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QUANTITATIVE ANALYSIS BY VARIOUS G.L.C. RESPONSE-FACTOR THEORIES FOR PARTIALLY METHYLATED AND PARTIALLY ETHYLATED ALDITOL ACETATES*

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ABSTRACT

Results are presented which demonstrate that the molar flame-responses of partially methylated and partially ethylated alditol acetates should be calculated on an effective carbon response (e.c.r.) basis. The relative responses of 2,3,4,6-tetra-*O*-ethyl-D-glucitol 1,5-diacetate, 2,3,6-tri-*O*-ethyl-D-glucitol 1,4,5-triacetate, hexa-*O*-ethyl-D-glucitol, hexa-*O*-methyl-D-glucitol, and α-D-galactopyranose pentaacetate were measured and compared to the predicted values from three theories: equal molar response, equal weight response, and effective carbon response. The observed values agree very well (±0–6%) with the e.c.r.-calculated values. The other theories of relative response can result in as much as 100% error in quantitation. The e.c.r-calculated, relative response-factors for all commonly found partially methylated and partially ethylated alditol acetates are presented, and their use is suggested for accurate quantitation.

INTRODUCTION

Both partially methylated[1–7] and partially ethylated alditol acetates[8,9] are used for quantitative determination of the glycosidic linkage-isomers present in unknown polysaccharides through integration of g.l.c. peak areas. In order to make such quantitative determinations, it is normally necessary to determine molar response-factors for the compounds in question. However, previously the assumption has been made that the molar response-factors for various partially methylated alditol acetates probably do not vary by more than ±5%, and therefore, each has been assigned a response factor of 1.00. The estimated 5% of error introduced in this way was assumed to be less than errors due to degradation and to losses due to evaporation during derivatization.

Response factors for partially methylated alditol acetates have also been assumed equal on a weight basis. This assumes that a given weight of any organic

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material will give the same g.l.c. peak area as the same weight of a similar organic material. Quantitation of the glycosidic linkage-isomers present in primary plant cell-wall polysaccharides, when derivatized as their partially methylated alditol acetates, has previously been accomplished in this laboratory on this weight basis[2–7].

The alternative to assumptions such as these is to isolate and purify each derivative and calculate each response factor, but this is a prohibitively long and difficult task. However, a third possible method of determining the response factors, without actually measuring them, would be to calculate values based on model compounds and empirical rules as established by Ackman[10–12]. To do this, it must be assumed that each type of carbon atom (for example, hydrocarbon, carbonyl, or ether) contributes to the flame response to the same extent in all compounds, regardless of the exact identity of the compound. The total response-factor is then obtained by summing all of the contributions for the various types of carbon atoms in the molecule. This effective carbon response (e.c.r.) approach has been shown to have some validity for certain systems, although it has not been used for carbohydrates. The e.c.r. approach is probably applicable especially with homologous series[12]. Using these empirical values, we have calculated response factors for the various linkage-isomers as their partially methylated and partially ethylated alditol acetates.

The validity of this e.c.r. theory for partially methylated and partially ethylated alditol acetates was determined for a limited number of specific compounds either by forming a known mixture or by isolating the pure compounds and determining their relative flame response-factors. The experimental results agree very well with the relative response-factors predicted by e.c.r., and therefore, this theory and the values calculated thereby are recommended for quantitative determinations.

EXPERIMENTAL

Validity of the e.c.r. approach to molar response-factors for partially methylated and partially ethylated alditol acetates. — A. Cellobiose derivatization and response factor determination. Various amounts of cellobiose (4-*O*-β-D-glucopyranosyl-α-D-glucopyranose) (Sigma, 1–6 mg) were derivatized as described[9] to the corresponding partially ethylated alditol acetates with a known and constant amount of *myo*-inositol (Calbiochem) added as an internal standard prior to hydrolysis. Each mixture was then chromatographed on a 120-cm packed column containing 0.2% poly(ethylene glycol succinate) (PEGS), 0.2% poly(ethylene glycol adipate) (PEGA) and 0.4% GE silicone XF-1150 on Gas Chrom P (100–120 mesh) operated from 110 to 185° at 1°/min after a post-injection interval of 6 min. The relative ratio of the 2,3,4,6-tetra-*O*-ethylglucitol 1,5-diacetate (terminal-Glc) to 2,3,6-tri-*O*-ethylglucitol 1,4,5-triacetate (4-substituted Glc) was determined by g.l.c. peak-area integration. A total of 25 cellobiose samples were derivatized in this way and the g.l.c. peak-area ratios obtained. This ratio should be the ratio of the molar response-factors for these two compounds, as cellobiose is equimolar in terminal-Glc and 4-substituted Glc.

B. Synthesis and isolation of glucitol hexamethyl ether and glucitol hexaethyl ether. Into each of two 100-ml, three-necked, round-bottom flasks fitted with a serum cap in one neck, was weighed 0.500 g of D-glucitol (Pfanstiehl). The flasks, with stirrer bar, were dried in a vacuum oven for 24 h, at 60° over phosphorus pentaoxide, and then stoppered and flushed with nitrogen. Dry dimethyl sulfoxide (60 ml) was added to each and the contents were stirred until the glucitol had dissolved. Dimethylsulfinyl anion, sodium salt, (3.0M, 6.7 ml), prepared as described by Sandford and Conrad[13], was added and the resultant viscous, blue solution was stirred at room temperature for 1.0 h. One of the samples was ethylated by adding 1.8 ml of ethyl iodide (Fisher) at room temperature and the other was methylated by adding 1.4 ml of methyl iodide (Fisher). The methyl iodide was added slowly during 45 min while the temperature was maintained at 20°. Both solutions were stirred for an additional 2.5 h at ~25° to complete the etherification. The alkylation procedure was repeated for each solution two more times by the addition of a second and third volume of both the "dimsyl" anion and alkyl iodide as in the first treatment to ensure complete alkylation. An excess of methyl iodide and ethyl iodide (2.0 ml of each) was used in the third alkylation.

Diethyl ether (75 ml) and water (75 ml) were then shaken with the dimethyl sulfoxide solution. The ether layer was separated and the aqueous layer reextracted twice with ether (75 ml). The combined ether layers were then extracted six times with water (75 ml). The ether layer was dried (magnesium sulfate), filtered, and evaporated to give 0.919 g of a yellow oil. This oil (250 mg) was resolved on a preparative-scale silica gel t.l.c. plate with 1:1 ether–hexane. Hexa-*O*-ethylglucitol had R_F 0.64, and was isolated from the silica gel by using ether. Evaporation of the extract gave 172 mg of colorless oil that was distilled at ~0.1 torr at 108° (oven temperature) to yield a colorless oil; ν_{max} 1120 (C–O stretch), 1350–1500, (C–H bending), 2800, and 2975 cm^{-1} (C–H stretch); no absorbance was observed at 3400 cm^{-1} (OH absent); n.m.r. δ 1.30 (triplet, 18 protons), 3.70 (multiplet, 20 protons); mass spectrum shown in Fig. 1.

The hexa-*O*-methyl-D-glucitol was isolated similarly, except that the combined ether extracts were back-extracted with water only once because hexa-*O*-methylglucitol is more soluble in water than is hexa-*O*-ethylglucitol. The isolated and dried oil (248 mg) was chromatographed on a preparative-scale, silica gel, t.l.c. plate with pure ether as eluant ($R_F = 0.56$). Ether was again used to separate the product from the silica gel. The resultant colorless oil (74 mg) was vacuum distilled (~0.1 torr) at 88° (oven temperature); ν_{max} 1100 (C–O stretch), 1350, 1450 (C–H bending), and 2900 cm^{-1} (C–H stretch); no absorbance at 3400 cm^{-1} (OH absent); n.m.r. (δ)3.4–3.6 (6 singlets, 3 protons each), 3.3–3.7 (multiplet, 8 protons); mass spectrum shown in Fig. 2.

C. Molar flame-response factor measurement for glucitol hexamethyl ether and glucitol hexaethyl ether. The hexamethyl and hexaethyl ethers of glucitol were assumed to be pure (a single g.l.c. peak was observed for each) and were used together with commercial α-D-galactopyranose pentaacetate (Sigma). The following amounts

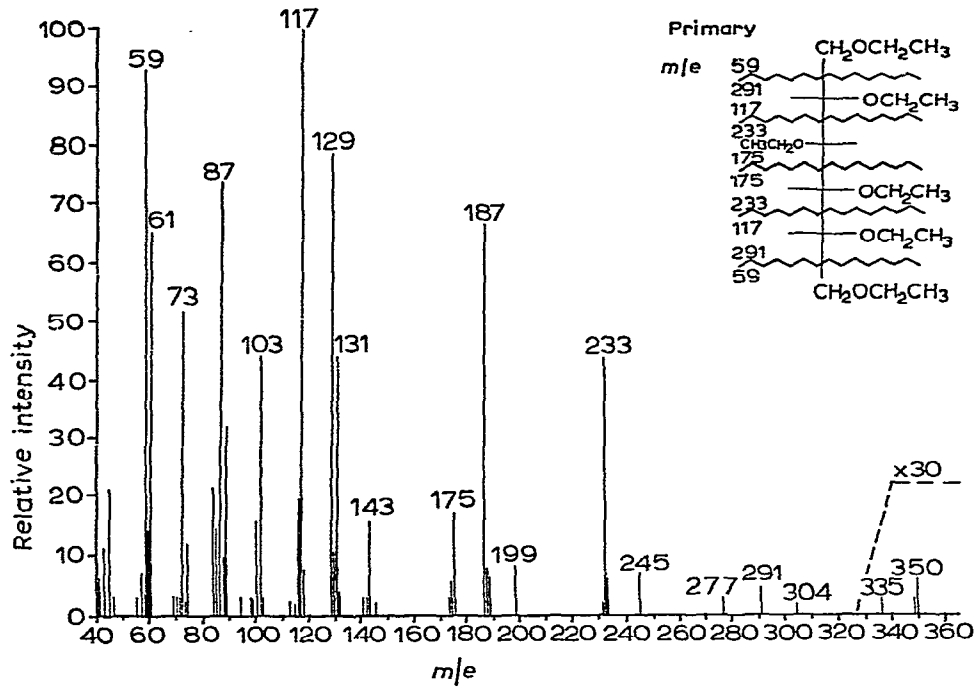

Fig. 1. The mass spectrum of hexa-*O*-ethylglucitol. (Primary mass-spectral fragmentations are shown in the insert. All other mass-spectral fragments can be explained by secondary fragmentations or by neighboring-group ethoxide shifts).

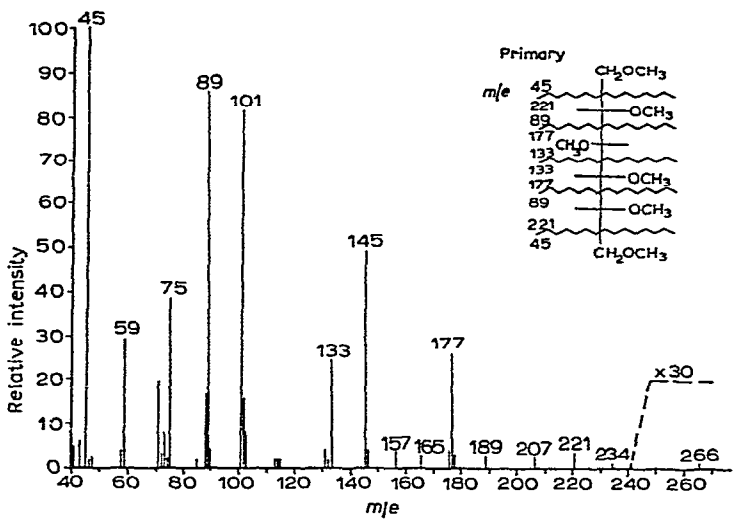

Fig. 2. The mass spectrum of hexa-*O*-methylglucitol. (Primary mass-spectral fragmentations are shown in the insert. All other mass-spectral fragments can be explained by secondary fragmentations or by neighboring-group methoxide shifts).

were weighed into a single 1.0-ml volumetric flask and dissolved in acetone (1.0 ml): hexa-*O*-ethylglucitol (11.06 mg); hexa-*O*-methylglucitol (9.57 mg); α-D-galactose pentaacetate (9.08 mg).

This mixture (1.0 μl) was injected several times onto two g.l.c. columns. The PEGS, PEGA, and XF-1150 columns were operated from 80–85° at 0.5°/min after a post-injection interval of 6 min and then heated from 85–180° at 10°/min. The second column was 180 cm long and contained 3% OV 1 on Chrom W(HP) (100–120 mesh). This column was operated from 105–235° at 4°/min after a post-injection interval of 6 min. The g.l.c. peak-areas were integrated. The relative peak-areas from these determinations, when compared with the absolute amounts of each sugar weighed into the volumetric flask, gave the relative molar-responses.

RESULTS

The results of the two experiments outlined in the experimental section should indicate which of the molar response-factor theories are correct for quantitation of partially methylated or partially ethylated alditol acetates, as the compounds synthesized represent the extremes of these series of alditol derivatives. The curve given in Fig. 3 summarizes the results of the first of these experiments, namely;

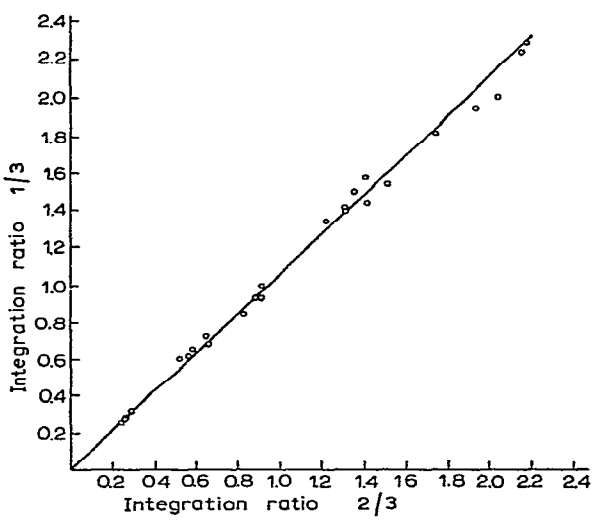

Fig. 3. Relative response-factors for terminal-Glc and 4-substituted Glc. [The integration ratio of the g.l.c. peak for 2,3,4,6-tetra-*O*-ethylglucitol 1,5-diacetate (**1**) is plotted versus 2,3,6-tri-*O*-ethylglucitol 1,4,5-triacetate (**2**) derivatized from various amounts of cellobiose. All peak areas are relative to the peak area of *myo*-inositol hexaacetate (**3**) formed by the addition of a known amount of *myo*-inositol prior to hydrolysis by trifluoroacetic acid.]

formation of the partially ethylated alditol acetates of terminal-Glc and 4-substituted Glc*. The slope of this plot, or the relative peak-area ratio of the partially ethylated

*See ref. 9 for definition of this terminology.

alditol acetates of terminal-Glc to 4-substituted Glc, is 1.066 ±0.041 for 24 separate determinations. Each of these mixtures was an equimolar mixture of terminal-Glc and 4-substituted Glc as the partially ethylated alditol acetates, because each was formed from cellobiose.

The result of the second experiment, in which the relative response-factors for hexa-*O*-ethylglucitol, hexa-*O*-methylglucitol, and α-D-galactopyranose pentaacetate were determined, is summarized in Table I. The relative response-factors for the three

TABLE I

RELATIVE PEAK-AREAS OF HEXA-*O*-ETHYL-D-GLUCITOL, HEXA-*O*-METHYL-D-GLUCITOL, AND α-D-GALACTOPYRANOSE PENTAACETATE ON G.L.C.[a]

	Relative peak areas		
	Glucitol $(OCH_2CH_3)_6$	*Glucitol* $(OCH_3)_6$	*Galactopyranose* $(OAc)_5$
Observed on PEGS, PEGA, XF-1150	2.153 ±0.055	1.313 ±0.054	1.000
Observed on OV-1	2.078 ±0.043	1.252 ±0.021	1.000
Theoretical			
(*a*) molar responses are equal	1.357	1.545	1.000
(*b*) responses are proportional to weight[c]	1.218	1.054	1.000
(*c*) responses are proportional to the effective carbon values[d]	2.201	1.252	1.000

[a]A standard mixture of hexa-*O*-ethyl-D-glucitol (11.06 mg), hexa-*O*-methyl-D-glucitol (9.57 mg), and α-D-galactopyranose pentaacetate (9.08 mg) was dissolved in acetone (1.0 ml), and 1.0-μl aliquots were injected into two g.l.c. columns as described in the text. The peak areas observed are expressed relative to the peak area of α-D-galactopyranose pentaacetate. Also shown are the peak areas expected for this standard mixture according to various molar response-factor theories. The effective carbon response theory uses the empirical values listed below. The total response is calculated by summing the contributions of the various carbon atoms. [b]The value reported is an average of the values calculated by assuming that either side of the ester group becomes the aldehyde in the flame. The uncertainty introduced is never > ±5% and is normally ±1–2%. [c]This theory assumes that equal quantities (by weight) of two compounds give the same relative peak area. [d]Effective carbon response: calculated by summing the contributions of the carbon atoms according to the empirical values formulated by Ackman[12] and shown in the legend below.

Type of carbon atom	*Effective carbon response (e.c.r.)*
hydrocarbon	0.100
primary alcohol	0.055
secondary alcohol	0.035
aldehyde	0.000
ketone (2 carbon fragment)	$CH_3-\overset{O}{\overset{\|}{C}}$ 0.100 $-CH_2-\overset{O}{\overset{\|}{C}}$ 0.135
ether	$-O-CH_2-$ 0.000
ester[b]	as a sum of an alcohol and aldehyde (either side being the alcohol)

compounds is, within experimental error, the same on both g.l.c. columns. Hexa-*O*-ethylglucitol gave a peak area 2.1 times the peak area of α-D-galactopyranose pentaacetate, whereas the peak area for hexa-*O*-methylglucitol was only 1.3 times that for α-D-galactopyranose pentaacetate.

DISCUSSION

Quantitation of polysaccharide components by derivatization and g.l.c. analysis requires either determination of molar response-factors or an assumption of their values. Molar responses and weight responses have hitherto been assumed equal in separate studies for the various partially methylated alditol acetates, and quantitation of polysaccharide components have been based on these assumptions. The results of two experiments, however, indicate that neither of these approaches is correct. The data of Table II present the relative peak-areas predicted by these two

TABLE II

RELATIVE PEAK-AREAS PREDICTED BY THREE THEORIES OR ASSUMPTIONS FOR AN EQUIMOLAR MIXTURE 2,3,4,6-TETRA-*O*-ETHYLGLUCITOL 1,5-DIACETATE AND 2,3,6-TRI-*O*-ETHYLGLUCITOL 1,4,5-TRIACETATE AND THE VALUE OBSERVED FROM THE SLOPE OF FIG. 3

Theory	*Terminal-Glc/4-substituted Glc*
Predicted	
Molar responses are equal	1.000
Molar responses are proportional to molecular weight	0.964
Molar responses are proportional to the number and type of carbon atoms (e.c.r.)	1.068
Observed	
Slope of Fig. 3	1.066 ±0.041

theories for an equimolar mixture of 2,3,4,6-tetra-*O*-ethylglucitol 1,5-diacetate and 2,3,6-tri-*O*-ethylglucitol 1,4,5-triacetate, together with the values predicted for this same mixture by summing the predicted response-contributions from the e.c.r. theory. The observed ratio, as given by the slope of Fig. 3, is 1.066 ±0.041, which agrees very well with the value (1.068) predicted by the e.c.r. theory, but does not agree with either of the other two theories within an arbitrary 5% limit. However, the observed value does have a fairly large standard deviation, and this uncertainty suggested an experiment in which the relative responses of hexa-*O*-ethylglucitol, hexa-*O*-methylglucitol and α-D-galactopyranose pentaacetate were measured. These compounds lead to a greater differentiation between predicted values, as they are the extremes of the partially methylated alditol acetates and the partially ethylated alditol acetates. Table I shows the relative peak-areas expected for the synthetic mixture of these three compounds under the three assumptions of molar response-factor calculations and the observed value. The e.c.r. theory is by far the most accurate, agreeing with the

observed values within 6%, whereas the other two theories are completely in error. The equimolar response theory even predicts that the hexa-*O*-methylglucitol should have the largest relative peak-area. The use of the equimolar response theory could account for an error of as much as 100% in the determination of these particular compounds.

The extremes of partially methylated and partially ethylated alditol acetates have been used here to demonstrate the accuracy of the effective carbon response theory for predicting molar g.l.c. flame-responses. However, as its accuracy has evidently been established, we consider that the theory can be applied to derivatives more-commonly encountered.

Table III gives the molar response-factors, according to the e.c.r. theory and according to the equal weight-response theory, for all partially methylated and partially ethylated alditol acetates commonly encountered. We recommend the values calculated by e.c.r. theory for quantitative determinations with these derivatives;

TABLE III

MOLAR RESPONSE-FACTORS[a] FOR PARTIALLY METHYLATED OR PARTIALLY ETHYLATED ALDITOL ACETATES ACCORDING TO TWO THEORIES, THE E.C.R. THEORY AND THE EQUAL WEIGHT-RESPONSE THEORY

Glycosidic linkage isomer	*E.c.r. theory*[b]		*Weight-response theory*	
	Ethyl	*Methyl*	*Ethyl*	*Methyl*
Hexose				
terminal	1.10	0.70	0.87	0.74
2,3, or 4-	1.03	0.74	0.90	0.81
6-	1.04	0.75	0.90	0.81
2,3 3,4 or 2,4-	0.99	0.79	0.94	0.87
2,6 3,6 or 4,6-	1.00	0.80	0.94	0.87
2,3,4-	0.94	0.84	0.97	0.94
2,3,6 2,4,6 or 3,4,6-	0.95	0.84	0.97	0.94
2,3,4,6-	0.89	0.89	1.00	1.00
Pentose				
terminal-(*f*)	0.90	0.60	0.74	0.64
terminal-(*p*)	0.91	0.61	0.74	0.64
2,3, or 4-(*p*) or 5-(*f*)	0.86	0.66	0.77	0.71
2 or 3-(*f*)	0.85	0.65	0.77	0.71
2,3 3,4 or 2,4-(*p*) 2,5 or 3,5-(*f*)	0.80	0.70	0.80	0.77
2,3-(*f*)	0.79	0.69	0.80	0.77
2,3,4-(*p*) or 2,3,5-(*f*)	0.75	0.75	0.83	0.83
6-Deoxyhexose				
terminal	1.00	0.70	0.77	0.67
2,3, or 4-	0.95	0.75	0.80	0.74
2,3 2,4 or 3,4-	0.89	0.79	0.83	0.80
2,3,4-	0.84	0.84	0.87	0.87

[a]Peak area÷response factor = relative moles. [b]Effective carbon response: calculated by summing the contributions of the carbon atoms according to the empirical values formulated by Ackman and shown in the legend of Table I.

however, Table III shows that little error (perhaps 5%) is introduced by quantitative determinations of partially methylated alditol acetates by the equal weight-response theory. The two theories predict the same order of change in molar responses as the derivatives become acetylated to a higher degree. However, the error introduced by the use of the weight-response theory for quantitative determination of partially ethylated alditol acetates, is quite large. The two theories predict the reverse trend in molar responses in proceeding to a more highly acetylated derivative.

The data shown in Table III demonstrate that, in general, weight response-factors or molar response-factors cannot be assumed to be equal for partially ethylated alditol acetates if the e.c.r. theory is correct without the introduction of significant error (as much as 50%) in quantitation. We consider that the responses predicted by the Effective Carbon Response theory are accurate and recommend their use for quantitative work.

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