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QUANTITATIVE METHODS FOR DETERMINING THE POINTS OF *O*-(CARBOXYMETHYL) SUBSTITUTION IN *O*-(CARBOXYMETHYL)-GUAR*

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ABSTRACT

Two methods are described for locating the *O*-(carboxymethyl) groups in *O*-(carboxymethyl)guar. In Method I, *O*-(carboxymethyl)guar was depolymerized by methanolysis, the *O*-(carboxymethyl) groups were reduced, and the mixture of methyl glycosides and *O*-(2-hydroxyethyl)-substituted methyl glycosides was converted into a mixture of per-*O*-acetylated alditols and partially *O*-(2-acetoxyethyl)ated, partially *O*-acetylated alditols. Analysis of these alditols by gas–liquid chromatography–mass spectrometry allowed the positions of substitution of the *O*-(carboxymethyl) groups on the galactosyl groups and mannosyl residues to be determined. However, this method did not distinguish between *O*-(carboxymethyl) substitution on 4-linked and 4,6-linked mannosyl residues. This limitation was overcome by the more-detailed analysis provided by Method II, in which *O*-(carboxymethyl)guar was carboxyl-reduced, the product methylated, the glycosyl residues hydrolyzed, the sugars reduced, and the alditols acetylated to yield a mixture of partially *O*-acetylated, partially *O*-methylated alditols and partially *O*-acetylated, partially *O*-(2-methoxyethyl)ated, partially *O*-methylated alditols. These derivatives, when separated and quantitated by g.l.c., and identified by g.l.c.–m.s., gave a quantitative measure of every type of carboxymethyl substitution in guar.

INTRODUCTION

Guar, a galactomannan isolated from the seed of guar (*Cyamopis tetragonolobus*), has a backbone of β-(1→4)-linked D-mannosyl residues, of which ~60 percent are substituted at O-6 with a single α-D-galactosyl group. Guar that is treated with chloroacetic acid yields *O*-(carboxymethyl)-substituted guar. Methods[1,2] that can determine the points of substitution in *O*-(carboxymethyl)cellulose are not applicable to *O*-(carboxymethyl)guar. We now present two methods for identifying and quantifying the positions of substitution of the carboxymethyl

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groups in *O*-(carboxymethyl)guar. These methods can readily be adapted for other *O*-(carboxymethyl)ated polysaccharides, including *O*-(carboxymethyl)cellulose.

EXPERIMENTAL

O-(Carboxymethyl)guar (CMguar) having a degree of substitution (d.s.) of 0.53 (mol of carboxymethyl group per mol of hexosyl residue) was obtained from Celanese, and methyl α-D-mannopyranoside, from Sigma.

Preparation of a mixture of partially O-*(carboxymethyl)ated methyl* α-D-*galactopyranosides.* — To a solution of methyl α-D-galactopyranoside (Koch–Light; 1.5 g) in 2-propanol (40 mL) was added NaOH (1.35 g in 4.5 mL) dropwise during 20 min at room temperature, and the solution was stirred for 1 h. Chloroacetic acid (1.8 g) was added during 15 min, and the mixture was stirred for 3 h at 55°, and cooled, and wet Dowex-50W X-12-400 resin (~100 mL) and water (50 mL) were added. The slurry was stirred for 15 min, the resin filtered off, and the filtrate evaporated, to yield a mixture of partially *O*-(carboxymethyl)ated methyl α-D-galactopyranosides (3 g).

Preparation of a mixture of partially O-*(carboxymethyl)ated methyl* α-D-*mannopyranosides.* — A mixture of partially *O*-(carboxymethyl)ated methyl α-D-mannopyranosides was prepared as for the galactoside derivatives, except that, to the filtrate resulting after removal of the Dowex 50 resin was added wet Dowex 3 resin (~150 mL); the pH was adjusted to 4.25, and the slurry stirred for 1 h, the resin filtered off, and the filtrate evaporated, to yield partially *O*-(carboxymethyl)ated methyl α-D-mannopyranosides (0.85 g).

Analysis of the points of substitution in O-*(carboxymethyl)guar. — Method I.* CMguar was subjected to methanolysis by adding 1 mL of a M solution of HCl in methanol [prepared by slowly adding acetyl chloride (0.71 mL) to methanol (10 mL), with stirring and cooling] to CMguar (1 mg), and heating the mixture overnight (16 h) at 85° in a Teflon-capped or glass-sealed tube. Then, a stream of filtered air was used to evaporate the solvent and the HCl, methanol (~0.5 mL) was added, and the solution was vigorously mixed, and evaporated, giving a mixture of unsubstituted and (methoxycarbonylmethyl)ated methyl mannosides and methyl galactosides.

The methoxycarbonylmethyl groups were converted into 2-hydroxyethyl groups by reduction, that is, by dissolving the methanolysis residue in 1:1 ethanol–water (0.25 mL) containing sodium borohydride (10 mg/mL). After 3 h at room temperature, acetic acid was added dropwise until evolution of hydrogen ceased, the solution was evaporated, 1:9 acetic acid–methanol (0.5 mL) was added, and the mixture was mixed thoroughly, and evaporated at 30°. The addition and evaporation of 1:9 acetic acid–methanol was repeated three times; then, methanol (~0.5 mL) was added and evaporated four times. A solution of the borate-free residue in water (0.5 mL) was placed on a column (prepared from a Dispo-pipet or small syringe) containing Dowex-50W X-12-400 (H^+) resin (0.5–1 mL), and the re-

sulting, desalted mixture of unsubstituted and *O*-(2-hydroxyethyl)-substituted methyl galactosides and mannosides was eluted into a test tube with water (2–4 mL), air pressure being used to speed the elution. The eluate was then blown dry with air. The methyl glycosides were hydrolyzed by adding 2M TFA (0.25 mL) and heating the solution for 1 h at 121°. The solution was cooled, and evaporated with a stream of air at 30°, and water (~0.1 mL) was added and evaporated, affording a residue that consisted of a mixture of unsubstituted and *O*-(2-hydroxyethyl)-substituted (galactose and mannose).

This mixture of sugars was reduced to the corresponding alditols by dissolving it in 0.25 mL of M NH_4OH containing 10 mg of $NaBD_4$/mL. The solution was thoroughly mixed, kept for 1 h at room temperature, acidified by adding acetic acid dropwise, and evaporated, and borate was removed by acetic acid and methanol evaporations as already described.

The resulting mixture of *O*-(2-hydroxyethyl)-substituted alditols and unsubstituted alditols was acetylated by adding acetic anhydride (0.1 mL) and heating for 3 h at 121°, cooling, and evaporating at room temperature, toluene (2 drops) being added to help remove the acetic anhydride. Water (0.5 mL) and then dichloromethane (1 mL) were added, the mixture was vigorously shaken, and, after several minutes, the organic layer was transferred to another tube and evaporated at room temperature. The residue was dissolved in dichloromethane (~50 μL), decane (50 μL) was added, and g.l.c. analysis was performed by means of split injection (10:1) on a 10-m, fused-silica, SP 2330 capillary column (Supelco) in a Hewlett–Packard 5880 chromatograph operated isothermally at 240°.

Method II. The carboxyl groups of polymeric CMguar were reduced by the carbodiimide method[3], which is a two-step, reduction method. The first step requires that the pH of the reaction be maintained near 4.75. The pH was kept in the proper range by adding, as a buffer, 2-(4-morpholino)ethanesulfonic acid (MES)[4] (obtained from Sigma). CMguar (d.s. 0.53; ~1 mg) was placed in a Teflon-lined tube that had a screw cap (Pyrex No. 9826), and 0.1M NaOH (0.1 mL) was added to assist in solubilization. The mixture was shaken gently and, after 2 min, H_2O (0.2 mL) was added. When dissolution was complete, 0.2M MES (pH ~3.4 upon dissolution; 1 mL) was added, and then 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC, Sigma; 40–45 mg). The mixture was thoroughly mixed to dissolve the CMC, and kept for 1–1.5 h at room temperature, the samples being thoroughly mixed every 20 to 30 min, and the pH remaining at 4–5.

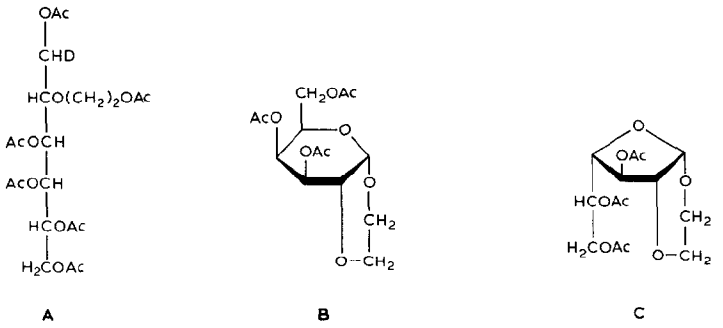
We found that there was no need to control[4,5] the pH during the second step of the reduction (reaction with sodium borohydride). 1-Octanol (1 drop) was added to the mixture and then, $NaBH_4$ (40–50 mg) was gradually added carefully (foaming occurs) in small portions during ~15 min, the mixture being thoroughly mixed after each addition. After the final addition, the mixture was kept for ~20 min at room temperature, and then acetic acid (~2.5 mL) was carefully added in small portions, with thorough mixing after each addition, and the mixture was

transferred to a dialysis tube, and dialyzed overnight against running tap water. The sample was transferred to a test tube, and evaporated to dryness with a stream of air at ~40°. Boric acid as its methyl ester was evaporated as follows. Water (0.1 mL) and then 1:9 acetic acid–methanol (0.5 mL) were added, and the solution was evaporated to dryness. 2M Trifluoroacetic acid (0.1 mL) and then 1:9 acetic acid–methanol (0.5 mL) were added, and the solution was evaporated. 1:9 Acetic acid–methanol (0.5 mL) was added, and the solution was evaporated, and this addition and evaporation was repeated four times. Finally, MeOH (0.5 mL) was twice added, and evaporated. It was important that the evaporations be performed as indicated, as otherwise, residual borate would have interfered with the methylation that followed.

The *O*-(2-hydroxyethyl)guar produced by reduction of the *O*-(carboxymethyl)guar was methylated, the product hydrolyzed, the sugars were reduced, and the alditols acetylated as reported[6] for *O*-(2-hydroxypropyl)guar. G.l.c. and g.l.c.–m.s. analyses were also performed as reported[6].

RESULTS AND DISCUSSION

Analysis of CMguar by Method I was initiated by methanolysis to yield a mixture of methyl-esterified, *O*-(carboxymethyl)ated methyl glycosides and unsubstituted methyl glycosides. On reduction to alcohols by $NaBH_4$, the methyl esters yielded *O*-(2-hydroxyethyl)ated methyl glycosides, and so a mixture with unsubstituted methyl glycosides resulted. These glycosides were converted, by successive hydrolysis, reduction, and acetylation into partially *O*-(2-acetoxyethyl)ated, partially *O*-acetylated alditols and peracetylated alditols. During hydrolysis, some of the mannosyl residues and galactosyl groups substituted at O-2 with a hydroxyethyl group formed cyclic glycosides[7] which were not reduced by the sodium borodeuteride and, after acetylation, yielded peracetylated cyclic glycosides (examples



A B C

Fig. 1. Three different derivatives produced from 2-*O*-(carboxymethyl)-substituted galactosyl groups during analysis of CMguar by Method I. Derivative A is the anticipated alditol. Derivatives B and C are the pyranoid and furanoid cyclic glycosides formed during acid hydrolysis from some of the galactosyl groups substituted at O-2 with a 2-hydroxyethyl group.

TABLE I

RESPONSE FACTORS FOR THE PER-*O*-ACETYLATED COMPOUNDS PRODUCED DURING ANALYSIS OF *O*-(CARBOXYMETHYL)GUAR BY METHOD I

Compound	*Response factor*[a]
Galactitol and mannitol hexaacetates	0.89
Mono-*O*-(2-acetoxyethyl)-galactitol and -mannitol pentaacetates	0.99
Cyclic galactoside and mannoside triacetates[b]	0.75

[a]To calculate the mol percent of each component, the areas of the peaks described by the g.l.c. flame-ionization detector were divided by their corresponding response factor. The resulting values were then normalized to 100 mol percent. [b]See text and Fig. 1.

of which are illustrated in Fig. 1). The formation of cyclic glycosides was not quantitative; some of the mannosyl residues and galactosyl groups substituted at O-2 with a 2-hydroxyethyl group yielded the expected derivatives, namely 2-*O*-(2-acetoxyethyl)-1,3,4,5,6-penta-*O*-acetyl-mannitol and -galactitol, respectively.

The mixture of peracetylated cyclic glycosides, partially *O*-(2-acetoxyethyl)ated, partially *O*-acetylated alditols, and fully acetylated alditols was analyzed by g.l.c. and g.l.c.–m.s. The locations in the alditols of the *O*-(2-acetoxyethyl) groups were determined by means of the e.i.-m.s. fragment-ions resulting from cleavage of the alditol chain[6,8]. The mannosyl and galactosyl derivatives were distinguished by comparison of their g.l.c. retention-times to those of appropriate standards. The standards were prepared by treating partially *O*-(carboxymethyl)ated methyl α-D-galactoside and, separately, partially *O*-(carboxymethyl)ated methyl α-D-mannoside (see Methods) by the procedure described in Method I.

The partially *O*-(2-acetoxyethyl)ated, partially *O*-acetylated alditols produced by the procedure of Method I were quantitated by using their molar-response values (see Table I), calculated as described[9]. The 3-*O*-(2-acetoxyethyl)-1,2,4,5,6-penta-*O*-acetylgalactitol and 4-*O*-(2-acetoxyethyl)-1,2,3,5,6-penta-*O*-acetylgalactitol derivatives are, as a result of the symmetry of galactitol, enantiomers (except for the deuterium atoms at C-1). Thus, these two derivatives co-chromatograph, that is, afford a single peak. To determine the relative amounts of the two derivatives, a comparison was made of the relative abundance of their respective e.i.-mass spectrometric fragment-ions that contained C-1 (which was labeled, by sodium borodeuteride reduction, with a deuterium atom). A gas–liquid chromatogram of a typical analysis is shown in Fig. 2. The results of three separate analyses of the same CMguar sample are given in Table II. Interestingly, O-2 of the mannosyl residues was the most frequently substituted position in the CMguar.

Method I offers the advantage of rapid analysis, as the derivatization and analysis can be completed within two days. However, Method I has several limitations. Formation of cyclic derivatives from some of the 2-*O*-substituted glycosyl residues complicates quantitative analysis. Furthermore, glycosyl residues substi-

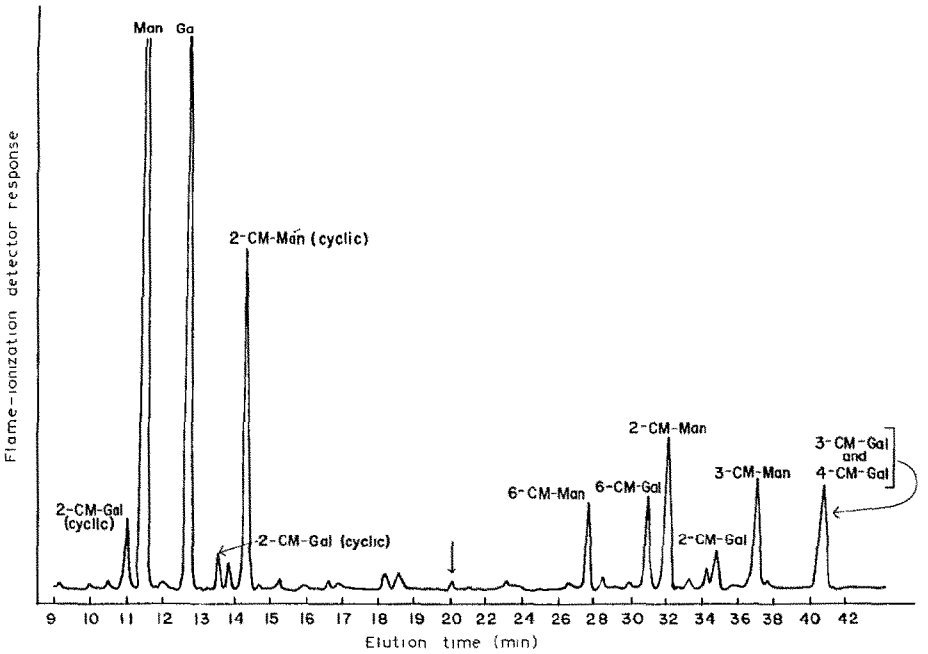


Fig. 2. The analysis, by gas–liquid chromatography, of the per-*O*-acetylated alditols, the partially *O*-(2-acetoxyethyl)ated, partially *O*-acetylated alditols, and the per-*O*-acetylated cyclic glycosides prepared from CMguar by Method I. The peaks were identified by their e.i.-mass spectra obtained during g.l.c.–m.s. analysis, and by comparison of their g.l.c. retention-times to those of appropriate standards. The g.l.c. chart-speed was decreased by a factor of 0.5 at 20 min (indicated by the arrow).

TABLE II

DISTRIBUTION OF CARBOXYMETHYL GROUPS IN *O*-(CARBOXYMETHYL)GUAR, AS DETERMINED BY METHOD I[a]

Glycosyl residue or group	*Sample 1*	*Sample 2*	*Sample 3*
Mannosyl	39	39	41
6-CM-Man[b]	4.5	4.6	4.4
2-CM-Man[c]	15	16	15
3-CM-Man	5.3	5.7	5.5
Galactosyl	19	19	19
6-CM-Gal	5.0	5.3	4.6
2-CM-Gal[c]	5.0	4.7	4.8
3-CM-Gal	3.0	3.2	2.9
4-CM-Gal	3.0	3.2	2.9
d.s.[d]	42	42	40

[a]The analysis was conducted on three different samples of the same batch of *O*-(carboxymethyl)guar (d.s. 0.53). [b]"6-CM Man" was a mannosyl residue originally substituted at O-6 with a carboxymethyl group, and so on. [c]This is the sum of the linear and cyclic derivatives resulting from carboxymethyl substitution at O-2 (see Fig. 1 and the text). [d]Degree of substitution calculated by addition of the mono-*O*-(carboxymethyl)-substituted glycosyl residues only. Di-*O*-(carboxymethyl)-substituted glycosyl residues were present (see Table IV), but were not determined by this method.

tuted with two *O*-(carboxymethyl) groups were not analyzed. Finally, Method I does not distinguish between mannosyl residues that are 4-linked in guar from those that are 4,6-linked, because the 2-*O*-(carboxymethyl)-substituted 4- and 4,6-linked mannosyl residues of guar yield the same derivative. The 3-*O*-(carboxymethyl)-substituted 4- and 4,6-linked mannosyl residues also yield the same derivative.

Method II requires significantly more time (5–7 days) than Method I for completion, but it yields a more complete analysis. In contrast to Method I, glycosyl residues substituted with two or more *O*-(carboxymethyl) groups are detected by Method II, and the derivatives of 4- or 4,6-linked mannosyl residues are distinguished. As with Method I, many samples can be derivatized simultaneously.

The first step in the analysis of CMguar by Method II was the reduction of the *O*-(carboxymethyl) groups (see Methods). The resulting *O*-(2-hydroxyethyl)guar was methylated and the product hydrolyzed, and the sugars were reduced and the alditols acetylated. This process yielded a mixture of partially *O*-acetylated, partially *O*-(2-methoxyethyl)ated, and partially *O*-methylated alditols which was analyzed by g.l.c. (see, for example, Fig. 3) and g.l.c.–m.s. The positions of *O*-(2-hydroxyethyl) substitution in the alditols were determined from their e.i.-mass

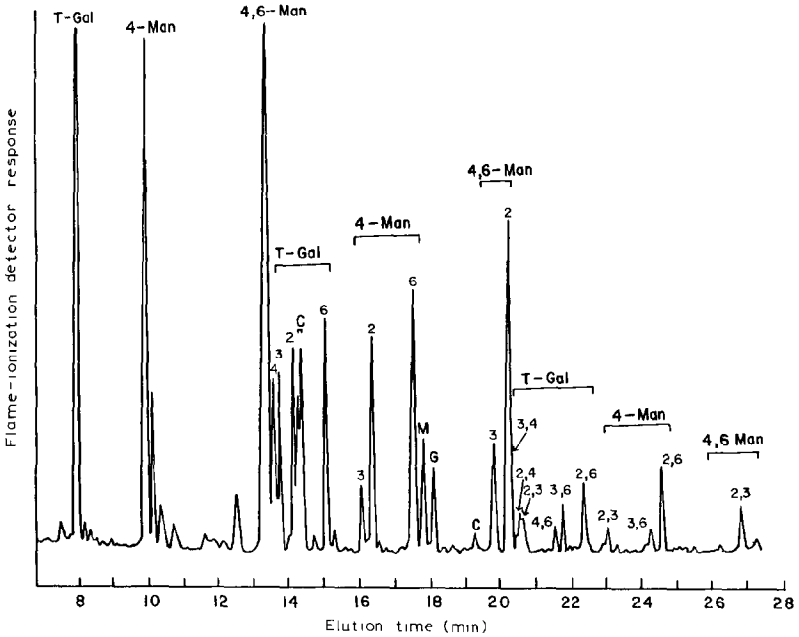


Fig. 3. A gas–liquid chromatogram of the partially *O*-acetylated, partially *O*-(2-methoxyethyl)ated, partially *O*-methylated alditols prepared from CMguar by Method II. The peaks were identified by their e.i.-mass spectra obtained during g.l.c.–m.s. analysis. The numbers above the peaks in the Figure refer to the position(s) of substitution of the 2-methoxyethyl groups. The letter "C" identifies peaks arising from contaminants present in the reagents; the letters "M" and "G" refer to hexa-*O*-acetyl-mannitol and -galactitol, respectively.

TABLE III

MOLAR-RESPONSE FACTORS FOR THE PARTIALLY *O*-ACETYLATED, PARTIALLY *O*-(2-METHOXYETHYLATED), AND PARTIALLY *O*-METHYLATED ALDITOLS PRODUCED DURING ANALYSIS OF *O*-(CARBOXYMETHYL)GUAR BY METHOD II

Compound	*Response factor*[a]
T-Gal[b]	0.70
4-Man[c]	0.74
4,6-Man[d]	0.80
T-Gal-mono-HE[e]	0.80
T-Gal-di-HE	0.90
4-Man-mono-HE	0.84
4-Man-di-HE	0.94
4,6-Man-mono-HE	0.90
4,6-Man-di-HE	1.0

[a]To calculate the mol percent of each component, the areas of the peaks described by the g.l.c. flame-ionization detector were divided by their corresponding response factor. The resulting values were then normalized to 100 mol percent. [b]T-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol. [c]4-Man = 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol. [d]4,6-Man = 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylmannitol. [e]T-Gal-mono-HE = T-Gal in which one *O*-methyl group was replaced by an *O*-(methoxyethyl) group. The names of the remaining compounds are abbreviated in an analogous manner.

spectra in a manner analogous to that used[6] to analyze the corresponding derivatives of *O*-(2-hydroxypropyl)guar. The number of *O*-acetyl groups on each derivative identified its origin: from a terminal galactosyl group, two; from a 4-linked mannosyl residue, three; and from a 4,6-linked mannosyl residue, four. The derivatives were quantitated by use of the molar-response theory[9] (see Table III). The results of the analysis of three separate aliquots of a single sample of CMguar (d.s. 0.53) are presented in Table IV. The relatively high degree of carboxymethyl substitution on O-2 of the mannosyl residues, which was detected by Method I, was confirmed. Interestingly, O-2 of the branched, rather than of the linear, mannosyl residues was the most frequently substituted position in the polymer.

In conclusion, two methods for determining the points of substitution of CMguar are presented. Method II yields the more detailed analysis, but Method I can be completed more rapidly. Both methods give quantitative data, and many samples can be derivatized simultaneously, and analyzed quantitatively in 30 min by g.l.c. Indeed, after the identity of the g.l.c. peaks has been confirmed by e.i.-m.s. analysis, routine analysis of samples can be completed simply by capillary-column g.l.c. with a flame-ionization detector.

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TABLE IV

DISTRIBUTION OF CARBOXYMETHYL GROUPS IN *O*-(CARBOXYMETHYL)GUAR AS DETERMINED BY METHOD II[a]

Glycosyl residue or group	*Sample 1*	*Sample 2*	*Sample 3*
Unsubstituted			
T-Gal	18	17	17
4-Man	13	13	13
4,6-Man	23	22	22
Monosubstituted			
T-Gal-4-CM[b]	3.1	3.2	4.0
T-Gal-3-CM	2.9	2.9	3.8
T-Gal-2-CM	3.6	3.9	3.6
T-Gal-6-CM	4.9	4.3	4.4
4-Man-3-CM	1.2	1.3	1.7
4-Man-2-CM	5.1	6.4	5.5
4-Man-6-CM	5.1	5.5	4.7
4,6-Man-3-CM	2.4	2.4	2.9
4,6-Man-2-CM[c]	9.2	8.8	9.3
Disubstituted			
T-Gal-3,4-CM[c]	—	—	—
T-Gal-2,4-CM[d]	0.60	0.56	0.65
T-Gal-2,3-CM[d]	0.60	0.56	0.65
T-Gal-4,6-CM	1.2	0.39	0.54
T-Gal-3,6-CM	1.6	1.4	1.1
T-Gal-2,6-CM	1.1	1.1	1.7
4-Man-2,3-CM	0.57	0.58	0.58
4-Man-3,6-CM	0.36	0.33	0.56
4-Man-2,6-CM	1.5	1.6	1.7
4,6-Man-2,3-CM	0.87	1.2	0.86
d.s.[e]	0.55	0.55	0.57

[a]Three different samples of the same batch of *O*-(carboxymethyl)guar (d.s. 0.53) were analyzed. [b]T-Gal-4-CM = a terminal galactosyl group substituted at O-4 with a carboxymethyl group. The other compounds are identified analogously. [c]These two derivatives were co-eluted; therefore, the value for the 4,6-Man-2-CM includes both derivatives. [d]These two derivatives were co-eluted; the amounts of each have been estimated by dividing their total by 2. The g.l.c. column used for these analyses was slightly different from that used for the chromatogram illustrated in Fig. 2, in which these two derivatives were separated. [e]D.s. is calculated by [O.S. + 2(T.S.)]/100, where O.S. is the total of the glycosyl residues substituted with one carboxymethyl group, and T.S. is the total of the glycosyl residues substituted with two carboxymethyl groups.

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