ester and carboxylate contents. The uronate values for sodium pectate and the ester derived therefrom were similar. The calibration lines were then redrawn to give absolute dry-weight values for the uronic acid methyl ester and carboxylate content for any pectin. Examples of the D_2O spectra between 1900–1550 cm^{-1} of high and low methoxy pectins are shown in Fig. 1 (A–C).

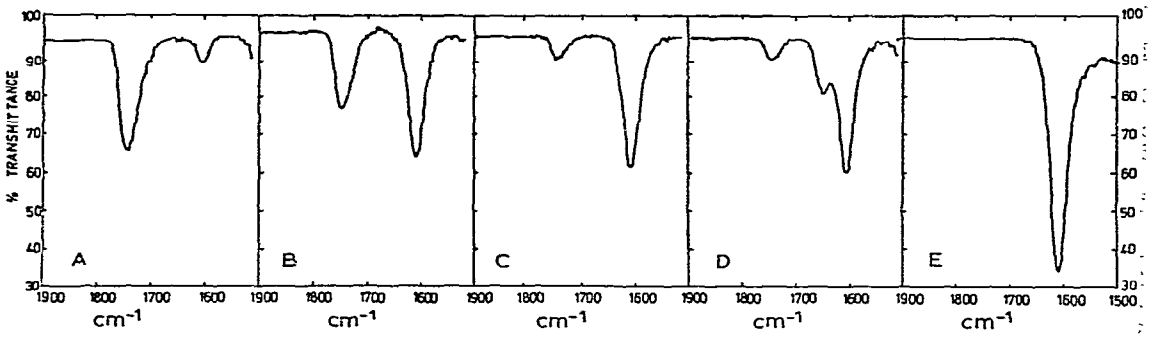

Fig. 1. Pectin and alginate spectra in D_2O solution. A, Pectin II containing ~82% of ester; B, pectin IV containing ~62% of ester; C, pectin III (2) containing ~27% of ester; D, pectin amide IV containing ~17% of ester, ~24% of amide; E, typical alginate (all values on sodium salt scale).

Amide content of pectins. — The amide content was determined by using the amide I band at 1650 cm^{-1}; this is sufficiently separated from the ester band for there to be no interaction, but there is an overlap between the amide I and the carboxylate bands, and due allowance must be made. In practice, as the salt band is generally larger than the amide band, the only significant interference is caused by the overlap of the salt band on the amide band. An example of a D_2O spectrum between 1900–1550 cm^{-1} is shown in Fig. 1 (D).

A potential amide calibration line was obtained from the spectrum of methyl α-D-galactopyranosiduronamide. However, the calibration was 20% high when compared to the amide absorbance values calculated from the nitrogen values of some commercial pectin amides. More-satisfactory answers were obtained from a calibration line based on a mean of the values calculated from the nitrogen contents of the commercial samples. This calibration line brought the i.r. values for the two samples for which we had independent estimates nearer to those reported by the suppliers.

The total uronate values can be calculated from the sum of ester, amide, and salt contents.

Uronate content of alginates. — Although there should be no difficulty in determining the uronate content of alginates, it is this step that has held back the successful application of more-sophisticated analyses of alginate structures. Without subsequent precautions, commercial sodium alginates can, after dialysis and freeze-drying, contain a small amount of free acid. Calculating the uronate values from the sodium contents obtained from ashing, titrating, and determining atomic absorption spectra will then give low figures.

As the freeze-dried material should contain only uronate and water, it might be expected that the uronate content could be obtained from a knowledge of the moisture content. Unfortunately, the alginate moisture contents obtained by any method were always too low (see below). The i.r. method of determining the uronate content has the advantage that, if a spectrum of the D_2O solution is taken in conjunction with the spectrum of a phosphate-buffered solution, the free-acid content can also be measured. It also ignores all sodium impurities, except for sodium carboxylates, and is more convenient than other methods. An example of a D_2O spectrum between 1900–1550 cm^{-1} is shown in Fig. 1E.

EXPERIMENTAL AND RESULTS

Sample preparation. — The pD of pectin/D_2O solutions varies between 2 and 4. As the pK_a value of pectins lies between 3 and 4, the unesterified uronate consists of an equilibrium mixture of the free carboxylic acid and the carboxylate anion. Because the i.r. carboxylic acid C=O stretching band ($\sim$1730 cm^{-1}) overlaps the ester C=O stretching band ($\sim$1740 cm^{-1}) used for measuring the ester content, it is essential that all the free acid is converted into ions and that the carboxylate content is measured from the well-separated antisymmetric COO^- stretching band ($\sim$1607 cm^{-1}).

The samples were prepared by dissolving 10–50 mg, preferably 30–40 mg, of the pectin or alginate in 0.28% NaH_2PO_4–0.86% Na_2HPO_4–D_2O buffer and making up to 2 ml. Using a "Whirlimix", pectin samples took up to 1 h to dissolve, depending on the weight and type of pectin. Amidated pectins were slower to dissolve. Dialysed alginates dissolved in 3–4 h, giving clear solutions. Undialysed alginates took longer to dissolve and often gave cloudy suspensions. The more-concentrated samples of sodium pectate needed heating for dissolution in the buffer; at times, it was easier to dissolve them in D_2O and measure them against a D_2O reference.

As a result of having to determine the free-acid content of dialysed alginates, it became apparent that these samples dissolved equally easily in 2 ml of D_2O. Accordingly, in order to conserve the samples and to increase the confidence in the determinations of free acid, the two determinations were performed on the same solutions. The D_2O solutions were converted into buffer solutions by the addition of $\sim$20 mg of the correct proportions of the solid phosphates.

I.r. sample handling. — The i.r. measurements were made on a Perkin Elmer 257 grating spectrometer, using conventional matched-cells made up with calcium

fluoride windows separated by polythene spacers of 52-μm pathlength. The reference cell was filled with the appropriate solvent. Each spectrum was scanned from 1900–1500 cm^{-1}. The base line from 1900–1800 cm^{-1} was extrapolated below the ester, amide, and carboxylate bands at 1740, 1650, and 1607 cm^{-1} to act as the base line for the absorbance measurement. All measurements were made from duplicate cell fillings, each of which was scanned twice at slow speed. It was preferable to dismantle and clean the cell between measurements. Care was taken to ensure that there were no air bubbles in the solution and no stains on the cell windows.

The hydrogen–deuterium exchange will form mainly HOD, giving no significant absorption between 1900–1500 cm^{-1}. If sufficient exchangeable hydrogen enters the system to give a significant amount of H_2O, which absorbs near 1600 cm^{-1}, then there will be sizeable errors in the results.

Preparation of alginate block samples. — The poly(α-L-guluronate) sequences (G blocks) and the poly[(β-D-mannosyluronate)-α-L-guluronate] sequences (alternating blocks) were prepared[9] by Dr. A. Penman using the method of Haug, Larsen, and Smidsrød[10], and the poly(β-D-mannuronate) sequences (M blocks) were prepared by Dr. D. Thom, by the same means in conjunction with the method of Szejtli[11].

Source of pectins and alginates. — Pectins I and II were obtained from Professor Pilnik (University of Wageningen), who gave ester values of 68.4 and ~81%, respectively. Pectin amide IV came from the same source. Pectins III 1, 2, and 3, which were all different samples of LM 320, pectin amide II (LM 400), and pectin amide VII (Frimulsion N.K.) originated from Caesalpinia (Italy). Pectin IV (Bulmers 230 Slow Set), pectins V 1 and 2 (Bulmers 135), pectin VII (Apple Pectin), and pectin amide I were all obtained from H. P. Bulmer Ltd. (Hereford, England). Pectin VI and pectin amide VI (Amidated Purple Ribbon) were supplied by Obipectin (Switzerland). Pectin amide III (Hi-Poly 3475) originated from Sunkist Growers Inc. Pectins VIII 1 and 2, IX, and pectin amide V were all commercial samples obtained through the Unilever Research Duiven.

The sodium pectate and fully esterified pectin samples were prepared from Bulmers 230 slow-set pectin by Dr. G. Grant. Alginates I, II, IV, V, and VI originated from Alginate Industries Ltd., alginate III from Alginate Australia Ltd., and alginate VII from Dr. I. Whyte, University of Edinburgh.

Standard calibrations of pectins. — Solutions of sodium pectate and totally methyl-esterified pectin from pectin IV were dialysed against deionised water and freeze-dried. The uronic acid contents were determined by (*1*) ashing and weighing, (*2*) titrating the ashed sodium carbonate against hydrochloric acid, and (*3*) measuring the sodium content of the ashed sample by atomic absorption spectroscopy. Moisture contents were measured by an automated Karl Fischer method.

(*a*) *Sodium pectate.* The sodium pectate was shown by high resolution liquid chromatography to be free of organic impurities of low molecular weight. The dialysis also ensured the removal of any excess ions. Using the foregoing methods, the uronate contents were: (*1*) 77.5, (*2*) 81.6, (*3*) 81.5% (average 80.2%). Since the moisture content was 8.0%, the % of uronate, dry-weight, was 87.2.

The i.r. absorbances for solutions of samples (corrected weights, *i.e.*, weight taken × 80.2) of sodium pectate were: 11.6 mg, 0.110; 16.8 mg, 0.158; 25.3 mg, 0.245.

The i.r. absorbances of saponified and dialysed samples (dry weight) of pectin IV were as follows:

Uronate (%)	*Sample (mg)*	*Moisture (%)*	*Uronate (mg)*	*Absorbance*
73.7	17.4	9.8	12.8	0.122
65.9	30.0	21.0	19.8	0.193
73.7	34.0	9.8	25.1	0.246
73.7	55.0	9.8	40.9	0.397

(*b*) *Totally methyl-esterified pectin.* The % dry-weight of the ester was calculated from the % dry-weight of the salt derived from the same source. The % uronate, dry weight, of the sodium pectate was 87.2. The % uronate, dry weight, of the ester was therefore 86.7.

As the moisture content of the dialysed ester was 5.9%, the wet-weight % uronate of the ester was 81.6. The absorbances for various weights of ester corrected (weight taken × 81.6%) for a very small amount of residual acid were as follows: 17.1 mg, 0.061; 24.7 mg, 0.088; 36.4 mg, 0.133; 49 mg, 0.180.

(*c*) *Amide pectins.* The amide band at 1650 cm^{-1} is overlapped by the carboxylate band at 1607 cm^{-1}, and the absorbance can be corrected by subtracting 5% of the peak absorbance value of the carboxylate band. The resulting ratio of absorbances 1607 cm^{-1}/1650 cm^{-1} for various samples of amide pectins were as follows: 31.6 mg, 1:20.4; 43.6 mg, 1:20.6; 63.5 mg, 1:22.0.

The total nitrogen contents of the amides were determined by the Kjeldahl method and included that for ammonium salts. Ammoniacal nitrogen contents were determined by a modified Kjeldahl technique which omitted the digestion stage. The amide nitrogen values of the pectin amides used for calibration purposes are included in Table I.

TABLE I

I.R. ABSORBANCE VALUES AND % AMIDE VALUES (BASED ON N_2 CONTENTS) OF THE PECTIN AMIDES USED FOR THE CALIBRATION

Pectin amide	*Amide (%)*	*Amide wt. in i.r. sample (mg)*	*Absorbances*		
			Carboxylate (1607 cm^{-1})	*Amide (1650 cm^{-1})*	*Corrected amide (1650 cm^{-1})*
I	15.6	6.6	0.179	0.063	0.054
II	8.4	3.8	0.113	0.036	0.030
III	12.1	5.4	0.164	0.051	0.043
IV	18.0	7.5	0.161	0.060	0.052
V	8.3	3.5	0.092	0.030	0.025

The mean straight-line for the calibration of the amide contents of the commercial pectins against their absorbance values was obtained by plotting the mean of the weights (13.2 mg) that corresponded to an absorbance value of 0.1 for the five pectins, against that absorbance value.

TABLE II

THE ESTER AND URONATE CONTENTS OF NON-AMIDATED PECTINS

Pectin sample	*Sucrose content (%)*	*Glucose/ fructose content (%)*	*Ester (%)*		*Uronate (%)*			
					Wet		*Dry*	
			Using salt	*Using acid*	*Based on salt*	*Based on acid*	*Based on salt*	*Based on acid*
I			66	69	71	68	80	77
			64	67	71	68	80	77
			65	68	73	70	83	79
II			82	84	83	81	92	90
			81	82	83	82	92	90
III (1)	29.2		27	29	49	45	80	73
			27	29	50	46	83	76
III (2)	31.6	1.5	27	29	50	46	87	80
			27	29	51	47	90	82
Dialysed and freeze-dried			26	29	84	77	94	86
III (3)	29.2	2.2	25	27	48	44	—	—
IV			61	64	79	75	87	83
			62	65	80	76	88	84
Dialysed and freeze-dried			60	63	79	76	86	82
			62	65	83	80	89	85
V (1)		22.8	30	33	63	58	90	83
			31	34	64	59	92	85
V (2)		25.1	27	30	51	46	—	—
VI			35	37	74	68	81	75
			36	39	76	70	83	77
			35	38	72	67	79	73
			36	39	76	70	83	77
VII			31	34	57	53	62	57
			28	30	59	54	64	59
			30	33	56	52	61	56
			30	33	60	55	65	60
			30	33	60	55	65	60
VIII (1)	27.6	4.7	71	73	52	50	84	81
VIII (2)	25.8	1.4	68	70	53	51	—	—
IX[a]	28.7	4.8	24	27	46	43	83	76
			24	26	48	44	86	79
			24	26	48	44	86	79

[a]Also contains 0.3% of solid.

(*d*) *Calculation of pectin results.* The percentage ester, amide, and salt contents are obtained by dividing each of their calculated weights by their total calculated weights. The results are also recorded in terms of free acid content, rather than salt content, by multiplying the salt weights by 176/198. The percentage total uronate is recorded as the total weight of the ester, amide, and salt/acid components divided by the weight of the sample that was taken.

Non-amidated pectins. — The % ester and % total uronate values of some commercial pectins are given in Table II. Although the calibration curves of the salt and ester are relative to each other, the sum of the errors due to the calibration moisture determinations and i.r. determinations make it realistic to report the % ester content to the nearest $\pm 2\%$. The % uronate values are additionally dependent on the uronate determinations for the original sodium pectate, which are given to $\pm 2\%$, giving a gross added error of $\pm 4\%$, but a more probable error of $\pm 3\%$. In both determinations, there can be additional fluctuations due to sample inhomogeneities.

Pectins VI and VII were tested for replication, and, for the latter, different weights of pectin were used.

The % ester values obtained for the pectins provided by, and independently measured by, Professor Pilnik of the University of Wageningen, Netherlands, were in good agreement with his results, *i.e.*, 64–66% on the salt scale or 67–69% on the acid scale, *cf.* 68.4% for Pectin I; and 81–82% on the salt scale or 82–84% on the acid scale, *cf.* 81% for Pectin II. The % ester values of the various Pectin III samples were in good agreement with each other. The different sub-groups of some of the pectins were obtained from different laboratories.

The % uronate values were self consistent, and the % moisture values were in the range 6–12%.

In Table II, the different samples of pectins III and VIII compare well, but the two samples of pectin V differ in every respect. The % uronate values of the saponified

TABLE III

THE ESTER, AMIDE, AND URONATE CONTENTS OF AMIDATED PECTINS

Pectin amide	*Determined on the salt*			*Determined on the free acid*			*Uronate (Wet impure) (%)*	
	Ester (%)	*Amide* (%)	*Salt* (%)	*Ester* (%)	*Amide* (%)	*Acid* (%)	*Na salt*	*Free acid*
I	26	20	53	28	22	50	82	77
II[a]	28	18	54	29	20	51	48	45
III[b]	26	19	55	28	20	52	68	64
IV	17	24	59	18	26	56	68	64
V[c]	23	20	57	25	21	54	39	36
VI	25	16	58	27	17	56	67	63
VII[d]	12	29	59	13	31	56	37	35

[a]Contains 27.5% of glucose/fructose. [b]Contains 0.2% of sodium organic salt. [c]Contains 23.8% of sucrose + 6.6% of sodium organic salt. [d]Contains 24.2% of sucrose, 2% of glucose/fructose + 20% LBG.

TABLE IV

URONIC CONTENTS OF ALGINATES

Alginate	*Wt. (mg)*	*Absorbance in buffer solution*	*Absorbance in D_2O solution*	*Approx. free acid (%)*	*Uronate (%) by ashing procedure*	*Wt. of uronate in D_2O solution (mg)*	*Corrected original wt. in buffer solution (mg)*	*Wt. of uronate from standard curve (mg)*	*Uronate (%)*
I	46.0	0.378[a]	0.339	10.3	69.4[b]	31.9	46.6	34.4	74
II	40.1	0.238[a]	0.301	10.9	70.5[b]	28.3	40.6	30.8	76
III	31.0	0.259[a]	0.252	2.7	72.9[b]	22.6	31.1	23.6	76
	26.9	0.236[a]	0.220	6.8	72.9[b]	19.6	27.1	21.5	79
IV	37.9	0.338[a]	0.270	20.1	61.3	23.2	38.9	30.8	79
M-Blocks[c]	43.0	0.405[a]	0.367	9.4	77.2	33.2	43.5	36.9	85
MG-Blocks I[d]	41.8	0.398[a]	0.364	8.5	79.6[b]	33.3	42.2	36.2	86
	36.3	0.345[a]	0.325	5.8	79.6[b]	28.9	36.6	31.4	86
I	43.6	0.361		10.3[e]			44.2	32.9	74
II	43.0	0.353		10.9[e]			43.6	32.1	74
IV	40.5	0.362		20.1[e]			41.5	32.9	79
V	42.3	0.361[a]	0.308	14.7			43.1	32.9	76
VI	50.5	0.443[a]	0.362	18.3			51.7	40.3	78
VII	44.1	0.419[a]	0.366	12.6			44.8	38.1	85
G-Blocks[f]	47.0	0.383[a]	0.320	16.4			48.0	34.9	73
	42.2	0.340[a]	0.290	14.7			43.0	30.9	72
MG-Blocks[d]	42.1	0.324[a]	0.279	13.9			42.8	29.5	69
	40.6	0.310[a]	0.268	13.5			41.3	28.2	68

[a]Absorbance values adjusted for addition of phosphate to D_2O solutions. [b]Mean values of two sets of determinations by the ashing procedure. [c]Blocks of mannuronate units. [d]Blocks of alternating mannuronate and guluronate units. [e]From the values given in the top part of the Table. [f]Blocks of guluronate units.

samples of Pectin III (2) and IV obtained by the i.r. method were 71 and 74, and the value given by the ash, titration, and atomic absorption methods was 74 for each sample. These values are different from those noted above, as some of the uronate was lost on saponification. In addition, % uronate for pectins I and II were 71 and 83 by the i.r. method, and 74 and 83 by the orcinol method.

Amidated pectins. — The results for the pectin amides are given in Table III. The only external comparisons possible were for pectin amides IV [26% (based on the acid scale) compared to 27% (Professor Pilnik)] and VI [ester 27%, amide 17%, compared to 31 and 19% (Obipectin)]. The accuracy of the amide determinations is probably within the range ± 2–3%.

Uronate content of alginates. — The dialysed and freeze-dried alginates used for calibration purposes contained free carboxylic acid groups. This meant that the ashing–titration–atomic absorption (ashing) procedure for uronate determinations accounted only for the uronate salt, and not the total uronate. The calibration line was therefore obtained by plotting the i.r. carboxylate salt absorbance values of the D_2O solutions against the corresponding weight of uronate salt calculated from the values obtained by the ashing procedure and the weight of sample dissolved in D_2O (Table IV). A mean straight-line was obtained by calculating the mean of the weights (36.4 mg) of the samples that corresponded to an absorbance of 0.4 in each case. A straight line was drawn between the mean of the weights at that absorbance, and the origin.

Also included in Table IV are the corrected absorbance values after the phosphate had been added. The difference between the two sets of absorbance values allows the percent free acid to be calculated, and from this the weight of uronate salt in the buffer solution can be estimated.

The uronate values obtained by the ashing procedure can differ by up to 4% between duplicate determinations, and individual readings from any of the ashing, titration, or atomic absorption results can differ also by up to 4% from the mean. The i.r. values recorded in Table IV can occasionally differ by up to 4%. If these errors are random, they should be reduced by the use of the mean calibration line, but they will still be within the range of ± 2–4% from the quoted value. It is not known how much of the error is due to the sample (inhomogeneity due to moisture, etc.) and how much is due to the technique.

The percent uronate values derived by different methods are given in Table V. The values in the last three columns are surprisingly close. The last column was obtained by subtracting from 100 the percent moisture values (Karl Fischer determinations) and then, if the uronate is postulated as being present as a stable monohydrate, multiplying by 198/216. The closeness of the different values suggests that the postulate may be correct. If so, it would explain why attempts to derive the percent uronate values of freeze-dried alginates from moisture values have previously been abortive, and why the moisture results themselves seemed unreliable.

Casu[12] has obtained some absorbance values (1%, 1 cm) for the 1607 cm^{-1} carboxylate band of the sodium salts of alginic and pectic acids. His result for alginic

TABLE V

ALGINATE URONATE VALUES (%) DERIVED FROM DIFFERENT METHODS

Sample	*By ashing procedure*	*By i.r. procedure*	*From Karl Fischer moisture values*
Alginate I	69 (77)[a]	74	75 (18.1%)[d]
II	71 (79)	75	74 (19.4%)
III	73 (77)	78	73 (19.9%)
IV	61 (77)	79	—
M-Blocks[b]	77 (85)	85	—
MG-Blocks[c]	80 (86)	86	83 (9.7%)

[a]Values in brackets are corrected (see Discussion). [b]Blocks of mannuronate units. [c]Blocks of alternating mannuronate and guluronate units. [d]Values in brackets are % H_2O.

acid (38.41), if modified for the presence of a monohydrate (41.9), agrees with ours (42.3). His result for pectic acid (37.43) agrees with ours (37.1) without modification. Thus, it appears that freeze-dried alginate exists as a monohydrate, but the freeze-dried pectate does not, and that the Karl Fischer reagent does not extract water of hydration. If the presence of monohydrated pectins is postulated and the percent uronate values adjusted accordingly, they would be too high to allow for the known presence of neutral sugars.

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