THE WATER SOLUBLE CARBOHYDRATES OF *PINUS RADIATA*: IDENTIFICATION AND QUANTITATION

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degree of M.Sc. in Chemistry
in the University of Canterbury,
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by

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INTRODUCTION

A wide range of techniques have been used in the analysis of carbohydrates from plant sources. Many of these techniques are fraught with difficulties and are not at all suitable for routine analytical procedures. A joint project with N.Z.F.R.I. was undertaken to develop suitable rapid methods for the routine analysis of the water soluble carbohydrates (w.s.c.) present in the various tissues of Pinus radiata. Seasonal variations of carbohydrate levels were also of particular interest to the plant physiologists at the N.Z.F.R.I.

The traditional extraction procedure uses 80% or 95% ethanol/water, (Holligan 1971 b, Ford 1974) and requires either a 'soak' or a soxhlet extraction of the plant tissue for a predetermined period. Unfortunately not all starch is removed by ethanol or water (Smith 1971) so a separate analysis in which the tissue is directly analysed for starch is usually necessary.

The techniques used for the analysis of carbohydrates range from paper chromatography to, more recently, carbon magnetic resonance (c.m.r.) spectroscopy. Paper chromatography is still used when a small number of samples are to be analysed, although it is more useful for identification than quantitation because the process becomes both complex and inaccurate when the chromatograms are required to be measured colorimetrically (Dubois 1956, Jeffery 1960). Thin layer chromatography (t.l.c.) using silica and cellulose absorbent phases and a wide range of solvent systems have been reported (Jeffery 1960, Hehl 1973), but the time involved in obtaining chromatograms is such that other methods are preferable.

Paper electrophoresis with borate buffer systems has been used with some success (Pettersson 1973), but it has the same limitations as paper chromatography.

Column chromatography has also been used to isolate certain w.s.c.'s. For example, a sugar-free mixture of the cyclitols was separated on a cellulose packing in conjunction with a Dowex ion-exchange column (borate form) (Dittrich 1971). Affinity chromatography, a more advanced form of column chromatography, enabled Kennedy (Kennedy 1973) to separate mono- and polysaccharides, but as with paper chromatography he had to rely on clumsy colorimetric methods for quantitation. Automation would be necessary for large numbers of samples.

Gas-liquid chromatography (g.l.c.) is the best of the chromatographic techniques available - offering rapid qualitative and quantitative analysis on relatively small amounts of material. The trimethylsilyl (TMS) ether derivatives of carbohydrates are easy to prepare and produce good chromatograms with compounds of similar or widely varying molecular weights (Holligan 1971 a, b). Acetate derivatives are more stable than their TMS counterparts, but separation of the aldose and ketose acetates was not satisfactory (Holligan 1971 a). G.l.c. relies largely on 'spiking' for identification of the peaks of a chromatogram. Linked to a mass spectrometer, the combined gc/ms provides mass spectra for all resolvable peaks and they can be identified by comparison with authentic spectra.

Proton n.m.r. spectroscopy has been attempted, but it was not possible to identify individual components due to the multiplicity and overlapping of peaks. In contrast, c.m.r.

has been shown to have great possibilities for qualitative and, more importantly for this work, quantitative analysis of aqueous solutions of carbohydrates.

Starch in plant tissue has been analysed by a number of methods (Bailey 1958, Stoddart 1966, Ebell 1969 a, MacRae 1968, 71), nearly all based on the analysis of the glucose formed by acid or enzymic hydrolysis. MacRae (MacRae 1971, 74) has reviewed the more traditional methods of starch analysis.

Identification and quantitation of the w.s.c. is, and probably will continue to be a problem as molecules of similar structure and molecular weight are involved. In P. radiata there are some 8 to 9 components to be considered. C.m.r. has greatly simplified the identification, and g.l.c. enables a large number of samples to be analysed accurately.

ISOLATION

Carbohydrates have usually been isolated from dry, finely ground plant material using 80% or 95% ethanol/water as solvent, extracting with either cold (Groce 1973) or hot solutions (Holligan 1971 b, MacRae 1971), or by using soxhlet apparatus (Ford 1974, Ebell 1969 a). The method adopted in this work was cold water extraction which, as experiments showed, quantitatively removed sugars and cyclitols together with organic and amino acids, although no work was done on these acids. The nonionic components are described as water-soluble carbohydrates (w.s.c.).

Although some workers have attempted to extract the starch from plant tissue (Bailey 1958, Stoddart 1966, Dekker 1971), many preferred to remove the soluble carbohydrates with 80% ethanol/water and only then analyse for starch (Ebell 1969 a, MacRae 1971). The simplest, most accurate methods of starch analysis rely on enzymic hydrolysis (Dekker 1971) and this was the technique adopted in this work.

The strategy for the analytical method adopted in this work was designed to include both carbohydrate and starch analysis from one tissue sample so that the number of steps in the analysis could be minimised. Since the starch analysis (see below) required a water medium, this solvent was selected for the extraction of the w.s.c. even though it was not practicable to combine the w.s.c. and starch analysis in this case. Another compelling reason for selecting water extraction was the dependence of the quantitative g.l.c. analysis, for certain components, on anomer ratios. The anomer ratios for carbohydrates in aqueous solution at 20°C are

known accurately. Therefore unless a subsequent water equilibration step is undertaken following an ethanol/water extraction, errors could be introduced in the analysis. Additionally, and most importantly, the percentage extraction of w.s.c. is greater for water extractions compared with 80% ethanol/water.

There are problems associated with water extractions. If the carbohydrates, in particular glucose and fructose, are to be in aqueous solution for any length of time the solution must be kept sterile. The antibiotic streptomycin sulphate was used when it became apparent the initial extracts had very reduced glucose and fructose levels due to fermentation. An alternative to streptomycin is to use fluoride, which is a potent inhibitor of glycolysis (Fales 1963). Higher temperatures (Aspinall 1963), or perhaps a low percentage of ethanol would also effectively sterilise the solution. However, higher temperatures result in extraction of a gelatinous polymeric material which interferes in the subsequent silylation steps as it is insoluble in pyridine (and no significant increase in w.s.c. was obtained). A convenient extraction temperature was 40°C.

The w.s.c. extraction was simplified to three basic steps:

(a) the soaking period, - the time taken for the extraction of w.s.c. was determined by monitoring glucose concentrations with the glucose oxidase enzyme as a function of time. The optimum extraction time for P. radiata tissue was determined as 36 hours.

(b) The extract, including the tissue was made up to volume with distilled water (the volume displaced by including the tissue was less than 0.6%). The supernatant was then treated with ion exchange resin to remove interfering ionic compounds.

(c) An aliquot was evaporated to dryness for TMS derivatisation, and subsequent g.l.c. analysis.

The starch analysis followed a similar analytical procedure, although the ion-exchange treatment was replaced with a charcoal wash, and enzymes rather than g.l.c. were used for quantitation. This was because the starch hydrolysates (glucose) could be measured more easily with glucose oxidase and the inhibitors of the enzymes could be removed with charcoal; there was no need for ion removal.

IDENTIFICATION OF W.S.C.

Before any meaningful work could be carried out on quantitation of the w.s.c. it was necessary to separate and identify the components of the mixture.

Of the various techniques available, such as liquid chromatography, partition chromatography, t.l.c. and g.l.c., it was g.l.c. with its very high resolving power that seemed to hold the greatest promise.

Successful separation of carbohydrates by g.l.c. requires the formation of volatile, heat-stable derivatives (Holligan 1971 a) which are chromatographed on an efficient column. For ease of preparation, acetate and TMS-derivatives are preferred and of these, the latter affords better separation. A drawback to the use of silyl ethers is their reactivity with water (Holligan 1971 b, Ford 1974) although up to 4% water (Pierce p. 9) can be tolerated.

The g.l.c. column packing selected for the analysis should ideally separate all components of the derivatised carbohydrate/cyclitol mixture to allow identification and accurate quantitation. A variety of columns were used in this phase of the work as the problem of overlapping peaks could not be avoided with any single column packing (Holligan 1971, Hamlen 1970). The choice of g.l.c. columns depended on the composition of the w.s.c. mixture, and unfortunately the degree of similarity between the components made separation difficult, so it was found necessary to use columns of different polarity (Farshtchi 1969, Ellis 1969 b, Sweeley 1963). Joint use of such columns produced complementary results as far as identification went and was sometimes useful in

quantitative analysis.

A table of retention times and peak ratios (p. 9) is given for a range of carbohydrates and cyclitols measured with respect to TMS-α-D-glucose, run on a linear temperature program: 140°-270°C at 8°C/min for OV-17 and SE-30

140°-250°C at 6°C/min for OV-225.

The retention times are summarised in (fig. 1, 2, 3). From this data, two basic trends are apparent:-

(a) Carbohydrates with anomeric hydroxyl groups in the equatorial position have longer retention times than the corresponding axial anomers. For example, the β-anomers of glucose, galactose and mannose, all with equatorial anomeric hydroxyls, have longer retention times than the corresponding α-anomers.

(b) Compounds with one or more O-alkyl substituents tend to have decreased retention times relative to the parent compound (Sweeley 1963).

Table of Retention Times

The retention times (R_t) for the following TMS-compounds, measured with respect to TMS-α--D-glucose, were taken from the columns; 3% SE-30, 2½% OV-17 and 2½% OV-225 and run on a linear temperature program:

140°-270°C at 8°C/min for SE-30 and OV-17

140°-250°C at 6°C/min for OV-225.

	SE-30	OV-17	OV-225
C_{16}	0.57	0.53	0.48
TMS-ribitol	0.80	0.65	0.59
-xylose	0.75,0.83	0.76,0.82	
	42 58	42 58	
-mannose	0.89,1.02	0.85,0.98	0.79,1.05
	74 26	74 26	76 24
-fructose	0.90,0.95,1.00	0.83,0.85,0.97,1.00	0.77,0.80,0.95,1.20,1.23
	87 9 4	57 43	54 16 29
-galactose	0.91,0.96,1.02	0.88,0.93,0.99,1.10	0.84,0.94,1.05,1.08
	10 21 69	4 36 57 3	7 23 t 70
-sorbose	0.89,98,1.02,1.06	0.94,0.99,1.09	0.76,0.92,1.12,1.47
	8 67 19 7	55 t 45	10 68 4 18
-glucose	1.00,1.11	1.00,1.12	1.00,1.24
	41 59	42 58	37 63
-mannitol	1.08	0.96	0.92
-chiro-inositol	1.10	1.01	
-pinitol	0.94	0.85	0.79
-dimethyl chiro-	0.88	0.88	0.87
-myo-inositol	1.27	1.22	1.41
-sequoyitol	1.10	1.04	1.15

Carbohydrates with 2 or more anomeric peaks have their percent ratios recorded under the appropriate retention time.

C_{16} - n-hexadecane ($C_{16}H_{34}$).

t - trace compounds too small to measure.

Figure 1.

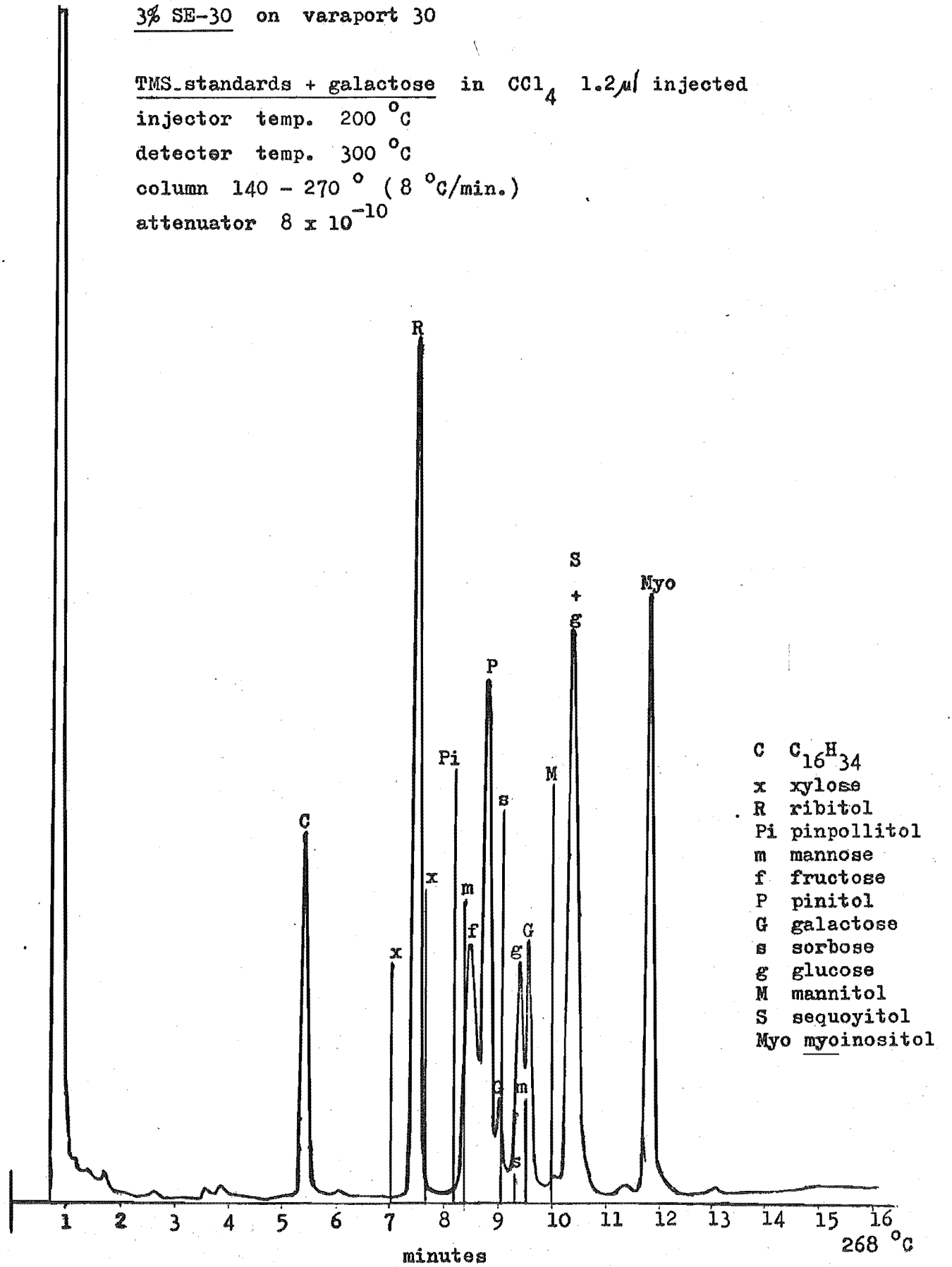

3% SE-30 on varaport 30
TMS-standards + galactose in CCl_4 1.2 µl injected
injector temp. 200 °C
detecter temp. 300 °C
column 140 - 270 ° (8 °C/min.)
attenuator 8 x 10^{-10}
C
x
R
Pi
m
f
x
P
s
G
g
G
m
s
M
S + g
Myo
C $C_{16}H_{34}$
x xylose
R ribitol
Pi pinpollitol
m mannose
f fructose
P pinitol
G galactose
s sorbose
g glucose
M mannitol
S sequoyitol
Myo myoinositol
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
minutes
268 °C

Figure 2.

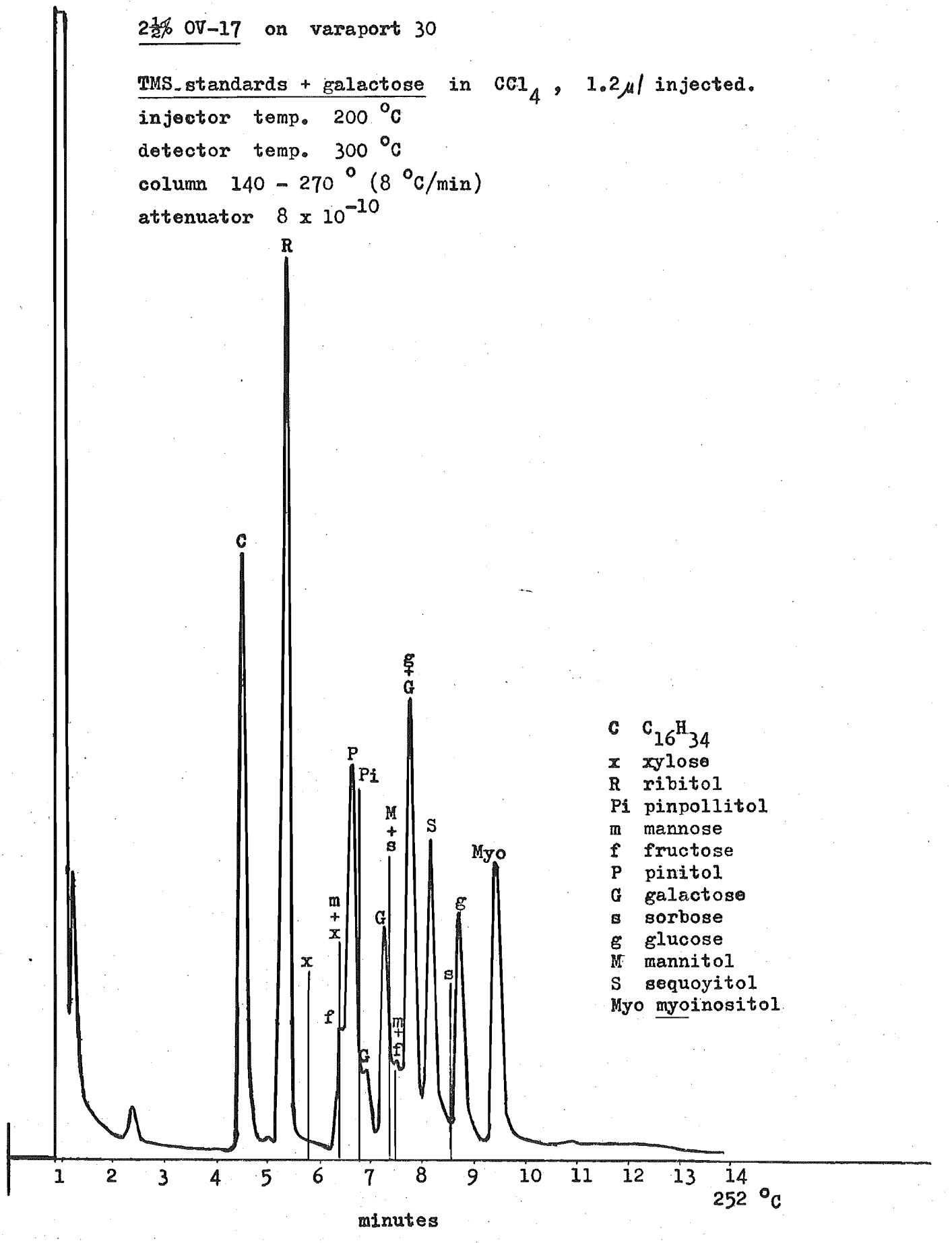
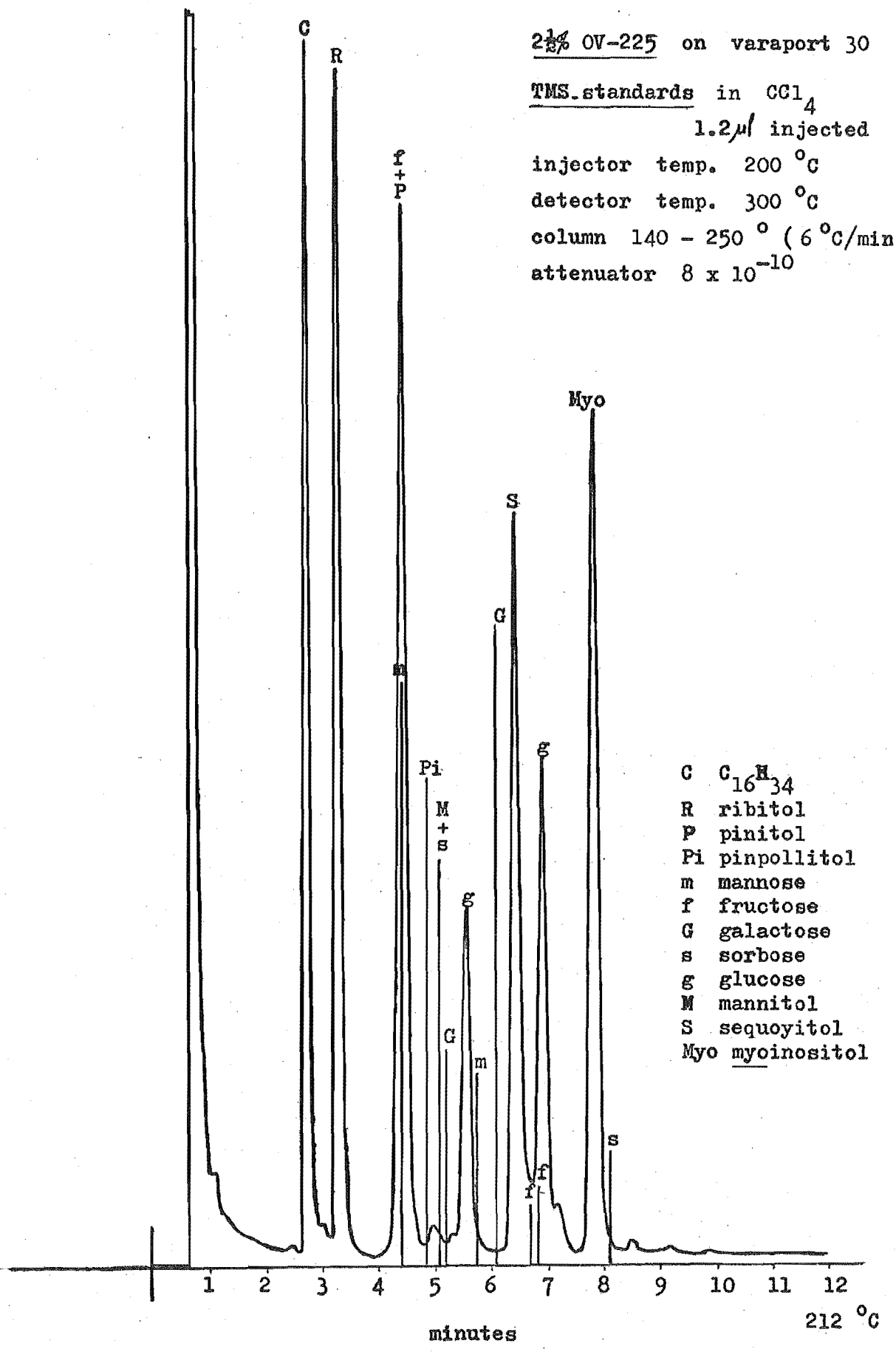

2½% OV-17 on varaport 30
TMS-standards + galactose in CCl_4, 1.2µl injected.
injector temp. 200 °C
detector temp. 300 °C
column 140 - 270 ° (8 °C/min)
attenuator 8 x 10^{-10}
R
C
P
Pi
M + s
g + G
S
Myo
m + x
x
G
f
s
g
C $C_{16}H_{34}$
x xylose
R ribitol
Pi pinpollitol
m mannose
f fructose
P pinitol
G galactose
s sorbose
g glucose
M mannitol
S sequoyitol
Myo myoinositol
1 2 3 4 5 6 7 8 9 10 11 12 13 14
252 °C
minutes

Figure 3.


2½% OV-225 on varaport 30
TMS. standards in CCl_4
1.2µl injected
injector temp. 200 °C
detector temp. 300 °C
column 140 - 250 ° (6 °C/min)
attenuator 8 x 10^{-10}
C
R
f+P
m
Pi
M+s
g
G
m
G
S
g
f
f
Myo
s
C $C_{16}H_{34}$
R ribitol
P pinitol
Pi pinpollitol
m mannose
f fructose
G galactose
s sorbose
g glucose
M mannitol
S sequoyitol
Myo myoinositol
1 2 3 4 5 6 7 8 9 10 11 12
minutes
212 °C

Identification was tentatively made by co-chromatography, or "spiking" of the derivatised water soluble extract with authentic TMS-derivatives of sugars or inositols to obtain coincident peaks on the g.l.c. trace. Fructose, galactose, glucose, mannitol, mannose and myo-inositol were thus identified.

As identification by co-chromatography is far from positive (as a means of identification) due to the problem of overlapping peaks, a gas chromatography/mass spectrometry (gc/ms) study on the TMS and acetate derivatives of the w.s.c. was undertaken.

The TMS derivatives of carbohydrates and cyclitols follow certain fragmentation patterns, the most common being loss of trimethylsilanol (TMSOH) and/or the trimethylsilyloxy radical ($\dot{TMSO}$) and the methyl radical ($\dot{CH_3}$). There are basic rearrangements of carbon-chain fragmentations which are found in all spectra, the most abundant being:-

m/e = 45/73/103/129/133/147/191/204/217/243/291

(Sherman 1970, De Jongh 1969). Peaks corresponding to carbon-chain fragmentations of saccharides occur at m/e values:-

89/117/189/317/319/332/435/437

Ions found at m/e 437 are characteristic of ketohexoses (M^+-CH_2OTMS). It has been observed that the abundance of m/e 437 > abundance m/e 435 for ketohexoses, and m/e 435 > m/e 437 for aldohexoses (Kamerling 1972). The abundance of the fragment ions, m/e 204 and m/e 217, formed usually from C_2-C_3 and C_2-C_3-C_4 carbon-chains respectively, is dependent on the ring structure of the saccharide. It is found that m/e 217 > 204 for furanose and m/e 204 > 217 for pyranose

(Curtius 1968).

On the basis of retention data obtained on OV-17 as liquid phase, and the fragmentation pattern, it was possible to positively identify the following carbohydrates in the w.s.c. from P. radiata wood (Fig. 4):-

fructo-	(peak 13)	fig. 4a
fructo-	(peak 19)	4b
α-D-glucopyranose	(peak 21)	4c
β-D-glucopyranose	(peak 24)	4d
sucrose	(peaks 34 and 35)	4e

The mass-spectrum obtained for myo-inositol (peak 26) (fig. 4f) was identical with that of an authentic sample of the TMS- derivative of myo-inositol. Characteristic peaks for inositols as compared to saccharides, are:-

m/e 507 (M^+-TMSOH-$\dot{C}H_3$)

432 (M^+-2TMSOH)

393

318

O-Methyl inositols have a $-CH_3$ in place of a TMS- group which is a difference of 58 m.u. Thus, characteristic peaks for O-methyl inositols will appear at 58 m.u. lower than for the corresponding inositol. Thus M^+_{554}-TMSOH-$\dot{C}H_3$ = m/e 449 which is m/e 507 - 58. Ions resulting from fragmentation of a TMS- derivative of an O-methyl inositol may or may not appear 58 m.u. lower depending on whether or not the fragment contains the $-OCH_3$ group or the -OTMS group. Typical ions found in methyl inositols are:-

m/e 374 (432)

Figure 4.

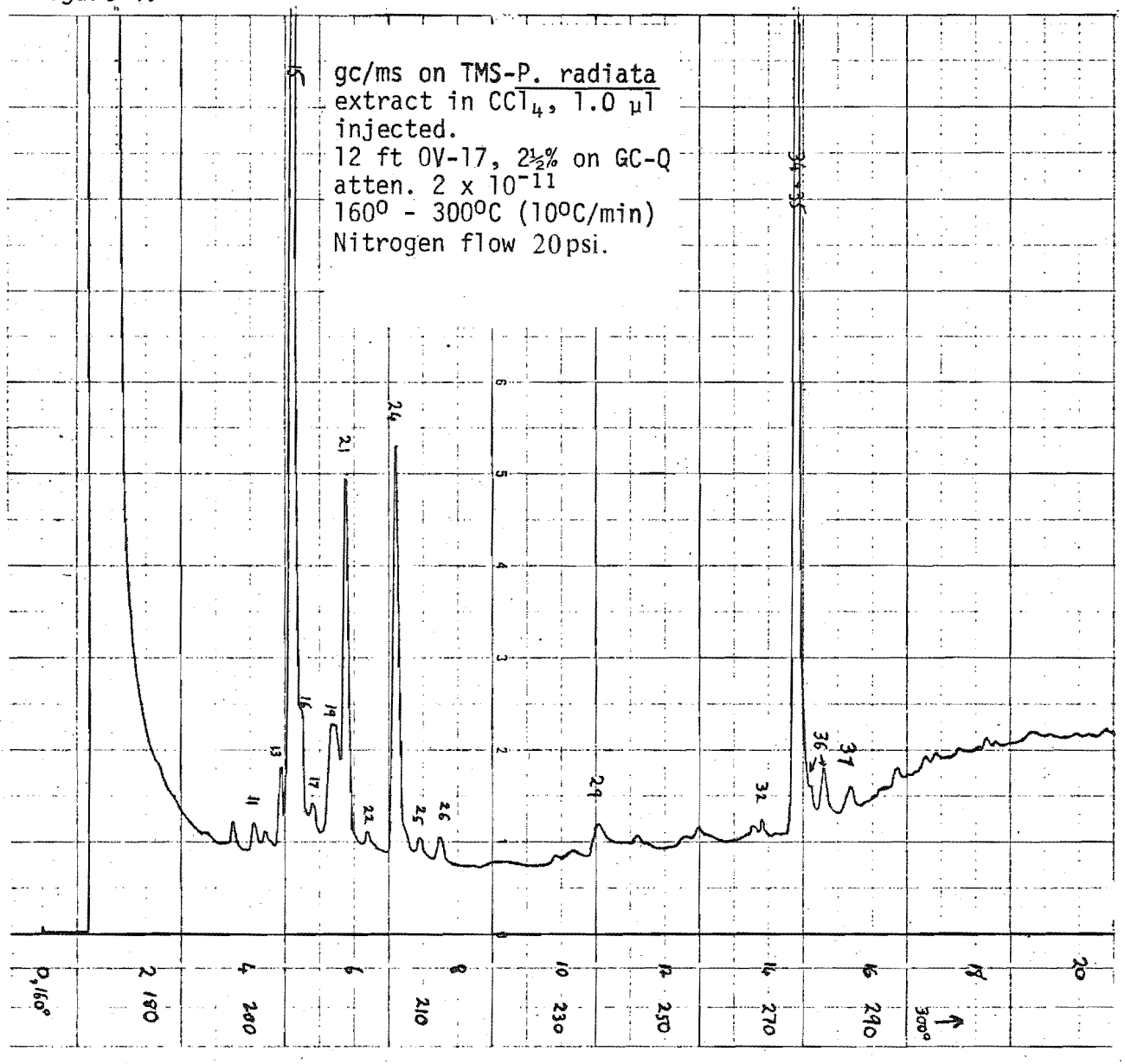

gc/ms on TMS-P. radiata
extract in CCl_4, 1.0 µl
injected.
12 ft OV-17, 2½% on GC-Q
atten. 2 x 10^{-11}
160° - 300°C (10°C/min)
Nitrogen flow 20 psi.
34.35
24
21
16
19
13
17
11
22
25
26
29
32
36
37
0
1
2
3
4
5
6
0,160°
2
190
4
200
6
210
8
220
10
230
12
250
14
270
16
290
18
300°
20

Figure 4a.

TMS-furanose

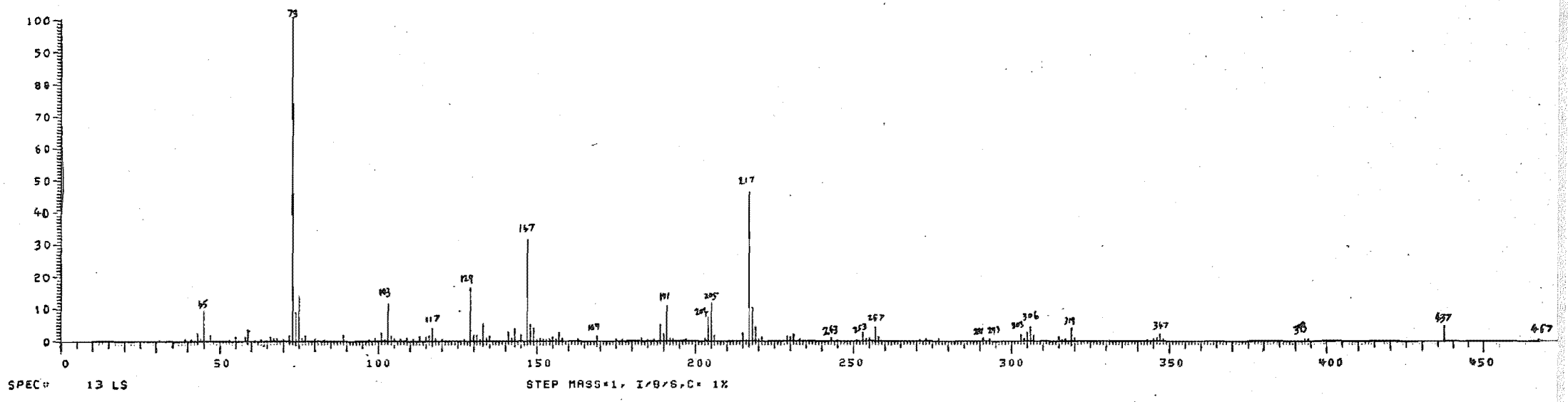


Figure 4b.

TMS-α-D-glucopyranose

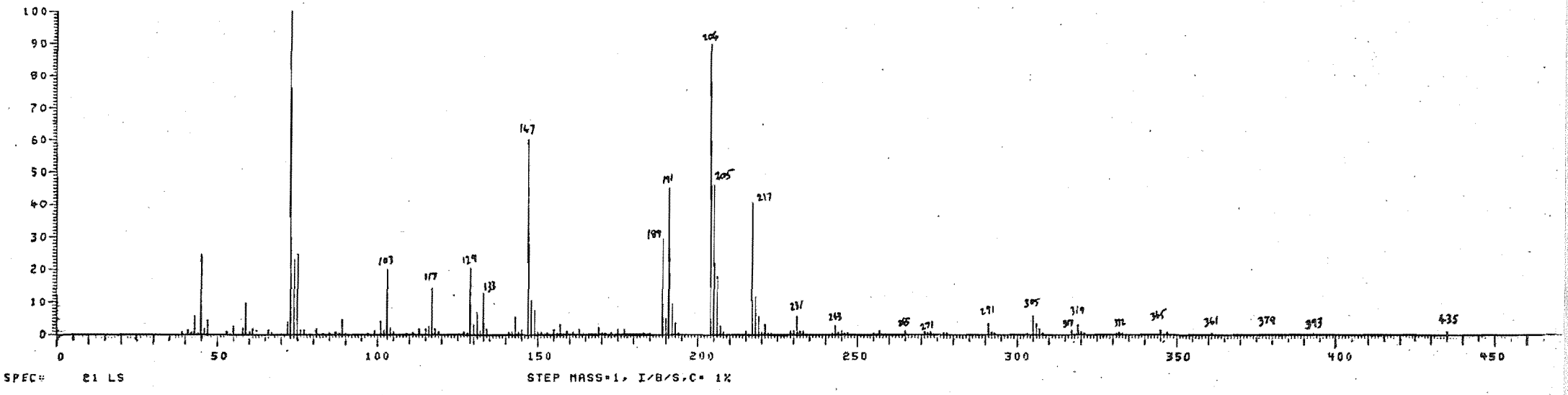


Figure 4c.

TMS-pyranose

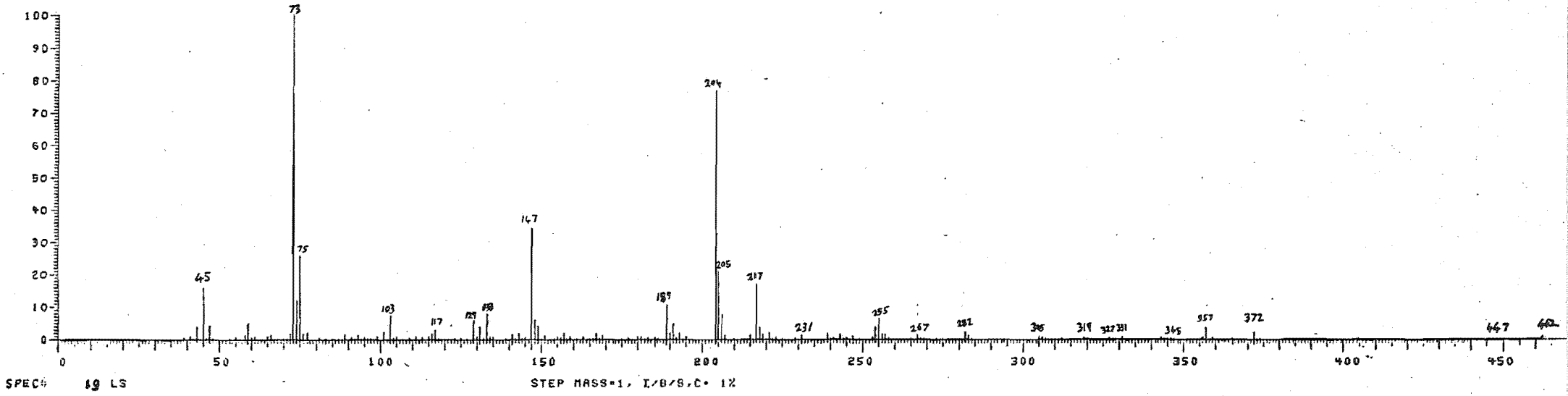


Figure 4d.

TMS-β-D-glucopyranose

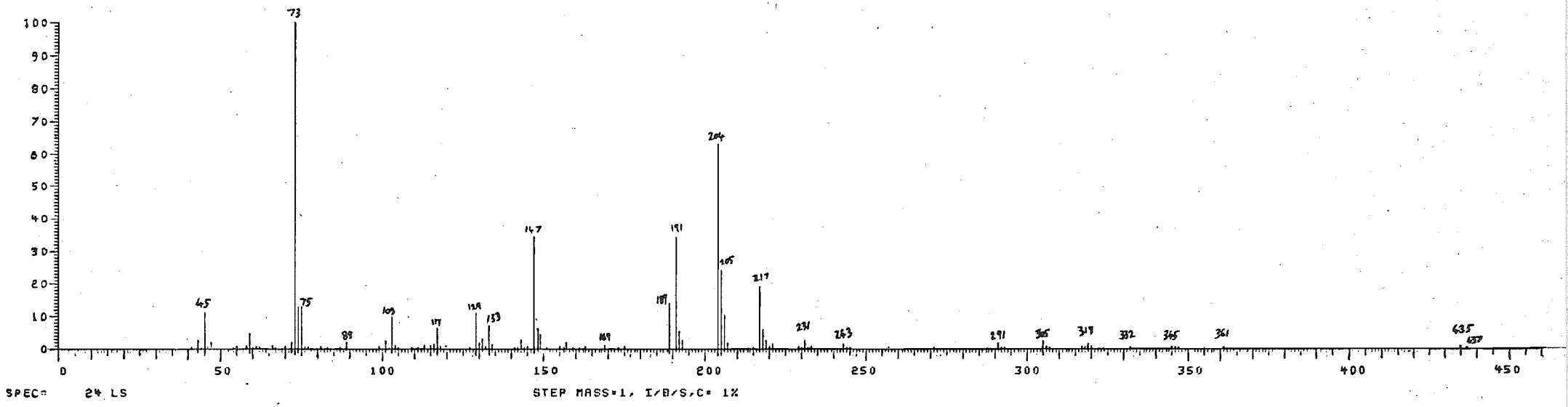


Figure 4e.

TMS-sucrose

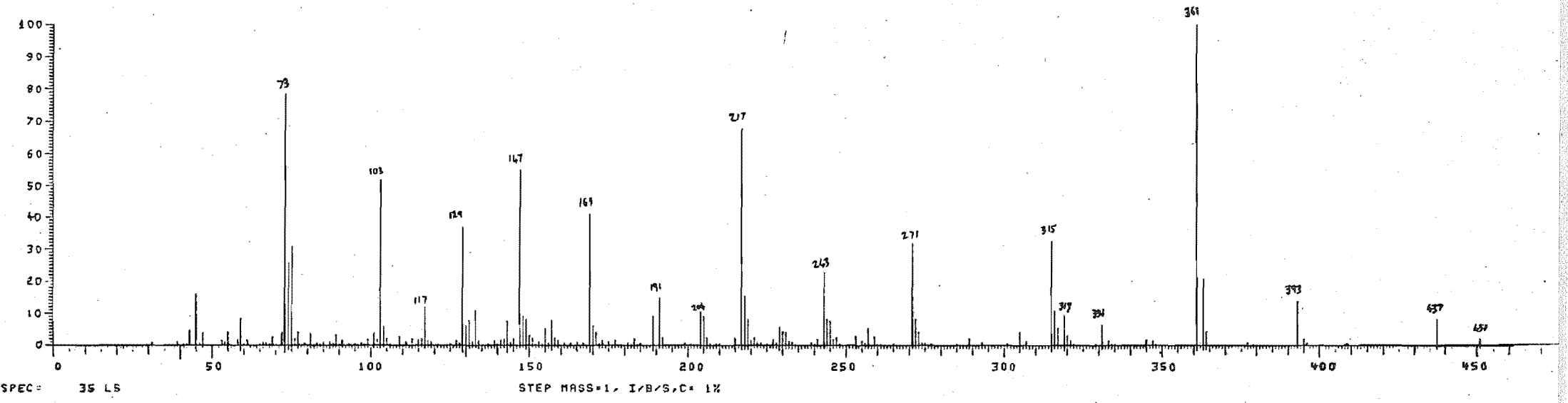


Figure 4f.

TMS-myo-inositol

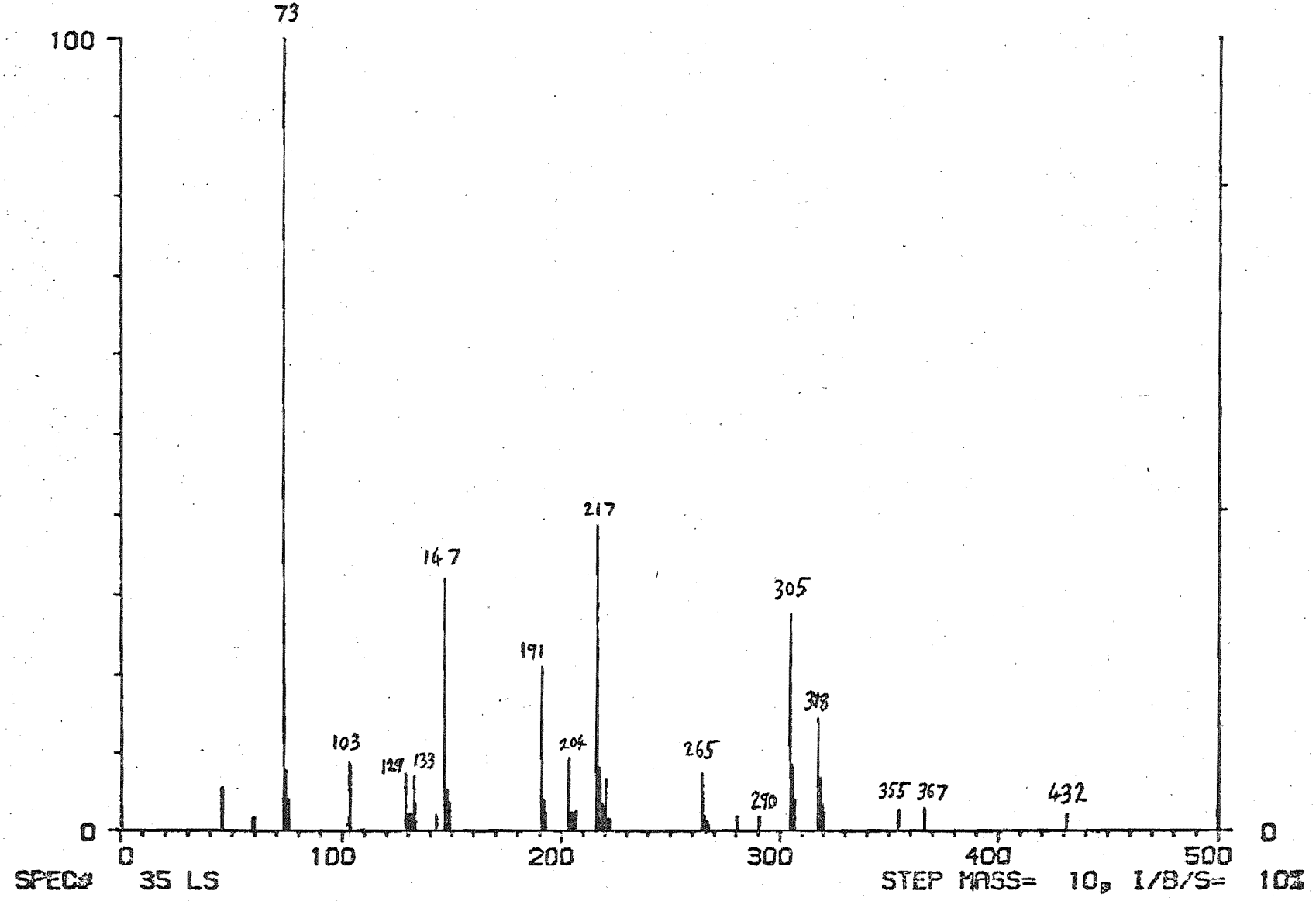


Figure 4g.

TMS-pinitol

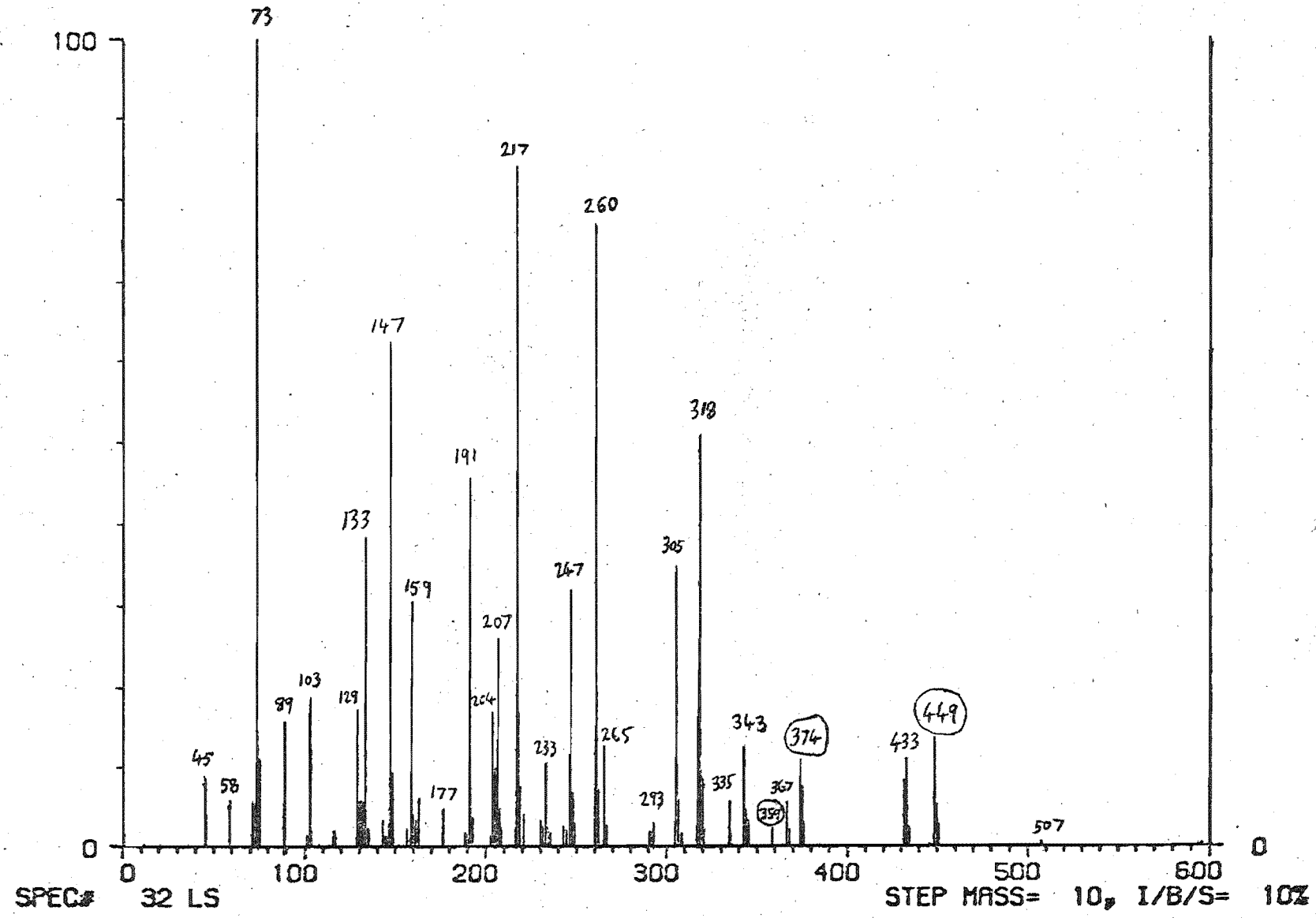


Figure 4h.

TMS-O-methyl-inositol

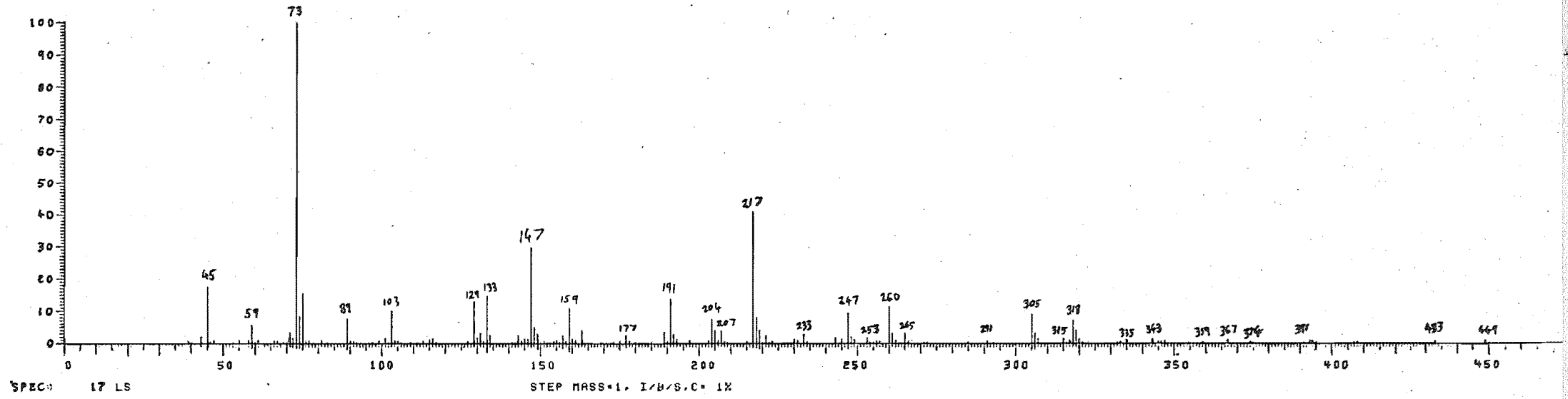


Figure 4i.

TMS-sequoyitol

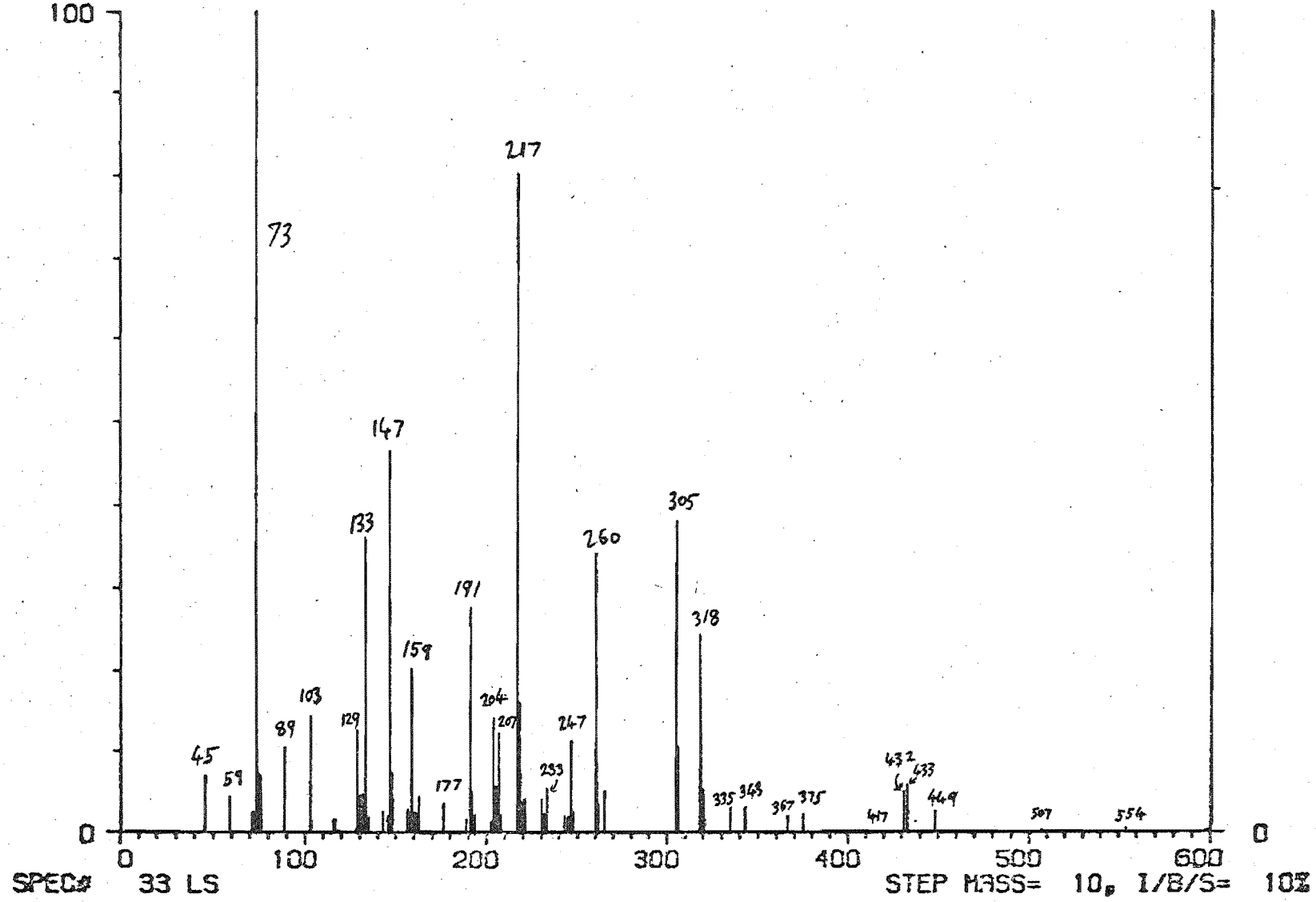
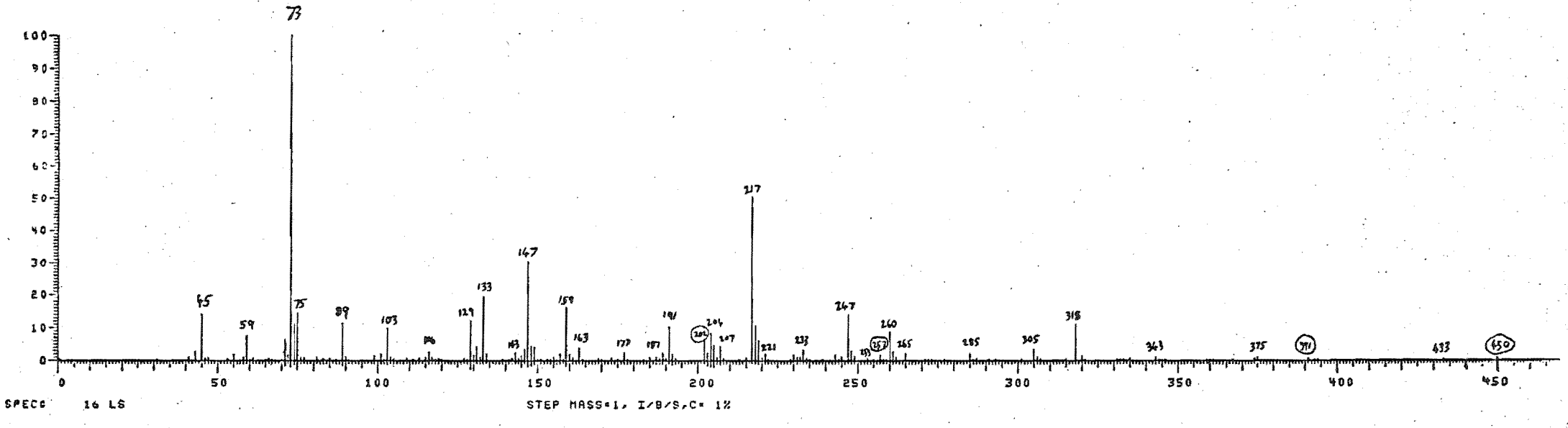


Figure 4j.

TMS-pinpollitol



335 (393)

260 (318)

247 (305)

233 (291)

159 (217)

The major fragment ions are found at m/e 318, 305, 260 and 217. From the m.s. it was possible to identify peaks 15, 17 and 22 (fig. 4) as O-methyl inositols (figs. 4g, h, i).

Furthermore, it was possible to identify peak 16 as an O,O-dimethyl inositol (fig. 4j). The m.s. showed only a low intensity m/e 305 ion, while ions at m/e 247 (305 - 58) and 260 (318 - 58) were strong. Peaks at m/e 391 (M^+-TMSOH-$\dot{C}H_3$) and m/e 450 (M^+-$\dot{C}H_3$-$\dot{O}CH_3$) and m/e 202 (318 - 58 - 58) confirmed the presence of an O,O-dimethyl inositol. A parent ion at m/e 496 was not observed, but this is not unusual as the parent ion in all TMS- derivatives is of very low intensity.

Isolation of the Cyclitols

The cyclitols were isolated from the water soluble extract by oxidation of the carbohydrates with 2,4-dinitrophenylhydrazine (2,4-DNP) followed by ion exchange treatment. Sequoyitol (5-O-methyl-myo-inositol), pinitol (3-O-methyl-chiro-inositol) and myo-inositol were selectively crystallised from the cyclitol mixture (see figs. 5a, b, c). At least 6 cyclitols or cyclitol derivatives remained in the mother liquors. All were easily separable on an SE-30 column as their TMS-derivatives (fig. 6a, b).

Initially pinitol had been obtained from a TMS-derivatised wood extract by preparative g.l.c. on a 2½% OV-17 column. The TMS- pinitol was contaminated with small quantities of TMS- fructose and the TMS- O,O-dimethyl inositol.

Figure 5. (a) TMS-cyclitols from P. radiata in CCl_4

1.0 µl injected onto
6 ft OV-17, 2½% on
Varaport 30.
atten. 16 x 10^{-10}.
140° - 240°C (8°C/min)

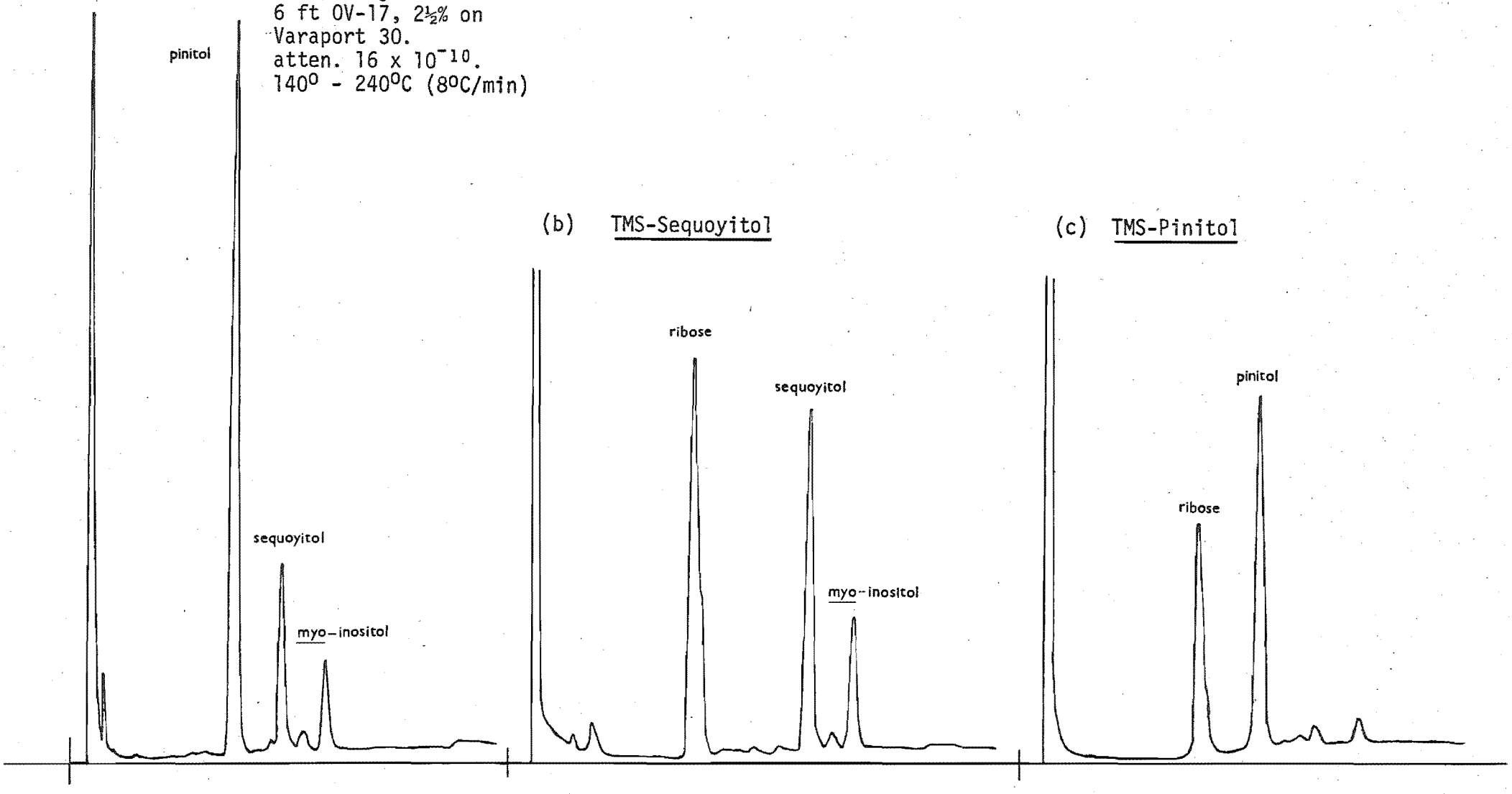
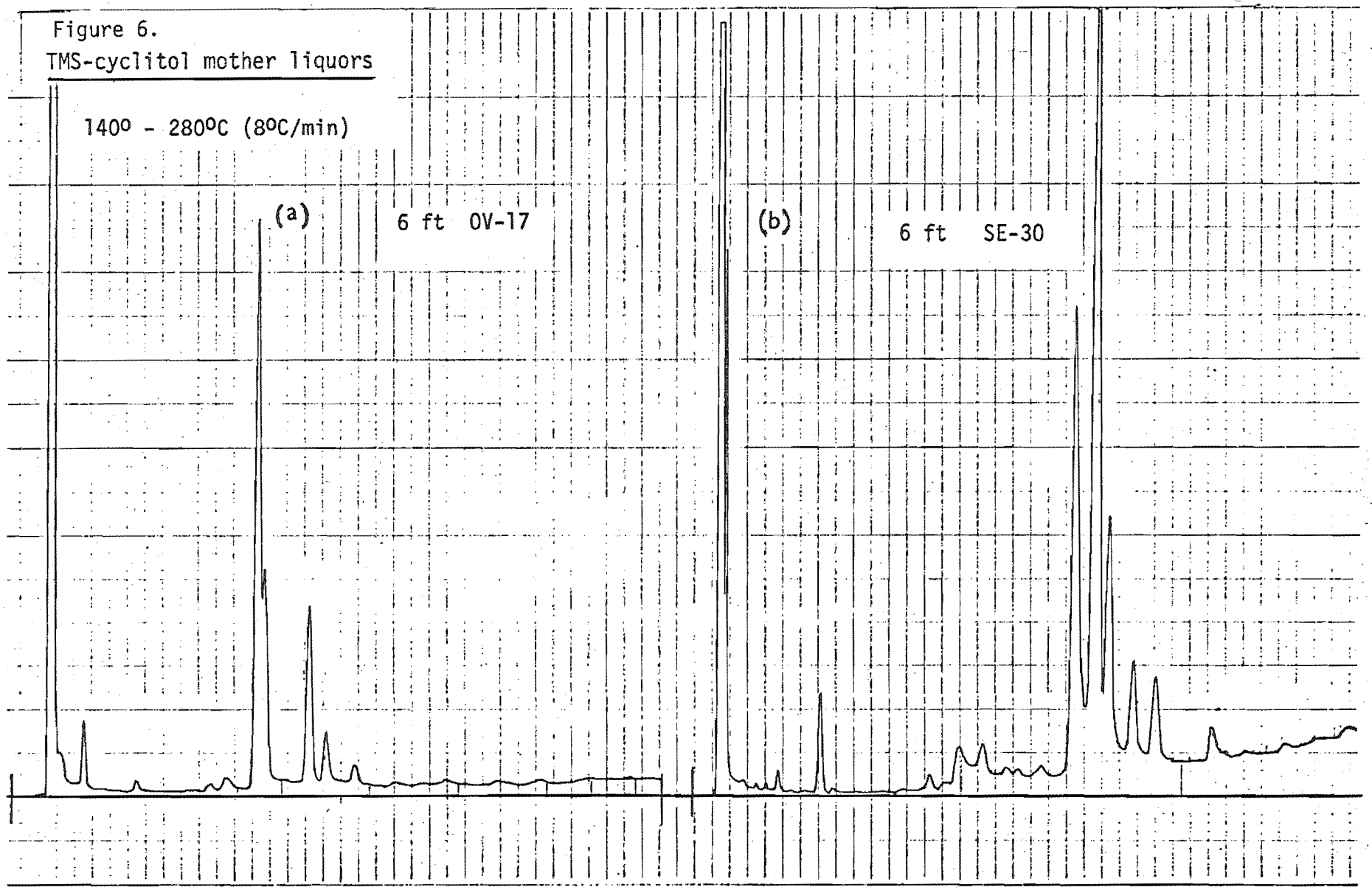


Figure 6.
TMS-cyclitol mother liquors



The p.m.r. spectrum of the isolated compound had confirmed its assignment as an O-methyl inositol (methoxy group, δ 3.40) and the single peak was retained (δ 3.15) after hydrolysis of the silyl ether groups to give the parent cyclitol.

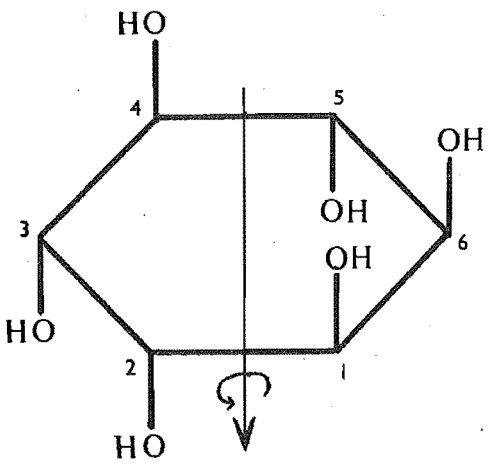
Identification of sequoyitol and pinitol was primarily based on m.p. determinations of the recrystallised cyclitols and their per-acetate derivatives. The mass-spectra and c.m.r. spectra of the pure compounds confirmed the assignments. The c.m.r. spectra of pinitol (fig. 7) and myo-inositol (fig. 8) were identical with previously published data (Dorman 1970).

Hydriodic acid hydrolysis of the cyclitol mother liquors (see above) gave a mixture of chiro- and myo-inositols, confirmed by a g.l.c. comparison of the TMS- derivatives with authentic samples. A comparison of the peak areas of the six components of the mother liquors (fig. 6b) with the peak areas of the two inositols formed on hydrolysis, indicated that the first four peaks were chiro-inositol derivatives, and the fifth and sixth peaks as myo-inositol derivative. The first peak was identified as pinitol, the second was the O,O-dimethyl-inositol, while the fifth and sixth peaks were sequoyitol and myo-inositol.

As an O,O-dimethyl-chiro-inositol had recently been isolated from P. radiata (Gallagher 1975) the procedure used in the isolation was repeated and a sample of pine-needle distillate was derivatised and analysed on both SE-30 and OV-17 columns (fig. 13 a, b). The distillate contained the O,O-dimethyl-inositol (R_t = 0.88 on SE-30) and pinitol (R_t = 0.94 on SE-30).

The c.m.r. spectrum of the mixture of the O,O-dimethyl-inositol (named pinpollitol) and pinitol, showed the resonances characteristic of pinitol and in addition, eight clearly resolved resonances due to pinpollitol (fig. 9). These resonances are also clearly seen in the spectrum of the cyclitol mother liquors (fig. 10).

The parent inositol, chiro-inositol, has a two-fold rotational axis of symmetry. Consequently only nine O,O-dimethyl isomers were possible:



1,6	1,2	2,3
2,5	1,3	2,4
3,4	1,5	3,6

The c.m.r. spectrum of chiro-inositol (Dorman 1970) displays only three resonances due to the rotational axis of symmetry. The 1,6-, 2,5- and 3,4 -O,O-dimethyl isomers are also symmetrical and would exhibit only four resonances. As the isolated dimethyl isomer had eight resonances, these isomers were not considered further.

The position (refer to diagram on p. 7) of resonance of the $-OCH_3$ group at 63.5 ppm is characteristic of an axial $-OCH_3$ grouping. This assignment of an axial $-OCH_3$ in the chiro-

inositol skeleton requires it to be flanked by an axial hydroxyl group. From Robert's work it is observed that for β-carbons bearing axial hydroxyl groups there is a large upfield shift on methylation by some 4.5 ppm. The resonance at 69.1 ppm is at high field for a chiro-inositol derivative and can only have resulted from such a methylation shift and confirms the assignment of an axial $-OCH_3$ group.

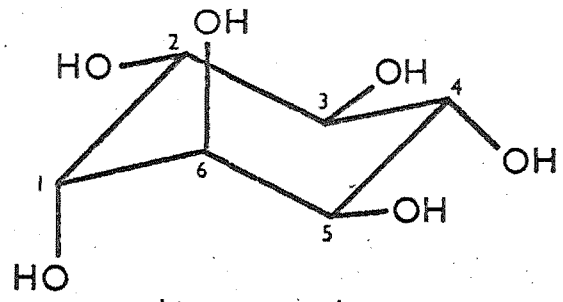
As the O,O-dimethyl isomer must contain an axial $-OCH_3$ group, the 2,3- and 2,4- isomers were also discarded. The $-OCH_3$ resonance of 60.4 ppm is characteristic of 3-O-methyl-chiro-inositol derivatives (and 4-O-methyl due to the rotational axis) and is quite distinct from the alternative 2- (or 5-) -O-methyl-chiro-inositols which appear downfield somewhat at 57.8 ppm. This limits the structure of pinpollitol to either 3,6- O,O-dimethyl chiro-inositol or 1,3- O,O-dimethyl chiro-inositol, and requires it to be a derivative of pinitol (3-O- methyl chiro-inositol).

The methylation shift for a β-carbon carrying an equatorial hydroxyl group has been described by Roberts to be small and variable. O-Methylation of the 1-position of pinitol would effect C-2 in a small, variable fashion and shift C-6 considerably upfield (but would have little effect on C-3, C-4 or C-5). Alternatively, O-methylation of C-6 of pinitol would shift C-1 considerably upfield and have a small effect on C-5 (and little effect on C-3, C-2 or C-4). Comparison of the resonances of pinpollitol and pinitol indicates a small β-methylation shift upfield in the C-5 resonance while C-2 remained constant. Additionally, there was the expected large upfield β-shift of C-1 by 3.45 ppm to 69.1 ppm and the

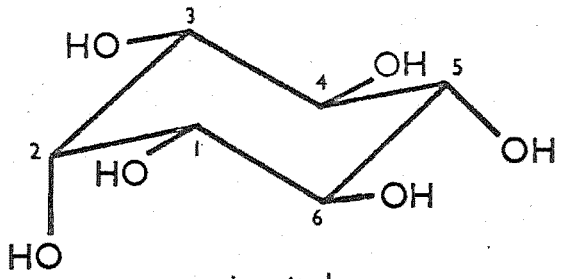
α-methylation shift of C-6 downfield by 9.55 ppm to 82.1 ppm. Thus pinpollitol can be assigned as 3,6- O,O-dimethyl chiro-inositol or more accurately 1,4- O,O-dimethyl chiro-inositol. This structure has subsequently been proven by synthesis (Gallagher, private communication).

It was demonstrated (see above) that hydriodic acid hydrolysis of sequoyitol (O-methyl inositol) yields myo-inositol. Since only five resonances were observed in the c.m.r. spectrum of sequoyitol (fig. 8) the O-methyl myo-inositol must retain the symmetry elements of the parent inositol by O-methylation at either C-2, or C-5. 2- O-Methylation was eliminated as the α-carbon shift (11.0 ppm) that would result, lies outside the reported range (7 - 10 ppm). 5-O-Methylation would give a 9.7 ppm shift. Thus sequoyitol can be assigned the structure 5- O-methyl myo-inositol.

The ms and gc/ms data on the acetate derivatives was in keeping with the assignments (see Appendix I). The remaining two chiro-inositol derivatives are the subject of further investigation.



chiro–inositol



myo–inositol

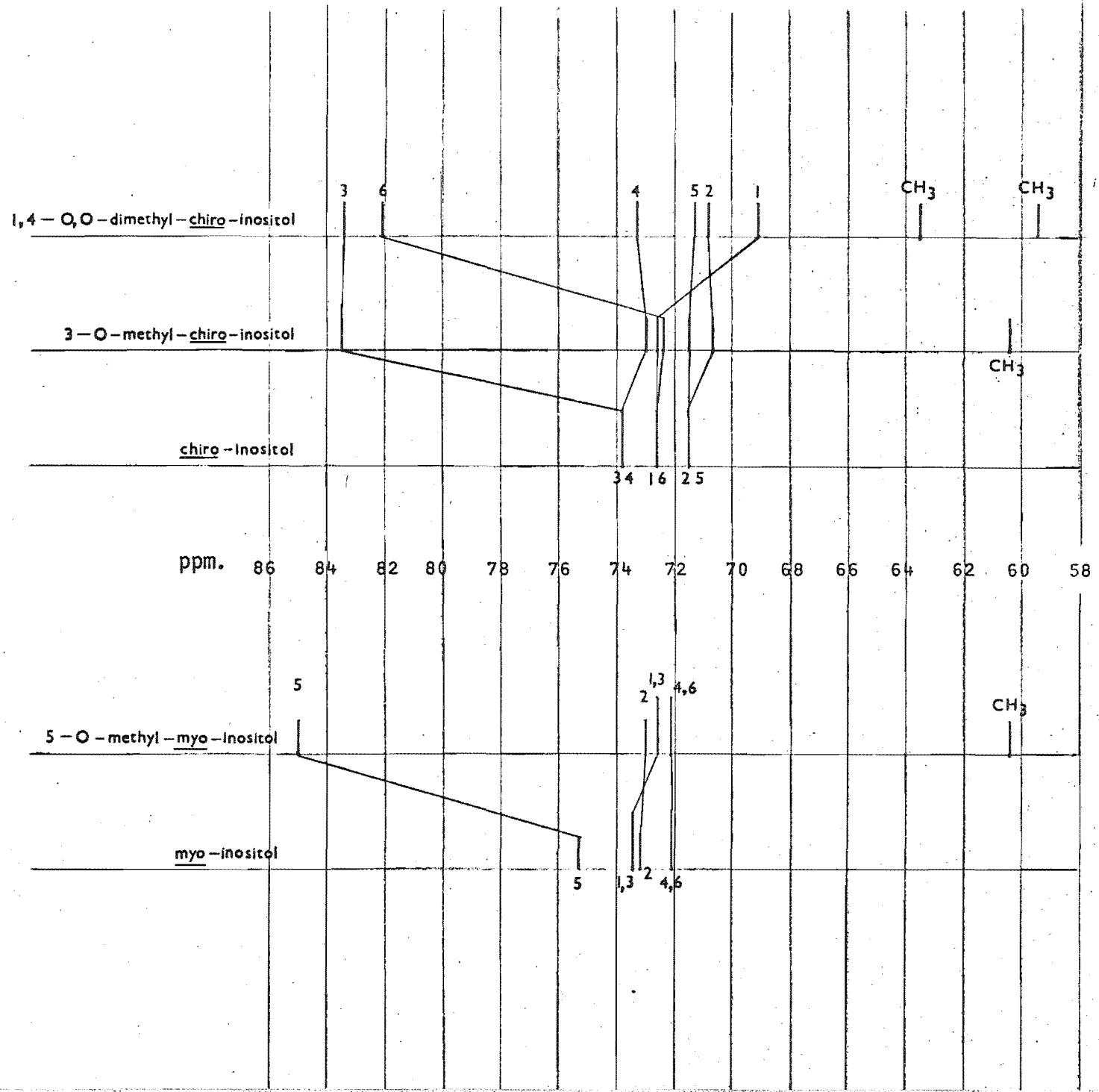


Figure 7. pinitol

(see Experimental for details)

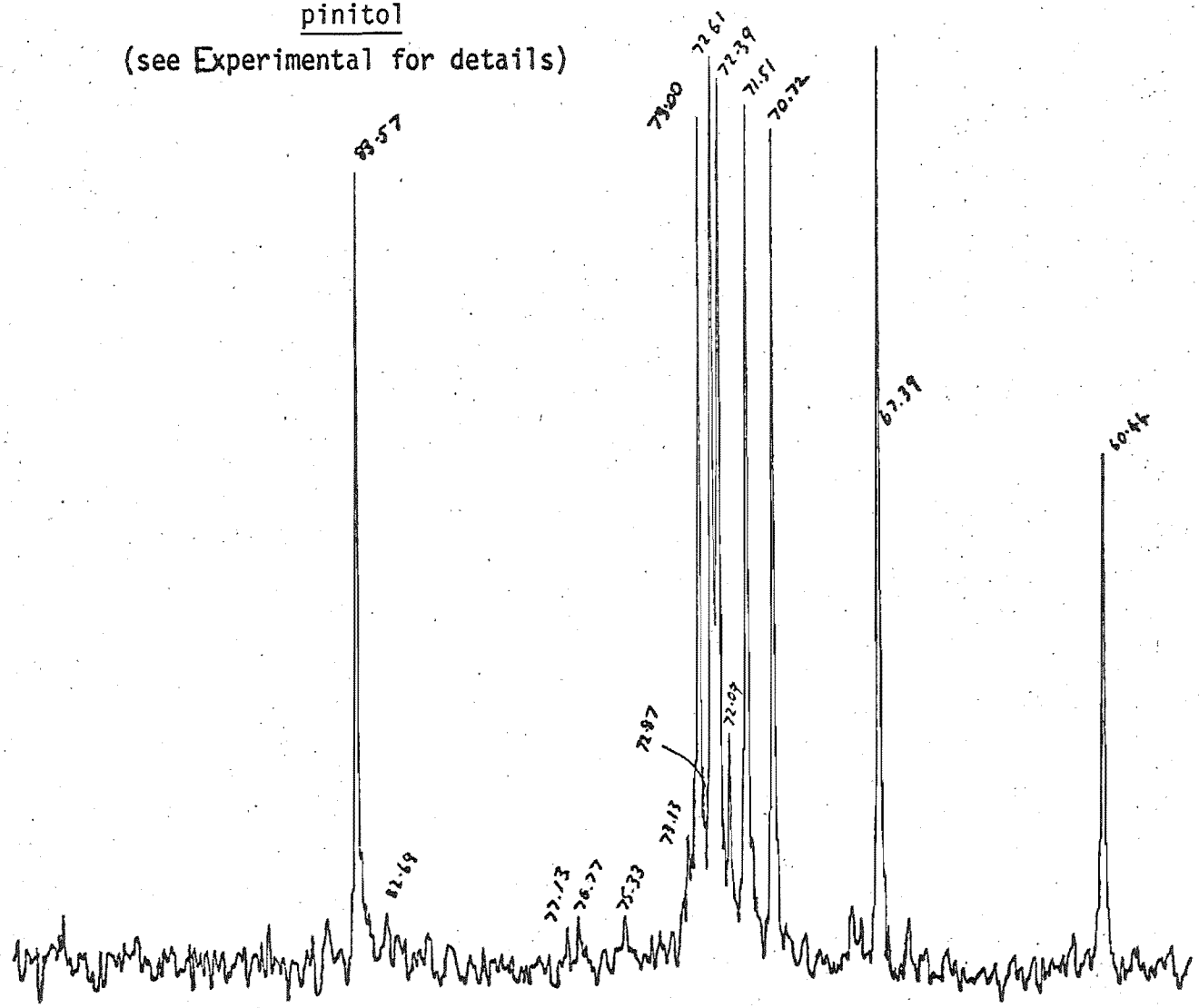
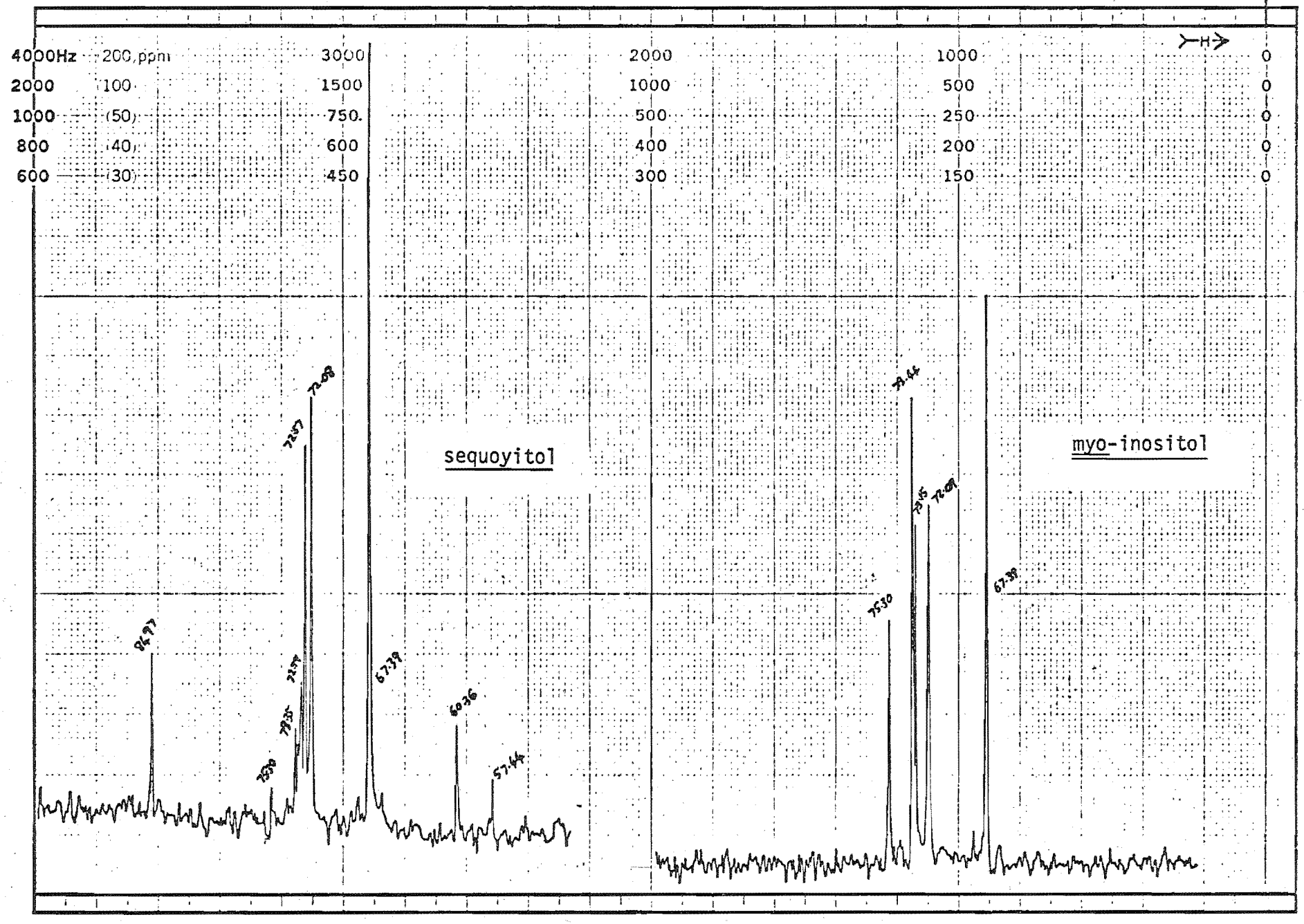


Figure 8.


4000Hz
2000
1000
800
600
200 ppm
100
(50)
40
(30)
3000
1500
750
600
450
2000
1000
500
400
300
1000
500
250
200
150
0
0
0
0
0
H
sequoyitol
myo-inositol
94.97
75.30
73.37
72.68
67.39
60.36
57.44
75.44
75.15
72.08
75.30
67.38

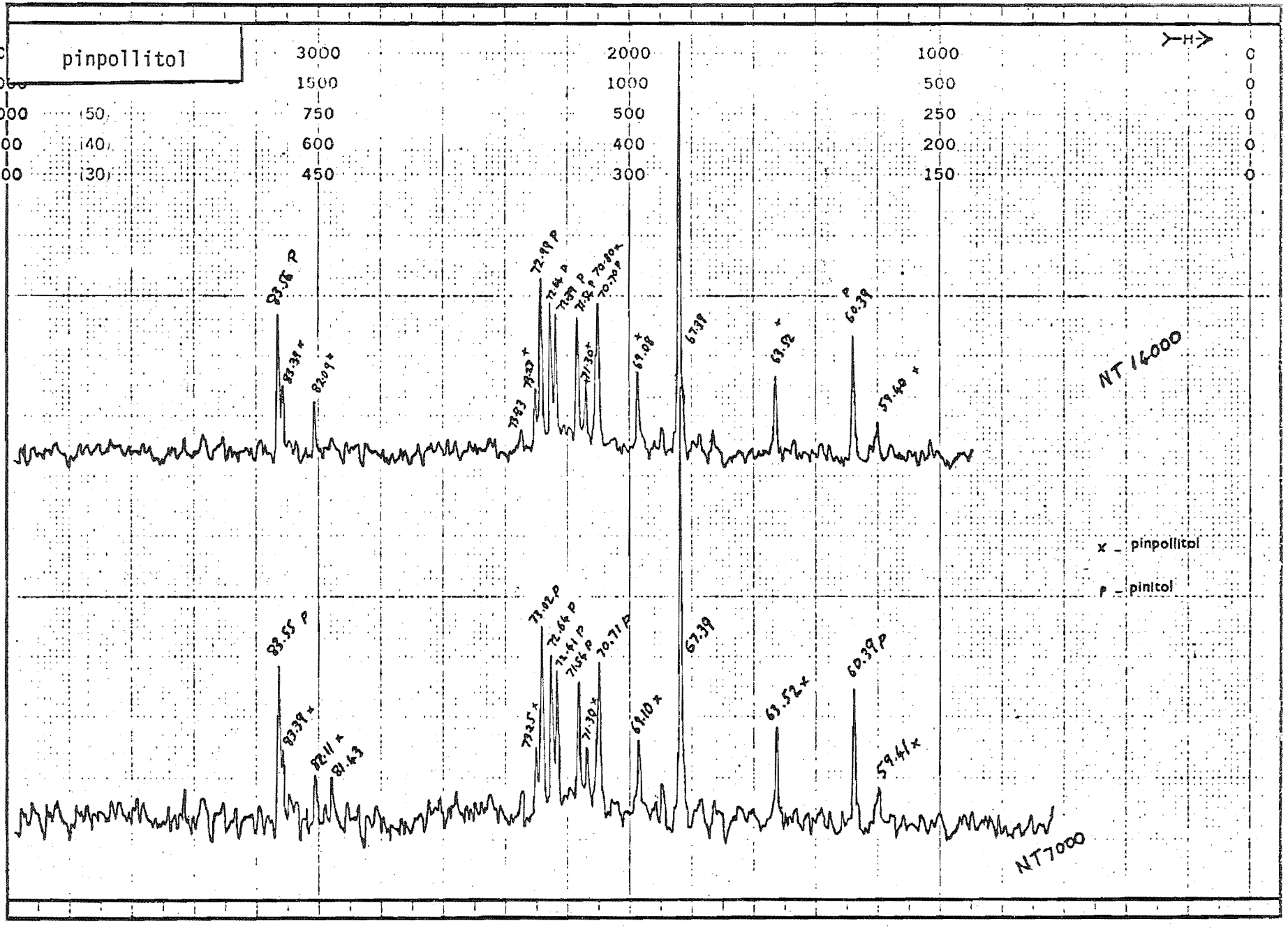


Figure 9.

Figure 10.

cyclitol mother liquors

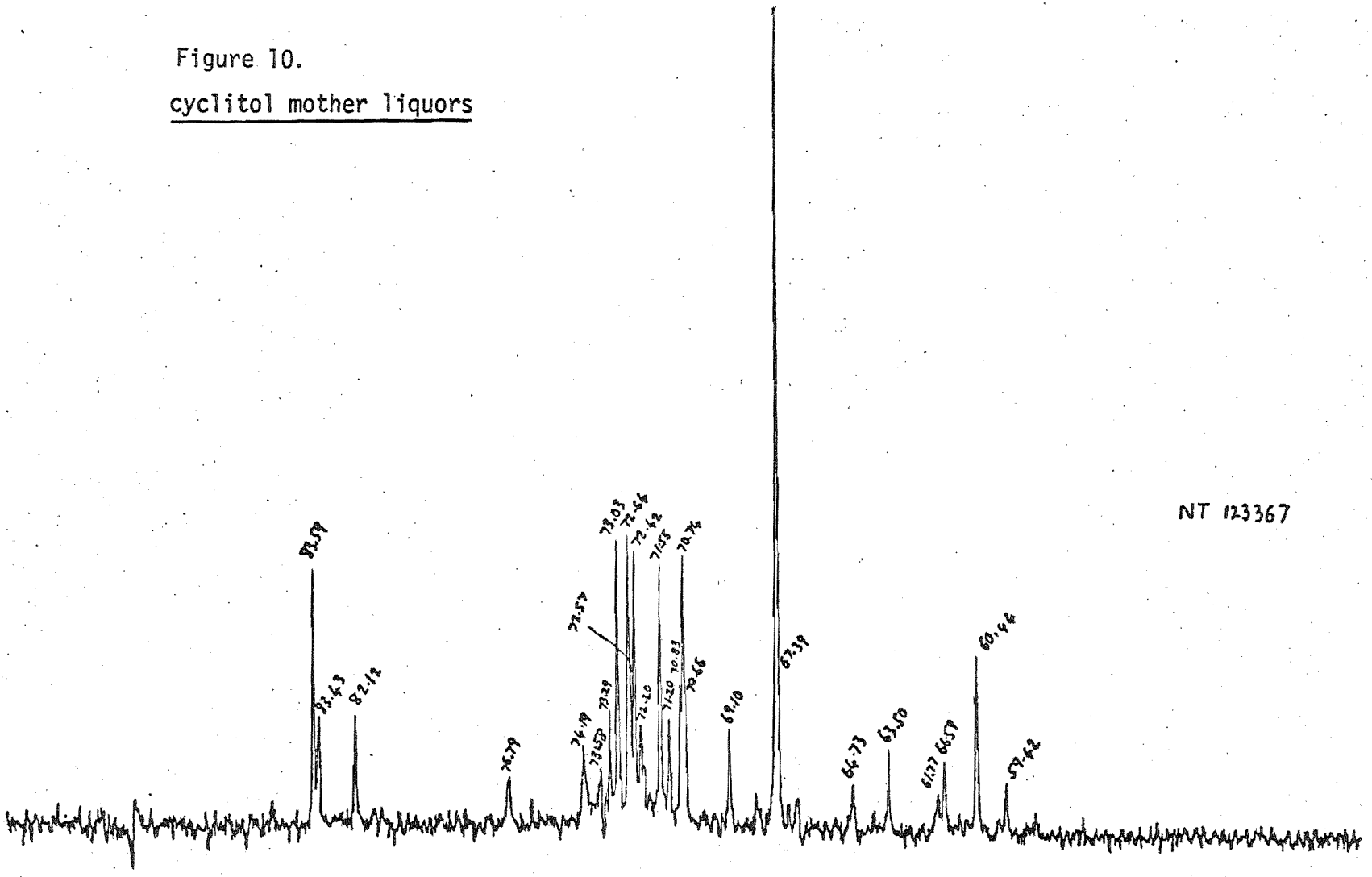


Figure 11.

cyclitol mixture

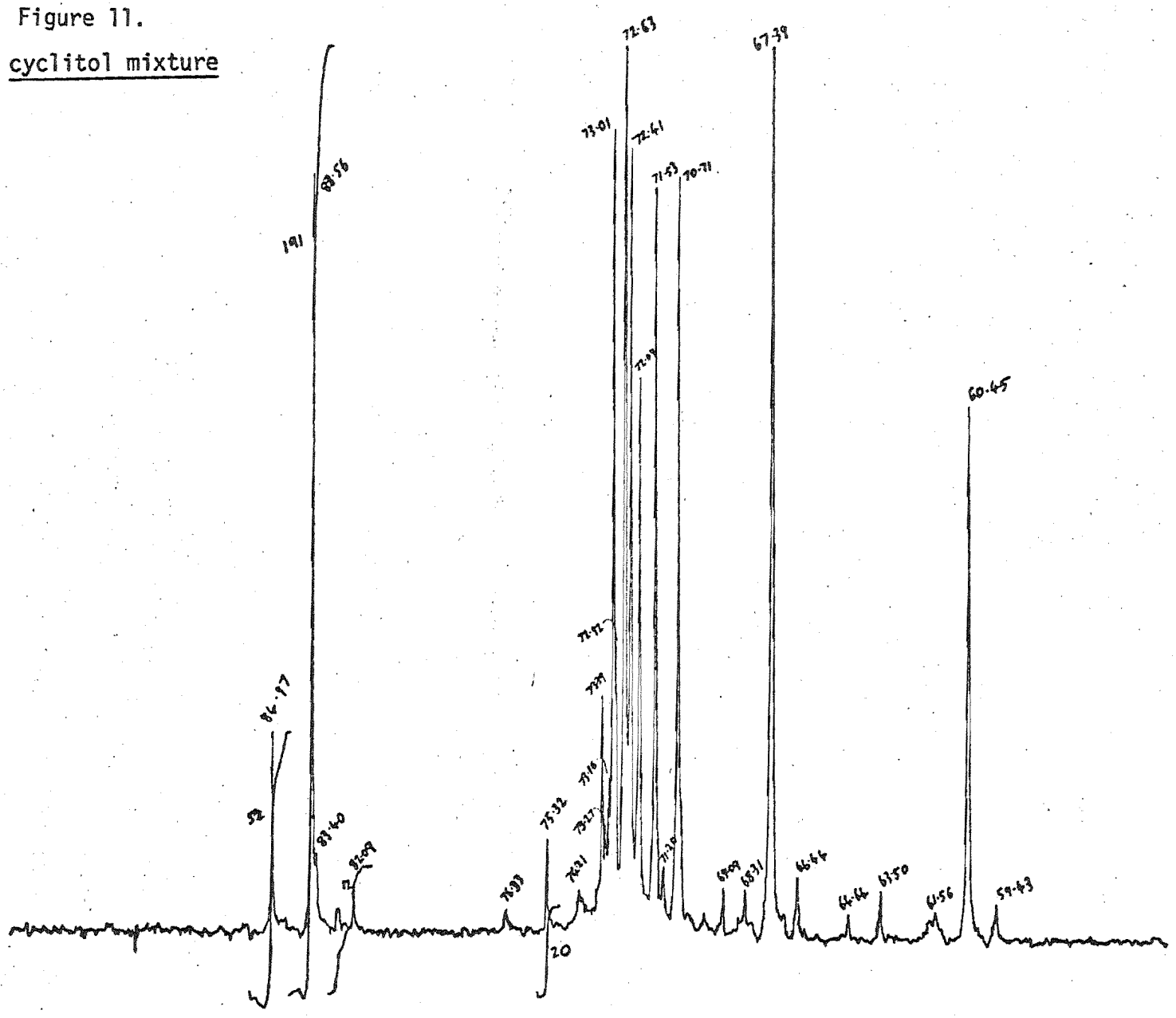
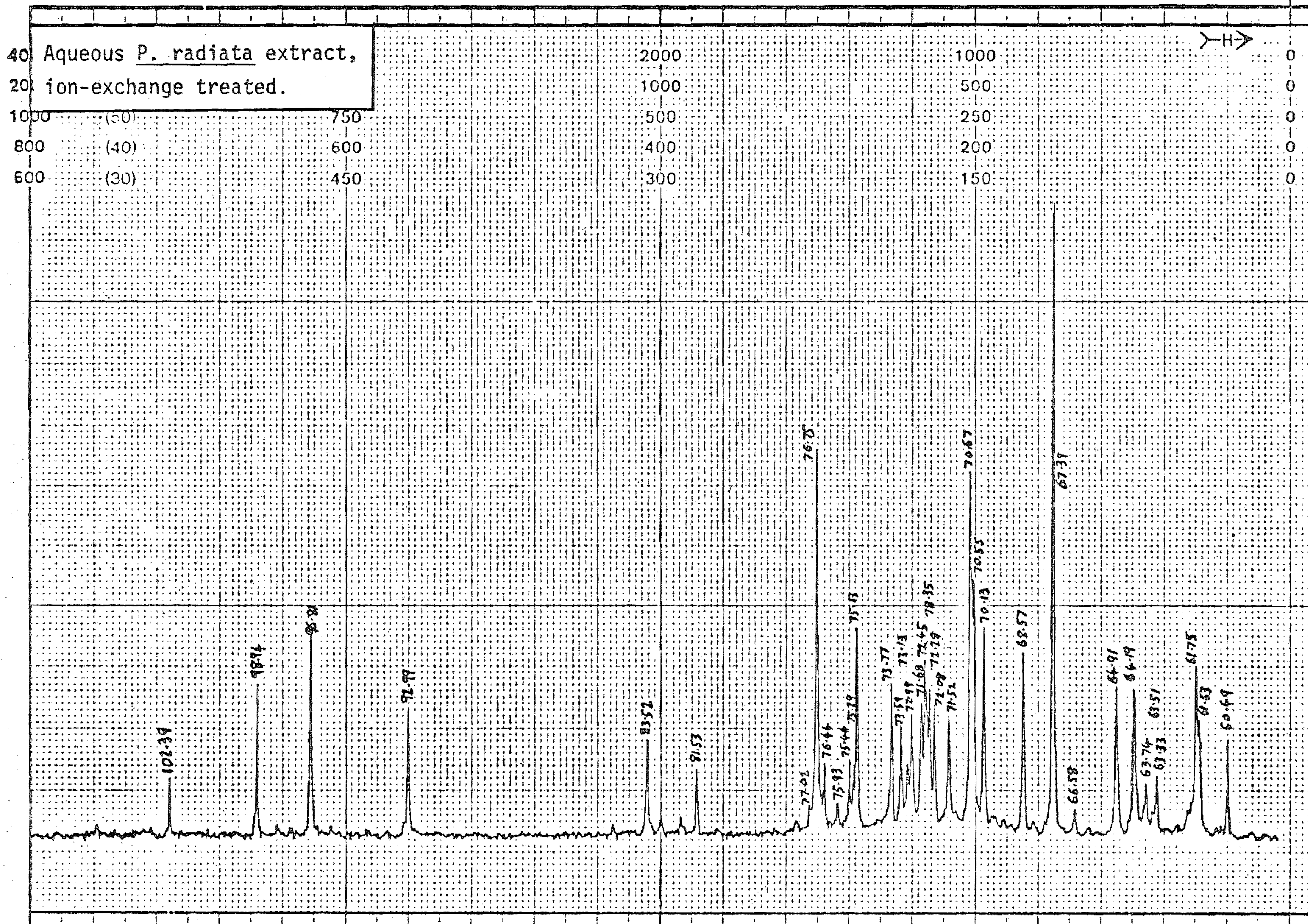
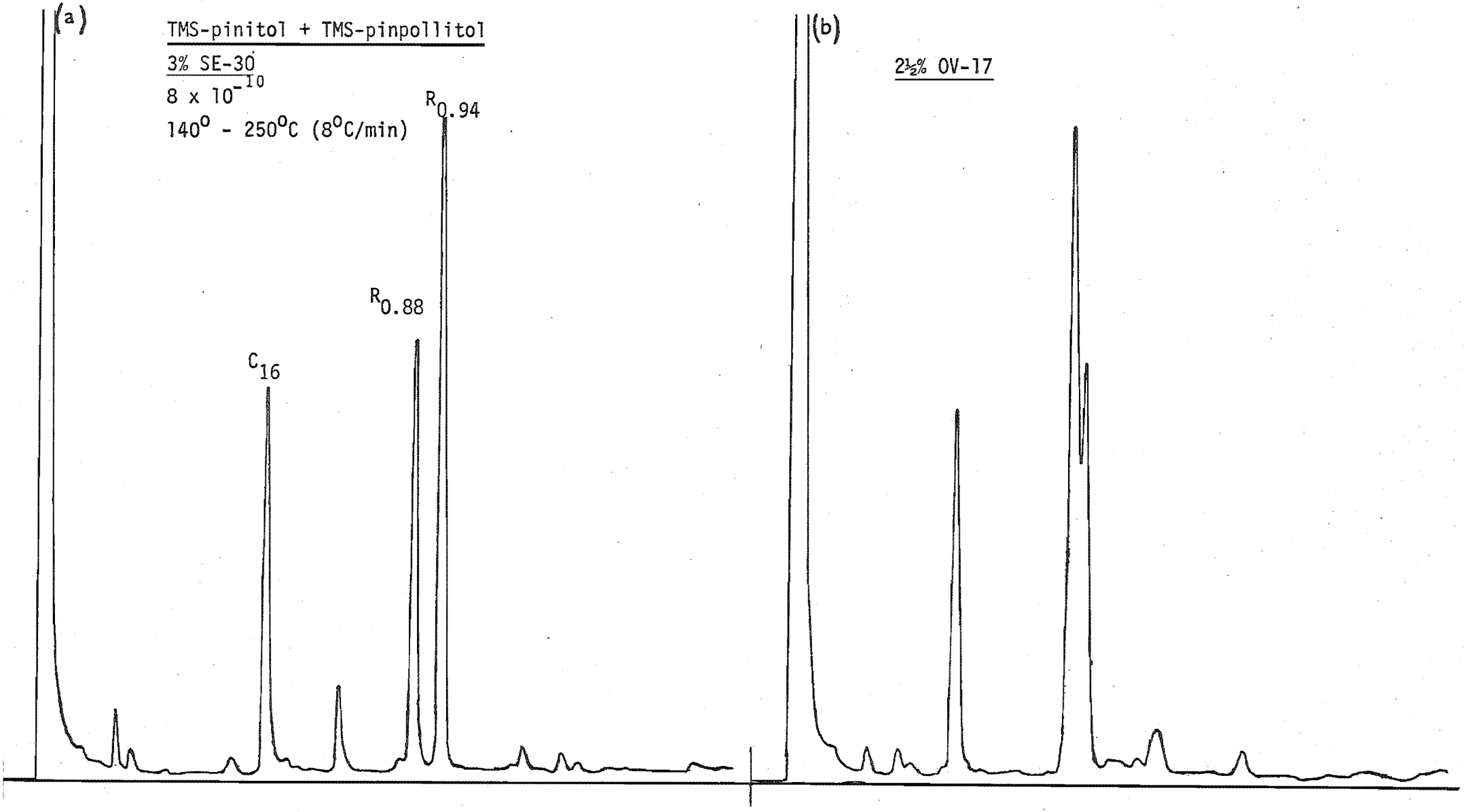


Figure 12.



Aqueous P. radiata extract, ion-exchange treated.

Figures 13a,b.



QUANTITATIVE ANALYSIS OF W.S.C.

Of the various techniques available, undoubtedly g.l.c. of a suitable carbohydrate or cyclitol derivative was the best method of quantitating the various components of the water soluble carbohydrates (w.s.c.). Again, of the various derivatives available the TMS- derivative is the most versatile.

As mentioned above a major problem in the g.l.c. of TMS-derivatives of the w.s.c. was the lack of resolution of all peaks on any one given column. For example, it was possible to obtain base-line resolution of sequoyitol and β-D-gluco-pyranose on OV-17 columns, but these peaks were either merged or barely resolved on SE-30 columns. Similar problems were encountered with glucose and fructose, and this particular problem was compounded when mannose was also present in the w.s.c.

Efficient OV-17 and SE-30 columns were difficult to prepare. The best results were obtained when using glass columns pre-treated with a 10% TMS/benzene solution for 3 days prior to packing. The efficiencies of all columns were measured as a function of the nitrogen carrier-gas flow. Typically they had 400 THEP/foot.

For some carbohydrate mixtures the chromatogram could be simplified by reducing the carbohydrates with $NaBH_4$ (Sweeley 1963). In the reduced sample each carbohydrate is represented by one peak only. In P. radiata where the principal sugars were glucose and fructose, borohydride reduction resulted in considerable simplification of the chromatograms, but in developing an analytical technique to deal with a large number of samples, this was simply not practicable.

The rapid preparation of TMS- derivatives was necessary for the development of a routine method to analyse a large number of samples. The method finally adopted was an amended version of Sweeley's in which the excess pyridine and excess TMS reagent were evaporated off under a stream of dry nitrogen. The TMS- derivatives were then dissolved in an inert solvent such as pentane or carbon tetrachloride. Using such solvents reduced the "tailing" produced when pyridine was the solvent. This produced a better chromatogram, better suited to integration of peak areas. Removal of the excess reagents and pyridine also enhanced column life.

The next consideration was the method of quantitating the raw integrator output. Two possibilities were considered. Either the use of a standard calibration curve plotting peak area against concentration for each component of the mixture or the use of an internal standard. As long as a suitable internal standard could be found, that system of quantitation was preferable. The technique of external calibration is too dependent upon factors such as detector response, column condition, oven temperature, detector temperature, etc. to be considered as an accurate method of quantitation.

An internal standard must have a similar chemical structure to the compounds being quantitated. This is an important consideration if there is decomposition on the column. Although the TMS- carbohydrates show no apparent signs of instability at the temperatures necessary for elution (Holligan 1971 a) from a 6 foot 2½% SE-30 column, the constant decomposition of TMS- derivatives during elution has been reported (Jansen 1968, Richey 1964). If the internal standard is of similar structure, then any decomposition during chromatography is compensated for.

The other requirement for an internal standard is a clean separation from other components of the mixture.

Ribitol, prepared by the reduction of ribose with $NaBH_4$, was the internal standard selected for the analyses of P. radiata water extracts. TMS- Ribitol is a single compound, similar in structure to the derivatised w.s.c. and is eluted clear of any other peaks on both SE-30 and OV-17 columns. Ribitol has not been observed to occur naturally in P. radiata.

The linearity of detector response (Richey 1964, Clamp 1971) for varying weights of glucose and fructose with respect to an internal standard demonstrates the validity of using an internal standard, as well as the principle of using response factors in the analyses. For example (fig. 14), the weight ratio of glucose to standard can vary from at least 0.3 to 2.0 and still be linearly related to the ratio of peak areas by the gradient or the response factor. Hence the weight of compound can be calculated if the weight of standard is known along with the peak ratio of compound/standard and of course the response factor which is unique for each compound. Not only did the response factor vary between compounds, it also depended on the type of column and the operating conditions. Even then a response factor, which is a function of the degree of ionization, could differ from day-to-day depending on the condition of the detectors, etc.

To overcome these severe problems the analysis included a standard sugar-cyclitol mixture (see Appendix III) which was injected before and after a series of sample runs (usually 6) giving up-to-date response data for each of the 51 samples analysed.

The hydrocarbon, n-hexadecane, was used as an additional internal standard. This was added at a late stage in the analytical procedure (see experimental p. 57) and served as a check on injection volumes and enabled normalisation of the repetitive injections for each sample. Just as importantly it also served as a check on the silylation reaction. Any fault in derivatisation was reflected in the changing ratio of ribitol to n-hexadecane since these two standards were present in fixed quantities - ribitol in pyridine (0.5012 mg/ml, 1 ml) was quantitatively added from a standard syringe to each sample prior to silylation, and the n-hexadecane in carbon tetrachloride (1.20 mg/ml, 0.50 ml) was added after silylation just prior to injection into the g.l.c. It was this n-hexadecane/ribitol comparison that illuminated the problem of the decomposition of the TMS-derivatives, apparent in extracts that had not been treated with an ion-exchange resin. When the ion-exchange step was excluded, the ribitol/n-hexadecane ratio decreased, the ribitol peak diminished with time and another peak of slightly different retention-time increased in size (fig. 15 a, b, c, d). The ion-exchange resin treated samples produced TMS-derivatives that were stable for some weeks (Hedgley 1960). Amberlite MB-3, a mixed anion (OH^-) and cation (H^+) resin, could be used without any effect on carbohydrate concentrations, whereas other resins, particularly Amberlite IRA 410 (OH^-), removed carbohydrates from solution so the use of the latter was avoided.

Another very strong point in favour of the ion-exchange treatment was the complete removal of at least 3 ionic components in the tissue extracts which interfered with the

Figure 14. Peak ratio Vs. weight ratio, (4 mg of ribose per sample, OV-17 Column used).

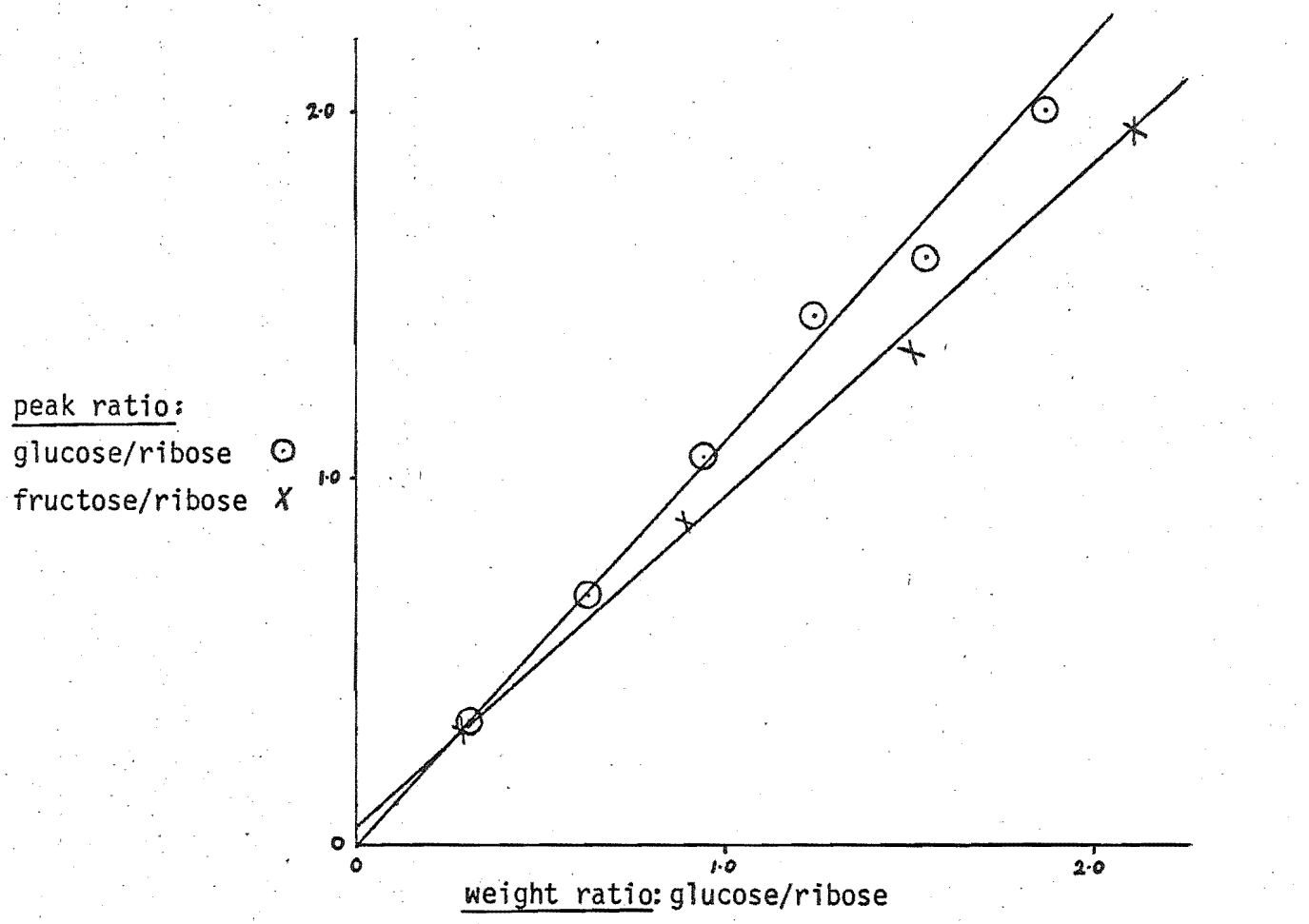
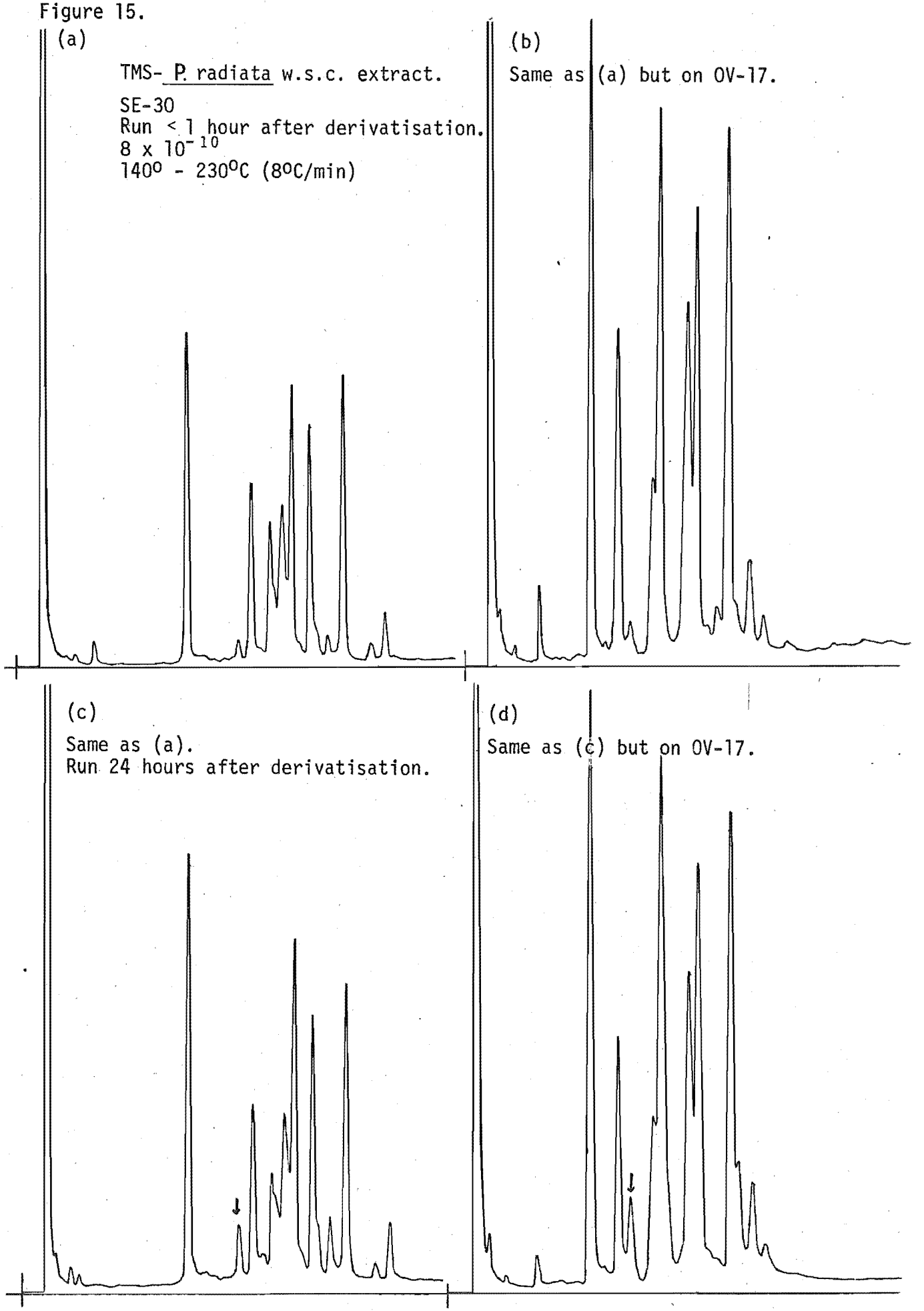


Figure 15.

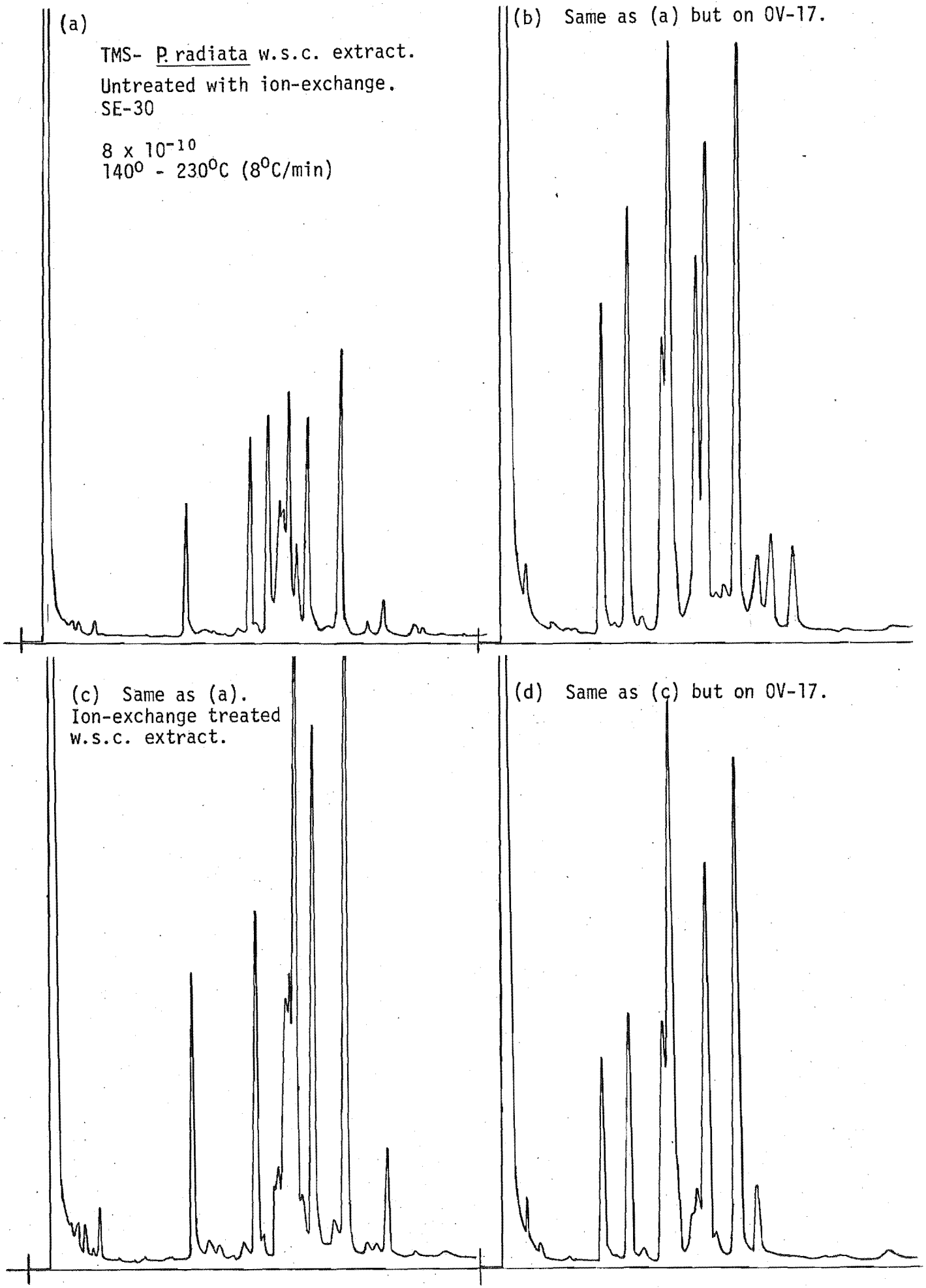

(a)
TMS- P. radiata w.s.c. extract.
SE-30
Run < 1 hour after derivatisation.
8 x 10⁻¹⁰
140° - 230°C (8°C/min)
(b)
Same as (a) but on OV-17.
(c)
Same as (a).
Run 24 hours after derivatisation.
(d)
Same as (c) but on OV-17.

quantitative analyses. Figs. 16 a, b, c, d give an indication of the extent of ion-exchange "cleaning". Although it is only speculation, one such peak due to an ionic component which had a retention time between pinitol and glucose (SE-30) was coincident with the extra peak produced when an extract was treated with glucose oxidase, the enzyme that converts glucose to gluconic acid.

Derivatisation was a weak point in the analysis. All other factors of sample handling and data analysis can be reasonably controlled but this chemical, silylation reaction is not so readily regulated. As a check, all silylations on the w.s.c. extracts were done in duplicate. Upon g.l.c. analysis, any widely contradicting samples were discarded and the process repeated with another 4 ml aliquot (see experimental p. 47) from the original extract. Furthermore, any failings of the derivatisation step are accounted for in the final statistical analysis by including results for both samples in the calculation of the mean and standard deviation (fig. 17). Because of their instability the TMS- reagents were mixed immediately before use (with glass syringes) and added in excess to the carbohydrate mixture in order to avoid the formation of partial derivatives. The reaction-time could be varied from $\frac{1}{2}$ to 24 hours with neglegible change observed in the g.l.c. results.

Solution of a dried w.s.c. extract in pyridine was difficult so alternative solvents (Ellis 1969 a, Pierce p. 10) for the TMS reaction were investigated. The samples were completely soluble in solvents such as dimethyl sulphoxide (DMSO), partially in dimethyl formamide (DMF) and barely soluble in pyridine. DMSO was tried as the silylating solvent but the resultant bad "tailing" was intolerable. It was found

Figure 16.


(a)
TMS- P. radiata w.s.c. extract.
Untreated with ion-exchange.
SE-30
8 x 10-10
140° - 230°C (8°C/min)
(b) Same as (a) but on OV-17.
(c) Same as (a).
Ion-exchange treated
w.s.c. extract.
(d) Same as (c) but on OV-17.

that pyridine could be used satisfactorally if the insoluble ionic residues were removed before-hand with ion-exchange resin.

For special analyses (like gc/ms) where purification was necessary, the TMS derivatives could be extracted by partitioning between water and chloroform (Sweeley 1966), but a simpler and safer method was to evaporate off the pyridine completely and extract with pentane. Since NH_4Cl (a product of the silylation reaction) is denser than this solvent the solid matter settles to the bottom of the vial and the supernatant is easily removed.

Problems inherent in the derivatisation procedure could usually be dealt with by the methods discussed (ion-exchange, internal standard, duplication, etc). The accurate quantitation of w.s.c. was, however, very dependant on the resolving capabilities of the columns used and it was this and not the derivatisation of samples which proved to be a problem. For example, on SE-30, pinpollitol, fructose and pinitol were badly resolved and this was even worse on OV-17. Consequently the errors associated with fructose and pinitol quantitation were substantial. Pinpollitol was not present in sufficient quantity to be measured accurately in routine analysis.

Some of the measurements were based on fixed anomer ratios of known sugars; that is, if one anomer were clearly resolved, the other anomer could be calculated based on set anomer ratios in aqueous solution at 25°C. Consequently the total carbohydrate could be determined (Oates 1967). This method was also valuable in detecting the presence of coincident peaks (Brower 1966). For example, galactose is coincident

with α-glucose on OV-17 and could therefore be detected by a consideration of the anomer ratio for glucose.

The rate of mutarotation of carbohydrates in pyridine at room temperature is low, so solution of the w.s.c. in pyridine prior to derivatisation has little effect on anomer ratios (Holligan 1971, Jacin 1968), and thus estimated concentrations. For glucose, the change in anomer ratio was < 2% or within experimental error.

Sequoyitol and β-glucose were unresolved on SE-30, but α-glucose was clearly resolved so the β-glucose could be calculated from the glucose anomer ratio (41:59) and hence sequoyitol determined by difference. This method was suitable for the standards, but the P. radiata extracts often had little sequoyitol so it was necessary to obtain sequoyitol concentrations from the OV-17 data.

Galactose, only present in two samples (4 and 44), was calculated from the "clean" anomer with retention time between that of pinitol and α-glucose (R_g 0.96 on SE-30).

Reproducability of results was an important quantitative requirement and this depended greatly on the g.l.c. operating conditions and injection techniques. Prior to injection the columns had at least 4 - 5 minutes equilibration after the cooling stage because any differences of temperature was likely to affect the retention times.

Modification of the dual electrometer of the gas-liquid chromatograph (Varian 2100) enabled analyses to be run simultaneously. Using a linear temperature program of 8°C/min. from 140°-220°C, necessitated quick injections and a minimum of delay between "simultaneous" injections. Injection volumes and concentrations were kept small so

electrometer attenuations (8×10^{-10}) could be as low as possible and still retain good stable baselines for integration. The integrators used were zeroed once, just prior to the elution of the n-hexadecane standard.

A full description of the analytical technique is included in the Experimental (p. 57).

The statistical manipulation of the crude integration data was done by computer (B 6718 using program PAN.). Briefly, for a particular tissue sample (51 samples analysed) on a given column there were two sets of data:- (a) standard response data from which the response factors were obtained for each w.s.c., and (b), sample data from the multiple injections carried out for each sample. The first step in the analysis was to normalise the data from each analysis. This was done by "scaling" the integral obtained for n-hexadecane in each analysis to a pre-determined standard value. This scaling factor was then applied to all integrals obtained from that particular analysis. From this adjusted data mean values and mean square standard deviations were derived. Application of the appropriate response factor, dilution factor, weight of ribitol in the standard and the tissue weight gave the weight and percentage of each sugar in the extract. A typical result is shown in fig. 17 with its g.l.c. trace fig. 18.

The "scaled data" was very useful for "troubleshooting". Any data entered incorrectly or obtained from a partially derivatised sample would be immediately obvious in the scaled data and could easily be traced back to the original chromatogram or sample.

"Unknowns 1, 2 and 3" were included in the analysis

program to accommodate any sugar or cyclitol that was present only sporadically in the tissues; e.g: galactose, mannose and mannitol. These peaks were analysed with an arbitrary response factor of 1.00 since they were not included in the standard mixture. The statistical accuracy achieved was about ±5% for most components of the w.s.c.

Significant errors in this analysis are estimated as follows:

Weighing tissue	±0.04%
Volume errors	±1.6%
Ion-exchange treatment	±1.0%
Preparation of ribitol and transfer	±0.2%
	±2.9%

Silylation errors are unmeasurable but can be accounted for in the statistical results by doing duplicate samples.

mean standard deviation ±5.0%

95% confidence level $\simeq$ ±10%.

Figure 17.

NEEDLES 11/7/74 SAM 39 SE30(A) ON 493.30 MG OF TISSUE

DILUTION FACTOR= 12.5 C16 REPONSE=100.0

RELATIVE STANDARD WEIGHTS:

C16	RIBITOL	FRUCTOSE	PINITOL	GLUCOSE	SEQUOYIT	MYOINOSI	UNKNOWN1	UNKNOWN2	UNKNOWN3
0.3936	1.0000	1.0377	0.8738	0.9284	0.9209	0.9473	1.0000	1.0000	1.0000

MG. OF RIBITOL=0.4964

CRUDE STANDARD INPUT:

C16	RIBITOL	FRUCTOSE	PINITOL	GLUCOSE	SEQUOYIT	MYOINOSI	UNKNOWN1	UNKNOWN2	UNKNOWN3
109.00	216.00	130.00	153.00	192.00	158.00	186.00	216.00	216.00	216.00
105.00	202.00	124.00	141.00	184.00	137.00	178.00	202.00	202.00	202.00
120.00	223.00	147.00	162.00	221.00	166.00	207.00	223.00	223.00	223.00
100.00	185.00	123.00	144.00	184.00	146.00	176.00	185.00	185.00	185.00

SCALED DATA:

100.00	198.17	119.27	140.37	176.15	144.95	170.64	198.17	198.17	198.17
100.00	192.38	118.10	134.29	175.24	130.48	169.52	192.38	192.38	192.38
100.00	185.83	122.50	135.00	184.17	138.33	172.50	185.83	185.83	185.83
100.00	185.00	123.00	144.00	184.00	146.00	176.00	185.00	185.00	185.00

MEAN AND STD. DEVIATION:

100.00	190.34	120.72	138.41	179.89	139.94	172.17	190.34	190.34	190.34
* 0.0000	* 6.1705	* 2.4062	* 4.6085	* 4.8591	* 7.1650	* 2.8352	* 6.1705	* 6.1705	* 6.1705
% ERROR:	3.2%	2.0%	3.3%	2.7%	5.1%	1.6%	3.2%	3.2%	3.2%

RESPONSE FACTORS :

1.3348	1.0000	0.6112	0.8322	1.0179	0.7983	0.9548	1.0000	1.0000	1.0000

CRUDE DATA INPUT

72.00	109.00	187.00	89.00	314.00	20.00	91.00	10.00	0.00	0.00
84.00	125.00	206.00	108.00	372.00	23.00	96.00	11.00	0.00	0.00
76.00	135.00	196.00	99.00	340.00	24.00	86.00	10.00	0.00	0.00
73.00	120.00	185.00	94.00	334.00	22.00	78.00	11.00	0.00	0.00
70.00	115.00	186.00	101.00	338.00	21.00	79.00	8.00	0.00	0.00

SCALED DATA:

100.00	151.39	259.72	123.61	436.11	27.78	126.39	13.89	0.00	0.00
100.00	148.81	245.24	128.57	442.86	27.38	114.29	13.10	0.00	0.00
100.00	177.63	257.89	130.26	447.37	31.58	113.16	13.16	0.00	0.00
100.00	164.38	253.42	128.77	457.53	30.14	106.85	15.07	0.00	0.00
100.00	164.29	265.71	144.29	482.86	30.00	112.86	11.43	0.00	0.00

MEAN AND STD. DEVIATION:

100.00	161.30	256.40	131.10	453.35	29.37	114.71	13.33	0.00	0.00
* 0.0000	*11.6123	* 7.6398	* 7.7859	*18.2431	* 1.7576	* 7.1451	* 1.3262	* 0.0000	* 0.0000
% ERROR:	7.2%	3.0%	5.9%	4.0%	6.0%	6.2%	10.0%	0.0%	0.0%

C16	RIBITOL	FRUCTOSE	PINITOL	GLUCOSE	SEQUOYIT	MYOINOSI	UNKNOWN1	UNKNOWN2	UNKNOWN3

MG OF EACH PRESENT:

2.882	6.205	16.138	6.060	17.133	1.415	4.621	0.513	0.000	0.000

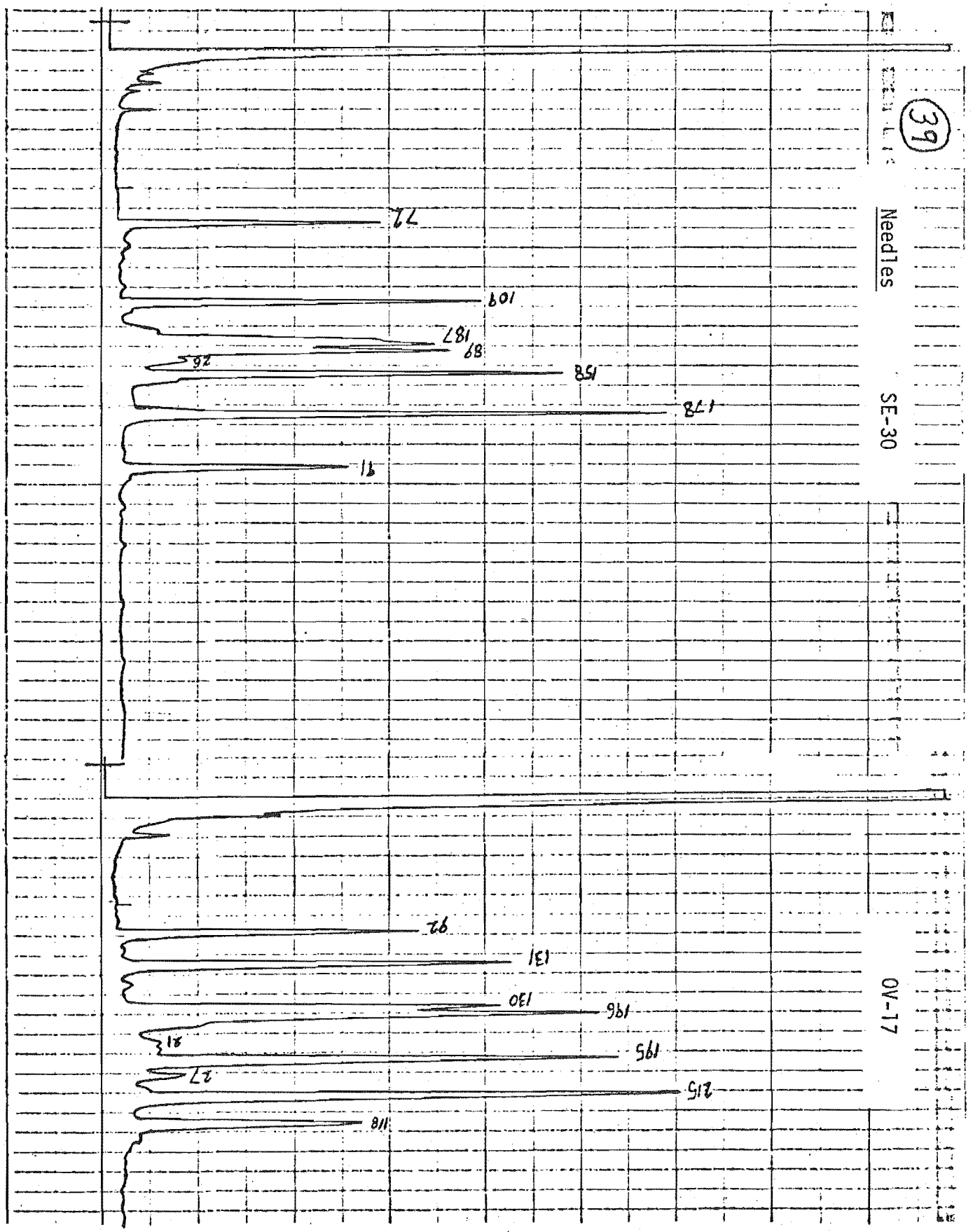
PERCENTAGE WOODHT OF EACH:

0.584%	1.258%	3.272%	1.229%	3.473%	0.287%	0.937%	0.104%	0.000%	0.000%

TOTAL ABSOLUTE ERROR:

0.000	0.131	0.163	0.114	0.234	0.032	0.074	0.014	0.000	0.000

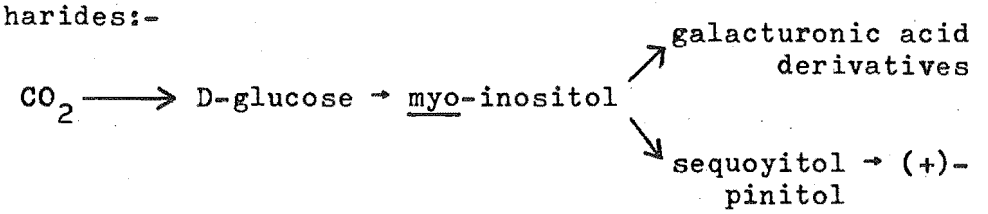
Figure 18.


39
Needles
SE-30
72
107
87
187
26
158
178
91
OV-17
92
131
130
196
31
195
27
215
118

SEASONAL VARIATION OF W.S.C.

A summary of results put in the form of a seasonal plot (using "omnitab " computer program) displays a variation of the carbohydrate levels - fructose, pinitol, glucose, sequoyitol and myo-inositol - with respect to the tissue type - buds, needles, wood, new needles and new wood. (See graphs 1 - 11). For example, graph 1 illustrates how the sugars and cyclitols fluctuate in buds over the season. Graph 6 gives fructose variation for all five tissue types over a fourteen month period. Graph 11 is a plot of the hexose/cyclitol ratio for the different tissues.

The key cyclitol in P. radiata is probably myo-inositol. It is a precursor for galacturonic acid derivatives, the monomer in cell-wall polysaccharides (Loewus 1973, Roberts and Loewus), and pinitol via sequoyitol (Anderson 1966, Dittrich 1971). Since myo-inositol is biosynthesised by the cyclisation of D- glucose, there exists a metabolic link between the primary photosynthates (e.g. glucose), pinitol (the most dominant cyclitol in P. radiata) and plant cell-wall polysaccharides:-



Graphs 1 - 5 show the low but constant levels of both myo-inositol and sequoyitol, and high but variable concentrations of pinitol and glucose. The latter two curves fluctuate in unison suggesting the existance of an equilibrium between glucose and pinitol (via myo-inositol). The following trends are observed:

1. The pinitol content in new needles and new wood is high

in springtime, but decreases rapidly as growth of tissue proceeds (graph 7).

2. Buds between February 1974 and August 1974 contain more pinitol than any other tissue (graph 7).

3. The glucose levels in old and new needle tissue were greater than the pinitol levels at corresponding times. The reverse was true with buds (graphs 2, 4).

This suggests that pinitol could well function as a stable "carbohydrate" store - buds store pinitol over winter converting to back to myo-inositol in spring when the new shoot growth demands the biosynthesis of galacturonic acid derivatives. Once the new needles have developed, the currently produced photosynthates are diverted to "sinks" or reserves such as buds and roots (Shiroya 1966, Ursino 1968, Loach).

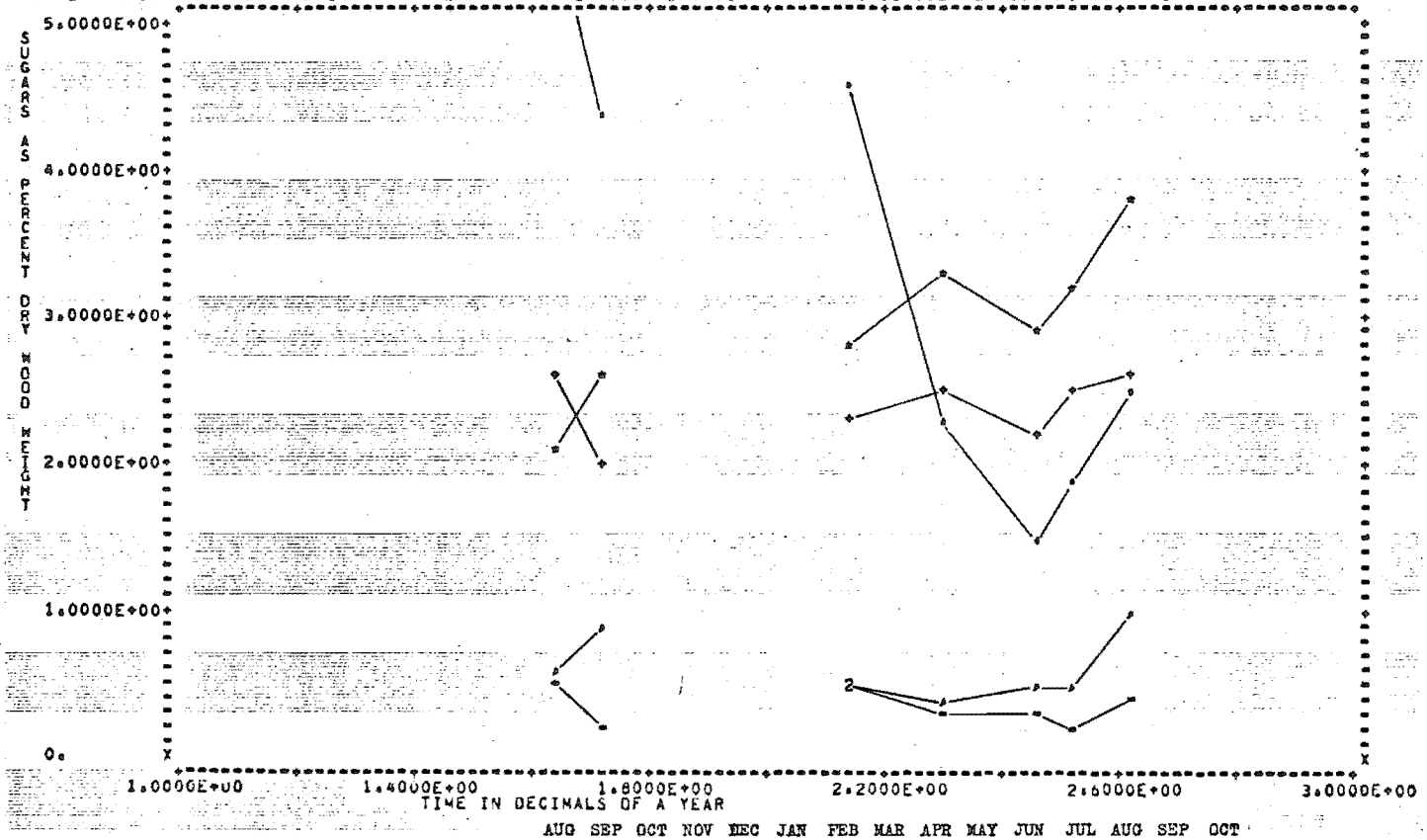
The above discussion is largely speculative and the true fate of photosynthates can really only be determined with carbon-14 (^{14}C) labelling experiments using for example: $^{14}CO_2$, ^{14}C-pinitol, ^{14}C-myo-inositol.

GRAPH 1.

OMNITAB SEASONAL VARIATION OF SUGARS + CYCLITOLS IN PINUS R PAGE 1

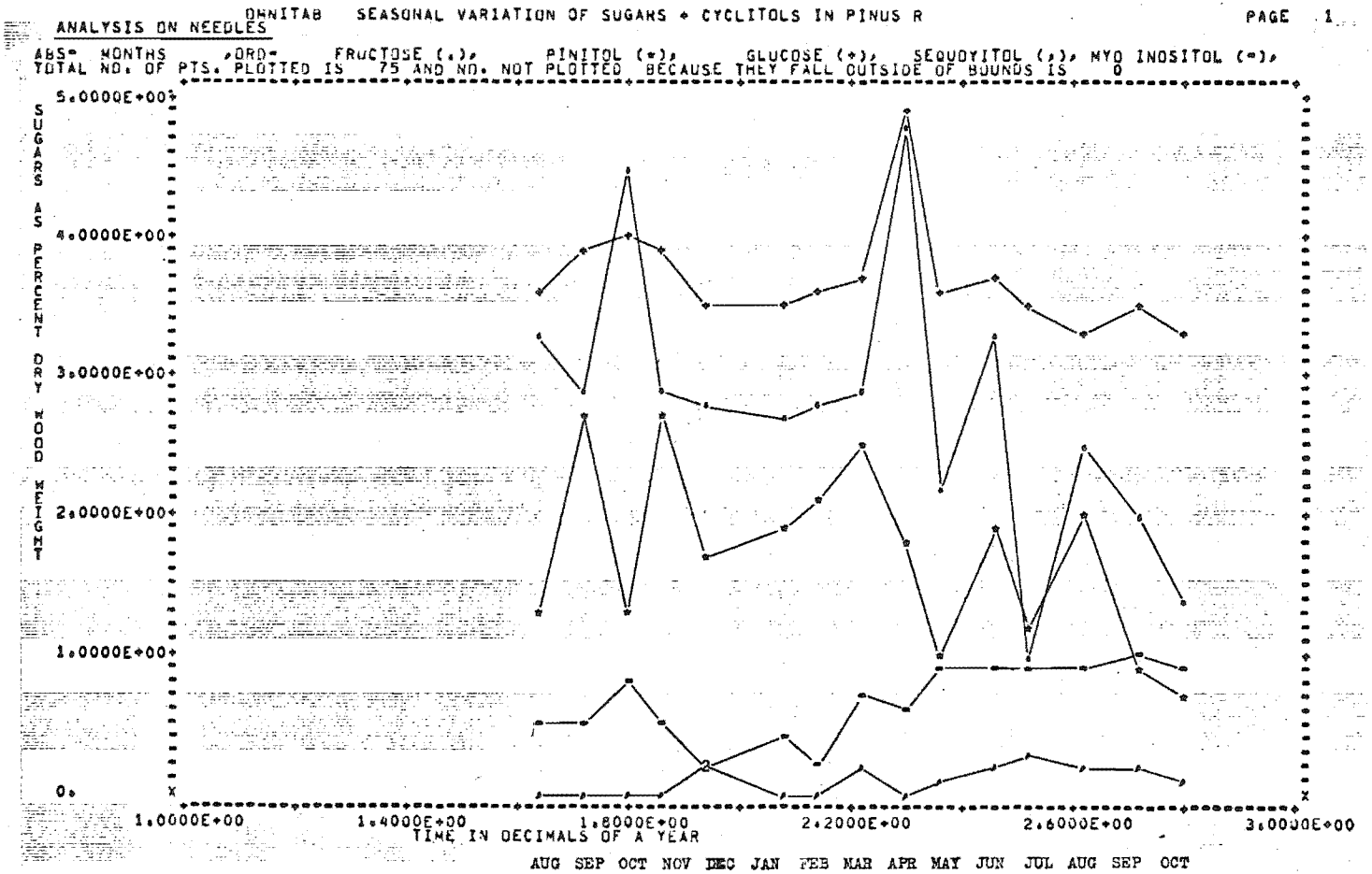
ANALYSIS ON BUDS

ABS= MONTHS ,ORD= FRUCTOSE (.), PINITOL (+), GLUCOSE (+), SEQUOYITOL (*), MYO INOSITOL (=),
TOTAL NO. OF PTS. PLOTTED IS 34 AND NO. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 1



SAMPLE	DATE	MONTHS	FRUCTOSE	PINITOL	GLUCOSE	SEQUOYITOL	MYO INOSITOL
1.0	240873	1.647	7.786	2.136	2.557	.6100	.5250
4.0	210973	1.723	4.432	2.649	1.968	.9350	.1680
23.	220274	2.145	4.569	2.834	2.336	.5200	.5140
29.	190474	2.299	2.297	3.341	2.507	.4370	.3140
35.	200674	2.468	1.480	2.942	2.167	.5310	.2740
38.	110774	2.526	1.928	3.237	2.454	.5300	.2390
41.	150874	2.622	2.543	3.781	2.602	1.024	.3750

GRAPH 2.

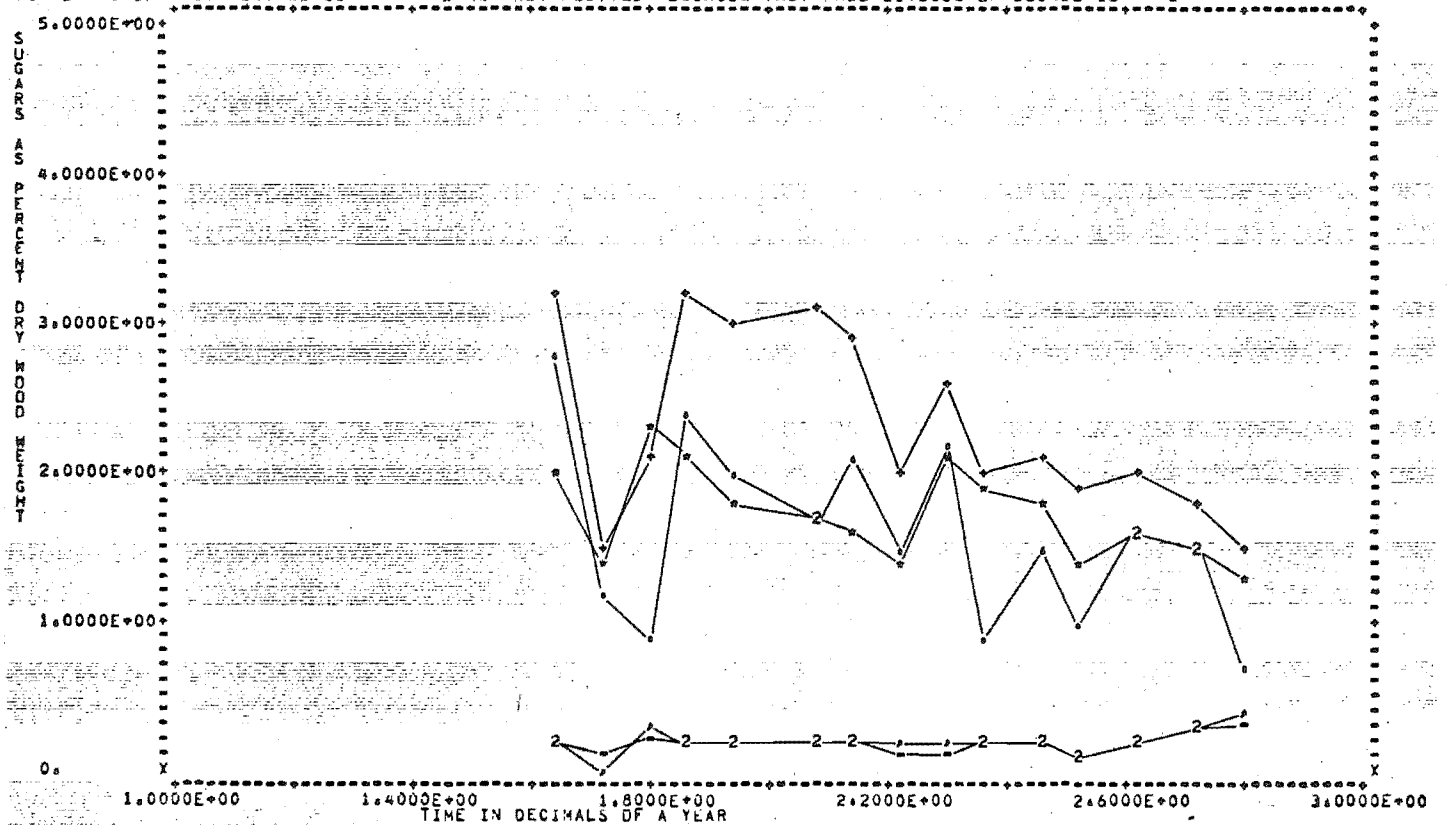


SAMPLE	DATE	MONTHS	FRUCTOSE	PINITOL	GLUCOSE	SEQUOYITOL	MYO INOSITOL
2.0	240873.	1.647	3.303	1.334	3.634	0.	.5440
5.0	210973.	1.723	2.933	2.736	3.850	0.	.4690
7.0	181073.	1.797	4.475	1.296	3.988	0.	.7960
11.	81173.0	1.855	2.919	2.716	3.877	0.	.5230
15.	71273.0	1.934	2.774	1.664	3.472	.1660	.2360
19.	290174.	2.079	2.693	1.866	3.497	0.	.3700
24.	220274.	2.145	2.790	2.076	3.579	0.	.2390
27.	210374.	2.219	2.904	2.505	3.710	.2000	.7270
30.	190474.	2.299	4.842	1.825	4.895	0.	.5980
33.	100574.	2.356	2.213	1.021	3.612	.1470	.8660
36.	200674.	2.468	3.260	1.900	3.650	.1900	.8500
39.	110774.	2.526	1.047	1.229	3.473	.2870	.9370
42.	150874.	2.622	2.463	1.995	3.339	.2270	.9340
45.	170974.	2.712	2.007	.8700	3.538	.2310	1.013
48.	181074.	2.797	1.399	.7440	3.255	.1360	.9100

GRAPH 3.

ANALYSIS ON WOOD OMNITAB SEASONAL VARIATION OF SUGARS + CYCLITOLS IN PINUS R PAGE 1

ABS= MONTHS ,ORD= FRUCTOSE (.), PINITOL (*), GLUCOSE (+), SEQUOYITOL (,), MYO INOSITOL (=),
TOTAL NO. OF PTS. PLOTTED IS 75 AND NO. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 0



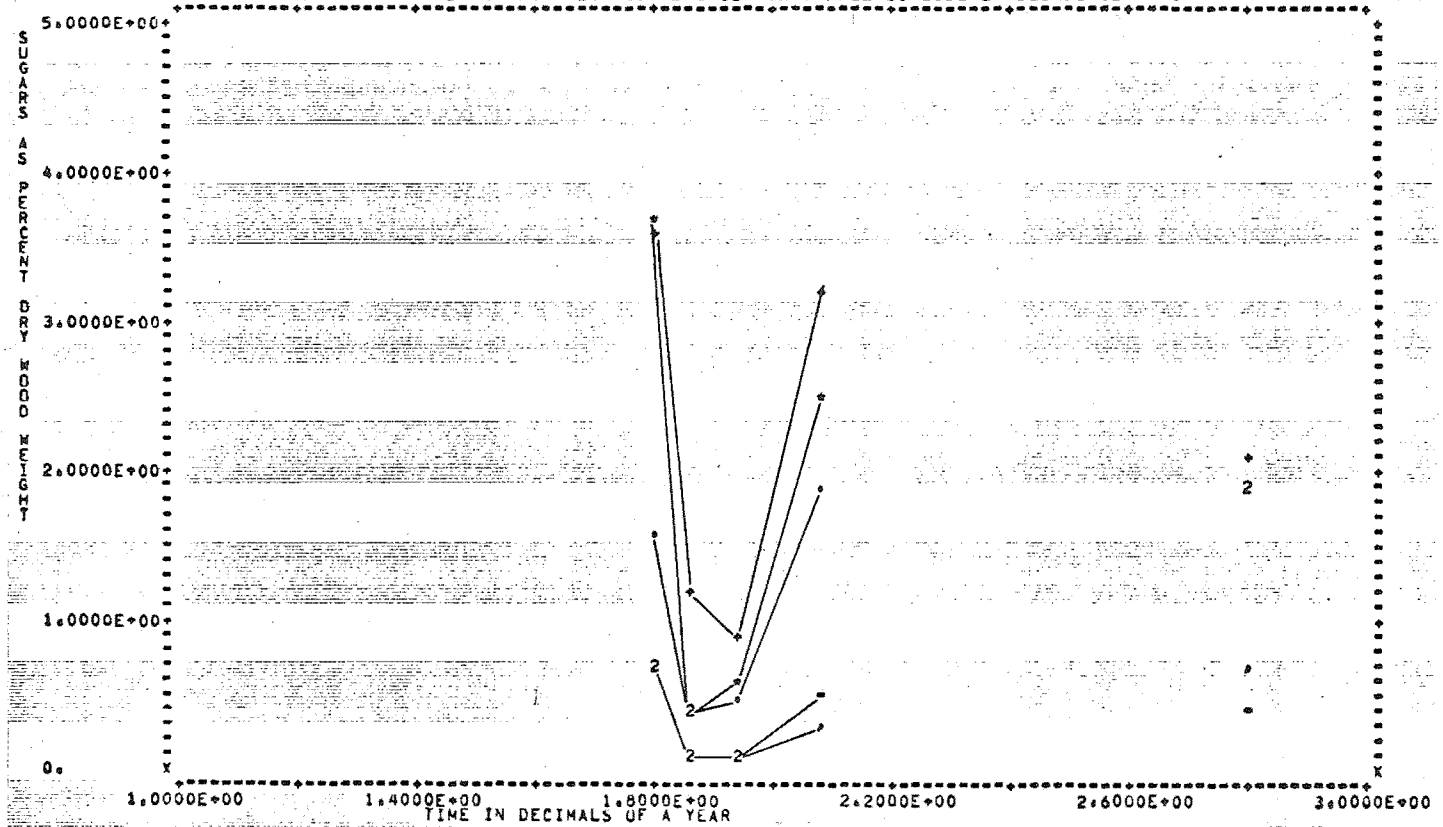
SAMPLE	DATE	MONTHS	FRUCTOSE	PINITOL	GLUCOSE	SEQUOYITOL	MYO INOSITOL
3.0	240873.	1.647	2.828	1.975	3.195	.1500	.2410
6.0	210973.	1.723	1.159	1.422	1.499	0.	.09200
9.0	181073.	1.797	.9180	2.287	2.054	.2600	.2150
13.	51173.0	1.855	2.431	2.098	3.180	.1870	.1620
17.	71273.0	1.934	2.019	1.765	3.024	.2290	.1550
21.	290174.	2.079	1.682	1.726	3.063	.2040	.1860
25.	220274.	2.145	2.091	1.601	2.903	.1960	.2230
28.	210374.	2.219	1.548	1.411	1.958	.2170	.1110
31.	190474.	2.299	2.248	2.073	2.599	.1670	.09700
34.	100574.	2.356	.9210	1.938	1.965	.1970	.2270
37.	200674.	2.468	1.478	1.799	2.081	.1990	.1830
40.	110774.	2.526	1.037	1.426	1.932	.09100	.1280
43.	150874.	2.622	1.637	1.624	1.992	.2350	.2210
46.	170974.	2.712	1.525	1.461	1.768	.2800	.3440
50.	161074.	2.797	.6580	1.326	1.534	.3500	.2680

GRAPH 4.

OMNITAB SEASONAL VARIATION OF SUGARS + CYCLITOLS IN PINUS R PAGE 1

ANALYSIS ON NEW NEEDLES

ABS= MONTHS ,ORD= FRUCTOSE (.), PINITOL (*), GLUCOSE (+), SEQUOYITOL (,), MYO INOSITOL (=),
TOTAL NO. OF PTS. PLOTTED IS 25 AND NO. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 0



SAMPLE	DATE	MONTHS	FRUCTOSE	PINITOL	GLUCOSE	SEQUOYITOL	MYO INUSITOL
8.0	181073.	1.797	1.602	3.736	3.602	.6900	.7160
12.	81173.0	1.855	.4410	.4370	1.212	.1000	.08300
16.	71273.0	1.934	.4580	.5990	.9310	.1140	.1340
20.	290174.	2.079	1.882	2.534	3.205	.2520	.4720
49.	181074.	2.797	1.919	1.901	2.149	.6730	.3650

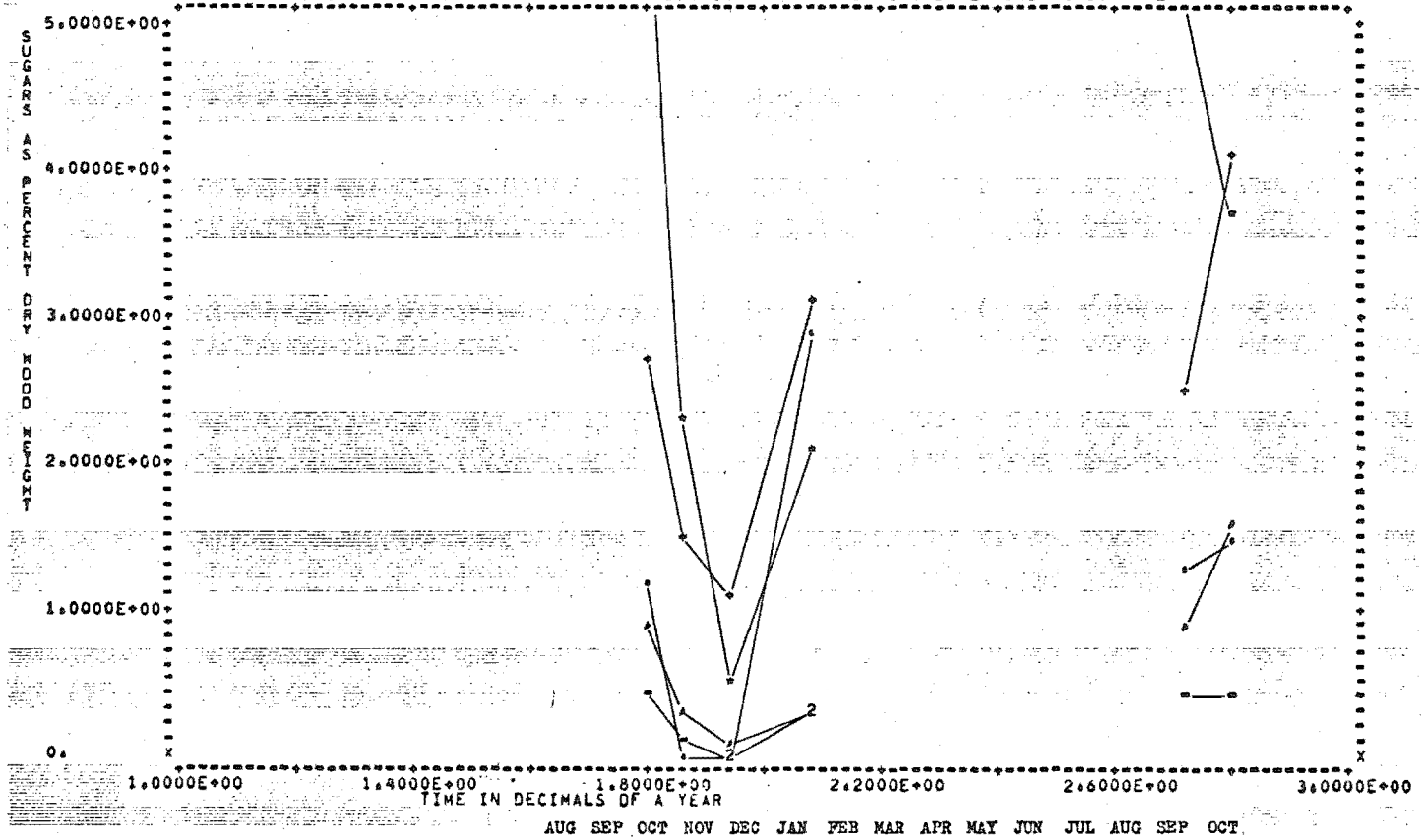
GRAPH 5.

OMNITAB SEASONAL VARIATION OF SUGARS + CYCLITOLS IN PINUS R PAGE 1

ANALYSIS ON NEW WOOD

ABS- MONTHS ,ORD- FRUCTOSE (.), PINITOL (+), GLUCOSE (+), SEQUOYITOL (,), MYO INOSITOL (=),

TOTAL NO. OF PTS. PLOTTED IS 28 AND NO. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 2



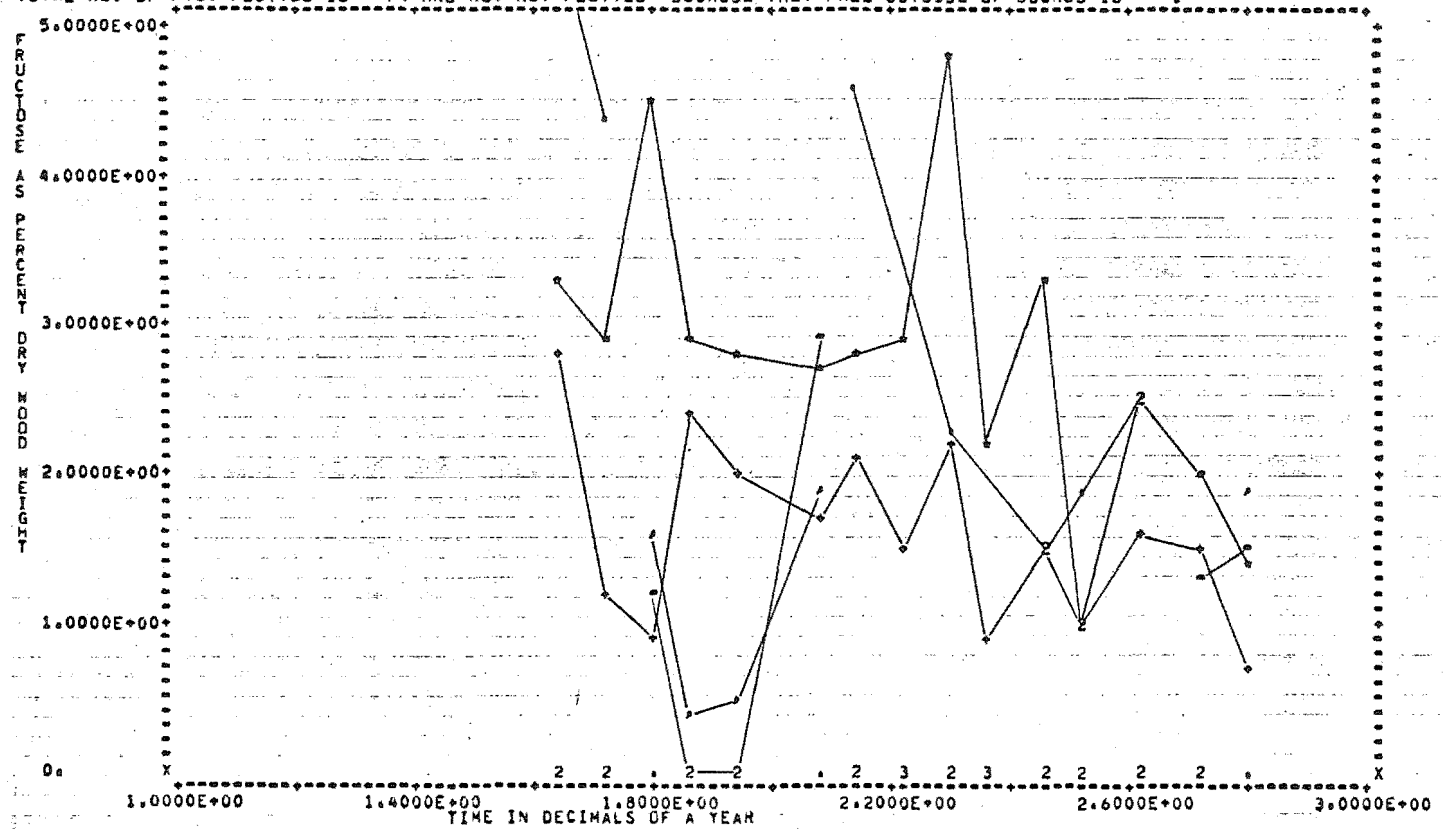
SAMPLE	DATE	MONTHS	FRUCTOSE	PINITOL	GLUCOSE	SEQUOYITOL	MYO INOSITOL
10	181073.	1.797	1.176	6.332	2.698	.9290	.3580
14	81173.0	1.855	0.	2.264	1.513	.2910	.08500
18	71373.0	1.934	0.	.5360	1.070	.1480	0.
22	290174.	2.079	2.875	2.116	3.125	.2610	.2660
47	170974.	2.712	1.303	6.433	2.491	.8600	.3790
51	181074.	2.797	1.525	3.713	4.097	1.612	.3640

GRAPH 6.

OMNITAB SEASONAL VARIATION OF SUGARS + CYCLITOLS IN PINUS R PAGE 1

GRAPH OF FRUCTOSE V TIME

ABS= MONTHS ,ORD= BUDS (.), NEEDLES (*), WOOD (+), NEW NEEDLES (,), NEW WOOD (=),
TOTAL NO. OF PTS. PLOTTED IS 74 AND NO. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 1

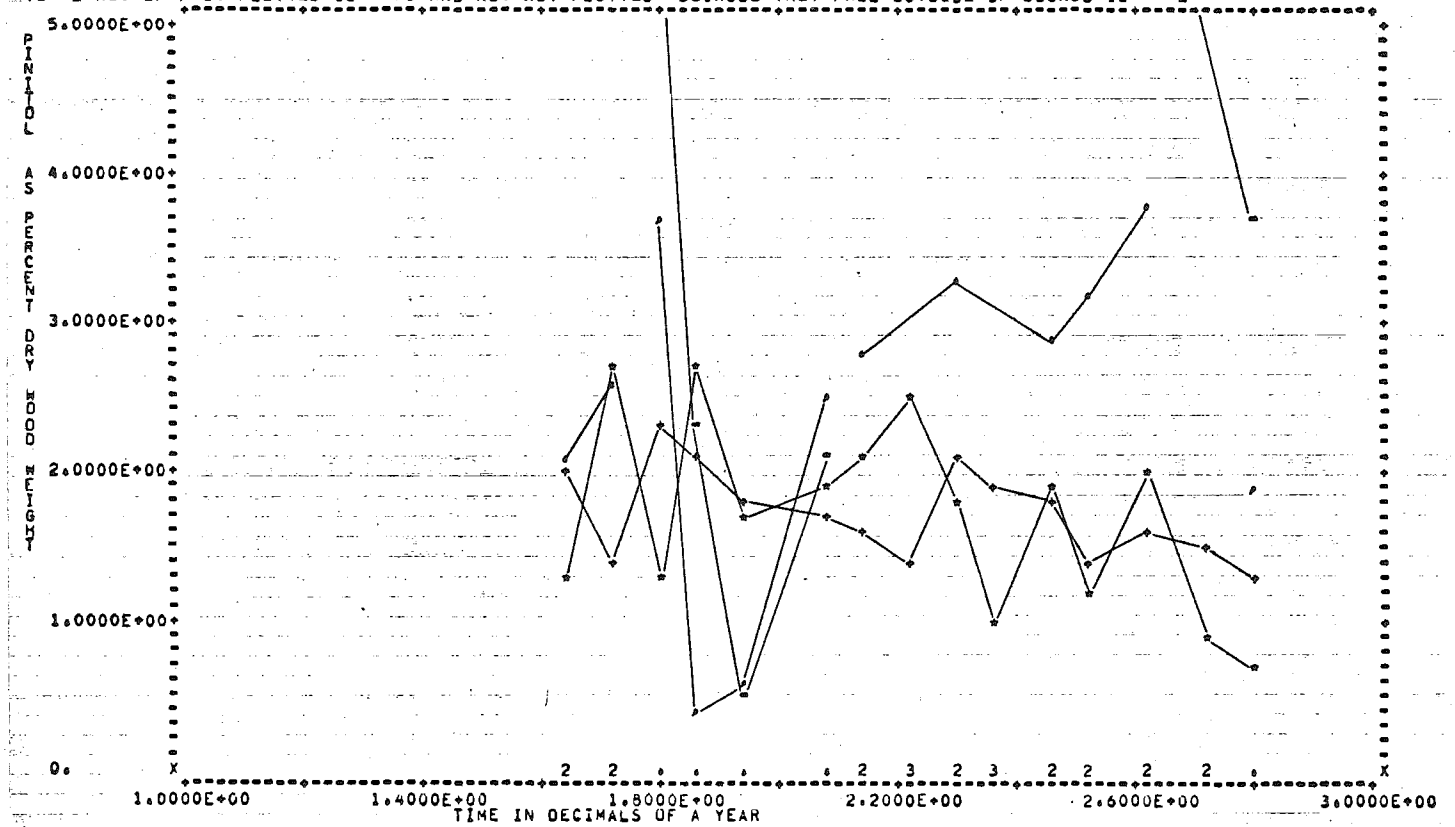


DATE	MONTHS	BUDS	NEEDLES	WOOD	NEW NEEDLES	NEW WOOD
240873.	1.647	7.786	3.303	2.828	0.	0.
210973.	1.723	4.432	2.933	1.159	0.	0.
181073.	1.797	0.	4.475	.9180	1.602	1.176
81173.0	1.855	0.	2.919	2.431	.4410	0.
71273.0	1.934	0.	2.774	2.019	.4580	0.
290174.	2.079	0.	2.693	1.682	1.882	2.875
220274.	2.145	4.589	2.790	2.091	0.	0.
210374.	2.219	0.	2.904	1.548	0.	0.
190474.	2.299	2.297	4.842	2.248	0.	0.
100574.	2.356	0.	2.213	.9210	0.	0.
200674.	2.468	1.480	3.260	1.478	0.	0.
110774.	2.526	1.928	1.047	1.037	0.	0.
150874.	2.622	2.543	2.463	1.637	0.	0.
170974.	2.712	0.	2.007	1.525	0.	1.303
181074.	2.797	0.	1.399	.6580	1.919	1.525

GRAPH 7.

GRAPH OF PINITOL V TIME OMNITAB SEASONAL VARIATION OF SUGARS + CYCLITOLS IN PINUS R

ABS= MONTHS ,ORD= BUDS (.), NEEDLES (+), WOOD (*), NEW NEEDLES (,), NEW WOOD (=),
TOTAL NO. OF PTS. PLOTTED IS 73 AND NO. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 2

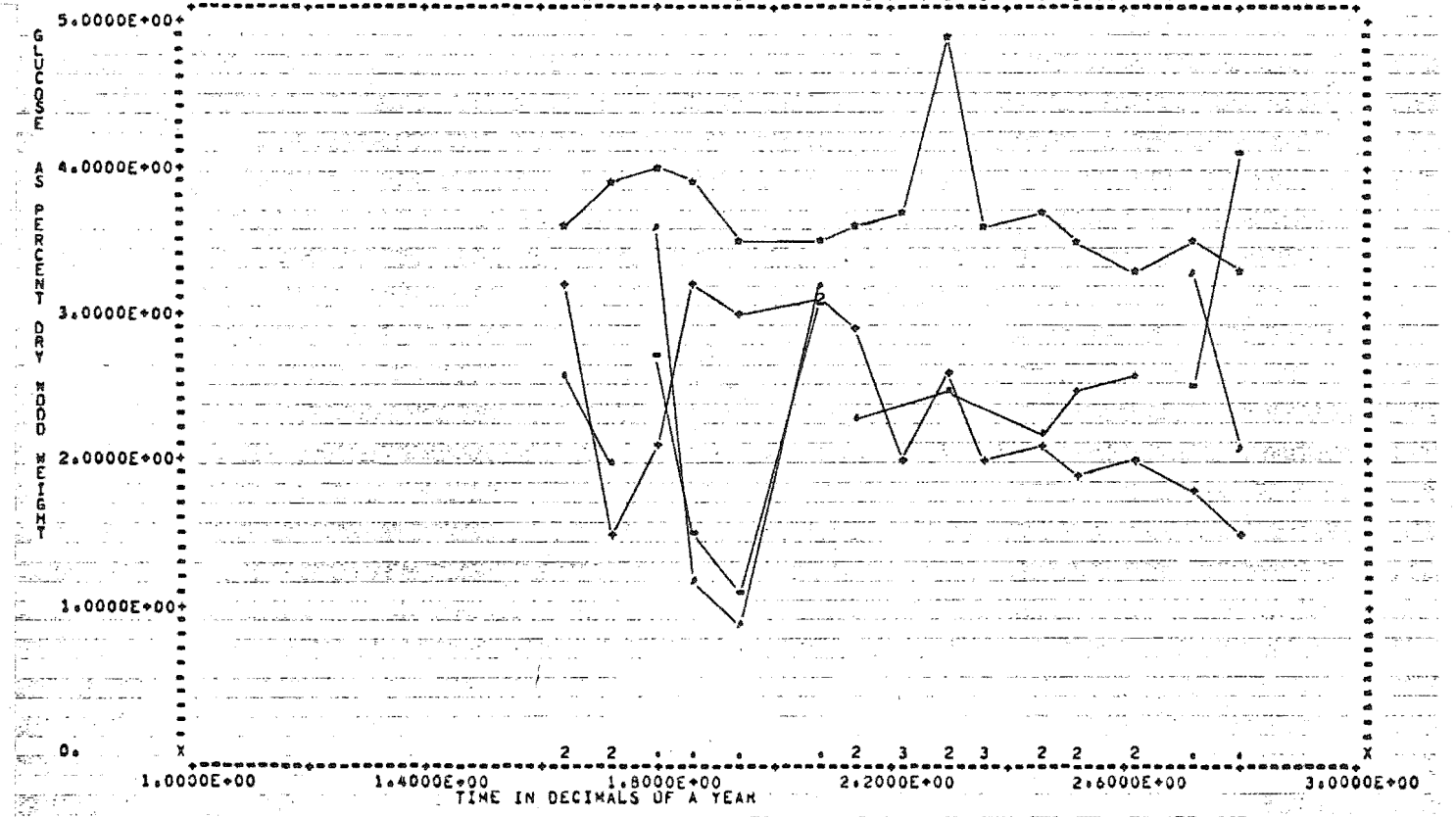


DATE	MONTHS	BUDS	NEEDLES	WOOD	NEW NEEDLES	NEW WOOD
240873.	1.647	2.136	1.334	1.975	0.	0.
210973.	1.723	2.649	2.736	1.422	0.	0.
181073.	1.797	0.	1.298	2.287	3.736	6.332
81173.0	1.855	0.	2.716	2.098	.4370	2.284
71273.0	1.934	0.	1.664	1.765	.5990	.5360
290174.	2.079	0.	1.896	1.726	2.534	2.116
220274.	2.145	2.834	2.076	1.601	0.	0.
210374.	2.219	0.	2.505	1.411	0.	0.
190474.	2.299	3.341	1.825	2.073	0.	0.
100574.	2.356	0.	1.021	1.938	0.	0.
200674.	2.468	2.942	1.900	1.799	0.	0.
110774.	2.526	3.237	1.229	1.426	0.	0.
150874.	2.622	3.781	1.995	1.624	0.	0.
170974.	2.712	0.	.8700	1.461	0.	6.433
181074.	2.797	0.	.7440	1.326	1.901	3.713

GRAPH 8.

GRAPH OF GLUCOSE OMNITAB V TIME SEASONAL VARIATION OF SUGARS + CYCLITOLS IN PINUS R PAGE 5

ABS= MONTHS ,ORD= BUDS (.), NEEDLES (*), WOOD (+), NEW NEEDLES (,), NEW WOOD (=)
TOTAL NO. OF PTS. PLOTTED IS 75 AND NO. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 0



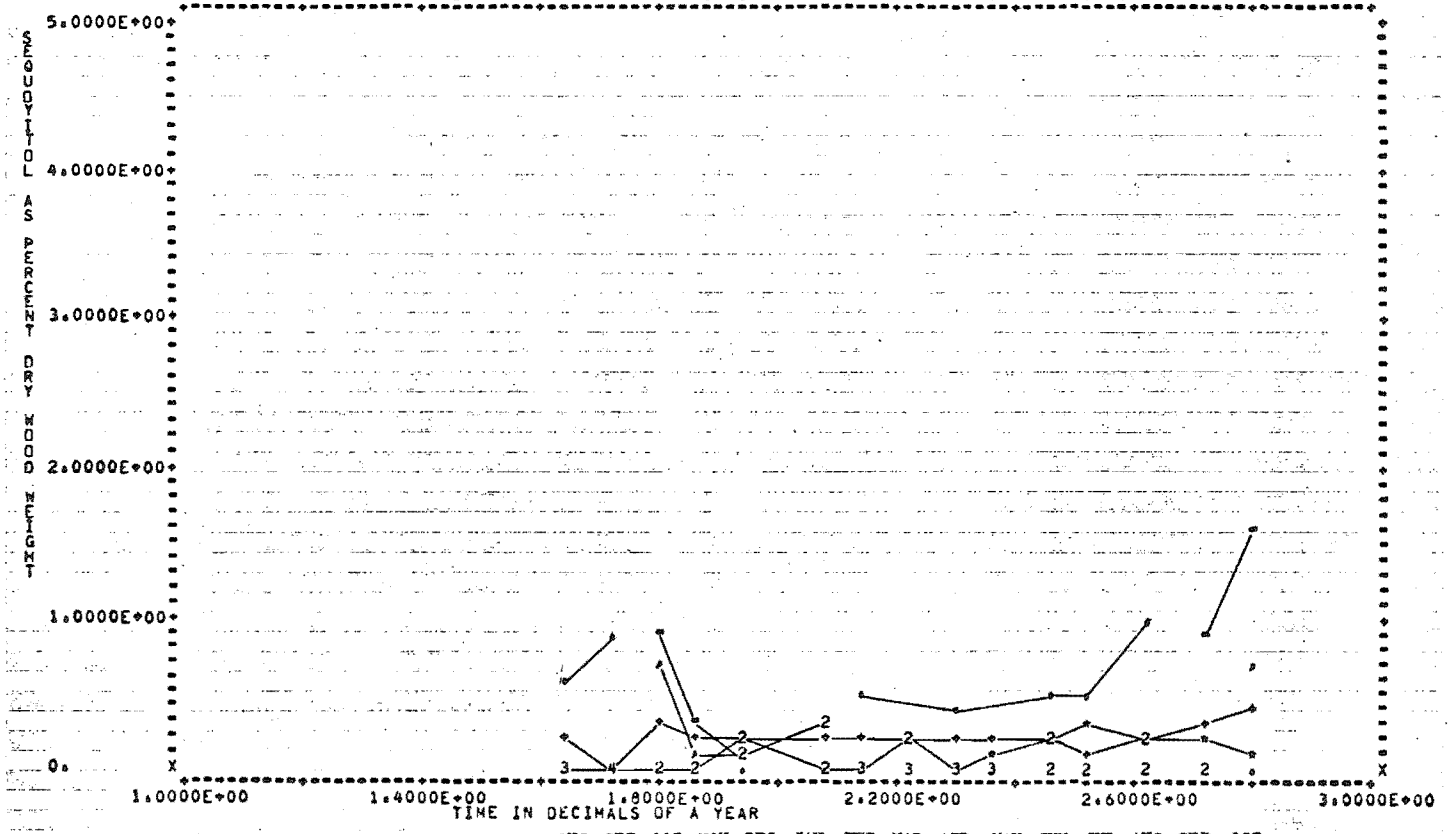
DATE	MONTHS	BUDS	NEEDLES	WOOD	NEW NEEDLES	NEW WOOD
240873.	1.647	2.557	3.634	3.195	0.	0.
210973.	1.723	1.968	3.850	1.499	0.	0.
181073.	1.797	0.	3.908	2.054	3.602	2.698
81173.0	1.855	0.	3.877	3.180	1.212	1.513
71273.0	1.934	0.	3.472	3.024	.9310	1.070
290174.	2.079	0.	3.497	3.063	3.205	3.125
220274.	2.145	2.336	3.579	2.903	0.	0.
210374.	2.219	0.	3.710	1.958	0.	0.
190474.	2.299	2.507	4.895	2.599	0.	0.
100574.	2.356	0.	3.612	1.965	0.	0.
200674.	2.468	2.167	3.650	2.081	0.	0.
110774.	2.526	2.454	3.473	1.932	0.	0.
150874.	2.622	2.602	3.319	1.992	0.	0.
170974.	2.712	0.	3.538	1.788	3.296	2.491
181074.	2.797	0.	3.255	1.534	2.149	4.097

GRAPH 9.

OMNITAB SEASONAL VARIATION OF SUGARS + CYCLITOLS IN PINUS R

GRAPH OF SEQUOYITOL V TIME

ABS= MONTHS ,ORD= BUDS (.), NEEDLES (+), WOOD (*), NEW NEEDLES (,), NEW WOOD (=),
TOTAL NO. OF PTS. PLOTTED IS 75 AND NO. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 0



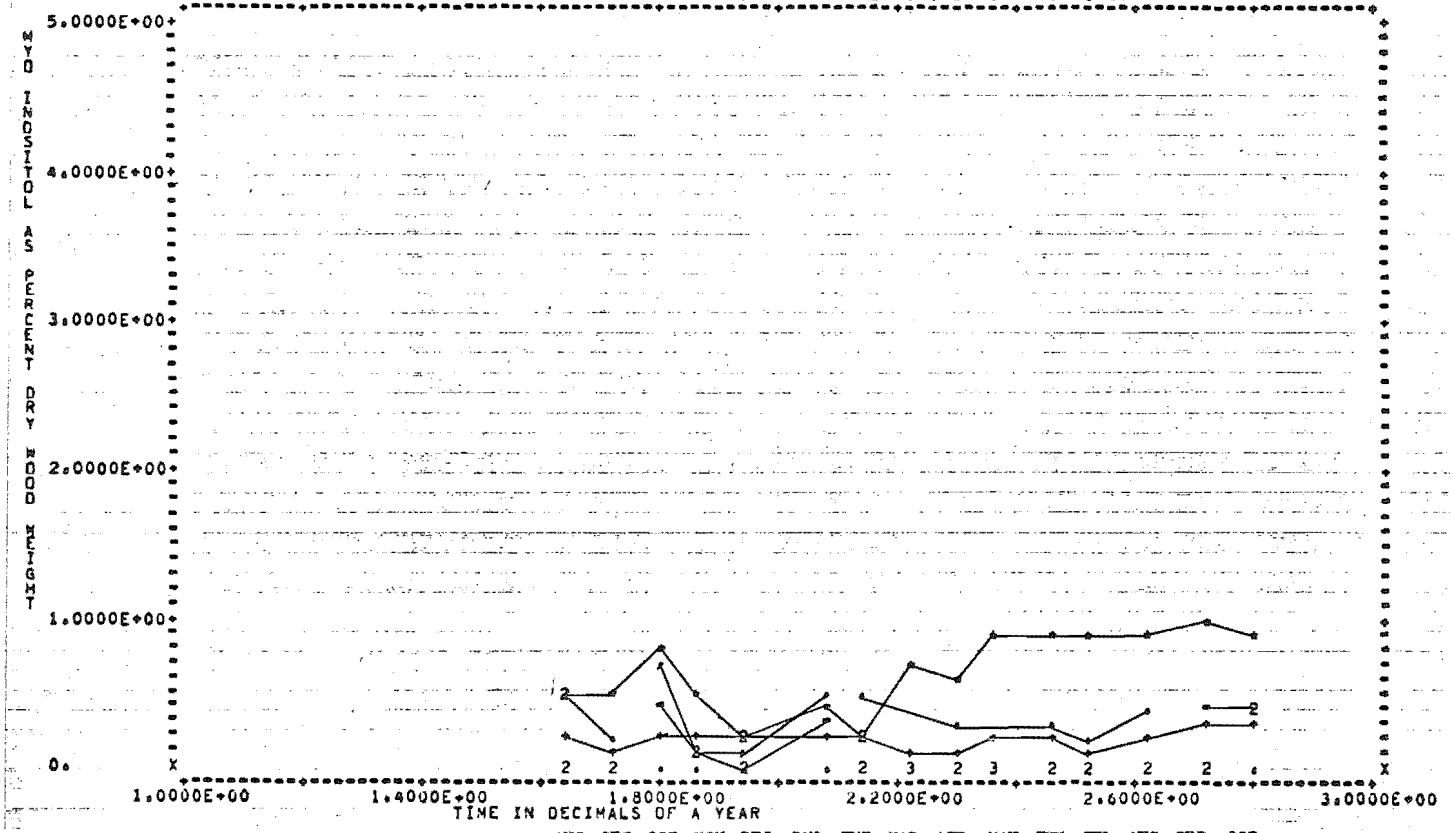
DATE	MONTHS	BUDS	NEEDLES	WOOD	NEW NEEDLES	NEW WOOD
240873.	1.647	.6100	0.	.1500	0.	0.
210973.	1.723	.9350	0.	0.	0.	0.
181073.	1.797	0.	0.	.2600	.6900	.9290
81173.0	1.855	0.	0.	.1870	.1000	.2910
71273.0	1.934	0.	.1660	.2290	.1140	.1480
290174.	2.079	0.	0.	.2040	.2520	.2610
220274.	2.145	.5200	0.	.1980	0.	0.
210374.	2.219	0.	.2000	.2170	0.	0.
190474.	2.299	.4370	0.	.1670	0.	0.
100574.	2.356	0.	.1470	.1970	0.	0.
200674.	2.468	.5310	.1900	.1990	0.	0.
110774.	2.526	.5300	.2870	.09100	0.	0.
150874.	2.622	1.024	.2270	.2350	0.	0.
170974.	2.712	0.	.2310	.2600	0.	.8600
181074.	2.797	0.	.1360	.3500	.6730	1.612

GRAPH 10.

OMNITAB SEASONAL VARIATION OF SUGARS + CYCLITOLS IN PINUS R PAGE 9

GRAPH OF MYO INOSITOL V TIME

ABS= MONTHS ,ORD= BUDS (.), NEEDLES (*), WOOD (+), NEW NEEDLES (,), NEW WOOD (=),
TOTAL NO. OF PTS. PLOTTED IS 75 AND NO. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 0

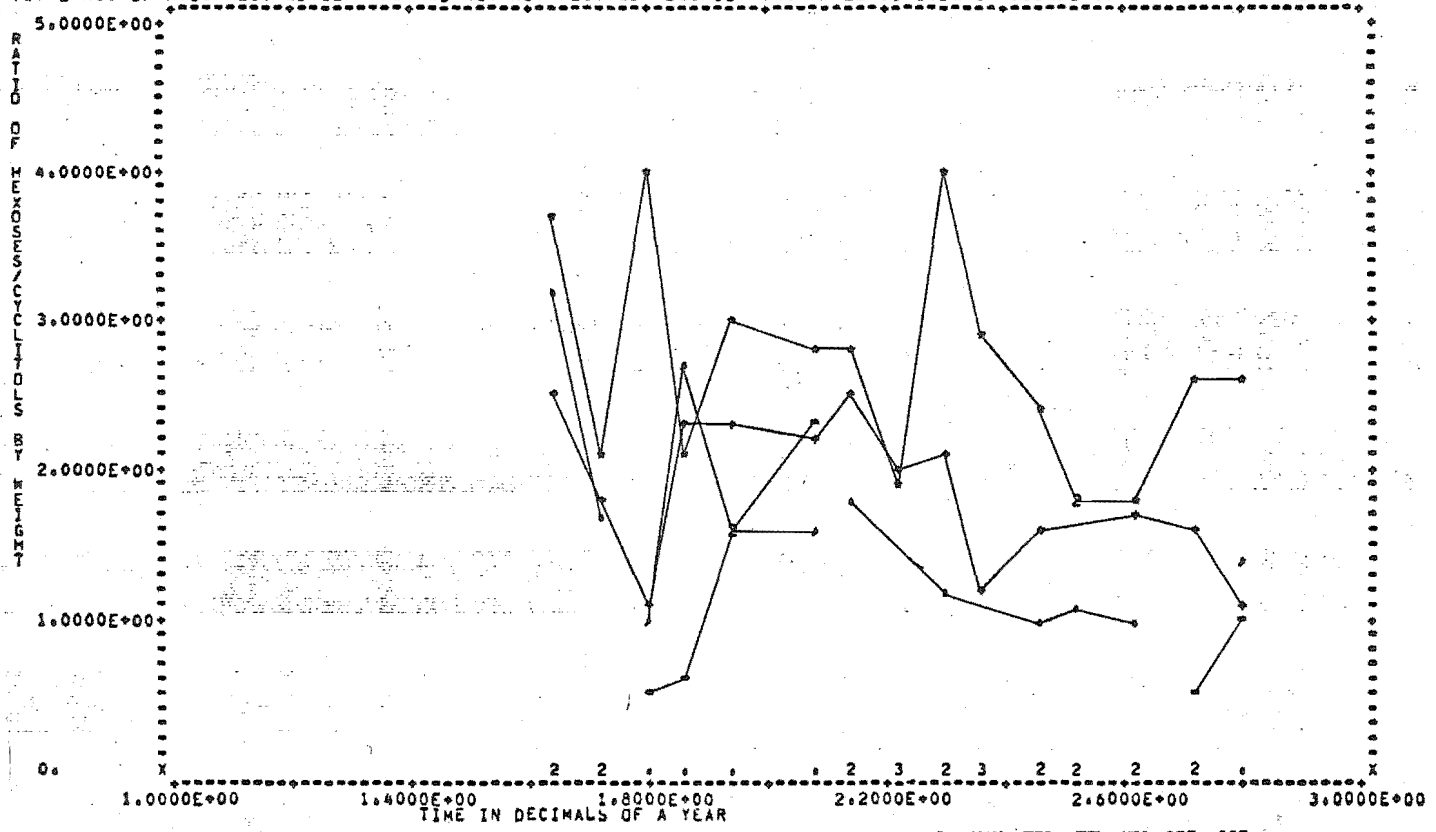


DATE	MONTHS	BUDS	NEEDLES	WOOD	NEW NEEDLES	NEW WOOD
240873.	1.647	.5250	.5440	.2410	0.	0.
210973.	1.723	.1680	.4690	.09200	0.	0.
181073.	1.797	0.	.7960	.2150	.7160	.3580
81173.0	1.855	0.	.5230	.1620	.08300	.08500
71273.0	1.934	0.	.2380	.1550	.1340	0.
290174.	2.079	0.	.3700	.1860	.4720	.2640
220274.	2.145	.5140	.2390	.2230	0.	0.
210374.	2.219	0.	.7270	.1110	0.	0.
190474.	2.299	.3140	.5980	.09700	0.	0.
100574.	2.356	0.	.8660	.2270	0.	0.
200674.	2.468	.2740	.8500	.1830	0.	0.
110774.	2.526	.2390	.9370	.1280	0.	0.
150874.	2.622	.3750	.9340	.2210	0.	0.
170974.	2.712	0.	1.013	.3440	0.	.3790
181074.	2.797	0.	.9100	.2680	.3650	.3640

GRAPH 11.

GRAPH RATIO OF HEXOSES TO CYCLITOLS V TIME

ABS= MONTHS ,ORO= BUDS (.), NEEDLES (*), WOOD (+), NEW NEEDLES (,), NEW WOOD (=),
TOTAL NO. OF PTS. PLOTTED IS 75 AND NO. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 0



DATE	MONTHS	BUDS	NEEDLES	WOOD	NEW NEEDLES	NEW WOOD
240873.	1.647	3.162	3.694	2.546	0.	0.
210973.	1.723	1.706	2.116	1.756	0.	0.
181073.	1.797	0.	4.045	1.076	1.012	.5085
81173.0	1.855	0.	2.098	2.293	2.666	.5688
71273.0	1.934	0.	3.020	2.347	1.640	1.564
290174.	2.079	0.	2.768	2.242	1.561	2.272
220274.	2.145	1.790	2.751	2.470	0.	0.
210374.	2.219	0.	1.927	2.016	0.	0.
190474.	2.299	1.174	4.019	2.074	0.	0.
100574.	2.356	0.	2.864	1.222	0.	0.
200674.	2.468	.9733	2.350	1.632	0.	0.
110774.	2.526	1.094	1.843	1.805	0.	0.
150874.	2.622	.9932	1.838	1.745	0.	0.
170974.	2.712	0.	2.623	1.589	0.	.4945
181074.	2.797	0.	2.600	1.128	1.384	.9882

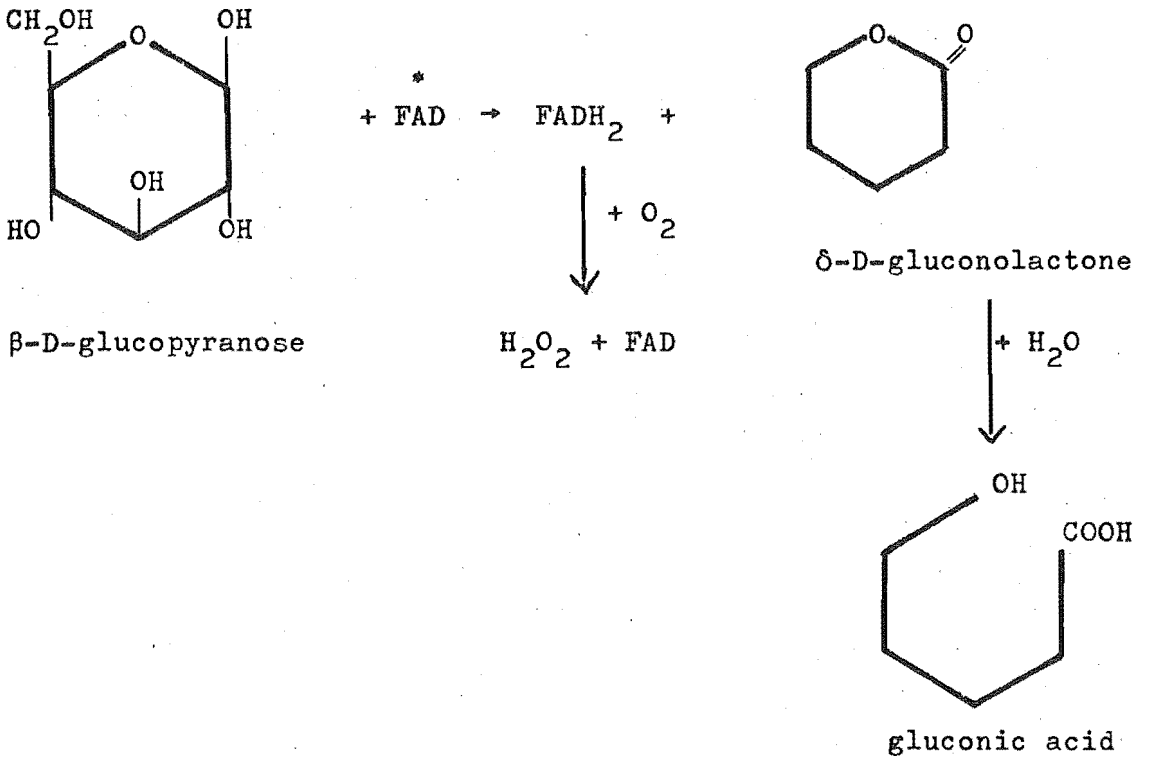
STARCH ANALYSIS

There are many tabulated methods of starch analysis. There are the specific techniques of Pucher, using perchloric acid/I_2 precipitation, or Steiner's optical rotation method. But these are lengthy and the large sample requirements make them impractical. The more easily applied techniques such as Neilsen's I_2 staining and McCready's anthrone methods (Ebell 1969 a), lack specificity which is always of concern when the starch concentrations are low relative to other carbohydrates.

Methods based on the use of amyloglucosidase/glucose oxidase (g.o.) fulfill the requirements for a rapid, accurate method suited to the analysis of a large number of small samples. Hydrolysis of starch by amyloglucosidase is rapid, quantitative, and not specific to 1,4- linkages only. Thus the end product is glucose.

This glucose can be rapidly and accurately analysed by using g.o. The principle advantage of using g.o. is its specificity for glucose, particularly in the presence of other reducing sugars which make the common reductometric methods (Dubois 1956) impossible to use.

The substrate, β-D-glucopyranose is oxidized to gluconic acid and hydrogen peroxide by the g.o. enzyme (Keilin 1952). (See diagram on following page).



* The prosthetic group of glucose oxidase.

An oxygen electrode can be adapted to measure indirectly the β-D-glucose consumed, assuming all O_2 present follows this reaction sequence. The "Beckman Glucose Analyser" used actually measures the rate of O_2 consumption, the rate being proportional to the initial glucose concentration. Alternatively, a chromogen (Fales 1963) which, in the presence of peroxidase and peroxide is oxidized, is used coupled with g.o. to measure glucose concentrations. α-D-Glucopyranose, which is also produced in the amyloglucosidase treatment of starch is not a substrate for g.o. and its mutarotation to β-D-glucopyranose at pH 7.0 is slow (Pigman 1970). However, this is not a problem as commercial glucose oxidase preparations contain a mutarotase as an impurity. In the presence of the mutarotase α- and β-D-glucose are oxidised at the same rate (Dahlqvist 1961).

Two chromogens were used in this work; O-dianisidine-HCl and 3-methyl-2-benz thiazolinone hydrazone-HCl (MBTH)/ N,N-dimethylaniline (DMA).

The g.o. -O-dianisidine-HCl reagent was buffered at pH 7.0 in a tris-glycerol buffer which stabilized the reagent and inhibited unwanted glycosidase activity (Dahlqvist 1961). Inhibitors of the g.o. sequence were a problem although most of them could be removed with a charcoal wash (Ebell 1969 a, b, Dekker 1971). The g.o. reagent was also very sensitive to w.s.c. extracts that had been treated with certain ion-exchange resins, in particular Amberlite IRA 410 (OH^-).

After the incubation period the g.o. reaction was quenched with acid and the absorbance of the solution measured (maximum absorption peak at λ420 nm). When the P. radiata needle extracts were analysed, the normal pink colour (λ532 nm) which was obtained after acid quenching, slowly faded to brown. This did not affect the absorbance measurements provided the transition in colour was complete since every set of analyses contained a series of glucose standards which produced a linear absorbance vs. glucose-concentration plot (fig. 19 b) despite the unusual colour change.

The MBTH/DMA-g.o. system was buffered with phosphates rather than tris, and the reagents mixed immediately prior to reaction for best results, but otherwise the same procedure applied as for g.o.-O-dianisidine. The MBTH/DMA method is said (Gochman 1972) to be considerably more resistant to the effect of inhibitors than other chromogens although it still suffers, like O-dianisidine, from inhibitors of the peroxidase reaction.

A major problem in any method of starch analysis is the mobilisation of the intact starch granules in the tissue. It

was found that soaking the tissue in an aqueous solution of amyloglucosidase for a period of 36 hours gave glucose levels comparable with those obtained by prior solubilisation of the starch. The enzyme "soak" procedure apparently acts directly on the starch granules without requiring pre-treatment of the granules. This was preferable to solubilisation or gelatinisation of starch by boiling (MacRae 1968, 1971) or treatment with alkali (Dekker 1971) prior to hydrolysis, as these caused varying degrees of inhibition of the subsequent g.o. analysis.

The effectiveness of the enzyme "soak" was demonstrated with dry potato tissue. The tissue contained 36% starch by weight, but only 4% of this starch was water soluble which emphasised the need for enzyme treatment in vitro. The results obtained by prior gelatinisation and by an enzyme "soak" were comparable showing that it was not necessary to solubilise the potato starch prior to enzyme attack.

In the procedure adopted for P. radiata samples, duplicate tissue samples were weighed into volumetric flasks. One was a normal water soak as in the w.s.c. analysis, the other an enzyme soak. Ten P. radiata samples could be handled at a time and the procedure was briefly:- water-soak/hydrolysis for 36 hours followed by a charcoal treatment and a settling period of approximately 4 hours. Removal of aliquots of the supernatant for g.o. analysis. The charcoal treatment was essential for the removal of inhibitors of the g.o. reaction (Ebell 1969).

The concentration of starch was determined by measuring the difference in glucose concentrations between the normal water-soak and the enzyme-soak solutions.

The concentration of the glucose solutions obtained from the P. radiata needles ranged from approximately 50-200 µg/ml, which was within the dynamic range (fig. 19 a) of the "Beckman Glucose Analyser". An extra dilution step was necessary before the O-dianisidine-HCl and MBTH/DMA methods came within their dynamic ranges (0-90 and 0-50 µg/ml respectively) (figs. 19 b, c).

A comparison of the results from the three glucose oxidase methods and those using g.l.c., based on 13 P. radiata samples is depicted in graph (12). All 4 methods with the exception of a few points, agree to within 20% of each other. The g.l.c.'s are consistantly lower than the other three and this has been attributed either to positive interference of the enzyme systems, or errors inherent in the g.l.c. analysis which do not account for all the glucose present.

Of the methods examined, that based on the "Beckman Glucose Analyser", was the most rapid with a throughput of about one sample/minute, and was least prone to the effects of inhibitors. The g.l.c. method was the most cumbersome and time consuming.

From the results obtained (graph 13) it was possible to draw the following conclusions:-

(a) high starch levels during summer,

(b) low starch in winter,

(c) starch build-up through spring.

Figure 19a.

Graph of $-\frac{d\,O_2}{dt}$ Vs. [glucose].

Calibration graph.

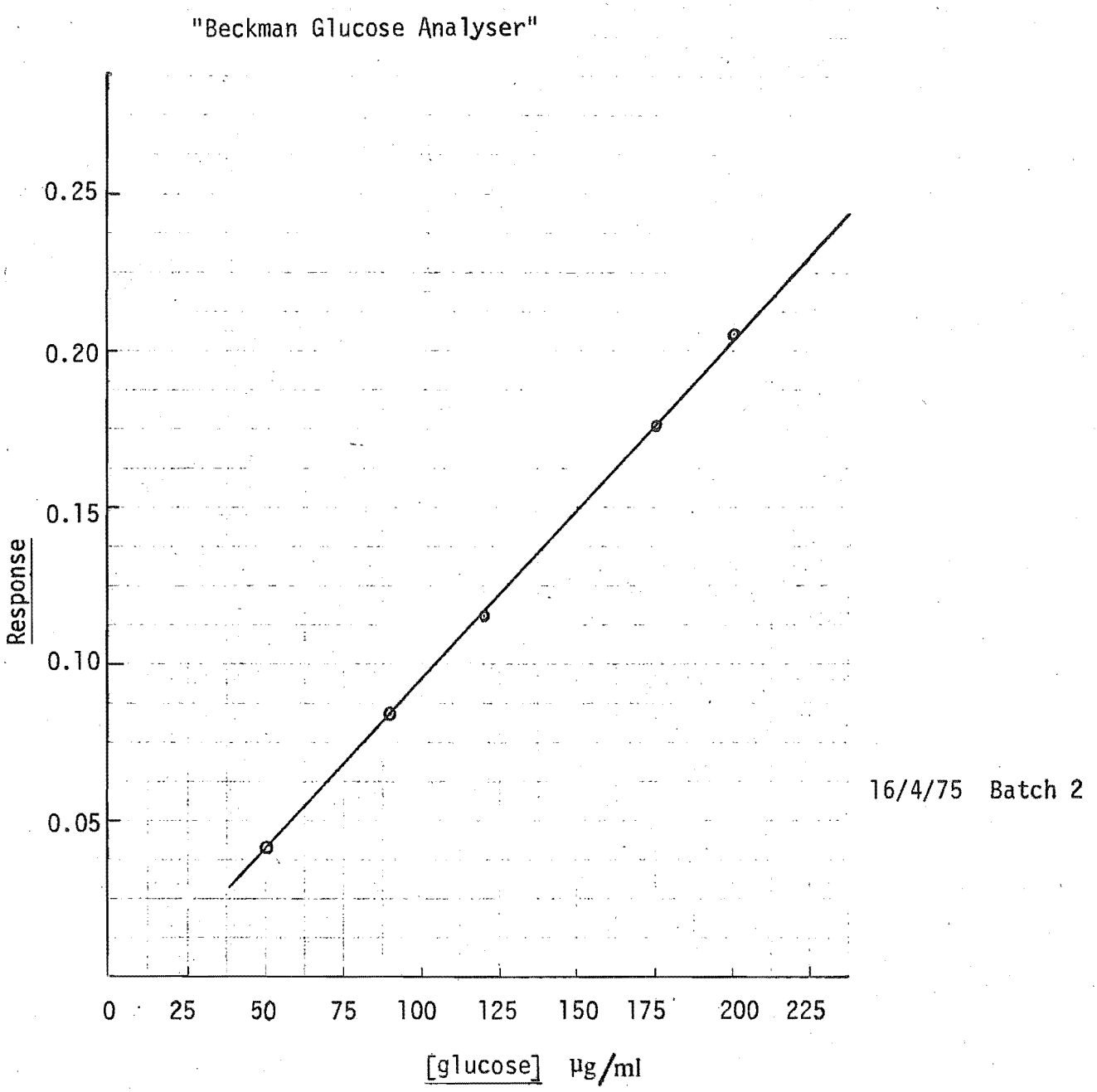


Figure 19b.

Graph of Absorbance Vs. [glucose]

Calibration graph.

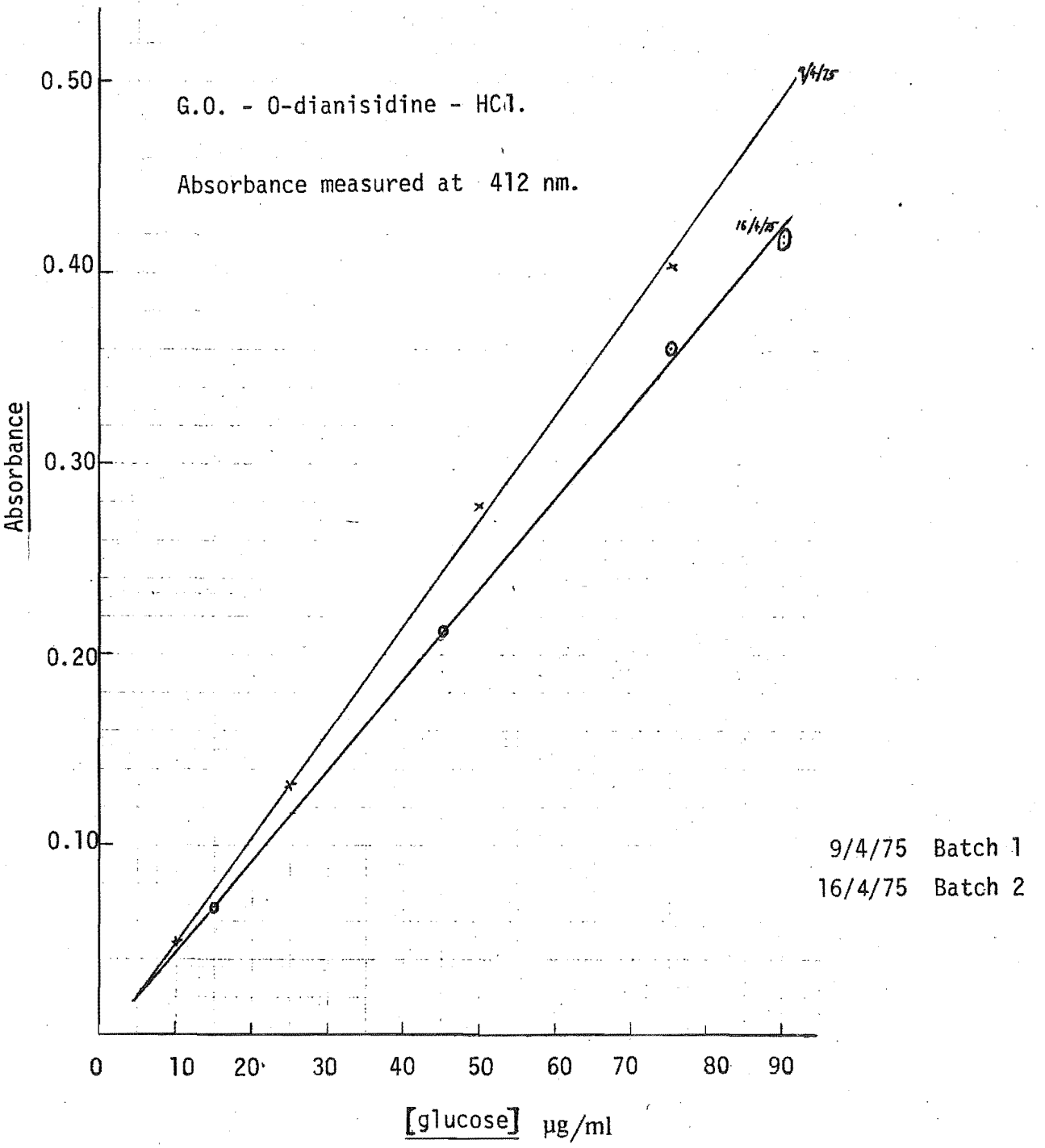
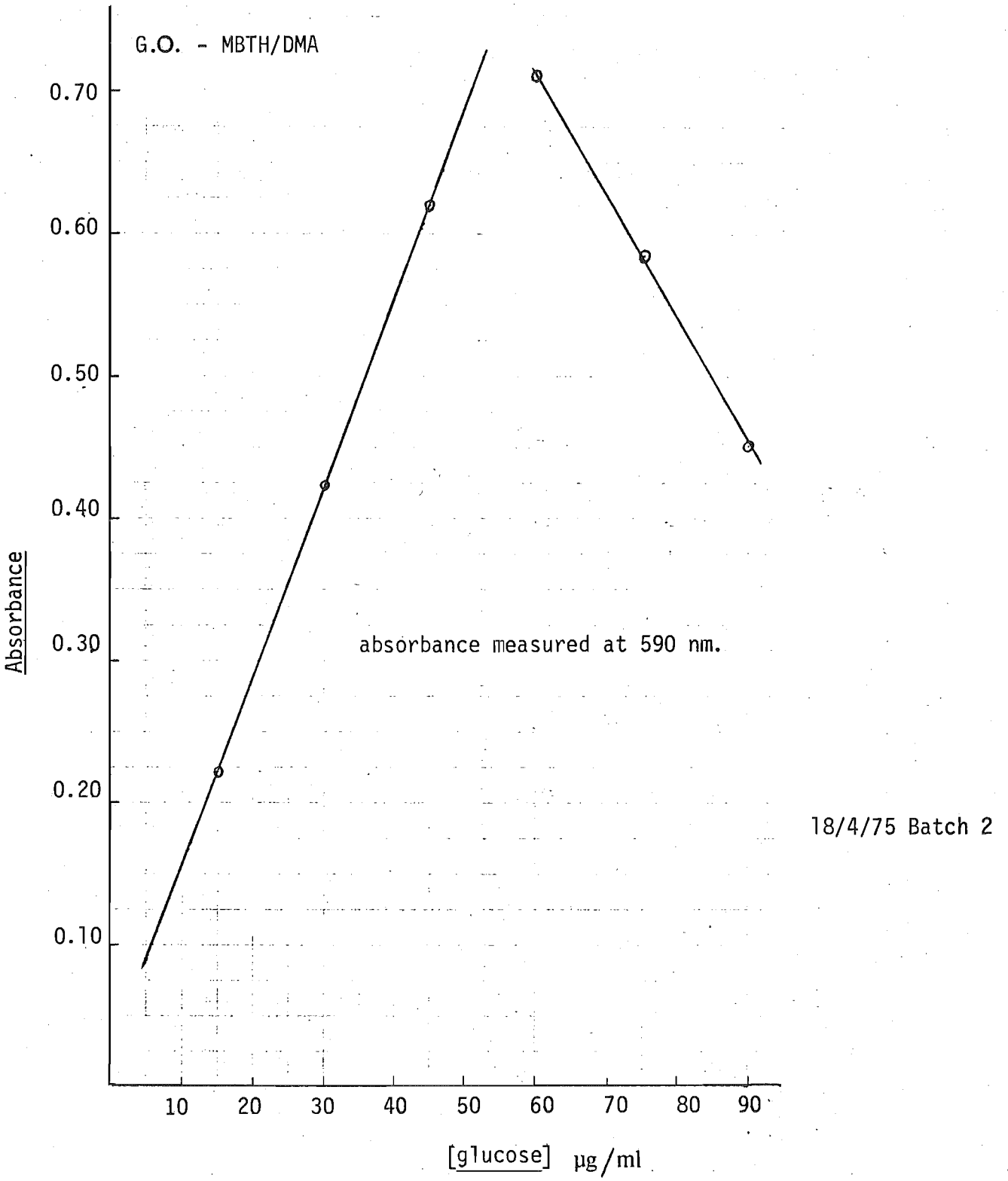


Figure 19c.

Graph of Absorbance Vs. [glucose]

Calibration Graph.



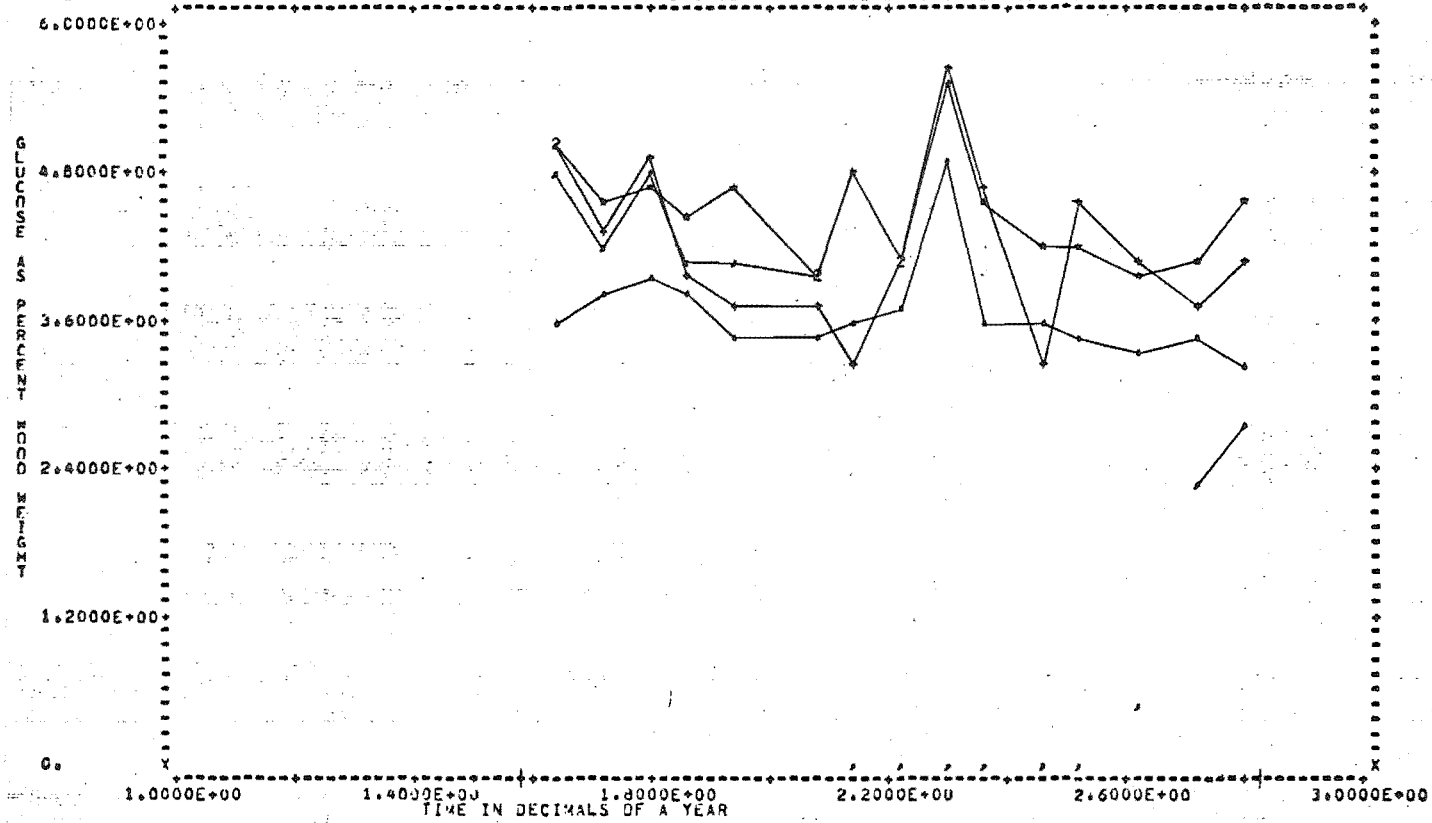
18/4/75 Batch 2

GRAPH 12.

OMNITAB GLUCOSE ANALYSIS WITH GLUCOSE OXIDASE 29/4/75 PAGE 1

GRAPH OF GLUCOSE V TIME

ABS= MONTHS ,ORD= GLC (.), GLUC ANALZR (*), D DIAN-GO (+), MBTH-GO (,),
TOTAL NO. OF PTS. PLOTTED IS 60 AND NO. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 0



AUG SEP OCT NOV DEC JAN FEB MAR APR MAY JUN JUL AUG SEP OCT

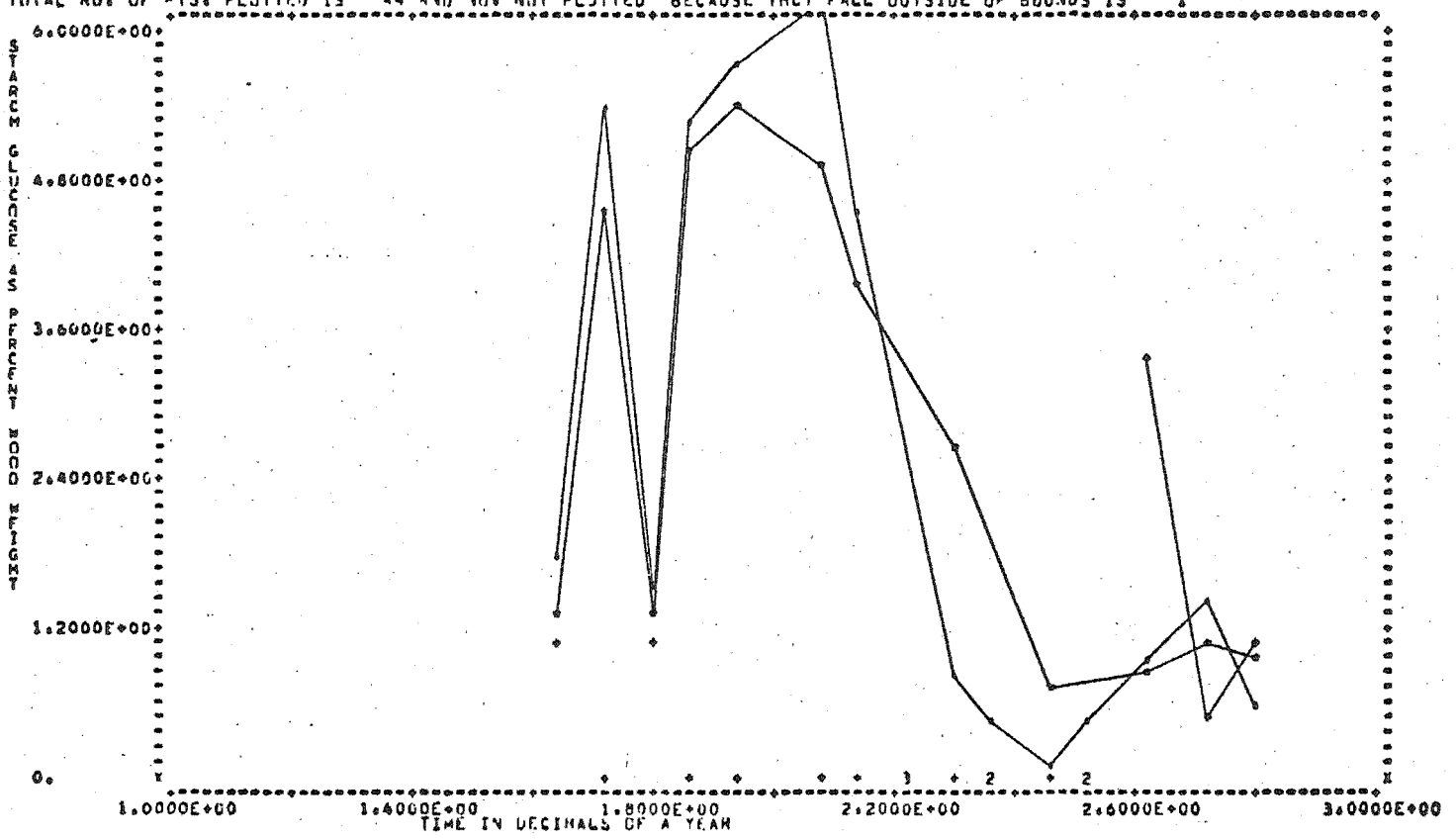
SAMPLE	DATE	MONTHS	GLC	GLUC ANALZR	D DIAN-GO	MBTH-GO
1.0	240873.	1.647	3.634	5.043	5.043	4.807
2.0	210973.	1.723	3.850	4.573	4.287	4.173
3.0	181073.	1.797	3.988	4.713	4.943	4.770
4.0	81173.0	1.855	3.877	4.477	3.972	4.041
5.0	71273.0	1.934	3.472	4.708	3.759	4.100
6.0	290174.	2.079	3.447	3.965	3.747	3.985
7.0	220274.	2.145	3.579	4.300	3.288	0.
8.0	210374.	2.219	3.710	4.030	4.132	0.
9.0	190474.	2.299	4.895	5.556	5.657	0.
10.	100574.	2.356	3.612	4.590	4.684	0.
11.	200674.	2.468	3.650	4.142	3.233	0.
12.	110774.	2.526	3.473	4.216	4.553	0.
13.	150874.	2.622	3.339	3.940	4.083	.4660
14.	170974.	2.712	3.538	4.036	3.699	2.251
15.	181074.	2.797	3.255	4.527	4.051	2.701

GRAPH 13.

OMNITAB STARCH ANALYSIS WITH GLUCOSE OXIDASE 29/4/75

GRAPH OF STARCH GLUCOSE V TIME

ARS= MONTHS ,2ND= GLUC ANALZR (.), Q-DIAN=GO (+), MBTH=GO (*),
TOTAL NO. OF PTS. PLOTTED IS 44 AND 40. NOT PLOTTED BECAUSE THEY FALL OUTSIDE OF BOUNDS IS 1



SAMPLE	DATE	MONTHS	GLUC ANALZR	Q DIAN-GO	MBTH-GO
1.0	240873.	1.647	1.759	1.341	1.098
2.0	210973.	1.723	5.403	4.620	0.
3.0	161073.	1.797	1.579	1.266	1.135
4.0	91173.0	1.835	5.281	5.095	0.
5.0	71273.0	1.904	5.797	5.355	0.
6.0	290174.	2.079	6.704	4.934	0.
7.0	220274.	2.145	4.548	4.018	0.
8.0	210374.	2.219	0.	0.	0.
9.0	190474.	2.299	.8930	2.651	0.
10.	100574.	2.456	.4810	0.	0.
11.	200674.	2.488	.1480	.6960	0.
12.	110774.	2.536	.4320	0.	0.
13.	150874.	2.622	.9050	.8040	3.377
14.	170974.	2.712	1.477	1.075	.4630
15.	181074.	2.797	.6080	.9650	1.041

W.S.C. QUANTITATION BY A C.M.R. TECHNIQUE

Rapid and quantitative as it is, g.l.c. does have some distinct drawbacks. Principal among these would be the necessity for derivatisation, always a time-consuming process. To obtain statistically accurate results multiple injections are necessary, and even then accuracy is not guaranteed because of overlapping, or badly resolved peaks and time-variable response factors. The problem of overlapping peaks is not necessarily resolved even by resorting to analysis on two or even three different liquid phases, e.g. mannose, fructose, glucose (figs. 1, 2, 3). A typical time for analysis of each sample by g.l.c. was at least two hours using an instrument modified to run two chromatograms simultaneously. This did not take into account the time taken for derivatisation.

G.l.c. could be said to be satisfactory but time consuming when dealing with simple mixtures of compounds of known identity. If the sample under study contains unknowns then considerable effort must be expended to identify the unknowns and obtain response factors.

C.m.r. spectroscopy had proven to be particularly useful in studying the mixture of cyclitols obtained by oxidation of the w.s.c. extract (p. 54). Some six cyclitols were present in the mixture, but because of the large chemical shift of the ^{13}C-nucleus (200 ppm) and the high resolution possible ($\geq$ 0.15 ppm), few coincident peaks were observed. In fact it was possible to distinguish resonances (fig. 11) due to myo-inositol, pinitol, sequoyitol and pinpollitol without purification. Integration of clearly resolved resonances for each of the above gave a ratio of 10:100:27:6 which compares

favourably with that obtained by g.l.c. analysis of the TMS- derivative (9:100:24:?). In this particular example it was possible not only to identify the components of the mixture by the unique position of the C-resonances for each component, but also to obtain quantitative data, albeit crude as all response factors were assumed to be equal (an invalid assumption due to differences in T_1's and NOE's for each carbon).

An examination of published c.m.r. data on carbohydrates indicated that they too would be amenable to identification in a complex mixture, and possibly quantitation as well.

If c.m.r. is to be used as a quantitative method certain requirements must be fulfilled:-

1. A suitable internal standard must be selected. This should be of low volatility, preferably symmetrical (to reduce the number of resonances) with carbon resonances that do not interfere with those of components in the mixture. It would be an advantage if the T_1's of the internal standard were of the same order as those measured for cyclitols or carbohydrates (0.3-0.6 sec. for protonated carbons).
2. Solutions should be of constant viscosity so that the T_1's of the components remain in a constant relationship with those of the selected internal standard. If this is not maintained the relationship between the intensity (and thus the area) of the resonances of the standard and the components could fluctuate.
3. Constant temperature in order to maintain constant viscosity (viz. 2).
4. Constant machine parameters. By altering the pulse width

and/or the acquisition time, variations in the intensity of the resonances are possible. The fluctuation in intensity is either in a +ve or -ve sense depending upon the T_1 of the resonance in question.

5. Constant response factors. It is necessary that the "response factor" of the resonances for each component be linear over a wide range of concentration.

Two suitable internal standards were selected. These were dioxan and 1,3-propanediol. Dioxan with its single resonance with shorter T_1 was preferred.

Constant or near constant viscosity was achieved by working at a constant concentration of 500 mg/ml.

The machine parameters and probe temperature, once optimised, were held constant. Typically a temperature of 32°C was used with an acquisition time of 1 sec and pulse width of 17 μsec.

The response factor for each resonance in a given carbohydrate or cyclitol was determined at a concentration of 500 mg/ml against a constant concentration of dioxan in water (5% w/v). Pure carbohydrates were allowed to mutarotate before measuring the spectrum. Mixtures of carbohydrates in varying proportions confirmed that the response factors for all the commonly occurring carbohydrates and cyclitols in P. radiata were constant over a wide range of concentrations, with the total concentration being held at 500 mg/ml.

The analysis of sugar mixtures was facilitated by writing a subroutine for the Varian CFT-20 whereby the resonances of known carbohydrates and cyclitols were put on file and could be compared with the resonances obtained in

the spectrum of an extract. The identification of any sugar or cyclitol was possible because of the unique resonances for each component. Once the unique and resolvable resonances for the mixture were identified, the areas were digitised, compared to the internal standard and the standard responses already on file. The concentration of each component could therefore be calculated on any of the resonances for that component that are discernible. In practice as many peaks as possible were utilised in the analysis, and the result expressed as the mean with its mean square deviation. The accuracy of this method depended on the number of resolvable peaks, - usually ranging from 3 - 6 for most components - the final results coming to well within ±10% uncertainty.

A variable in the integration sub-routine allowed the "degree of resolution" of resonances to be selected. The "degree of resolution" selected determines the number of resonances utilized and the accuracy of the analysis.

The method worked well for carbohydrate mixtures and was extended to ion-exchange treated P. radiata w.s.c. solutions (500 mg/ml) where the major components could be identified and quantitated (fig. 12), although initially not without some difficulty. Polymeric material, in particular an arabino-galactan, created a more viscous solution and affected response factors. The results, after removal of the galactan by precipitation, compared favourably with those obtained from the g.l.c. analysis.

The c.m.r. method offers a number of convincing advantages over g.l.c. as a method of quantitation. In c.m.r. the position and area of the resonances observed for each sugar enables certain identification and greater reliability in

quantitation than can ever be achieved with g.l.c. where retention times are not related to structure.

The uncertainty inherent in TMS-ether preparation and the variability of g.l.c. detector response is avoided in the c.m.r. techniques where no chemical handling is necessary, and standards do not need to be checked even after long intervals.

The total sugar analysis of a w.s.c. using c.m.r. may only take 2 - 3 hours from the water extraction stage. Only one "run" is necessary - each peak is the mean of thousands of signals and the results calculated from each of these peaks can be averaged out for each component. In contrast, g.l.c. analyses can take up to a day for the accumulation of statistically meaningful results.

Unfortunately the expense and the present lack of availability of c.m.r. spectrometers limits its usefulness somewhat. Lack of sensitivity in the need for large samples (125-250 mg) could be limiting in certain circumstances.

Problems due to enhanced viscosity of solution because of the presence of polymeric material are diminished by suitable preparation of the samples.

On balance it would appear that the c.m.r. quantitative method has advantages over the more traditional g.l.c. method, but is probably best suited for the investigation and quantitation of mixtures of unknown composition rather than routine quantitative analysis.

APPENDICES

APPENDIX I

gc/ms Data on Acetate Derivatives

As with TMS- derivatives, polyacetates fragment in definite patterns, usually by removal of an acetate group from the parent ion:-

e.g. myo-inositol, M^+-OAc (432-59) = m/e 373

pinitol and sequoyitol, M^+-OAc = m/e 345

Apart from the ions at m/e 43/103/145, common to all acetate spectra, there are two basic modes of fragmentation for acetylated cyclitols (figs. 21 a, b, c):-

e.g. myo-inositol-Ac_6 (Sherman 1970),

(a) m/e 432 $\xrightarrow{-CH_2CO}$ 390 $\xrightarrow{-CH_3COOH}$ 330 → 270 → 210 → 168 ↗ 109 / ↘ 126

(b) m/e 241 $\xrightarrow{-CH_2CO}$ 199 → 157 → 115

In the O-methyl cyclitols pinitol and sequoyitol, a $-OCH_3$ group replaces an -OAc so the above two series become:-

(a) m/e 404 → 345 → 285 → 224 → 182 → 140

(b) m/e 213 → 171 → 129 → 87

The structures of pinitol -Ac_5 and sequoyitol -Ac_5 are different at only one carbon (fig. 20) so the mass spectra are very similar. In accordance with the mode of fragmentation (Sherman 1970) just discussed, the removal of the 4th and 5th acetate groups from carbons 1 and 5 from pinitol and carbons 1 and 3 from sequoyitol, will give the ion fragments m/e 182 and m/e 140 (series a). The ratio of the sum of the abundances

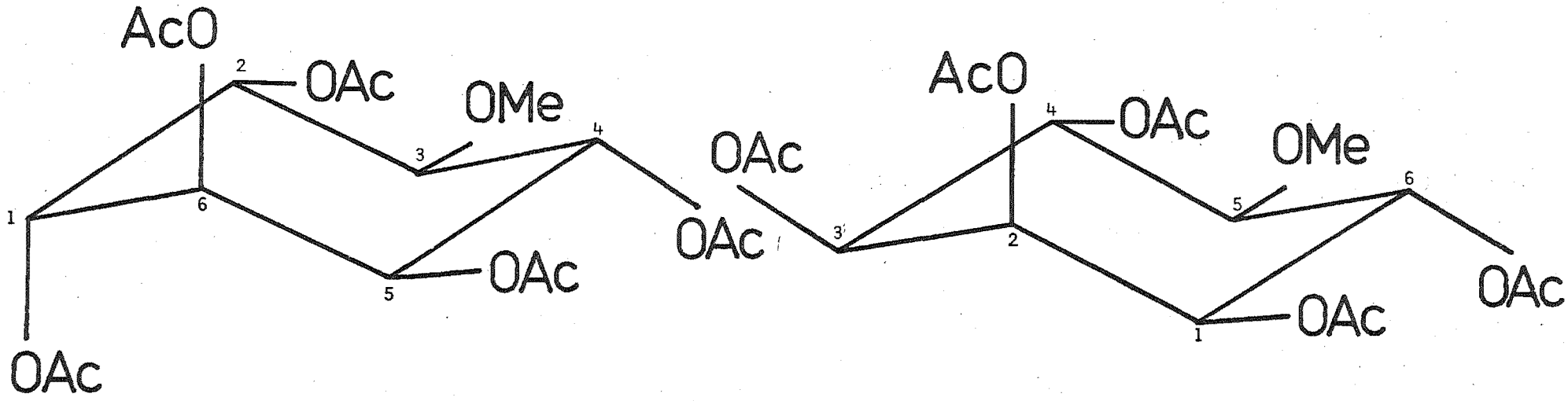
of these ions to m/e 109 (an ion formed independantly of the O-methyl cyclitol series) is considered for pinitol and sequoyitol:-

ms Source		$\frac{182 + 140}{109}$
Cyclitol mixture	pinitol	6.87
cyclitol mixture	sequoyitol	3.91
recrystallised	sequoyitol	3.31
auto-prepared	pinitol	7.81

1. m/e 109 abundance indicates the tendency for the parent to lose -OMe, (the ion m/e 109 doesn't have -OMe).
2. m/e 182, 140 abundance is proportional to, among other things, the ease with which the remaining two -OAc groups are removed from the parent O-methyl cyclitols.

Although it is impossible to say what exactly affects the ratio $\left(\frac{182 + 140}{109}\right)$, it is significant that in the observed spectra (figs. 21 b, c), pinitol and sequoyitol are affected to different extents and this could be due to the difference between the -OAc groups on carbon-1 axial in pinitol, and C-2 equatorial in sequoyitol.

Figure 20.



pinitol-penta-acetate
(3-O-Me-chiro-inositol)

sequoyitol-penta-acetate
(5-O-Me-myo-inositol)

Figure 21a.

Myo-inositol-hexa-acetate

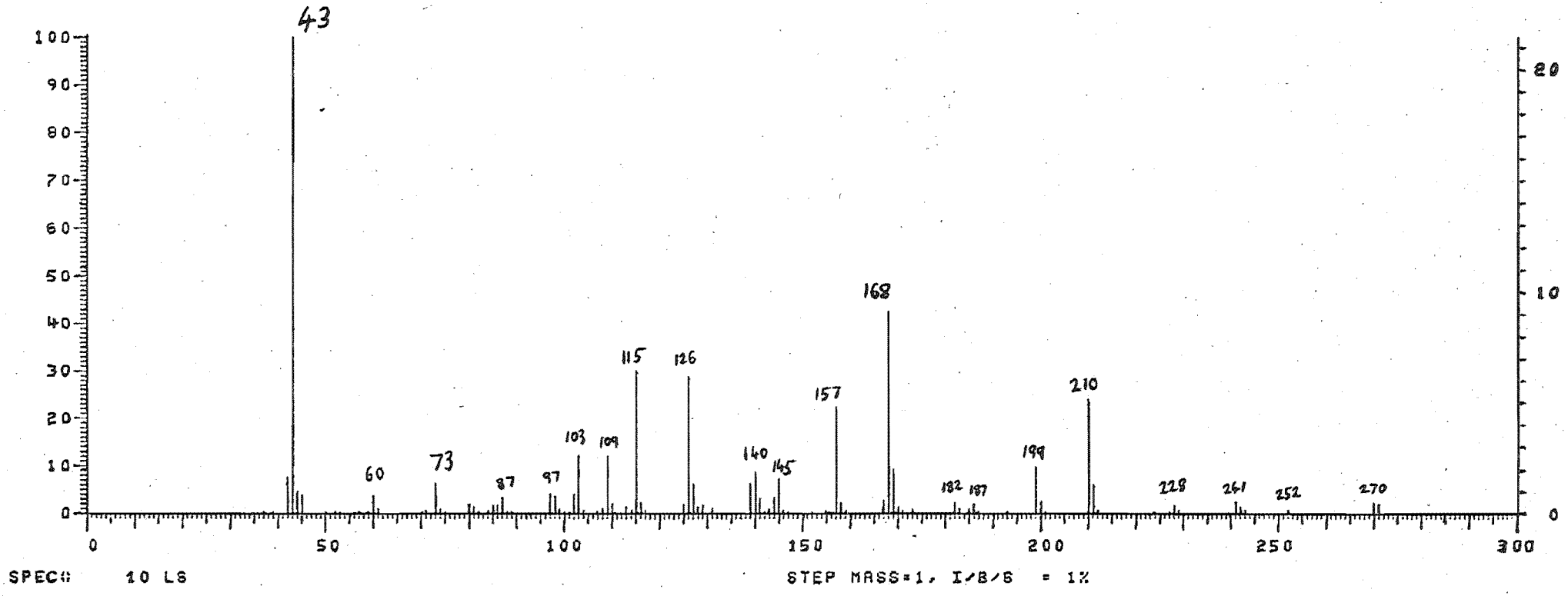


Figure 21b.

Pinitol-penta-acetate

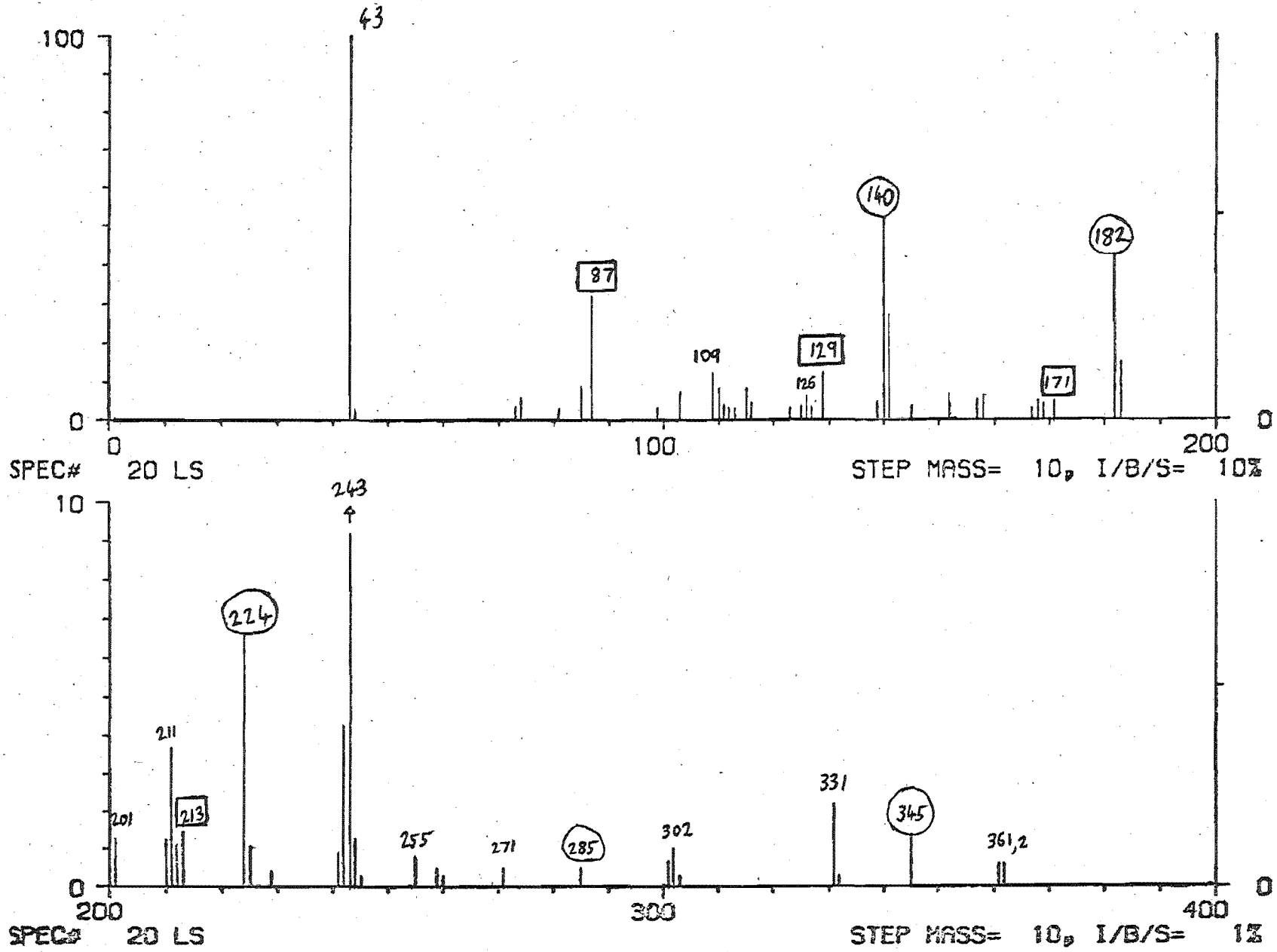
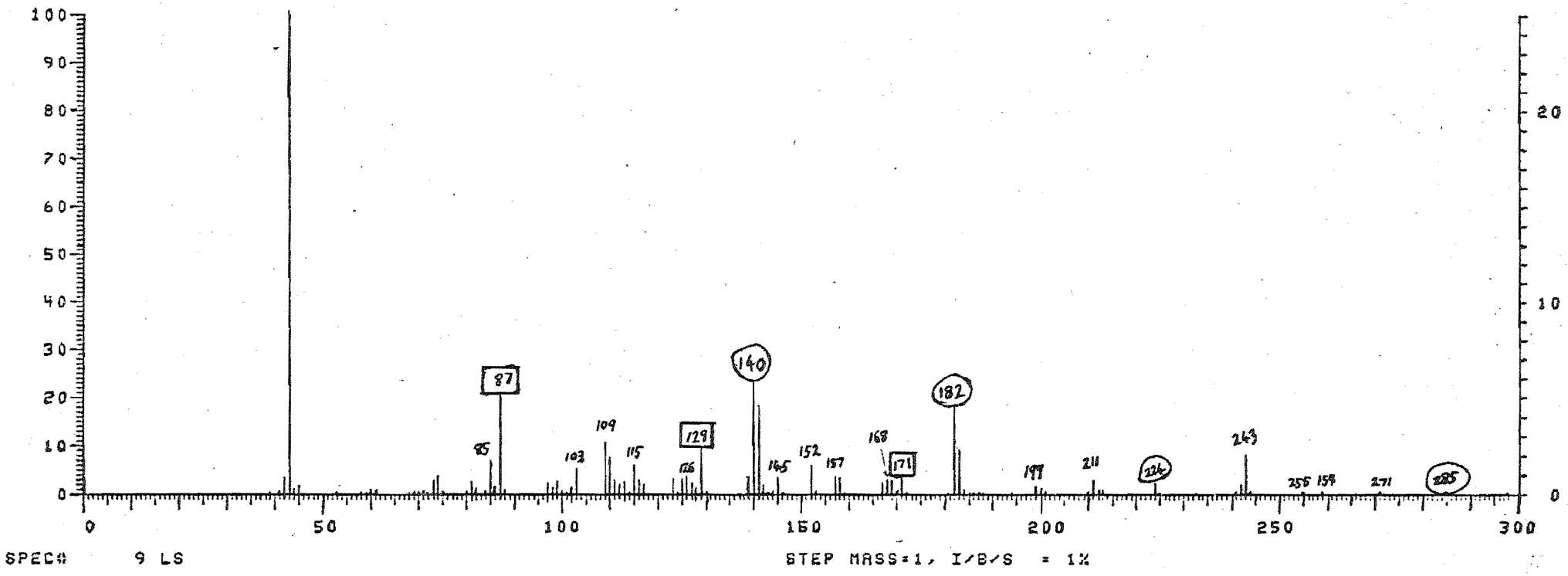


Figure 21c.

Sequoyitol-penta-acetate



APPENDIX II

gc/ms Data on Some TMS- Derivatives

Peak 19 (fig. 4) was not representative of fructose. This peak was known to disappear on ion-exchange treatment so it may well be an organic acid on top of the fructose peak. The characteristic TMS- fragmentations (p. 10) were present, but the higher molecular weight ions did not coincide with any recognised fragmentations. A dicarboxylic hexose could fit the pattern (fig. 4c):

e.g. M^{+}_{642}-2(TMSOH) = m/e 462 $\xrightarrow{-\dot{C}H_3}$ 447

462 $\downarrow$ -TMSOH

372

372 $\downarrow$ -C(=O)-OTMS

255

Peak 35 was a disaccharide (m/e 361) and it probably contained fructose (m/e 437) (Kotchetkov 1968, Kamerling 1971). There was no reason to say this peak was not TMS- sucrose.

The ms. of TMS-pinitol, sequoyitol, myo-inositol chiro-inositol and pinpollitol can be categorised in terms of the abundance of the m/e 305 and 318 ion fragments:-

chiro-inositol, pinitol, pinpollitol m/e 318 > 305

myo-inositol, sequoyitol m/e 305 > 318

This was true for all cyclitol spectra studied.

APPENDIX III

Carbohydrate/cyclitol Standard Mixture

The standard mixture of carbohydrates and cyclitols (p. 50) consisted of:-

ribitol	26.8 ± 0.4 mg
fructose	27.8 ± 0.2
glucose	24.9 ± 0.2
myo-inositol	25.4 ± 0.2
pinitol	23.4* ± 0.6
sequoyitol	24.7* ± 0.3

* Pinitol and sequoyitol contained impurities:-

pinitol contained 7% sequoyitol
+ 7% myo-inositol

sequoyitol contained 8% myo-inositol

23.4 and 24.7 mg represent the adjusted weights for pinitol and sequoyitol respectively. The standard integrals of sequoyitol and myo-inositol had to be reduced by the appropriate factor. For example, the true integral of sequoyitol was the recorded value, less the 7% from pinitol.

APPENDIX IV

g.l.c. Data on the Lesser Peaks

Small peaks that occasionally appeared on the chromatograms of the P. radiata analyses were found at retention times:- R_G = 0.62, 0.66, 0.96, 1.02, 1.07 measured with respect to TMS- α-glucose on SE-30. However, they were usually too small to be measured quantitatively.

Galactose was found in 2 samples (4, 44). A pollen sample (44) is depicted in fig. 22, the galactose peaks labelled as 170 and 264. The data for galactose is recorded under "unknown 2" (fig. 23) with an arbitrary standard response factor of 1.00. The response factor of galactose relative to ribitol was determined at a later date (0.660). Hence the real galactose concentration in pollen tissue is

$$\frac{6.633}{0.660} = 10.05\%$$

Figure 22.

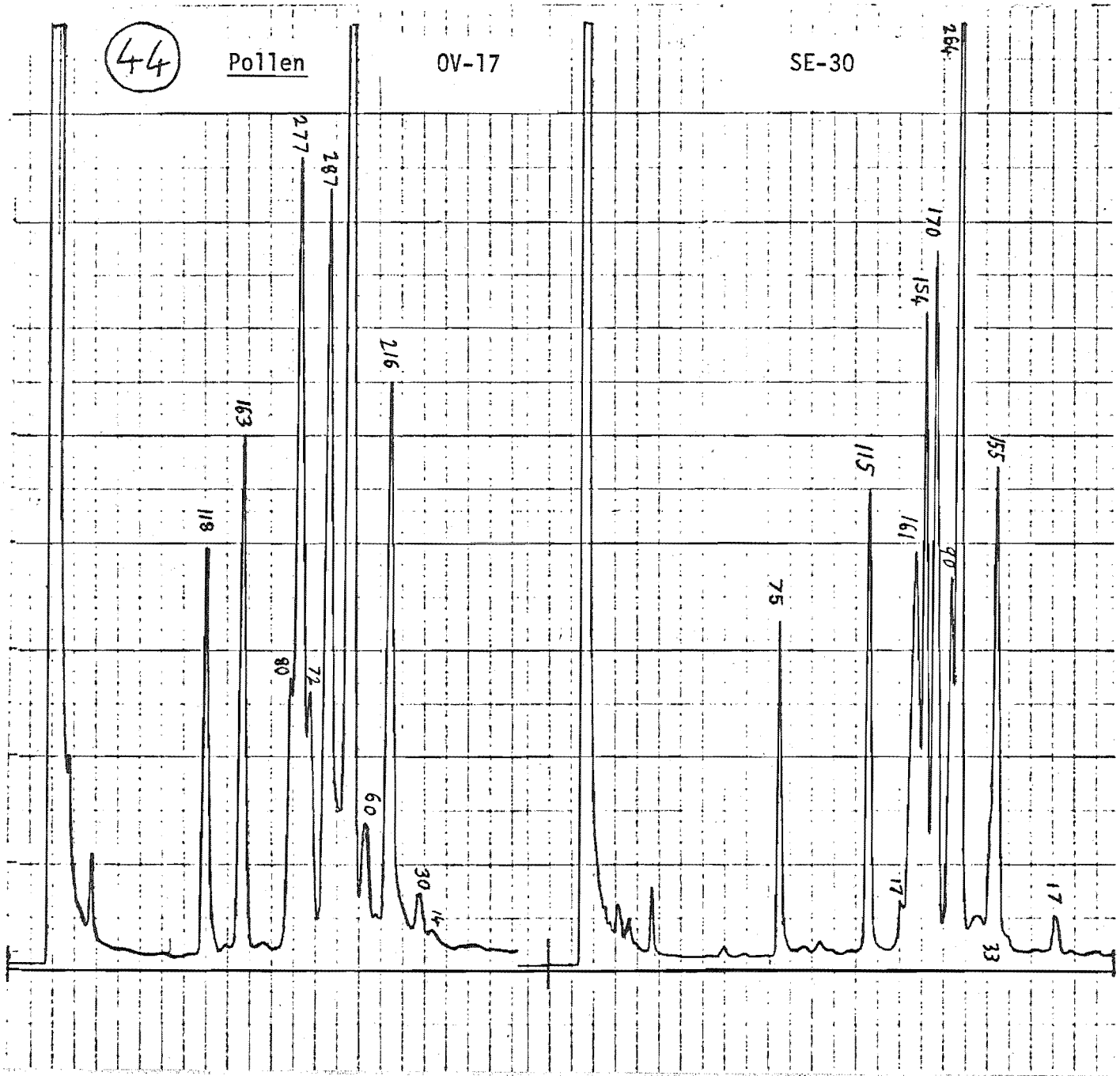

44
Pollen
OV-17
SE-30
277
287
216
163
118
80
72
60
30
14
284
170
154
155
115
191
90
75
17
33

Figure 23.

MALE FLOWER 17/9/74 SAM 44 SE30(A) ON 367.10 MG OF TISSUE

DILUTION FACTOR= 12.5 C16 REPONSE=100.0

RELATIVE STANDARD HEIGHTS:

0.3936	1.0000	1.0377	0.8738	0.9284	0.9209	0.9473	1.0000	1.0000	1.0000

MG. OF RIBITOL=0.4964

C16	RIBITOL	FRUCTOSE	PINITOL	GLUCOSE	SEQUOYIT	MYOINOSI	UNKNOWN1	UNKNOWN2	UNKNOWN3
CRUDE STANDARD INPUT:									
100.00	185.00	123.00	144.00	184.00	146.00	176.00	185.00	185.00	185.00
84.00	188.00	110.00	131.00	155.00	140.00	155.00	188.00	188.00	188.00
90.00	190.00	114.00	133.00	170.00	135.00	153.00	190.00	190.00	190.00
80.00	161.00	106.00	127.00	150.00	131.00	145.00	161.00	161.00	161.00
SCALED DATA:									
100.00	185.00	123.00	144.00	184.00	146.00	176.00	185.00	185.00	185.00
100.00	223.81	130.95	155.95	184.52	166.67	184.52	223.81	223.81	223.81
100.00	211.11	126.67	147.78	188.89	150.00	170.00	211.11	211.11	211.11
100.00	201.25	132.50	158.75	187.50	163.75	181.25	201.25	201.25	201.25
MEAN AND STD.DEVIATION:									
100.00	205.29	128.28	151.62	186.23	156.60	177.94	205.29	205.29	205.29
* 0.0000	*16.3795	* 4.2985	* 6.8904	* 2.3499	*10.1387	* 6.3537	*16.3795	*16.3795	*16.3795
% ERROR:	8.0%	3.4%	4.5%	1.3%	6.5%	3.6%	8.0%	8.0%	8.0%
RESPONSE FACTORS :									
1.2376	1.0000	0.6022	0.8452	0.9771	0.8284	0.9150	1.0000	1.0000	1.0000
CRUDE DATA INPUT									
63.00	98.00	148.00	135.00	197.00	28.00	16.00	12.00	408.00	0.00
56.00	90.00	129.00	120.00	180.00	26.00	13.00	11.00	358.00	0.00
84.00	125.00	171.00	158.00	230.00	36.00	19.00	15.00	484.00	0.00
64.00	95.00	132.00	125.00	178.00	27.00	12.00	13.00	368.00	0.00
73.00	111.00	151.00	146.00	206.00	32.00	17.00	16.00	430.00	0.00
75.00	115.00	161.00	154.00	212.00	33.00	17.00	17.00	434.00	0.00
SCALED DATA:									
100.00	155.56	234.92	214.29	312.70	44.44	25.40	19.05	647.62	0.00
100.00	160.71	230.36	214.29	321.43	46.43	23.21	19.64	639.29	0.00
100.00	148.81	203.57	188.10	273.81	42.86	22.62	17.86	576.19	0.00
100.00	148.44	206.25	195.31	278.13	42.19	18.75	20.31	575.00	0.00
100.00	152.05	206.85	200.00	282.19	43.84	23.29	21.92	589.04	0.00
100.00	153.33	214.67	205.33	282.67	44.00	22.67	22.67	578.67	0.00
MEAN AND STD.DEVIATION:									
100.00	153.15	216.10	202.89	291.82	43.96	22.66	20.24	600.97	0.00
* 0.0000	* 4.5888	*13.4097	*10.4914	*20.0050	* 1.4631	* 2.1671	* 1.7981	*33.3839	* 0.0000
% ERROR:	3.0%	6.2%	5.2%	6.9%	3.3%	9.6%	8.9%	5.6%	0.0%
C16	RIBITOL	FRUCTOSE	PINITOL	GLUCOSE	SEQUOYIT	MYOINOSI	UNKNOWN1	UNKNOWN2	UNKNOWN3
MG OF EACH PRESENT:									
3.274	6.205	14.540	9.726	12.101	2.150	1.003	0.820	24.349	0.000
PERCENTAGE WOODWT OF EACH:									
0.892%	1.690%	3.961%	2.649%	3.296%	0.586%	0.273%	0.223%	6.633%	0.000%
TOTAL ABSOLUTE ERROR:									
0.000	0.186	0.378	0.257	0.268	0.057	0.036	0.038	0.898	0.000

APPENDIX V

The 51 *P. radiata* samples analysed by g.l.c. were made up of the following types:- buds (fig. 24), needles (fig. 18), wood (fig. 25), new needles (fig. 26), new wood (fig. 27) and pollen (fig. 22). In general, all samples contained all the carbohydrates and cyclitols that were in the standard mixture (Appendix III); pinitol and glucose dominated.

Figure 24.

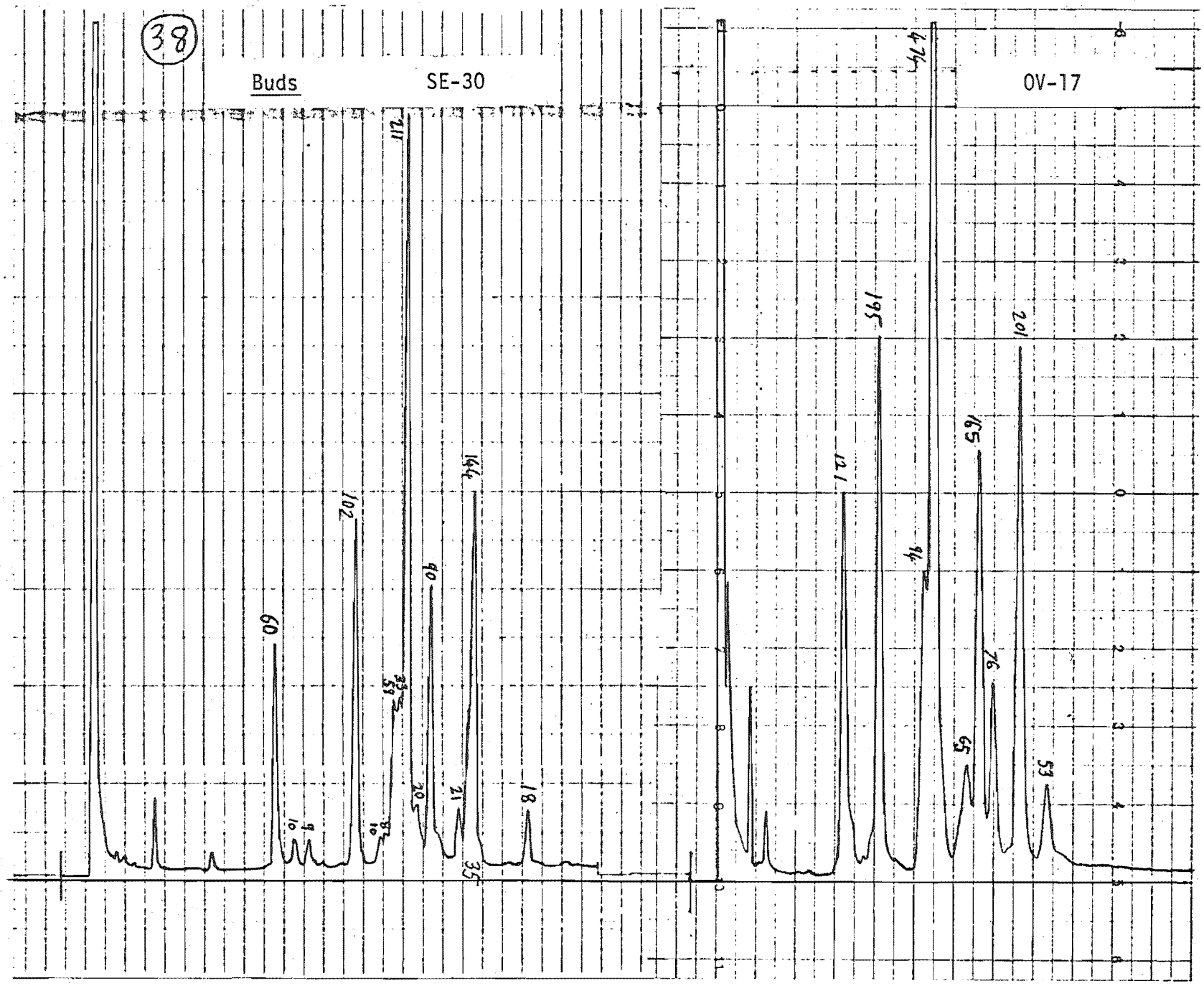

38
Buds
SE-30
OV-17
60
10
9
102
10
8
35
211
20
40
21
144
35
18
474
195
94
121
165
76
65
201
53

Figure 25.

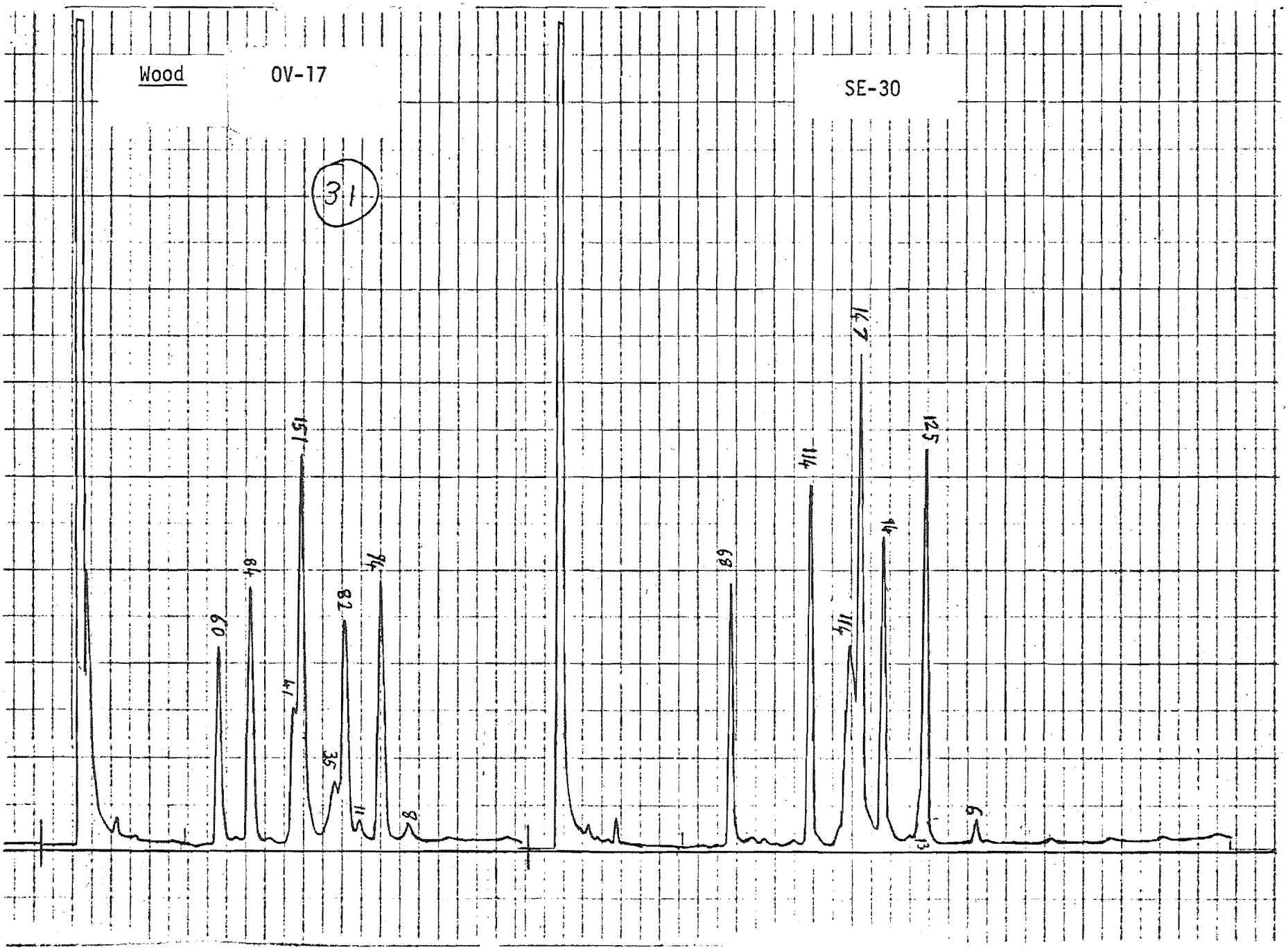

Wood
OV-17
SE-30
31
60
48
151
41
82
35
74
11
8
89
114
114
147
14
125
13
9

Figure 26.

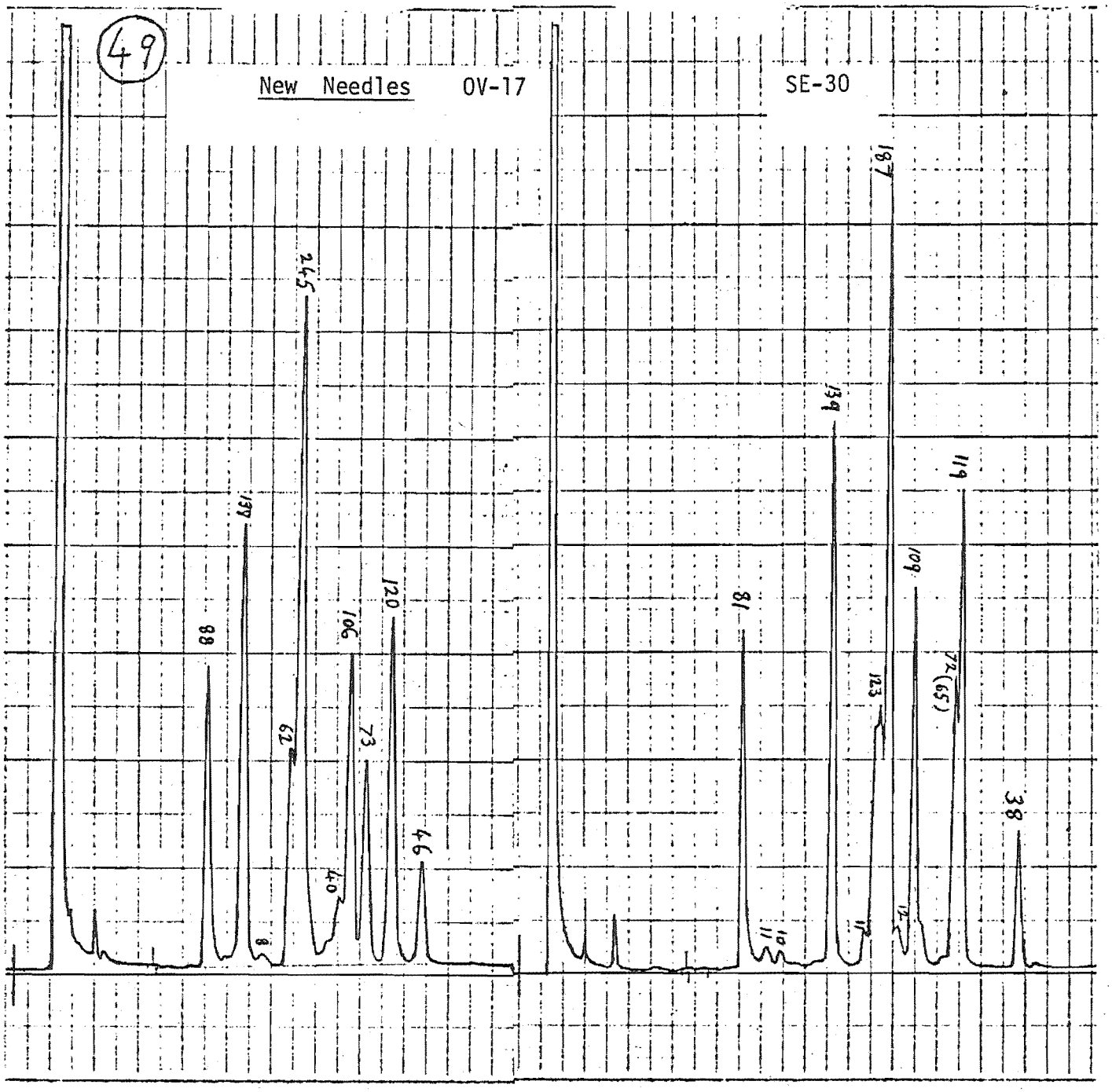
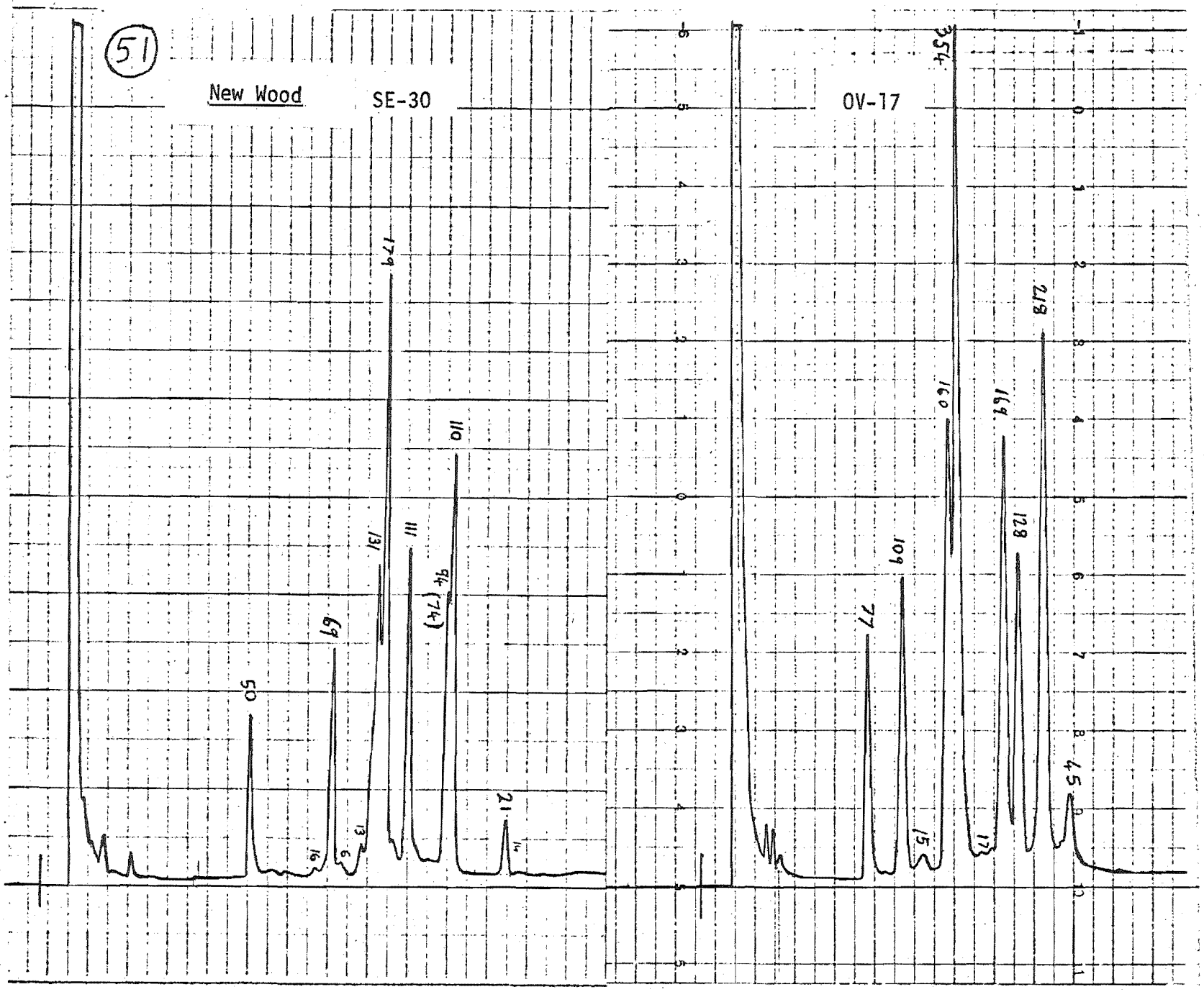

49
New Needles
OV-17
SE-30
245
137
88
62
106
73
120
40
46
8
187
139
119
109
81
72 (65)
123
38
12
11
10

Figure 27.


51
New Wood
SE-30
OV-17
50
69
179
131
111
94 (74)
110
21
354
160
109
77
169
128
219
45
15
17

EXPERIMENTAL

SAMPLE PREPARATION

I Sampling

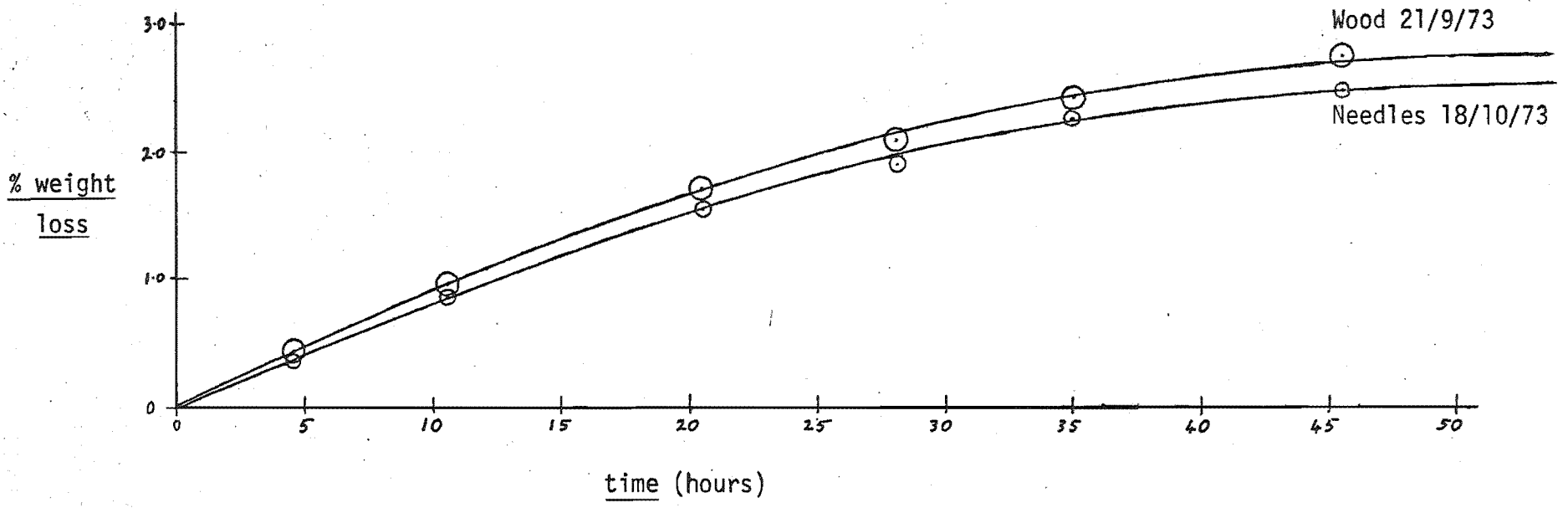
The Pinus radiata samples were collected monthly from Ashley Forest using trees of the same clonal stock. The weather conditions, sunshine and wind (bright sunshine and wind less than 5 knots), and the time-of-day collected (mid-afternoon) were kept constant. Each collection consisted of 4 of the top lateral branches cut 1 foot from the top of each branch. These were immersed in liquid air either at the time of collection or less than 1 hour after harvest, separated into needles, wood, buds or new wood and new needles depending on the time of year. The plant material was then air-dried (Hehl 1973). The separated plant material was ground in a mechanical mill (A. H. Thomas Co. Philadelphia) to pass through a 60 mesh sieve. The resins and/or pigments were extracted with dichloromethane in a soxhlet extractor. Typically, needles were extracted for 12 hours; buds and wood for 8 hours.

All P. radiata samples, 51 altogether, were stored in jars until required.

II Isolation of W.S.C.

All P. radiata samples (500 mg) were dried over concentrated H_2SO_4 in a desicator for a period of 48 hours. This "drying period" was determined for ground (60 mesh) needles (500 mg) and a similar sized sample of wood tissue. These were placed over concentrated H_2SO_4 in standard volumetric flasks (50 ml) and periodically weighed until a constant weight was attained (fig. 28). Water loss from all 51 samples was approximately

Figure 28. Water Loss Vs. Drying Time.



3% of the dry weight.

Two identical tissue samples (8.00 g) were extracted, one with 80% ethanol/water and the other with water. The 2 extracts were lyophilysed (1.111 g, 1.667 g) and 500 mg of each analysed by Carbon -13 (c.m.r.). The intensities of the known peaks were about the same for both spectra indicating that the ethanol extraction had pulled out only 2/3 of the important carbohydrates relative to the cold water extraction.

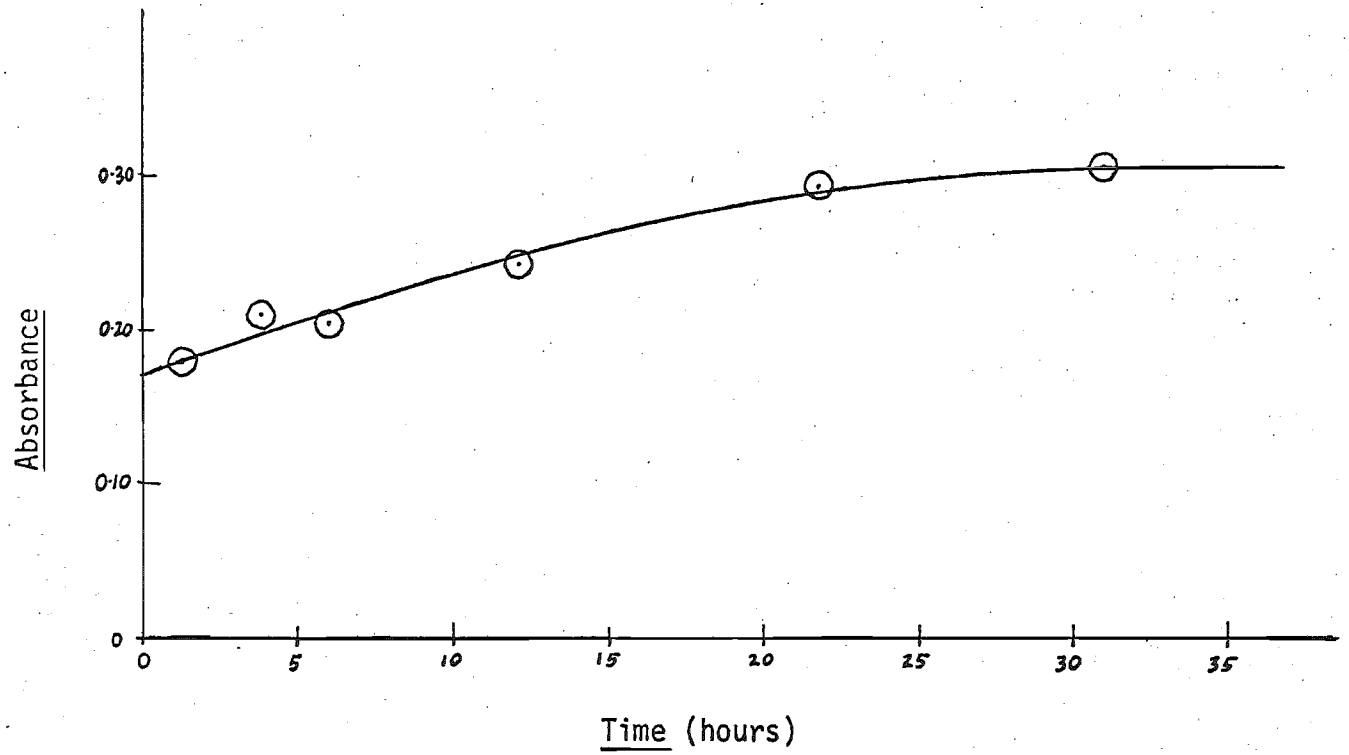
A ground (60 mesh) wood tissue sample (5.00 g) was extracted 3 times with cold water (60, 60, 30 ml) for a period of 12, 18 and 21 hours. Each time the mixture was vacuum filtered and the filtrate evaporated to dryness (3.50g, 30 mg, 5 mg respectively) and analysed by gas-liquid chromatography (g.l.c.) (see below). A fourth extraction (3 hours) using hot water (30 ml, 80-90°C) produced gelatinous material (50 mg). No carbohydrates were observed in extractions 3 and 4 and results indicated :

(a) 99% of carbohydrates were extracted in the first extraction;

(b) the hot water extract produced no more of the important compounds - only polymeric material which interfered with the silylation procedures.

The most suitable extraction time was determined by monitoring the glucose concentrations over a period of 30 hours. The glucose oxidase method was used. From 2 volumetric flasks (50 ml) each containing ground needle tissue immersed in water (30 ml), aliquots (2 ml) were taken from alternate samples after 1, 3 3/4, 6, 12, 22, 31 hours. Treatment with activated charcoal (25 mg) and dilution (1/12) was necessary before glucose estimation (fig. 29). The curve of absorbance v. time leveled off at about 30 hours. The extraction time

Figure 29. Glucose Oxidase Absorbance Vs. Extraction Time.



decided on for this analysis was 36 hours.

The analytical procedure adopted for the quantitative extraction of w.s.c. from P. radiata was this: Tissue (approximately 500 mg) was weighed into a standard volumetric flask (50 ml), dried over concentrated H_2SO_4 for 48 hours and reweighed. The dry sample was mixed with streptomycin sulphate solution (10 ml, 2 mg/ml), shaken vigorously to break up the clumps and the sides of the flask washed with further distilled water (10 ml). The tissue was extracted at 40°C for 36 hours, cooled, left for 6 hours to settle before making up to volume. A sample of supernatant (approximately 40 ml) was drawn off by pipette and treated with damp Amberlite MB-3 ion-exchange resin (5 g) to remove ionic material such as amino acids and low molecular weight carboxylic acids.

Two aliquots of the aqueous extract (2 x 4 ml) were pipetted into 2 reaction vials and evaporated to dryness with compressed air and gentle warming (not exceeding 50°C). A 10-port gas manifold supplied air to 10 vials mounted in a ½-inch thick block of aluminium, heated by a temperature regulated hotplate. The time for evaporation was about 4 hours.

The vials were stored below 0°C until required for g.l.c. analysis.

IDENTIFICATION OF W.S.C.

I Gas Chromatography

Equipment

The gas chromatograph used was a Varian Aerograph Series 2100 with dual electrometers and flame ionization detection. Modifications enabled 2 channels to be run simultaneously. The outputs were connected to digital integrators; a Kent Chromalog Integrator with printout (span 0.5 mv, cut-off 0.1%, dip detector "V"), and a Townsend Digital Integrator.

The columns ('U' shaped glass, 6 ft. x 1/8 in. I.D.) used in this work were packed with the liquid phases; 3% SE-30, 2½% OV-17, 2½% OV-225, coated onto Varian Aerograph Varaport-30 (100-120 mesh) as support. Typically, the packings were prepared (Holligan 1971 b, Sawardeker 1965) by dissolving a pre-weighed amount of the liquid phase in warm chloroform (10 ml). This solution was poured over the Varaport-30 (5 g) in a round bottomed flask and rotated intermittantly under vacuum until all the chloroform had evaporated. Pre-silanised (10% TMS/Benzene for 3 days) glass columns were packed with freshly prepared material under pressure and vibration. All columns required conditioning at 290°C for at least 24 hours with nitrogen carrier gas flow (approximately 12 ml/min). An efficiency versus nitrogen flow rate curve was plotted for each column. A flow rate of about 12 ml/min (equivalent to '5' on the carrier gas flow gauge of "2100") gave the greatest efficiency.

Some injector septums (Pierce Chemical Co. Silicon Teflon-Lined High Temperature Septums, Neoprene Sheet) produced extraneous peaks on columns at high temperatures.

The teflon-coated S.G.E. septums (P.T.F.E.) did not give any trouble.

To ensure a steady base line, and a reproducible response, it was necessary to clean the flame tips and the towers at regular intervals.

Reagents

Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were mixed in a ratio of 2:1 respectively to give the trimethylsilane (TMS) reagent (Sweeley 1963).

Analytical grade pyridine (500 ml) was dried by refluxing over KOH pellets (50 g) for 2 hours followed by distillation. Stored over KOH.

The sugars : Xylose, fructose, galactose, glucose are B.D.H. reagents; mannose, L-sorbose, mannitol, D-ribose from L. Light and Co.; maltose, sucrose, Hopkin and Williams and myo-inositol from Sigma Chemical Co.

The solvents carbon tetrachloride and pentane were dried over molecular sieves (type 5A).

Streptomycin sulphate from the American Cyanimid Co.

Preparation of Standards

(a) n-Hexadecane (1.200 g) was made up to 1000 ml with dry CCl_4. The resultant 1.20 mg/ml solution was stored over molecular sieves.

(b) Sodium borohydride (2.00 g) was dissolved in distilled water (50 ml) and D-ribose (2.00 g) added . The solution was kept chilled for 24 hours, water added (to 500 ml) and the pH adjusted to 4.5 with glacial acetic acid. Chromatography on Amberlite MB-3 (200 g) ion-exchange resin removed all ionic material. The eluant was taken to dryness and crystallised from ethanol to give ribitol (1.14 g, 57% yield), m.p. 101.5°C

(literature: 102°C). A sample of ribitol (125.3 mg) was made up to 250 ml with dry pyridine (0.5012 mg/ml).

(c) Samples (≏ 25 mg) of ribitol, pinitol, fructose, glucose, sequoyitol and myo-inositol were accurately weighed into a 50 ml volumetric flask, dissolved in dry pyridine and made up to volume. Not all the myo-inositol went into solution so homogeneity of the mixture was ensured by shaking the solution prior to aliquot removal.

Four aliquots of the standard solution (1 ml) were derivatised (see below) and the TMS ethers extracted with dry pentane (5 ml). The 4 samples were combined, evaporated to dryness and dissolved in the n-hexadecane/CCl_4 standard (2 ml). This standard solution was stable if stored below 0°C.

(d) Aqueous solutions of : xylose, mannose, fructose, galactose, sorbose, glucose, mannitol, maltose and sucrose were prepared by dissolving the carbohydrates (12.5 mg) in distilled water and making up to volume (25 ml). After standing at room temperature for 24 hours to reach equilibrium (Jacin 1968), an aliquot (2 ml) of each sample was evaporated to dryness; dissolved in pyridine (1 ml). The TMS ethers were prepared and extracted with pentane as before.

Silylation

The basic method adopted was that of Sweeley (1963). In all derivatisations, the excess TMS reagent and pyridine were evaporated at room temperature by a stream of nitrogen. All mixing of the TMS reagents was done with glass syringes. Silylation will be discussed more fully in the quantitation section.

Injection

Accurate analyses by g.l.c. require reproducibility of chromatograms which in turn are dependant on the column conditions and injection techniques. Using the standard carbohydrate and cyclitol solutions (see above c, d), retention data was accumulated using a temperature program $140^{\circ} \rightarrow 250^{\circ}$ at $8^{\circ}C$/min, commencing the program at injection. The sensitivity of the instrument was such that a carbohydrate sample (1 mg/ml), when derivatised, produced a chromatogram with a good stable baseline (attenuator 8×10^{-10}). Once the retention times for the major carbohydrates were obtained, the peaks in the derivatised P. radiata extract could be tentatively identified by co-chromatography. Glucose, fructose, mannose, mannitol, sucrose and myo-inositol were found this way.

II gc/ms

A TMS derivatised P. radiata wood (May 1973) extract was run on a gas chromatograph/mass spectrometer (Ch. 7 Atlas Varian M.A.T.) giving mass spectra of all the components resolved on an OV-17 column (fig. 4). Spectra were also obtained for the TMS and Acetate derivatives of the cyclitols (see below), pinitol, sequoyitol and myo-inositol, using the instrument, but with O V-1 as liquid phase. G.C. Conditions:- Injector $290^{\circ}C$; detector $310^{\circ}C$; carrier gas flow 20 psi; programmed $120^{\circ} \rightarrow 300^{\circ}C$ at $10^{\circ}C$/min; 6 ft. O V-1, 0.75% on G.C.-Q.

Mass spectrometer:- accel. voltage 3 kV
electron energy 70 eV
emission current 300 μa
analyser pressure 10^{-8} Torr

source pressure 6×10^{-6} Torr

source temperature 250° C

capillary temperature 300°C

separator temperature 300°C

L.O.S. Probe Temperature 300°C

resolution 1000

The concentrations of the gc/ms samples were 60-80 μg/μl and 1-2 μl was injected for each run.

III Carbon-13 n.m.r. (c.m.r.)

Analyses were carried out on a Varian CFT-20 spectrometer under the following conditions:

spectral width	2k Hz.
no. of transients for a typical *P. radiata* w.s.c. extract	15,000-24,000
acquisition time	1 sec.
pulse width	7 μ sec.
data points	4k/8k.
transmitter offset	57.
receiver gain	3
decoupler mode	1
decoupler offset	52
noise band width	1 k Hz.
Sens. enhancement	-1.0
width of plot	1k Hz.
width of chart	1k Hz.
reference line	1348.

The 1 ml samples of carbohydrates and/or cyclitols usually consisted of 500 mg of the appropriate sample. Dioxan (5% W/V) was used as the internal standard and weighed into the aqueous solution immediately before the analysis. A precise quantity of dioxan is required for accurate quantitation. Preparation of a P. radiata sample for c.m.r. was basically : water extraction of approximately 4-5 g tissue using 2 x 200 ml distilled water; vacuum filtration; ion exchange treatment of filtrate (Amberlite MB-3); evaporation (rota-evaporation) followed by lyophilisation.

IV Isolation of Pinpollitol (1,4-O,O-dimethyl chiro-inositol)

An air-dried P. radiata needle sample (60 mesh) (99.2 g) collected May 17 1974 was stirred vigorously with distilled water (1 l) at 34°C for 22 hours. The aqueous extract was filtered through a bed of celite 545 which was then washed with fresh water (500 ml). The total aqueous extract (1.21 l) was freeze dried on an Edwards Centrifugal freeze drier to give a gummy powder that smelt strongly of pine needles. Dissolved in water (200 ml) and saturated with Na_2CO_3. Extracted 3x with ethanol (200 ml). The total ethanol extract was back-washed with saturated Na_2CO_3 solution (200 ml) and evaporated to dryness on a "rotavap". The solid was dissolved in CH_3OH/H_2O (1:1) and filtered. The residue was discarded. The process was repeated a further 3x with methanol in order to remove as much ionic material as possible. Weight of gum = 9.35 g.

A sample of this was placed in a distillation tube (30 cm x 15 mm I.D.) and vacuum (10^{-4} Torr) distilled in a 160°C furnace. After about 24 hours the distillate that collected along the cooler parts of the glass was washed from

the tube and a sample of this derivatised (TMS) and put through the g.l.c. (fig. 13).

The total needle extract was similarly distilled (436 mg) and a sample of this was used to obtain a c.m.r. spectra (fig. 9). There was about 1½x as much pinitol as there was pinpollitol.

V Isolation of Cyclitols

Ground P. radiata wood tissue (60 mesh), (May 1974, 22.26 g) was stirred with distilled water (200 ml) for 3 hours in a conical flask (250 ml). After filtering the extract through a sintered-glass funnel, the extraction was repeated on the residue. The total filtrates and washings were combined and evaporated to dryness on a "rotavap" at low temperatures to give an oil (1.960 g). Redissolved in water (10 ml) and ethanol (90 ml) added. A white solid precipitated out. The supernatant solution was decanted into a round-bottomed flask (500 ml). The precipitate was washed with water (3 ml) and these washings combined with the bulk solution.

2,4-Dinitrophenylhydrazine (2,4-DNP) (8.0 g) was partially dissolved in ethanol (200 ml) and acidified with concentrated H_2SO_4 (5 ml). This mixture was added to the wood sugar extract. A thick gelatinous orange precipitate formed. After 24 hours at room temperature, the crystals were filtered off and the filtrate concentrated to approximately 100 ml. The addition of water precipitated out further osazones. The ethanol was removed on a "rotavap" and the orange crystals (4.61 g) filtered and washed. The remaining aqueous solution (300 ml) was deionized by chromatography on 2 ion-exchange

columns linked in series. The first was Amberlite IR 120 (250 g, in the H^+ form) followed by Amberlite IRA 410 (250 g, in the OH^- form). The now colourless eluant was evaporated to dryness to give a cyclitol mixture (320 mg).

Crystallisation from methanol (3 ml) gave small white rhombic crystals of sequoyitol (38 mg) which were recrystallised from methanol : m.p. = 236 - 238°C (literature 239°C). The mother-liquors were taken to dryness and recrystallisation from ethanol (3 ml) gave small granular crystals of pinitol (137 mg) which were recrystallised from ethanol : m.p. = 178 - 179°C (literature : 186°C). The cyclitol mother liquors (96 mg) contained pinitol and sequoyitol in reduced amounts, pinpollitol, myo-inositol and 2 other O-methyl-inositols (fig. 6). The g.l.c. traces of the purified sequoyitol are seen in fig. 5.

The cyclitol mother liquors were treated with 2,4-DNP to remove any possible remaining traces of monosaccharides that might be present. The method used was as above scaled down by 1/20. No change was observed for the treated mother liquors, consequently all peaks in the trace must belong to inositols, methylated or unmethylated.

VI De-methylation of O-methyl- and O,O-dimethyl-inositols

A sample of pinitol (17.8 mg) was refluxed in 50% hydriodic acid (0.4 ml). After 1½ hours the HI and I_2 were evaporated off under a stream of nitrogen and the residues taken up in methanol (2 ml). The dark-brown oil slowly crystallised to give white crystals of chiro-inositol.

The cyclitol mother-liquors were similarly treated to

produce crystals of mainly chiro-inositol (12.5 mg). Of the total material in the cyclitol M.L.'s some 90% was hydrolysed to chiro-inositol. By comparing peak ratios, the first 4 peaks of the M.L.'s run on S E -30 have chiro-inositol parentage; the last 2 peaks are from myo-inositol (the 6th one is actually myo-) (fig. 6).

QUANTITATIVE ANALYSIS OF W.S.C.

I Calibration of Syringe

Calibration of the 1 cc "stopped" syringe was done by repeated weighings of dry pyridine. The mean weight after eleven injections was 0.9726 g per injection. The density of pyridine at 20°C is 0.9819 g/ml.

$\Rightarrow$ Volume of pyridine at each injection = $\frac{0.9726}{0.9819}$

Hence "1 cc" of standard ribitol (0.5012 mg/ml)

= 0.9905 x 0.5012

= 0.4964 mg

II Sample Preparation

A standard solution of ribitol in pyridine (1 ml, 0.5012 mg/ml) was injected into each of the vials (7 ml) containing dry P. radiata w.s.c. samples (See "Isolation of W.S.C."), using the calibrated "stopped" syringe. The capped vials were vigorously shaken for $\frac{1}{2}$ hour. Freshly prepared TMS reagent (HMDS : TMCS, 2:1, 1 ml), (Sweeley 1963) was added and the vial occasionally shaken.

After 1 hour the excess TMS and pyridine were evaporated by a stream of nitrogen. Ten samples were evaporated simultaneously. The silyl ethers were dissolved in n-hexadecane/CCl_4 standard solution (0.50 ml, 2.09 mg/ml).

III Injection

An SGE 10 µl syringe was used to inject 1.0 µl samples. All analyses were done under the following conditions:

Detector temperature 300°C, injector temperature 200°C, Column temperature 140° → 270°C at 8°C/min, starting the linear temperature program at the time of injection; the nitrogen carrier flow rate was 12 ml/min; the hydrogen pressure 12 p.s.i.; attenuator setting at 8, with range 10^{-10}; chart speed 60 cm/hr.

Two samples were injected onto columns A and B within 10 seconds of one another then the temperature program commenced. After 3 runs the duplicate samples were similarly analysed on columns A and B. A standard mixture was analysed before and after each set of duplicate samples. 47 P. radiata samples were analysed on S E -30 columns and 4 on OV-17.

IV Results

The integration data was processed on a Burrows 6718 computer using a program developed for the analysis. Copies of 2 work sheets are seen in fig. 17, 23.

An explanation of terms follows:-

1. Dilution Factor: only 4 ml of the 50 ml aqueous carbohydrate solution taken.
2. C_{16} Response: 1.0 µl of C_{16} standard in CCl_4 gives a response of 100.
3. Relative Standard Weights: ratio of weights of standard carbohydrates relative to ribitol.
4. M.G. of Ribitol: weight of ribitol added to each tissue sample.
5. Crude Standard Input: actual integral responses for standards.

6. Scaled Data: all above data scaled on $C_{16} = 100$.

7. Mean and Standard Deviation: read * as $\pm$.

8. Response Factors:

$$\text{e.g. } \frac{\text{Mean Resp. fructose}}{\text{Mean Resp. ribitol}} \times \frac{1}{\text{Relative wt. fructose}}$$

$$\text{e.g. } = \frac{120.72}{190.34} \times \frac{1}{1.0377} = \underline{0.6112}$$

9. Crude Data Input: same as before.

10. M.G. of Each Present:

$$\frac{\text{Mean Resp. fructose}}{\text{Mean Resp. Ribitol}} \times \frac{\text{Dilution Factor x Wt. Ribitol}}{\text{Resp. Factor Fructose}}$$

$$\text{Same e.g. } \frac{256.40}{161.30} \times \frac{12.5 \times 0.4964}{0.6112} = \underline{16.138}$$

11. Percent Wood Wt. of Each: $\frac{\text{Mg of Each Present}}{\text{Tissue Wt.}} \times 100$

$$\text{e.g. } \frac{16.138}{493.3} \times 100 = \underline{3.272\%}$$

12. Total Absolute Error: $\frac{\text{Sum of \% errors}}{100} \times \text{\% wood wt.}$

$$\text{e.g. } \frac{(2.0 + 3.0)}{100} \times 3.272 = \underline{0.163}$$

STARCH ANALYSIS

I Reagents

Glucose oxidase - Sigma Chemical Co., Type II from Aspergillus niger.

Peroxidase - Sigma Chemical Co., Type II from horseradish.

o-Dianisidine-HCl - Sigma Chemical Co. (Purified).

3-Methyl-2-benzothiazolinonehydrazone.HCl, (MBTH) - Technicon Instruments Corp. New York.

N,N-Dimethylaniline, (DMA) - B.D.H.

Amyloglucosidase - Sigma Chemical Co., Grade II from Rhizopus.

a). Buffer Solutions

Tris(Hydroxymethyl)methylamine (30.5 g) was dissolved in 5M HCl (42.5 ml) and made up to 500 ml with water. Glycerol (660 ml) was added to make a tris/glycerol buffer (0.5M, pH 7).

A Phosphate buffer (0.1M, pH 7) was prepared by dissolving hydrated Na_2HPO_4 (10.71 g) and KH_2PO_4 (2.65 g) in water and making up to 500 ml with water.

Sodium acetate (4.10 g) was dissolved in distilled water and made up to 500 ml. Glacial acetic acid was slowly added until a pH of 4.8 was attained. This reagent was referred to as the acetate buffer (0.1M pH 68).

b). Glucose oxidase (30 mg), peroxidase (3 mg) and o-dianisidine (10 mg) were dissolved in the tris/glycerol buffer solution (100 ml, 0.5M) and the reagent, which will henceforth be described as glucose oxidase (g.o) - O-dianisidine-HCl reagent, was stored in a brown bottle below 4°C (Dekker 1971). This solution was stored for several months.

c). MBTH (50 mg) was dissolved in HCl (0.1M) and made up to 50 ml with water to be stored ($<4^{o}C$) in a brown bottle as stock MBTH reagent. A stock solution of DMA was prepared by dissolving a sample (1 ml) in HCl (5 ml, 2M) and making up to volume (100 ml) with water. Stored as for MBTH solution. A "working" MBTH/DMA solution consisted of - MBTH : DMA : HOAc (10M) = 2 : 3 : 5.

Glucose oxidase (50 mg) and peroxidase (1 mg) were dissolved in phosphate buffer (125 ml, 0.1M) and stored below $4^{o}C$. This reagent is described as g.o. reagent.

The working glucose oxidase - MBTH/DMA reagent is mixed immediately before use (g.o. : MBTH/DMA = 4:1). (Gochman 1972).

d). Glucose standards were prepared by successive dilutions of 2 stock solutions (1000 µg/ml, 120 µg/ml) saturated with benzoic acid. Appropriate aliquots of the above stock solutions were made up to volume with H_2O saturated with benzoic acid. The following range of concentrations was obtained:- 15, 30, 45, 50, 60, 75, 90, 100, 105, 120, 150, 200, 250, 300 µg/ml.

e). Amyloglucosidase (250 mg) was dissolved in acetate buffer (250 ml, 0.1M) and the reagent stored below $4^{o}C$.

II Glucose Estimation

a). Glucose concentrations of a wide range (10 - 200 µg/ml) could be successfully analysed on the Beckman Glucose Analyser. Standard glucose solutions were used for calibration, the plot of response vs. concentration (fig. 19a) was linear over the range tested (50 - 200 µg/ml). As the reproducibility was good (±2%) only 2 injections were used per sample. All samples

and standards were injected with a 100 µl dispensing syringe. The glucose concentrations of each analysis were obtained from the calibration graph and the results summarised in a computer plot comparing the different methods of glucose estimation graph 12.

b). An aliquot of glucose solution (1 ml) to be analysed was transferred to a volumetric flask (10 ml) with a calibrated "stopped" syringe and reacted with g.o. - o-dianisidine-HCl reagent (2 ml). The flask was placed in a 37°C temperature - bath for 1 hour. At the end of this time the reaction was quenched with 2.5M H_2SO_4 (5 ml), generating a pink/brown coloured solution which was immediately made up to volume and the absorbance at 412 nm measured on a "635 Varian Techtron" spectrophotometer. The standard calibration plot of absorption vs. concentration was linear over the range 0 - 90 µg/ml (fig. 19b) so this necessitated a dilution (1/3) of the P. radiata samples for all samples to come within the linear range.

c). The g.o. - MBTH/DMA method was similar. The colour formed was blue (λ 590 nm), but the dynamic range was too narrow (0 - 45 µg/ml) for it to be a viable alternative. That is, the absorption vs. glucose plot had a very steep gradient so that small changes in glucose concentration resulted in large changes in absorption.

The results obtained by the methods are summarised in graph 12.

III Starch Analytical

Duplicate samples of dry P. radiata tissue (125 mg) were weighed into volumetric flasks (50 ml). Acetate buffer

solution (10 ml, 0.1M, pH 4.8) was added to one sample; amyloglucosidase/acetate buffer solution (10 ml) to the other. Both flasks were vigorously shaken, and the tissue adhering to the sides of the flask washed down with distilled water (10 ml). The flasks were placed in 55°C temperature bath for 36 hours. After removal from the water bath and cooling (20°C) the solutions were made up to volume. Activated charcoal (100 mg) was added and the solution left for several hours to allow the charcoal to settle. An aliquot (10 ml) of this solution was filtered through a "millipore" filter, and the filtrate analysed by the three glucose oxidase methods described (see above). Up to 10 P. radiata samples could be handled simultaneously.

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