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GAS CHROMATOGRAPHY OF CARBOHYDRATES

THE QUANTITATIVE DETERMINATION OF THE FREE SUGARS OF PLANTS AS THEIR TRIMETHYLSILYL ETHERS

P. K. DAVISON AND R. YOUNG
Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford (Great Britain)
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SUMMARY

A gas chromatographic system for separating the trimethylsilyl ethers of the common free sugars of plants is described. Using myoinositol as an internal standard the technique has been used for the direct quantitative determination of fructose, glucose and sucrose. Its accuracy, as assessed by the analysis of synthetic sugar mixtures, was within 5 %. Some difficulties encountered in the analysis of plant extracts have been overcome by modifying the preparation of the samples. The technique has been applied to a range of plant tissues and found to be simple, fast and to give high reproducibility.

INTRODUCTION

A technique was needed for the quantitative determination of the free sugars of plants, a fraction which commonly includes the aldohexose glucose, the ketohexose fructose and the non-reducing disaccharide sucrose.

The free sugars are generally concentrated in storage organs and fruits, such tissues often being used as food, and may comprise up to 50 % of the dry weight of these tissues[1]. They contribute to the flavour of the raw material and can affect cooking properties both by acting as flavour precursors and by causing browning by the Maillard reaction. Only the reducing sugars take part in this reaction and it has been shown that pentoses are more active than hexoses, and aldoses more than ketoses[2]. A knowledge of the amounts of individual sugars is therefore preferable to an estimation of total and reducing sugars.

Existing methods for the quantitative determination of the individual free sugars include fractionation by column[3] or paper[4] chromatography or by ion exchange resins[5], followed by colourimetric estimation of the separate fractions[6,7]. Gas chromatography offered an alternative technique which was preferred for the rapid, simple handling of a large number of small samples.

Since carbohydrates can be simply and quantitatively converted to volatile derivatives which are readily analysed by gas chromatography, the use of this analytical technique has been widespread[8]. The most commonly used derivatives are the

alditol acetates[9] and the trimethylsilyl (TMS) ethers[10]. The alditol acetates are less suitable when fructose is to be determined in the presence of glucose. This is because on reduction fructose yields a mixture of mannitol and glucitol, and as glucitol is also derived from glucose, direct quantitation is impossible.

In both aqueous solutions and plant tissues, equilibrium is established between the anomers of a reducing sugar. The anomers give rise to separate TMS derivatives so that more than one peak is obtained from each reducing sugar. For accurate quantitation these peaks must either be completely separated or the derivatives of one sugar must be eluted as a single, symmetrical peak. Using pure sugars SWEELEY *et al.*[10] achieved separation of a large number of mono- and oligosaccharides including α- and β-glucose, fructose and sucrose. No attempt was made to separate α- and β-fructose. LUDLOW *et al.*[11] reported a technique for the quantitation of the free sugars of ferns by gas chromatography of their TMS ethers. The relative amount of each sugar was determined by cutting out and weighing incompletely resolved peaks, a procedure which we have found to be inaccurate. We have developed a gas chromatographic system which achieves better resolution and improved quantitation.

EXPERIMENTAL

Apparatus

Instrument. The instrument used in this investigation was a Pye 104-24 gas chromatograph (10^{-12} F.S.D.) with dual flame-ionisation detectors, equipped with a Smith-Kelvinator Servoscribe recorder. The carrier gas was argon at a flow rate of 60 ml/min. The attenuation setting was generally 5×10^3 (5×10^{-9} F.S.D.), and the chart speed 600 mm/h.

Columns. Standard, twin, coiled glass columns 1/4 in. I.D. $\times$ 3, 5 or 7 ft. long were used.

Columns for the analysis of TMS ethers: several liquid phases and solid supports were used during preliminary experiments. On most of these columns and under the experimental conditions α- and β-fructose were partially but incompletely resolved. In one instance β-fructose was eluted with α-glucose. Columns included: 3 % SE-52 on 80–100 mesh Chromosorb W, 5 ft. and 3 ft.; 5 % XE-60 on 80–100 mesh Chromosorb W, 5 ft. and 3 ft.; 2 % QF-1 on 80–100 mesh Gas-Chrom P, 3 ft.; 5 % Silicone oil on 80–100 mesh Gas-Chrom P, 5 ft.; 3 % SE-52–5 % XE-60 (1:1) on 80–100 mesh Chromosorb W, 5 ft.; 3 % SE-52–5 % XE-60 (1:2) on 80–100 mesh Chromosorb W, 5 ft.; 5 % Apiezon M on 80–100 mesh Gas Chrom P, 3 ft. and 5 ft.; 5 % Apiezon L on 80–100 mesh Gas-Chrom P, 5 ft.; 3 % SE-54 on 80–100 mesh Gas-Chrom P, 7 ft. and 5 ft.

The most suitable column was 3 % SE-54 on 80–100 mesh Gas-Chrom P, 3 ft. All data reported here for TMS ethers were obtained on this column. All columns were conditioned by heating to 250–300° for 24 h with the outlets disconnected from the detectors, and an argon flow rate of 60 ml/min. Separations were generally achieved under the following temperature programme conditions: 10 min isothermal hold at 160–165° followed by 3°/min increase to 295°, final hold of *ca.* 15 min, if necessary.

Columns for the analysis of alditol acetates: 3 % ECNSS-M on 100–120 mesh Gas-Chrom Q, 5 ft. Analytical conditions: isothermal at 184°.

Sugars standards

D(+)Glucose (Hopkin and Williams Ltd., Analar) recrystallised from methanol–ethanol (1:1); D(—))fructose (British Drug Houses Ltd., General purpose) recrystallised from R/R ethanol; sucrose and mannose (Hopkin and Williams Ltd., Analar); myoinositol (British Drug Houses Ltd., Analar) ; D(+)raffinose pentahydrate and stachyose tetrahydrate (Sigma Chemical Co., practical grade).

Syrups: the crystalline sugars were dissolved in distilled water and shaken overnight at room temperature to allow equilibrium to be attained. In equilibrated aqueous solution glucose is known to exist almost exclusively as α- and β-glucopyranose and fructose as α- and β-fructofuranose. It has been shown[10] that the TMS ether of α-glucose has a shorter retention time on a non-polar column than the TMS ether of β-glucopyranose.

Trimethysilylation reaction

Sugar (5–30 mg) either crystalline or as a syrup dried on a rotary film evaporator, was taken. Redistilled pyridine stored over potassium hydroxide pellets (2 ml) was added followed by hexamethyldisilazane (HMDS, Koch Light Ltd.) (0.2 ml) and trimethylchlorosilane (TMCS, Koch Light Ltd.) (0.2 ml). The mixture was shaken vigorously for 5 min at room temperature then taken to dryness on a rotary film evaporator. The residue was taken up in carbon tetrachloride and taken to dryness again to remove the last traces of reagent. Finally the residue was suspended in carbon tetrachloride and aliquots of this preparation were injected into the gas chromatograph. This procedure differed from that used by SWEELEY *et al.*[10] in that the reagents were removed before chromatography. This practice obviated the problem of solvent tailing without affecting the stability of the TMS ethers.

Peak areas were calculated, after triangulation, as the product of the height $\times$ the width at half height.

Preparation of alditol acetates

Alditol acetates were prepared by the technique of SAWARDEKER *et al.*[9].

Paper chromatography

Paper chromatography was carried out on Whatman No. 1 paper using developing solvent 1 to separate neutral sugars and solvent 2 to separate uronic acids. Solvent 1 = ethyl acetate–pyridine–water (8:2:1); solvent 2 = ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Alkaline silver nitrate was used to detect the spots.

Plant extracts

Extracts (2 l) were prepared by macerating washed, roughtly chopped tissue in a Waring Blendor with sufficient ethanol to give a final concentration of 80 % with respect to the intrinsic water in the tissue[1]. Each macerate was filtered and the residue washed with 80 % ethanol. The washings and filtrate were combined and concentrated on a rotary film evaporator to 250 ml. Details of the tissues used are summarised in Table I.

Aliquots of these standard solutions (10 ml) were partitioned between chloroform–methanol–water (64:32:24). As no free sugars were detected in the methanol–

TABLE I

FRUIT AND VEGETABLE ETHANOLIC EXTRACTS

Common name	*Systematic name*	*Solids (%)*	*Fresh weight (g/250 ml)*
Tomato (fruit) cv Craigella full size green fruit	*Lycopersicon esculentum* Mill.	6	425.5
Potato (tuber) cv King Edward	*Solanum tuberosum* L.	20	500
Apple (fruit) cv Granny Smith	*Malus sylvestris* Mill.	15	470.6
Cabbage (leaf)	*Brassica oleracea* var. *capitata*	5	421
Carrot (root) cv Chantenay red core	*Daucus carota*	10	444.4

chloroform (lower) layer this was generally discarded. The methanol–water (upper) layer was concentrated to dryness and analysed as described in the text.

RESULTS AND DISCUSSION

Analysis of the TMS ethers of pure sugars

Table II shows the retention times of the TMS ethers of five common free sugars on a 3 ft. column of 3 % SE-54 at optimal temperatures. It will be noted that α- and β-fructose were eluted as a single symmetrical peak. Two higher oligosaccharides are included as these are frequently found as free sugars in plants, often with sucrose[12]. Each was eluted as a single regular peak in a reasonable retention time. However as neither was found in the plant materials examined they were not further investigated.

TABLE II

RETENTION TIMES OF THE TMS DERIVATIVES OF FIVE COMMON FREE SUGARS ON A 3 ft. COLUMN OF 3 % SE-54

Sugar	*Molecular weight*	*Optimum temperature*	*Retention time (min)*
Fructose	180	164	5.4
Glucose	180	164	7.6, 12.2
Sucrose	342	233	5.3
Raffinose	504	271	7.7
Stachyose	666	290	23.5

The temperature programme of a 10 min initial isothermal hold at 160–165° followed by 3°/min increase to 295° was selected. This achieved adequate separation of the monosaccharides while retaining reasonable elution volumes for each oligosaccharide.

Myoinositol was chosen as an internal standard for quantitative work[9]. This gives a single TMS ether and was not found in the free sugar fractions of the tissues investigated. It had a convenient retention time in that it was eluted at the start of the temperature increase between β-glucose and sucrose, with good separation from both (see Fig. 1).

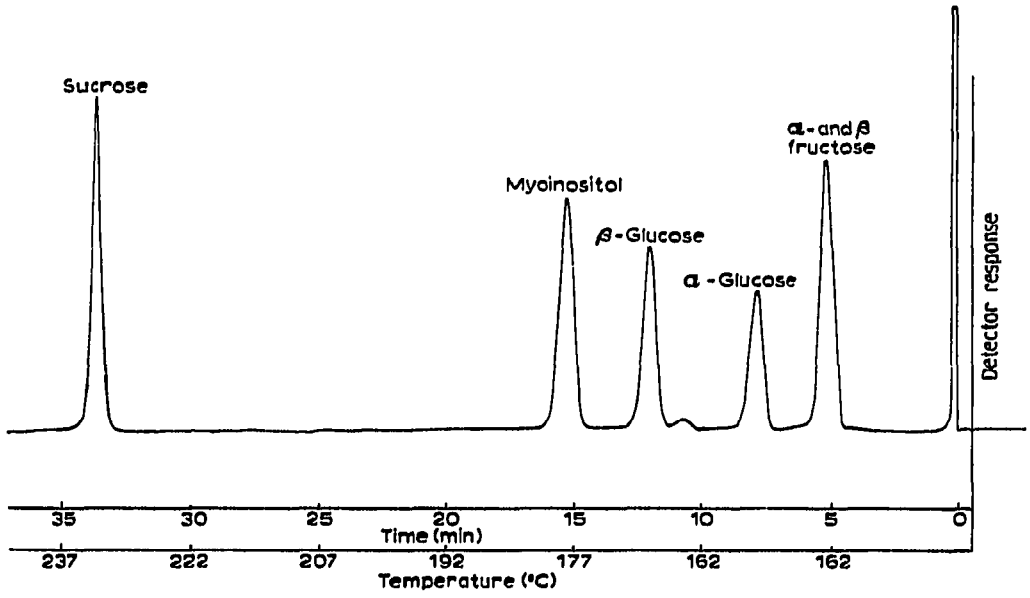

Fig. 1. Gas–liquid chromatogram of the TMS derivatives of a mixture of pure, equilibrated sugars. 3 ft. column of 3 % SE-54. Argon flow rate 60 ml/min. Temperature programme as shown.

Preliminary investigation of the free sugars from tomatoes

It was found necessary to take additional care when the TMS ethers of the plant extracts were prepared. Preliminary paper chromatography of the tomato extract (developing solvents 1 and 2) had shown the major free sugars to be fructose and glucose with sucrose as a minor component and a faint trace of galacturonic acid. The absence of other monosaccharides was confirmed by the preparation and analysis

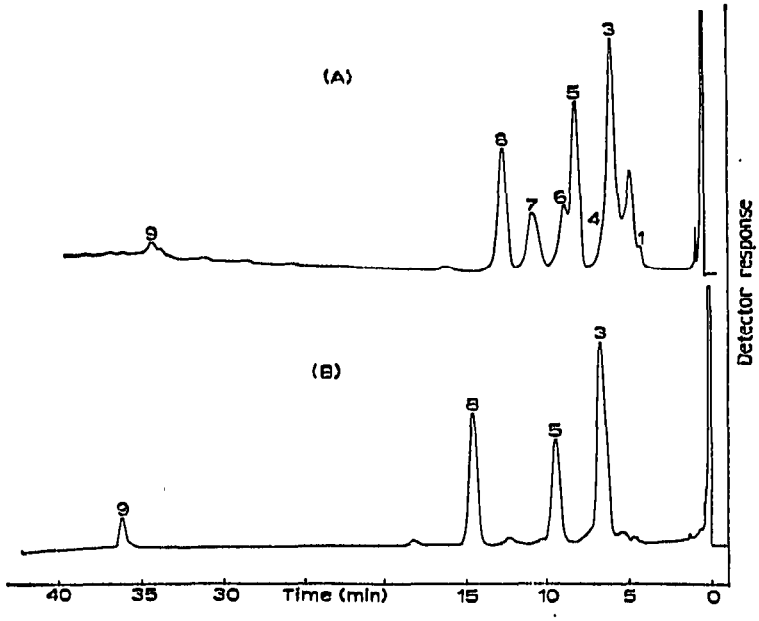

Fig. 2. Gas–liquid chromatograms of the TMS derivatives of an 80 % ethanolic extract of tomato. (A) sample dried on a rotary film evaporator before silylation; (B) sample azeotroped with benzene. 3 = α- and β-fructose; 5 = α-glucose; 8 = β-glucose; 9 = sucrose. 3 ft. column of 3 % SE-54. Argon flow rate 60 ml/min. Temperature programme as in Fig. 1.

of the alditol acetates. Gas chromatograms of these showed only peaks corresponding to mannitol and glucitol. It was therefore expected that gas chromatography of the TMS ethers would give four peaks, corresponding to α- and β-fructose, α-glucose, β-glucose, and sucrose. However a minimum of nine partly and fully resolved peaks were found, see Fig. 2A. On the basis of retention times on SE-54 peaks were tentatively identified thus: peak 3, as fructose; 5, as α-glucose, 8, as β-glucose and 9, as sucrose. The remainder were thought likely to be partial TMS derivatives of these sugars[13].

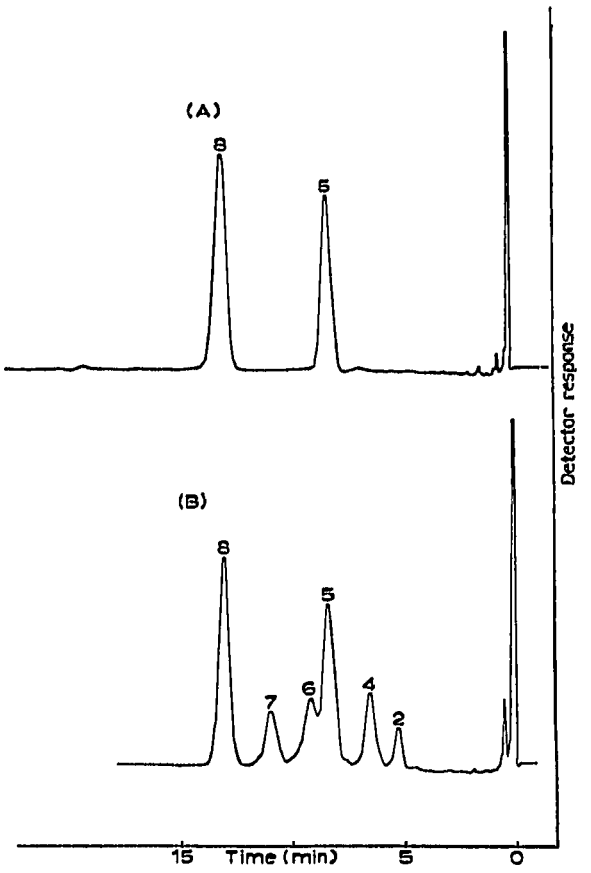

Fig. 3. Gas–liquid chromatograms of the TMS derivatives of an equilibrated glucose syrup. (A) prepared with excess TMS reagents; (B) prepared with added water and limiting TMS reagents. 3 = α- and β-fructose; 5 = α-glucose; 8 = β-glucose; 9 = sucrose. 3 ft. column of 3% SE-54. Argon flow rate 60 ml/min. Temperature programme as in Fig. 1.

Partial silylation is known to occur when insufficient reagent HMDS is provided[13]. Such deficiency could have resulted from competition for reagent from residual water after inadequate drying, and so a more rigorous dehydration step was introduced. The plant extract was azeotroped with benzene before silylation, gas chromatography of these derivatives gave a chromatogram showing only peaks for fructose, glucose and sucrose (Fig. 2B).

Chromatograms similar to that in Fig. 2B were also obtained when the extracts were dried over phosphorus pentoxide or treated with a ten-fold increase in the volumes of HMDS, TMCS and pyridine.

Confirmation of the origin of the anomalous peaks was obtained with a glucose

syrup divided into two equal volumes (A and B). Both aliquots were dried on a rotary film evaporator using benzene as an azeotrope. The TMS derivatives of (A) were prepared directly. A measured volume of water was added to sample (B), the mixture then being treated with sufficient HMDS to react with all the water and half the sugar[14]. The TMS reaction was completed as above. The two samples were analysed and the chromatograms shown in Figs. 3A and B obtained. The dry sample gave only peaks with the retention times of α- and β-glucose, while the sample treated with limiting reagent gave peaks corresponding to those designated 2, 4, 5, 6, 7 and 8 in Fig. 2A.

TABLE III

ANALYSES OF SYNTHETIC SUGAR MIXTURES AS TRIMETHYLSILYL DERIVATIVES

3 ft. column of 3 % SE-54 temperature programme and carrier gas as given in the text.

Mixture	*Sugar*	*Weight taken*	*Weight found (average of 3 analyses)*	*Standard deviation*	*% error*
I (9.97 mg inositol)	Fructose	10.05	10.52	0.24	+4.7
	Glucose	11.96	12.15	0.23	+1.6
	Sucrose	10.53	10.99	0.14	+4.4
II (9.97 mg inositol)	Fructose	5.02	4.78	0.47	—4.8
	Glucose	5.98	5.94	0.25	—0.7
	Sucrose	21.05	21.37	0.94	+1.5
III (9.97 mg inositol)	Fructose	20.10	19.12	0.47	—4.9
	Glucose	23.92	23.93	0.44	+0.0
	Sucrose	5.26	5.21	0.08	—1.0
IV (9.97 mg inositol)	Fructose	5.02	5.18	0.49	+3.2
	Glucose	17.94	17.75	0.84	—1.0
	Sucrose	10.53	10.86	0.19	+3.1

Similar experiments showed that peaks 1, 2 and 3 were obtained from fructose trimethylsilylated with insufficient reagent, and that a series of unresolved peaks with retention times close to that of peak 9 were derived from sucrose.

Detector calibration and assessment of the technique

Linear calibration graphs were obtained experimentally[9] for fructose, glucose and sucrose using myoinositol as an internal standard. Varying amounts of each sugar were mixed with constant amounts of myoinositol. The TMS derivatives of the mixtures were prepared and chromatographed. The ratio of the area of each TMS-sugar to that of the TMS-internal standard was plotted with the ratio of the molar weight of the sample sugar to that of the internal standard. Molar detector constants (K) previously found to be the same for both detectors, were determined from the slopes of these curves: fructose, 0.73; glucose, 0.90; sucrose, 1.43. The difference in the values for these constants for the different sugars was greater than expected and stressed the importance of carrying out this calibration step.

To analyse a sugar mixture quantitatively a known amount of myoinositol was added to the sample. The TMS derivatives were prepared and chromatographed. The

peak areas of the unknowns were then related to their amounts by the expression:

$$\text{weight of sugar} = \frac{\text{peak area sugar} \times \text{weight standard/molecular weight standard}}{\text{peak area standard} \times \text{appropriate } K} \times \times \text{molecular weight sugar}$$

To investigate the accuracy of the method a series of mixtures were prepared of solutions of fructose, glucose, sucrose and inositol in known amounts. These mixtures were dried and the TMS derivatives prepared. Each preparation was analysed in triplicate. The amounts of the individual sugars in each mixture were determined from the experimental data and these values are compared with the known true composition in Table III.

TABLE IV

DETERMINATION OF THE FREE SUGARS OF SOME FRUITS AND VEGETABLES

Tissue	*Standard solution (ml)*	*Added inositol (mg)*	*g Sugar/100 g fresh tissue*		
			Fructose	*Glucose*	*Sucrose*
Tomato	1	5.98	1.16	0.99	0.15
	1	5.98	1.26	1.05	0.12
Cabbage	1	5.25	1.38	1.95	0.11
	1	4.98	1.43	2.12	0.11
Apple	1	19.94	6.57	2.60	2.54
	1	19.94	6.58	2.71	2.29
Carrot	1	4.98	1.13	1.01	0.63
	1	9.97	1.17	1.17	0.60
Fresh potato	5	1.99		0.16	
	5	1.99		0.14	
	1	9.97			0.72
	1	9.97			0.66
Cold stored potato	5	1.99		0.23	
	5	1.99		0.25	
	1	9.97			2.10
	1	6.80			2.05

The low standard deviations indicate that the errors incurred between successive injections together with those involved in the measurement of the peak areas were generally small, even when a relatively minor component was estimated. The total errors were such that the experimentally determined data were within 5 % of the true concentrations.

Quantitative analysis of the free sugars of plant extracts

The technique was used to determine quantitatively the free sugars of green tomato, cabbage, carrot, apple, freshly harvested potatoes and cold stored potatoes (1 week at 2°).

Paper chromatography (solvents 1 and 2) showed that the only detectable sugars were fructose, glucose and sucrose. As a further precaution the alditol acetate derivatives were prepared and analysed. The presence of only glucitol and mannitol acetates

in these gas chromatograms was further evidence that glucose and fructose were the only monosaccharides in the extracts.

Replicate aliquots of the methanol–water soluble fraction of each standard solution were taken to dryness with suitable volumes of a standard inositol solution using benzene as an azeotrope. The TMS derivatives were prepared and chromatographed, the results being given in Table IV. Typical chromatograms of apple, cabbage and carrot free sugars are shown in Fig. 4.

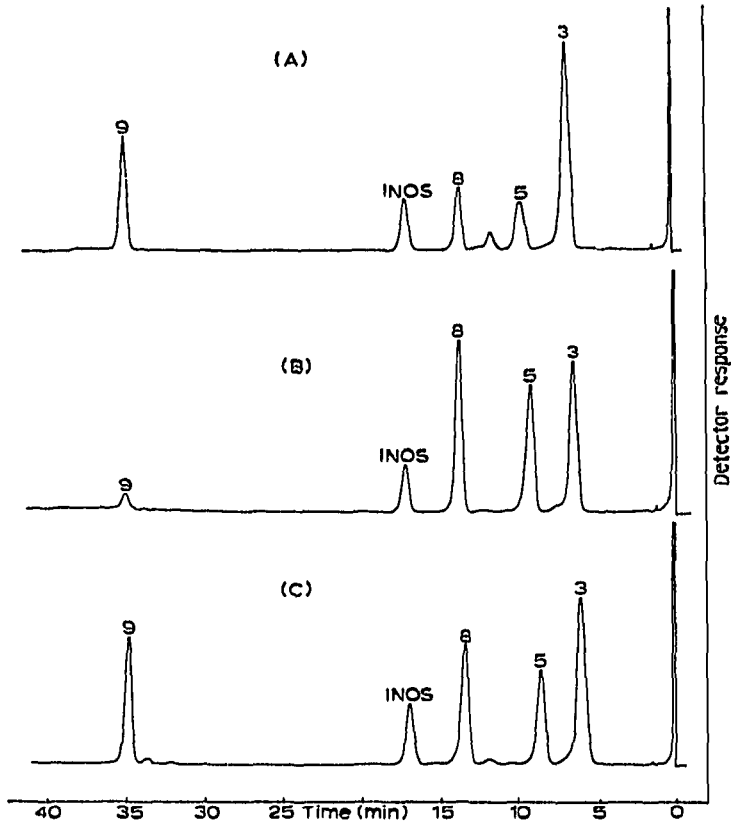

Fig. 4. Gas–liquid chromatograms of the TMS derivatives of 80% ethanolic extracts of plants. (A) apple; (B) cabbage; (C) carrot. 3 = α- and β-fructose; 5 = α-glucose; 8 = β-glucose; 9 = sucrose. 3 ft. column of 3% SE-54. Argon flow rate 60 ml/min. Temperature programmes as in Fig. 1.

The weights of monosaccharide in both potato samples were very low in relation to sucrose. It was therefore necessary in each case to evaluate them on separate aliquots, using a smaller volume of inositol solution when the monosaccharide was estimated.

Evidence for the identification of the sugars in the plant extracts had been obtained by comparison with pure standards using paper chromatography with two different developing solvents, and on the basis of the retention times of the TMS ethers on SE-54. In addition the monosaccharides were identified by gas chromatography of their alditol acetates.

Satisfactory quantitative results were obtained from ethanolic extracts of a range of morphologically different plant tissue.

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