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THE GAS CHROMATOGRAPHIC DETERMINATION OF POLYHYDROXY MONOCARBONIC ACIDS OBTAINED BY OXYGENATION OF HEXOSES IN AQUEOUS ALKALINE SOLUTIONS

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SUMMARY

A qualitative and quantitative method of analysis for the acidic reaction products resulting from the oxidation of hexoses in aqueous alkaline solutions with oxygen is described.

After the addition of hydrochloric acid to the aqueous reaction mixture, the sample is evaporated nearly to dryness. In addition to the unconverted hexoses, the residue consists, mainly, of the lactone structures of the acidic oxidation products. The residue is then dissolved either in pyridine or dimethylsulfoxide and silylated by means of a mixture of trimethylchlorosilane and hexamethyldisilazane. The silylated products are analysed by gas chromatography.

Retention times have been determined on three stationary phases. A method is described for identifying an unknown compound by plotting the logarithms of the net retention times on two stationary phases of different polarity against each other. This method is applied to 25 compounds with different functional groups and varying molecular weights.

A quantitative method has been developed based on calculated molar responses, relative to normal alkanes used as internal standards. The contribution of the primary and secondary silyl ether groups and the silyl ester group is determined from the molar responses of pure compounds.

INTRODUCTION

We have studied the mechanisms and the kinetics of the oxidation of hexoses with oxygen in aqueous alkaline solutions. As a kinetic investigation is only practicable when the concentrations of the hexoses and the reaction products (mainly the potassium salts of hydroxy-acids) can be measured, methods for determining these products had to be developed.

A schematic survey of the expected products is given in Table I.

The following methods for determining the oxidation products were investi-

TABLE I

SCHEMATIC SURVEY OF THE OXIDATION PRODUCTS OBTAINED BY THE OXYGENATION OF HEXOSES IN AQUEOUS ALKALINE SOLUTIONS

Monosaccharides	*Oxidation products*	
	Non-catalytic	*Catalytic*
Aldoses	Polyhydroxy-monocarbonic acids with a number of carbon atoms varying from 2 to 5 Formic acid Carbon dioxide Saccharic acids	Polyhydroxy-monocarbonic acids with 6 carbon atoms

gated: thin layer chromatography; zone electrophoresis; gas–liquid chromatography; displacement electrophoresis. Of these the latter two gave results within an acceptable level of accuracy. The investigation into the gas chromatographic method will be reported here in three parts, *viz.* sample preparation, qualitative aspects, and quantitative aspects.

SAMPLE PREPARATION

Extensive surveys of the applications of gas–liquid chromatography to carbohydrates have been given by BISHOP[1] and BERRY[2].

Several techniques are known to convert the carbohydrate molecules into volatile derivatives:

(a) Methylation

The formation of polymethyl ethers and/or esters has been described by KUHN AND TRISCHMANN[3]. The method is not quantitative and takes a long time.

(b) Acetylation

The formation of acetate ethers and/or esters has been described by WOLFROM AND THOMSON[4]. The reaction can be about 95% quantitative and can be performed with aqueous samples. However, rigorous acetylation may cause unwanted changes in the composition of the sample.

(c) Trimethylsilylation

SWEELEY *et al.*[5] in 1963 applied trimethylsilylation in qualitative carbohydrate analysis. They proposed a mixture of trimethylchlorosilane (TMS-Cl) and hexamethyldisilazane (HMDS) in a volume ratio of 1:2. A possible reaction mechanism is given by:

$$ROH + (CH_3)_3SiCl \longrightarrow ROSi(CH_3)_3 + HCl$$

$$2\,ROH + (CH_3)_3SiNHSi(CH_3)_3 \longrightarrow 2\,ROSi(CH_3)_3 + NH_3$$

$$3\,ROH + (CH_3)_3SiCl + (CH_3)_3SiNHSi(CH_3)_3 \rightarrow 3\,ROSi(CH_3)_3 + NH_4Cl$$

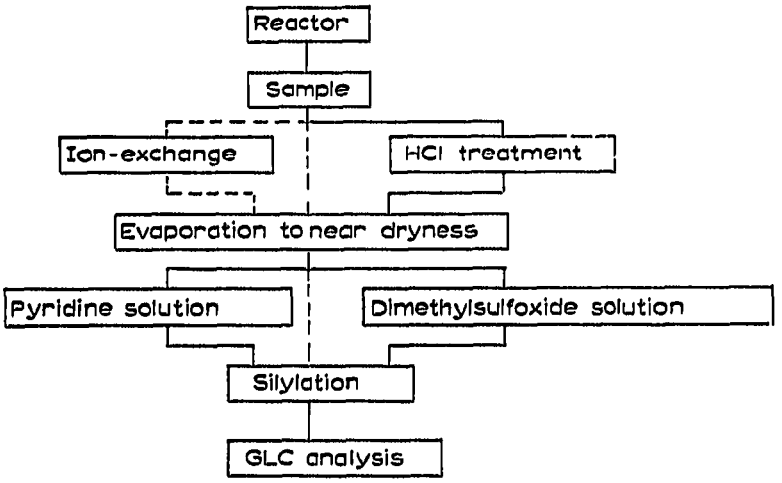
Acids can be converted in the same way into silyl esters. Water reacts very rapidly to yield hexamethyldisilyl ether. Besides TMS-Cl and HMDS, some other reagents such as N,O-bis-trimethylsilylacetamide (BSA) and N-trimethyl-diethylamine[6] have been studied for special purposes such as protein and steroid chemistry.

The silyl derivatives of carbohydrates are stable when stored under completely dry conditions.

An important advantage of (c) with respect to (a) and (b) is the relatively large increase in molecular weight per active hydrogen atom owing to the introduction of the trimethylsilyl group. This favours the separation of compounds with different numbers of active hydrogen atoms.

Experimental

Each sample to be analysed was available as an aqueous alkaline solution of the unconverted carbohydrates and the potassium salts of the acids formed. We investigated various ways of converting these samples into samples which were suitable for GLC analysis, as indicated in the following diagram and discussed below.



The dry residue obtained after the evaporation of the water contains the potassium salts of the polyhydroxy-carbonic acids and could not be silylated directly, because these salts do not dissolve in the trimethylsilyl reagent and their conversion into the trimethylsilyl ether and/or ester is only possible in a solution. It was found that the silyl ester bond is less stable than that of the silyl ether. Thus it was expedient to convert the potassium salts of the polyhydroxy-monocarbonic acids into the lactones of the free acids. This can be done either with a cation exchanger or with hydrochloric acid. The first method requires a rather large sample, which is further diluted by the water needed to remove the lactones and acids quantitatively from the ion exchanger.

For the second method only a small sample is needed, *viz.* 0.1 ml of a 4% by weight solution. Addition of an equal volume concentrated hydrochloric acid gives an almost complete conversion of the potassium salts, *via* the free acids, to the lactones.

The subsequent concentration of the acidic sample must take place at a low temperature and must be stopped at a solid/water ratio of about 1 (nearly dry). Evaporation of all the water makes it difficult to dissolve the products, subsequently, in the solvents used. The remaining quantity of water is taken up by the silylating agent but does not cause undue loss of silylating agent.

For quantitative silylation it is necessary that the reactants should be in solution. The most widely used solvent is pyridine, in which carbohydrates and their corresponding silyl derivatives are reasonably soluble. The metal salts of the acids constitute an exception. This difficulty is overcome by the use of dimethyl sulfoxide (DMSO). The potassium salts of polyhydroxy-monocarbonic acids can be converted into their trimethylsilyl esters without previous acidification when DMSO is used. DMSO forms a two-phase system. The upper layer consists of the silylated compounds and the excess of silylating agent. When using an apolar internal standard, this forms an additional component. The lower layer consists only of the DMSO and the non-silylated compounds. The mixture should therefore be shaken vigorously to complete silylation. The advantages of DMSO are that the TMS-derivatives are concentrated in the upper layer and there is diminished tailing as compared with pyridine.

The silylation was carried out in a sealed vial for 12 h with Trisil-concentrate (Pierce Chemical Company cat. no. 490.057) at room temperature. The Trisil was added in a ratio of about 0.5 ml (3 mequiv.) to one mequiv. of active hydrogen. By this means, the active hydrogen of both the hydroxyl and carboxyl functions is replaced by the trimethylsilyl group.

Procedure

The standard procedure for sample preparation adopted as a result of this study is as follows:

Approximately 100 mg of the aqueous sample (containing 4 mg of carbohydrates and their reaction products) is weighed into a vial with a capillary neck, and 0.1 ml of a 6 *N* hydrochloric acid solution is added. The vial is left for one hour at room temperature and cooled in ice to a temperature of 0°; the vial is then connected to a vacuum pump. The vial is kept in the air at room temperature during the evaporation until it is a nearly dry residue. The residue in the vial is dissolved in about 0.3 ml of pyridine or DMSO. A known quantity of an internal standard and then about 250 μl Trisil-concentrate is added and the vial sealed. If the residue was dissolved in pyridine, this solution was shaken for 10 min and then stored for 12 h at room temperature*. If DMSO was used, this solution was shaken vigorously for 12 h at room temperature. The mixture was then analysed on a gas chromatograph.

APPARATUS

Both qualitative and quantitative experiments were performed with a Philips (Pye) type PV 4000 gas chromatograph, equipped with a 401 type 1 mV W & W recorder. The coiled columns consisted of type AISI 321 stainless steel with an outside diameter of 1/8 in.

The carrier gas, argon, was previously dried with silica gel and had a flow rate of about 20 ml/min controlled by a pressure control unit. The pressure drop over the different columns used varied between 25 to 30 p.s.i. A hydrogen flame ionisation detector was used with a hydrogen flow rate of about 20 ml/min and an air flow rate of about 250 ml/min. The F.I.D. was constructed as a vertical flame centrally mounted in an open vertical cylindrical electrode, so that the contamination of the electrode

* When using pyridine the solution becomes cloudy owing to precipitation of ammonium chloride. This does not interfere with the GLC analysis.

due to silicon dioxide was negligible. The sensitivity of the detector remained constant during the analysis. Injection took place on the stationary phase. The sample volume varied between 0.2 and 1 μl dependent on the concentration of silylated material.

QUALITATIVE ANALYSIS

For the qualitative analysis three columns were used, which, in order of increasing polarity, were packed as follows:

(a) 1% by weight of GESE 52 (Hewlett Packard) on Chromosorb G-AW-DMCS*, 60–80 mesh (Becker Delft N.V.). Length: 3.7 m.

(b) PO 17 (OV 17). A column material manufactured by Pierce Chemical Company. The stationary phase is a 50% phenyl substituted methylsilicone. Support unknown. Length: 2.0 m.

(c) 3% by weight of polyphenyl ether-5 ring (Hewlett Packard) on Chromosorb G-AW-DMCS*, 60–80 mesh (Becker Delft N.V.). Length: 4.5 m.

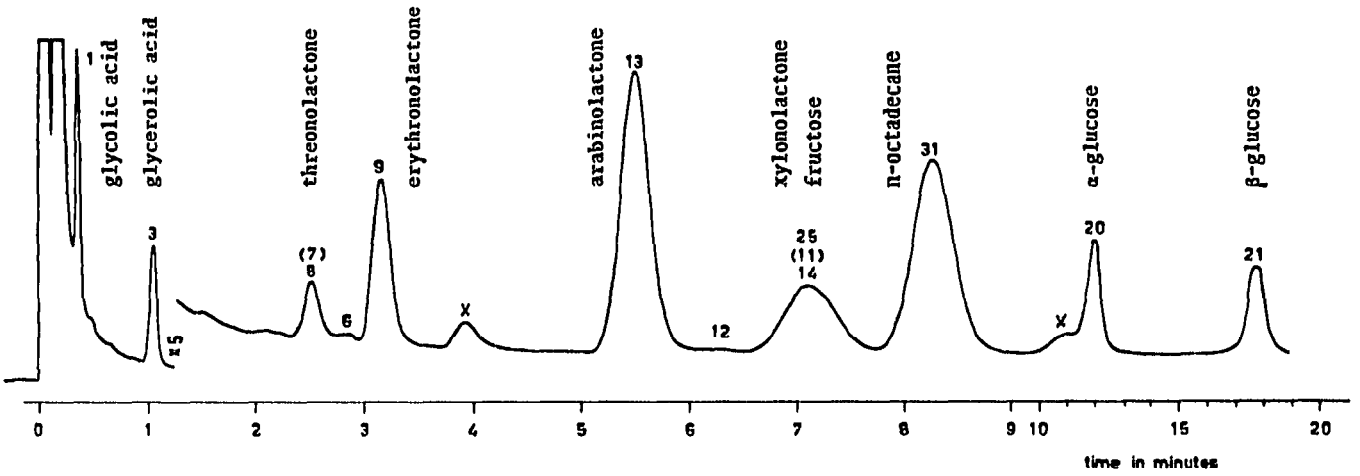


Fig. 1. Analysis of the oxidation products of glucose, obtained from an aqueous alkaline solution by means of the standard procedure. Column: PO 17 isothermal, 175°. The various compounds are indicated by numbers which refer to Table II. The brackets indicate very low concentrations of the compounds concerned.

Fig. 1 illustrates the product distribution of the oxidative degradation of glucose in aqueous alkaline solutions. Several methods have been used to identify the various peaks in the chromatogram:

(a) Injection of pure substances

(b) Variation in the sample pre-treatment

(c) Plotting the values of log t_r obtained on two stationary phases of different polarity against each other

(d) Comparison of t_r with the data of SWEELEY *et al.*[5]

(e) Comparison of t_r with the data of PETERSSON *et al.*[8]

* According to OTTENSTEIN[7] the best de-activation of the support material was achieved by combined acid washing (AW) and dimethylchlorosilane (DMCS) treatment.

TABLE II

RELATIVE NET RETENTION TIMES (α-GLUCOSE = 100) FOR A NUMBER OF COMPOUNDS ON DIFFERENT STATIONARY PHASES

	Compound	*Stationary phase, temperature and net retention time*					
		GESE 52 175° *23.9 min*		*PO 17* 175° *11.8 min*		*PPE 5-ring* 200° *15.8 min*	
		t_0	*I.M.*[a]	t_0	*I.M.*	t_0	*I.M.*
C_2							
1	Glycolic acid	2.1	a.c.	2.9	a.c.	7.4	a.c.
C_3							
2	Glycerol	5.5	a.c.	5.4	a.c.	9.1	a.c.
3	Glycerolic acid	7.2	a.c.	8.9	a.c.	16	a.c.
4	Tartronic acid	9.6	a.	15	a.		
5	Lactic acid	2.0	a.	2.6	a.	5.6	a.
C_4							
6	Threonic acid	21	b.c.	26	b.c.	30	b.c.
7	Erythronic acid	20	b.c.	21	b.c.	27	b.c.
8	Threonolactone	10	b.c.	21	b.c.	56	b.c.
9	Erythronolactone	13	b.c.	27	b.c.	74	b.c.
10	Tartaric acid L+	32	a.c.	42	a.c.	56	a.c.
C_5							
11	Arabonic acid	61	a.b.c.	59	a.b.c.	55	a.b.c.
12	Xylonic acid	56	b.c.	52	b.c.	48	b.c.
13	Arabinolactone	30	a.b.c.	47	a.b.c.	97	a.b.c.
14	Xylonolactone	38	b.c.	60	b.c.	126	b.c.
15	Arabinose	28	a.	28	a.	35	a.
C_6							
16	Sorbitol	124	a.c.	89	a.c.	59	a.c.
17	Mannitol	120	a.c.	86	a.c.	57	a.c.
18	Gluconic acid	163	a.b.c.	139	a.b.c.	100	a.b.c.
19	Gluconolactone	95	a.b.c.	136	a.b.c.	198	a.b.c.
20	α-Glucose	100	a.	100	a.	100	a.
21	β-Glucose	150	a.	149	a.	150	a.
22	α-Mannose	68	a.	62	a.	55	a.
23	β-Mannose	104	a.	92	a.	92	a.
24	Sorbose	89	a.	84	a.	79	a.
25	Fructose	72	a.	60 63	a. a.	54 57	a. a.
26	Dihydroxyacetone dimer	62	a.	66	a.	82	a.
27	*n*-Undecane	2.4	a.c.	3.2	a.c.	7.3	a.c.
28	*n*-Tridecane	6.2	a.c.	7.7	a.c.	18	a.c.
29	*n*-Pentadecane	15	a.c.	19	a.c.	42	a.c.
30	*n*-Heptadecane	37	a.c.	45	a.c.	98	a.c.
31	*n*-Octadecane	56	a.c.	70	a.c.	149	a.c.

[a] I.M. = identification method, see text.

(b) Variation in the sample pre-treatment

The acid lactone equilibrium is dependent on the pH of the solution and can be influenced by the amount of HCl added to the reaction sample. In this way it can be decided which of the peaks in the chromatogram emanate from polyhydroxy-monocarbonic acids and which from lactones.

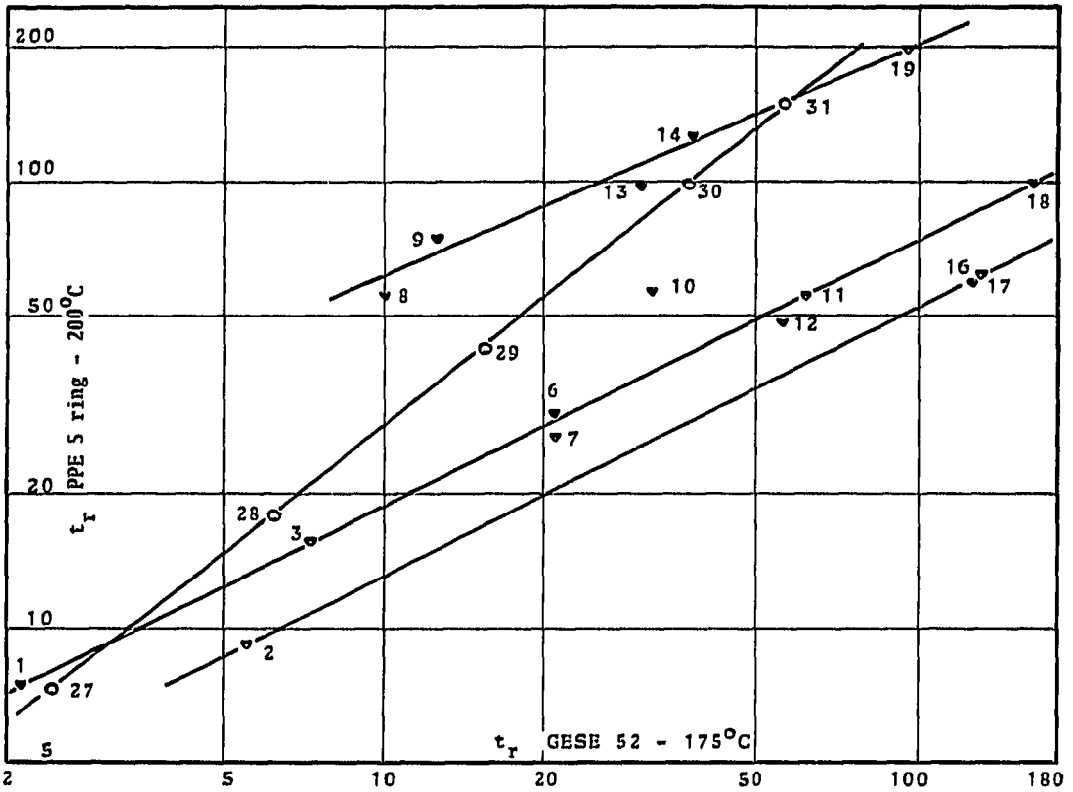


Fig. 2. Log t_r on stationary phase PPE-5-ring *vs.* log t_r on stationary phase GESE 52. The numbers refer to Table II.

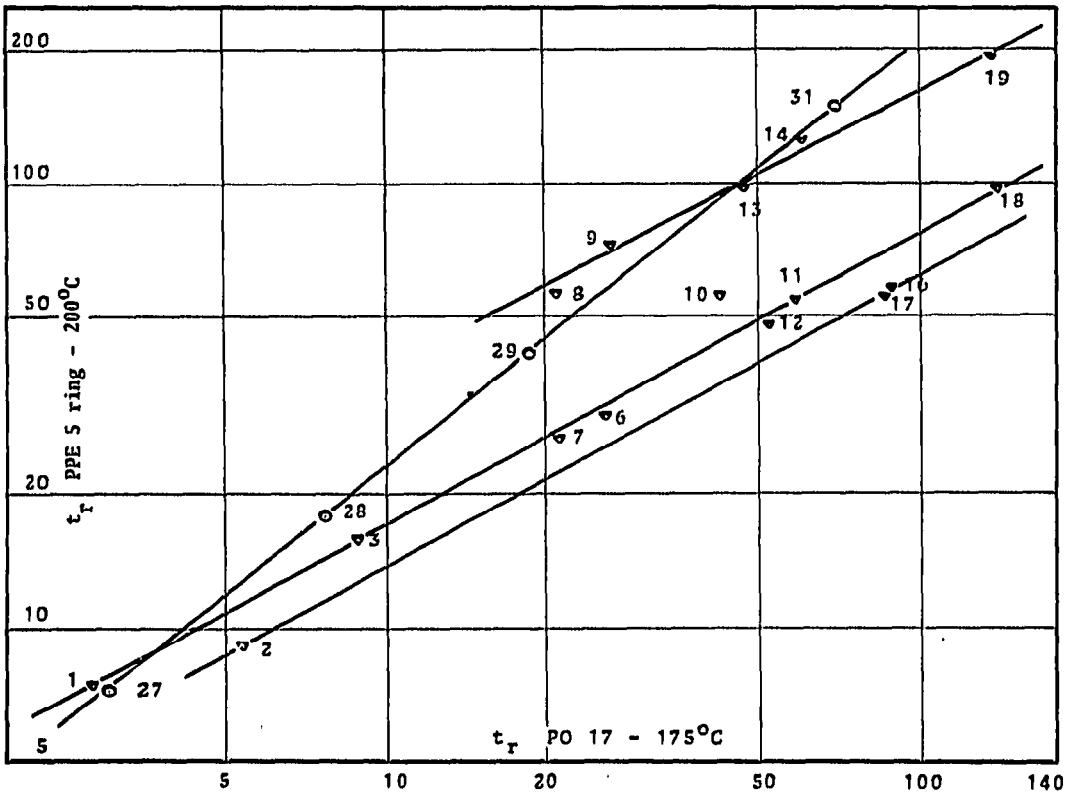


Fig. 3. Log t_r on stationary phase PPE-5-ring *vs.* log t_r on stationary phase PO 17. The numbers refer to Table II.

(c) Comparison of log t_r values obtained on two stationary phases

With this graphic method of representation homologous series give rise to straight lines of which the slope is dependent on the type of the structural unit and the level fixed by the nature of the attached groups[9]. Both the slope and the level are dependent on the two stationary phases used.

(d) and (e) Comparison of t_r values with published data

We found good qualitative agreement between the data of SWEELEY *et al.*[5] and PETERSSON *et al.*[8], obtained at a column temperature of 140 and 150°, and our data obtained at a column temperature of 175°.

A survey of the qualitative results is given in Table II. Next to each compound the method(s) of identification is (are) mentioned. The compounds are listed in order of their number of carbon atoms with a subdivision in alcohols, acids and lactones. The retention times (t_r) given in the subsequent text and tables are the relative net retention times based on α-glucose = 100.

Discussion

The lactones mentioned are most probably the 1,4-lactones as a result of the pre-treatment of the sample with hydrochloric acid.

As will be shown for a quantitative determination the difference between the 1,4 and the 1,5 forms is of no interest, because they have little or no difference in molecular detector response.

Several authors have tried to find relations between the retention time and the molecular structure. SWEELEY *et al.* suggested a rule for the hexose anomers[5]. Similar rules are proposed by GUNNER[10] and discussed by FERRIER[11].

PETERSSON *et al.*[8] found that on a great number of stationary phases lactones

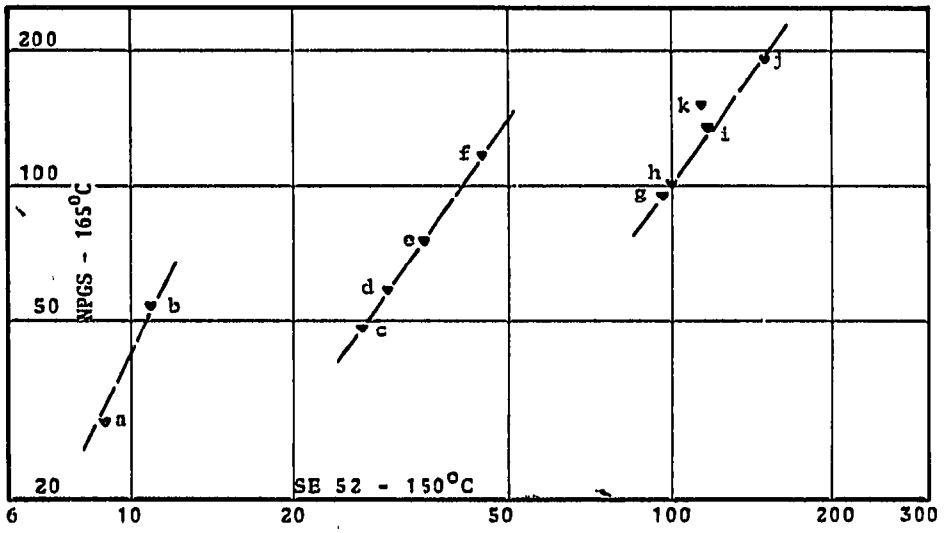


Fig. 4. C_4 lactones: (a) threonolactone; (b) erythronolactone. C_5 lactones: (c) arabinolactone; (d) xylonolactone; (e) ribonolactone; (f) lyxonolactone. C_6 lactones: (g) galactonolactone; (h) gluconolactone; (i) talonolactone; (j) mannonolactone; (k) gulonolactone. The X and Y axes give the relative retention times based on gluconolactone (= 100) reported by PETERSSON[8]. SE-52 = 1% SE-52 silicone gum on Chromosorb W silan, 80/100 mesh. NPGS = 1% neopentylglycolsuccinate polyester on Chromosorb W silan, 80/100 mesh.

with TMS-groups in a *trans* position on the number 2 and 3 carbon atoms have shorter retention times than the corresponding *cis* isomers.

All these rules are only applicable to isolated cases where closely related compounds are compared and are not based on a physical interpretation of experimental data, although the hexoses and lactones in Table II satisfy the rules of SWEELEY *et al.* and of PETERSSON.

On the other hand, relationships which are applicable to almost all members of a homologous series of compounds can be obtained by plotting log t_r on stationary phase X *versus* log t_r on stationary phase Y. In Figs. 2 and 3 our data given in Table II are treated in this way.

Parallel levels can be distinguished for poly-alcohols, polyhydroxy-monocarbonic acids and for lactones. A polyhydroxy-dicarbonic acid, like tartaric acid, shows a distinct difference, again, from the level given by the polyhydroxy-monocarbonic acids.

In the lactone series, the "fine structure" of the stereo isomers merits particular attention. This phenomenon is comparable with the roofing-tile effect of skeleton isomers as described by WALRAVEN *et al.*[12] for a great number of petrochemicals.

The fine structure of the stereo isomers with a roofing-tile formation becomes more marked with an increasing difference in polarity of the two stationary phases, as can be shown in Fig. 4 where the data obtained by PETERSSON[8] for the lactones are plotted.

In this way it is possible to identify a compound—the origin of which is known—with respect to its type and structure by the determination of the retention times on two standard stationary phases only and by plotting the relative net retention times in a standard graph.

QUANTITATIVE ANALYSIS

Only a small number of the compounds which are formed during the oxidation of hexoses in aqueous solution were available with a satisfactory grade of purity. This made it impossible to draw empirical standard curves for each of the components present. For this reason a quantitative approach by means of calculated molar responses was developed.

With flame ionisation detection the molar response can be calculated from the contribution of each of the component groups within the molecule. KAISER[13] summarises a number of data on C–H–O compounds based on the studies of ACKMAN[14] and others. As the influence of the silicon atom is unreported, we determined its contribution by silylating various types of compounds and measuring their responses in a flame ionisation detector.

We used normal alkanes as internal standards because they were readily obtainable with a high degree of purity. They do not react on the Trisil-reagent and are sufficiently soluble in the Trisil-reagent and in pyridine. They are insoluble in DMSO and are therefore found quantitatively in the upper layer which contains the silyl derivatives.

The response of this type of internal standard is independent of the level of silylation of the sample, and it is not influenced by the decomposition of the silylated compounds during the analysis.

The relative molar responses on the PO 17 column were constant and were not affected by external parameters. Therefore this column was used for all the quantitative experiments.

The responses on the PPE-5 ring column fluctuated widely and were always found to be lower than those on the PO 17 column; this was probably owing to the interaction between the silylated compounds and an incompletely deactivated support material.

The relative molar response (r.m.r.) is defined as:

$$\text{r.m.r. (X)} = \frac{\text{(molar response of compound X)}}{\frac{1}{y}\text{(molar response of a normal alkane with } y \text{ carbon atoms)}} \cdot 100$$

For a known product distribution the most suitable normal alkane can be derived from Figs. 2 and 3.

Table III lists a number of compounds suitable for r.m.r. determination. The purity of the compounds used was over 99% and the degree of silylation was assumed

TABLE III

MEASURED RELATIVE MOLAR RESPONSE OF STANDARD COMPOUNDS

Compound	*Measured r.m.r.*	*Standard deviation (%)*	*Number of component groups*		
			$-CH_2OSi(CH_3)_3$	$=CHOSi(CH_3)_3$	$-COOSi(CH_3)_3$
Glycerol	1095	2.2	2	1	—
Mannitol	2168	1.3	2	4	—
Tartaric acid	1288	1.0	—	2	2
1,5-Pentanediol	1045	0.5	2 (+3 $=CH_3$ groups at 100)		

to be 100%. From these data the contribution of the $-CH_2OSi(CH_3)_3$ group, the $=CHOSi(CH_3)_3$ group and the $-COOSi(CH_3)_3$ group could be calculated. These three unknowns are mutually related by four independent equations, the solution of which was obtained by calculating by means of the method of least squares (Table IV).

TABLE IV

Group	*Calculated units*
$-CH_2OSi(CH_3)_3$	370
$=CHOSi(CH_3)_3$	355
$-COOSi(CH_3)_3$	290

TABLE V

Group	*Number of units*
$-COOSi(CH_3)_3$	290
$-CH_2OSi(CH_3)_3$	370
$=CHOSi(CH_3)_3$	355
An alcohol group combined with a carboxyl group by means of a lactone oxygen bridge	50
—CHO (aldehyde)	0
=CO (ketone)	35

TABLE VI

COMPARISON BETWEEN MEASURED AND CALCULATED RELATIVE MOLAR RESPONSES FOR THE STANDARD COMPOUNDS

Compound	*Measured r.m.r. (a)*	*Calculated r.m.r. (b)*	*(a)/(b)*
Glycerol	1095	1095	1.000
Mannitol	2168	2160	1.004
Tartaric acid	1288	1290	0.998
1,5-Pentanediol	1045	1040	1.005

According to KAISER[13] the response of the group $-CH_2O- = 55$, the group $=CHO- = 35$, the group $-COO- = 0$ and the group $-CH_3 = 100$. So we found that in the ether function the silicon atom has a positive effect on the response but in the ester function a small negative one. The calculated difference of about 15 units

TABLE VII

CALCULATED MOLAR RESPONSES

No.	*Compound*	*Calculated r.m.r.*
1	Glycolic acid	660
2	Glycerol	1095
3	Glycerolic acid	1015
4	Tartronic acid	[a]
5	Lactic acid	[a]
6	Threonic acid	1370
7	Erythronic acid	1370
8	Threonolactone	760
9	Erythronolactone	760
10	Tartaric acid	1290
11	Arabonic acid	1725
12	Xylonic acid	1725
13	Arabinolactone	1130
14	Xylonolactone	1130
15	Arabinose	1435
16	Sorbitol	2160
17	Mannitol	2160
18	Gluconic acid	2080
19	Gluconolactone	1485
20 to 23	Aldoses	1790
24	Sorbose	1840
25	Fructose	1840[a]
26	Dihydroxy acetone dimer	1700
27 to 31	*n*-Alkanes with *y* carbon atoms	$100 \cdot Y$
	Oxalic acid	[a]

[a] No signal could be obtained from oxalic acid. The responses of tartronic acid and lactic acid are much lower than the calculated ones. In this study, it was not determined which of the active hydrogen atoms was not replaced by a TMS-group. According to HENGLEIN[15] this was most probably the carboxylic acid function. The quantitative determination of fructose was only successful when crystalline fructose was dissolved in anhydrous pyridine. The r.m.r. became too low after evaporation of an aqueous solution nearly to dryness, depending on the degree of evaporation. This is probably caused by the formation of dianhydrides, produced by the action of strong mineral acids on ketoses[16].

between a primary and a secondary silyl ether group agrees reasonably with the difference between a primary and a secondary alcohol group of about 20 units as reported by KAISER.

The standard calculation procedure adopted as a result of the above considerations is based on the data given in Table V.

The measured and calculated r.m.r. are compared for the 4 standard compounds in Table VI. Table VII lists the calculated r.m.r. of the compounds which were studied in the qualitative part (Table II).

The compounds numbered 1, 11, 16, 18, 19, 20, 21, 22 and 23 proved to be fully silylated, for their measured r.m.r. was almost equal to the calculated one.

By using two samples, one of which was not treated with any hydrochloric acid at all and the other with the reported quantity, we were able to measure the response ratio between the acidic and the lactone structure of the compounds: 6/8, 7/9, 11/13, 12/14 and 18/19. This ratio agreed well with the calculated one.

The number of carbon atoms, determined as a function of time during the oxidation of glucose in aqueous alkaline solutions, balanced within 2%. About 85% of the number of carbon atoms was determined by the reported gas chromatographic method. The results agreed very well with those obtained by an electrophoretic method.

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