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AN ANALYSIS OF THE FREE SUGARS OF FERNS BY GAS–LIQUID CHROMATOGRAPHY

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Abstract—The free sugars of twelve species of fern have been determined by gas–liquid chromatography of their trimethylsilyl derivatives. Sucrose was the principal sugar in most species examined; in certain species the level of glucose, fructose, galactose or a combination of these was also high. The sensitivity and accuracy of this method, which are considerably greater than those of other techniques, commend its usefulness in analyses of plant materials.

INTRODUCTION

QUALITATIVE and quantitative analyses of individual sugars in plant materials have in the past been made principally by paper chromatographic techniques.[1] Recently Sweeley *et al.*[2] described a method for determination of mono- and disaccharides by gas–liquid chromatography of trimethylsilyl derivatives.

The trimethylsilyl derivatives are formed essentially quantitatively under mild conditions. They have good thermal stability, relatively high volatility and produce sharp, symmetrical peaks on the gas chromatograph, making them well suited for use in analyses. Earlier workers had experimented with gas chromatography of methylated and acetylated sugars, but the results with these derivatives were poor.[3]

Although fairly extensive use has been made of this new method for determination of sugars in animals[4–7] and micro-organisms,[8–11] only very limited use has been made of it in the study of plants. One such study was concerned with the structure of the xylan of *Acacia mollissima* Willd.[12] The polysaccharide was methylated and hydrolyzed, and the partially methylated xyloses were chromatographed as the trimethylsilyl derivatives. In a study of apiose in parsley,[13] its trimethylsilyl derivative was used for its identification and isolation.

In this paper we describe the use of gas–liquid chromatography of trimethysilyl derivatives for determination of the spectrum of free monosaccharides and sucrose in several species of pteridophytes (ferns). This investigation is part of a larger study of the metabolism of this plant group.[14]

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RESULTS

In Fig. 1 is shown the chromatogram of a mixture of a tetrose, three pentoses, three hexoses, and the disaccharide, sucrose. Positive identification of the peaks was made by chromatographing authentic samples of individual members of the group. It will be noted that reducing sugars, such as glucose, can give multiple peaks corresponding to α- and

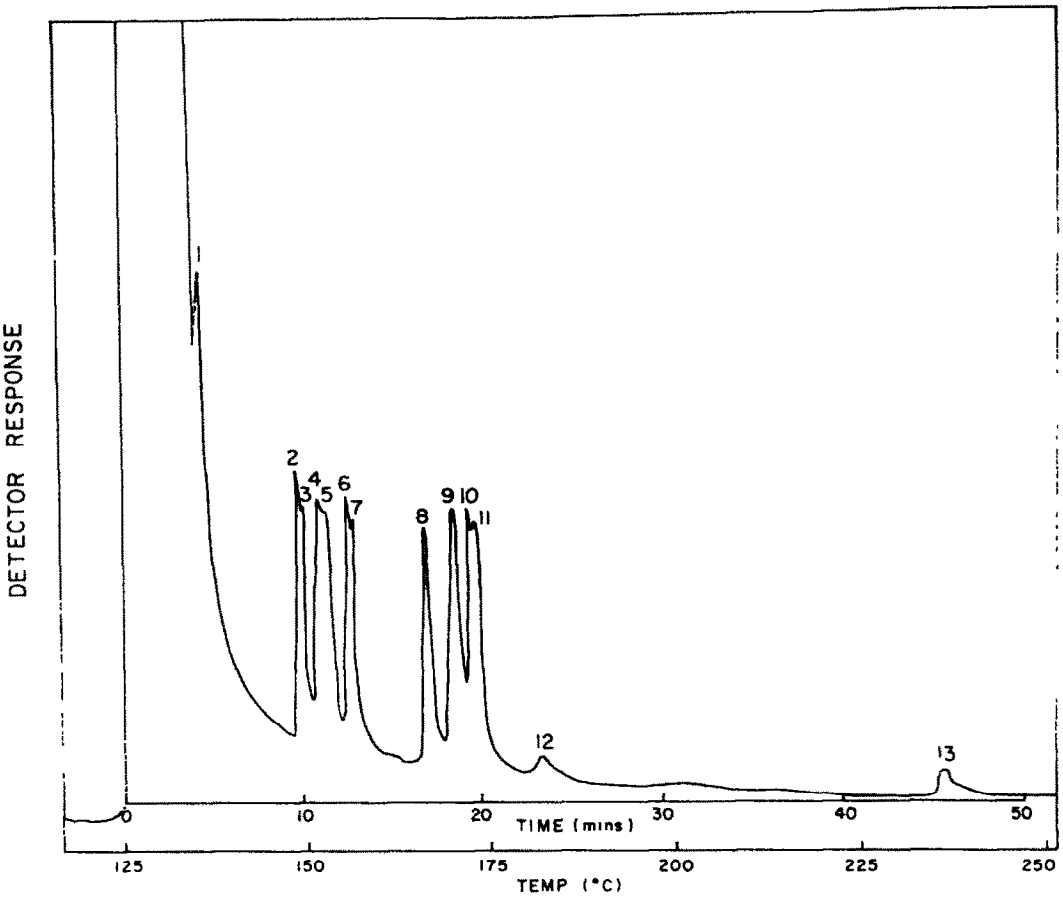

FIG. 1. CHROMATOGRAM OF AUTHENTIC SAMPLES OF SUGARS.
1, Erythrose; 2 and 3, Arabinose; 4 and 5, Xylose; 6 and 7, Ribose; 8, Fructose; 9, Galactose; 10–12, Glucose; 13, Sucrose.

β-pyranose and "γ"[2] (furanose or open chain) forms. Sweeley *et al.*[2] observed that little or no equilibration among these forms occurred during the silylation process if the solutions were not heated; thus they were able to make specific assignment of isomer structures to these peaks. The programmed temperature increase during each sample analysis permitted the observation of both monosaccharides and sucrose in a single analysis.

Figures 2 and 3 are typical chromatograms of the trimethylsilyl derivatives of sugars from extracts of ferns. The three peaks eluted at about 154° are due to arabinose which was added to the plant extract as an internal standard for the quantitative determination of the

other sugars. The absence of arabinose in the plant extracts was ascertained in previous experiments. Figure 2 was obtained with the extract of *Nephrolepis exaltata* var. *bostoniensis* and Fig. 3 with *Woodsia obtusa*. The small initial peak observed with the latter sample was tentatively identified as erythrose, although the high volatility of its trimethylsilyl derivative makes the identification less certain than that of the other sugars. This peak was also seen in *Ophioglossum engelmanni* and a possible trace was found in both *Polypodium polypodioides* var. *Michauxianum* and *Botrychium virginianum*.

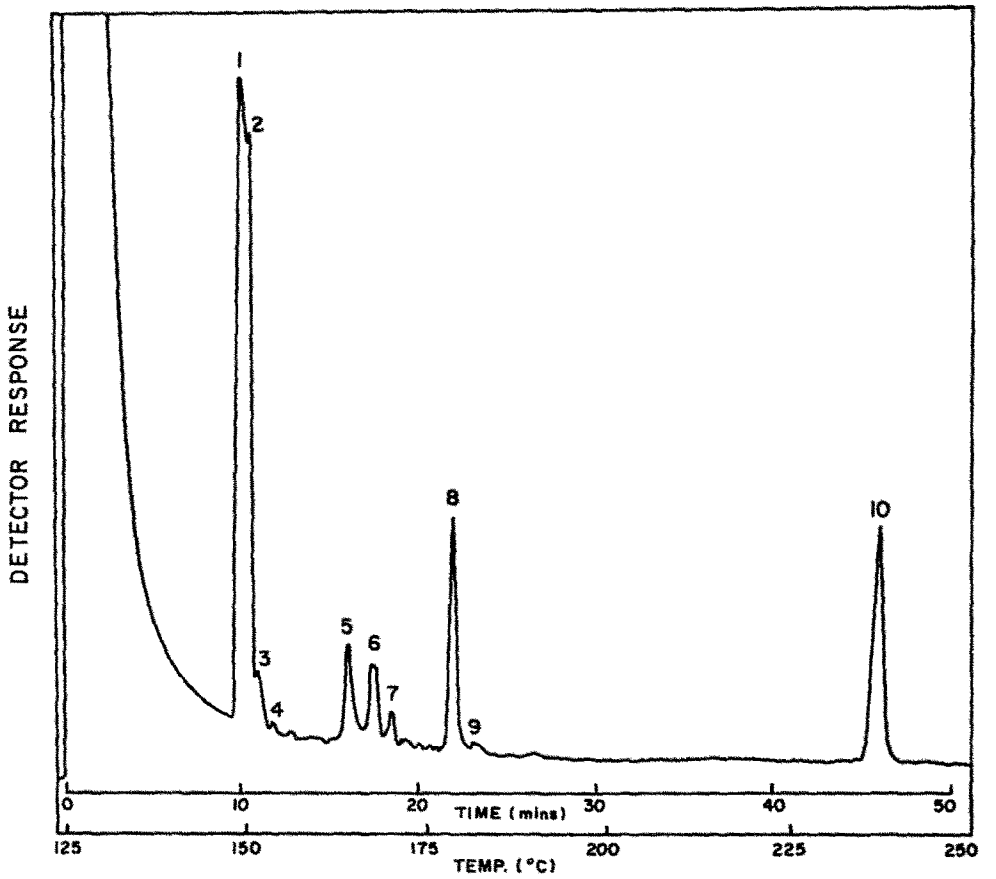

FIG. 2. CHROMATOGRAM OF THE SUGARS OF *Nephrolepis exaltata* VAR. *bostoniensis*.
1–3, Arabinose (added to sample); 4, Xylose; 5, Fructose; 6 and 7, Galactose; 8 and 9 ,Glucose; 10, Sucrose.

The elution temperatures and the retention times of the trimethylsilyl derivatives of known sugars relative to that of glucose are shown in Table 1. At a given gas-flow rate these values were highly reproducible. Small changes in flow rate that occurred from one day to another did not cause a comparable change in retention times because of the overriding effect of column temperature on elution rate.

In Table 2 the results obtained with the extracts from the twelve species of ferns are shown. It can be seen that all species contained monosaccharides and sucrose. The concentration

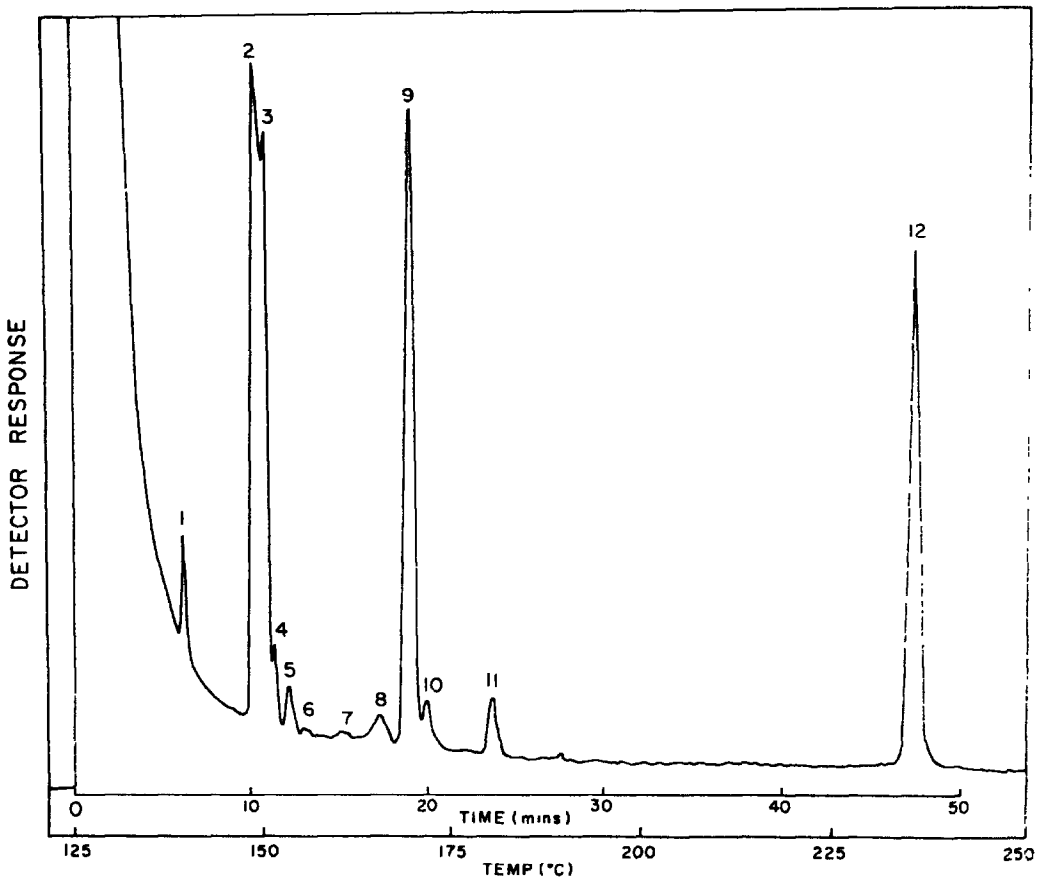

FIG. 3. CHROMATOGRAM OF THE SUGARS OF *Woodsia obtusa*.
1, Erythrose; 2–4, Arabinose (added to sample); 5 and 6, Xylose; 7, Unknown; 8, Fructose; 9 and 10, Galactose; 11, Glucose; 12, Sucrose.

TABLE 1. GAS–LIQUID CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF KNOWN MONOSACCHARIDES AND SUCROSE

Sugars	Relative retention times*	Elution temperatures, C
D-Erythrose	0·23	140
D-Xylose	0·50†, 0·52†	151†, 152†
L-Arabinose	0·56†, 0·58†	154†, 155†
D-Ribose	0·65†, 0 67†	157†, 158†
D-Fructose	0 87	170
D-Galactose	0·95	173
D-Glucose	1·00‡, 1·02†, 1·21†	176†, 177†, 184†
Sucrose	2·43	236

* Retention times are expressed relative to α-glucose.
† Multiple peaks, indicating presence of more than one isomer
‡ α-Glucose.

TABLE 2. THE SUGARS OF TWELVE SPECIES OF FERNS EXPRESSED ON A FRESH WEIGHT BASIS

Species	Water content (%)	Sugars (mg/g)					
		Erythrose	Xylose	Fructose	Galactose	Glucose	Sucrose
Asplenium platyneuron	77	—	tr.	1·42	0·90	2·10	11·07
Asplenium resiliens	61	—	0·86	0·66	1·82	0·80	11·32
Botrychium dissectum	75	—	tr.	6·28	9·90	12·18	18·79
Botrychium virginianum	82	tr.	—	0·62	1·44	25·80	7·15
Camptosorus rhizophyllus	65	—	—	0·59	0·72	1·48	23·58
Cyrtomium falcatum	81	—	—	3·84	0·88	15·60	28·29
Nephrolepis exaltata var. *bostoniensis*	80	—	tr.	2·18	2·28	5·50	7·89
Pellaea atropurpurea	79	—	tr.	9·14	4·24	4·90	19·55
Polypodium polypodioides var. *Michauxianum*	63	tr.	tr.	tr.	12·40	1·76	47·23
Polystichum acrostichoides	76	—	—	0·76	3·90	1·68	13·82
Ophioglossum engelmanni	85	2·20	—	6·42	7·20	6·56	19·66
Woodsia obtusa	81	1·21	0·74	0·94	19·00	1·32	16·21

of sucrose was high in all species examined, ranging from 7·15 mg/g of fresh tissue in *Botrychium virginianum* to 47·23 in *Polypodium polypodioides* var. *Michauxianum*. Fructose and glucose were found in all species. Fructose varied from a trace in *Polypodium polypodioides* to 9·14 mg/g of fresh tissue in *Pellaea atropurpurea*. The glucose concentration likewise varied considerably, ranging from 0·80 mg/g of fresh weight in *Asplenium resiliens* to 25·80 mg/g in *Botrychium virginianum*. About half of the species contained small amounts of xylose, and small amounts of erythrose were observed in four species.

In some cases additional small peaks were observed with elution temperatures higher than that of sucrose. These were in the range expected for maltose and cellobiose, but the peaks were not positively identified.

DISCUSSION

This investigation clearly indicates the usefulness of the gas–liquid chromatographic method for qualitative and quantitative determination of free sugars in plant materials. The method is characterized by its simplicity and reproducibility.

The limit of detection under the conditions employed was approximately 0·5 mg/g of fresh plant material. However, the sensitivity can be greatly increased by several means. These include sampling a larger quantity of plant tissue or operation of the gas chromatograph at a higher level of sensitivity. The flame ionization detector system that was employed in these analyses was capable of at least 100-fold greater sensitivity. The sensitivities observed in the present study were of the same order of magnitude as those observed by Bidwell *et al.*[1] in paper chromatographic studies of carbohydrates from plant tissue.

At the other end of the sensitivity scale, Grisebach[13] has very recently employed preparative gas chromatography of the trimethylsilyl derivative to isolate pure samples of the monosaccharide, apiose, from parsley.

The accuracy and reproducibility of the gas chromatographic analyses appear very good, particularly with the major components (±5%). The chief limitation of the accuracy of the method is the measurement of peak areas. Many of the peaks are very sharp and narrow. The accuracy with which such peaks can be measured would be much better with an integrator incorporated into the chromatograph recorder.

In the early stages of this work some difficulty was experienced in obtaining quantitative conversion of several sugars, particularly sucrose, to silyl derivatives. Incomplete conversion of sucrose was indicated by a secondary spike in the trailing edge of the peak. Additional evidence of incomplete conversion was provided by the fact that larger peaks were obtained with longer silylation periods.

The accuracy of the gas chromatographic method should far exceed that of paper chromatographic methods. Bidwell *et al.*[1] described a method of visual comparison of color spots resulting from different sample sizes with color spots resulting from known amounts of the sugars. Their method depends upon reproducibility of sample application to the paper and reproducibility of spraying the paper with developing agents. In their skilled hands, they estimated an accuracy of 20 per cent. This element of subjective judgement is not a part of the gas chromatographic method.

The principal finding to emerge from the present study is the very high sucrose content of all the species examined. Other sugars may also attain high concentrations: e.g. galactose, glucose and fructose in *Botrychium dissectum*, galactose and fructose in *Ophioglossum engelmanni*, glucose in *Botrychium virginianum* and *Cyrtomium falcatum*, fructose in *Pellaea atropurpurea*, and galactose in *Woodsia obtusa* and *Polypodium polypodioides* var. *Michauxianum*.

In agreement with the findings of Bidwell *et al.*[1] concerning representatives of twenty-seven different families of Spermatophytes, it was found in the present study of ferns that the majority of the species investigated show a sucrose hexose ratio greater than one and a higher concentration of glucose than fructose.

It is anticipated that the gas chromatographic technique using trimethylsilyl derivatives will be used widely in the future for the study of carbohydrates and other polyhydroxy compounds in plant materials.

EXPERIMENTAL

Plant Material

Specimens of twelve species of ferns were collected: ten were found wild (*Asplenium platyneuron* (L.) Oakes, *Asplenium resiliens* Kunze, *Botrychium dissectum* Spreng. forma *obliquum* (Muhl.) Fern., *Botrychium virginianum* (L). Sw., *Camptosorus rhizophyllus* (L.) Link, *Pellaea atropurpurea* (L.) Link, *Polypodium polypodioides* (L.) Watt var. *Michauxianum* Weatherby, *Polystichum acrostichoides* (Michx.) Schott, *Ophioglossum engelmanni* Prantl and *Woodsia obtusa* (Spreng.) Torr.); the remaining two (*Cyrtomium falcatum* Presl. and *Nephrolepis exaltata* Schott var. *bostoniensis* Davenport) were obtained from the greenhouse. The plant material for each extraction was taken from fifteen to twenty individual plants, with the exception of the greenhouse species, of which only three plants of each were sampled. Portions of the roots, stems, and leaves were combined. The material was harvested in mid-afternoon during May.

Dry Weights

Samples of fresh plant material were dried in an oven at 90 for 12 hr preparatory to determination of dry weights.

Extraction Procedure

Fresh plant material (10 g) was ground in 100 ml of 70% ethanol in a Waring blendor. The slurry was transferred to a 500 ml round bottom flask and the blendor rinsed

with another 100 ml portion of 70% ethanol. The mixture was refluxed for 14 hr. The mixture was then filtered and the filtrate was concentrated to a thick syrup. The residue was take up in 2·0 ml of distilled water and quantitatively transferred to a 15 ml centrifuge tube by repeated emulsification with 10 ml portions of ether. The emulsions were resolved by centrifugation. The ether layer was removed and the aqueous solutions were stored at $-20°$ until analyzed.

Trimethylsilyl Derivatives

Hexamethyldisilazane and trimethylchlorosilane were obtained from Aldrich Chemical Co., Milwaukee, Wis., and used without purification.

Aliquots (0·1 ml) of the plant extract were placed in small test tubes and evaporated with a vacuum pump to dryness. One milliliter of anhydrous pyridine (stored over potassium hydroxide pellets), 0·2 ml of hexamethyldisilazane and 0·1 ml of trimethylchlorosilane were added. The mixture was stirred thoroughly, and then allowed to stand at room temperature for at least 60 min before use. Precipitation of ammonium chloride occurred during the reaction period. No attempt was made to remove the precipitate since it did not interfere with the subsequent use of the derivatives. Derivatives of known sugars were used to identify the chromatographic peaks. These were prepared from 5–10 mg of the finely powdered dried sugar.

Gas–Liquid Chromatography

Chromatograms were obtained with a dual column, temperature-programmed gas–liquid chromatograph equipped with hydrogen flame ionization detectors (Aerograph Model 204, Wilkins Instrument and Research, Inc., Walnut Creek, Calif.). The samples were analyzed with a 10 ft × ⅛ in. column packed with 3 per cent silicone gum rubber on 80–100 mesh Diatoport S (F and M Scientific Corp., Avondale, Pa.).

Approximately 2 μl of the derivative solution was injected at 125° and the temperature was raised at about 2·4°/min to 250°. Identification of the components was made by comparison of elution times and temperatures with those of the derivatives of known sugars.

Quantitation was obtained by using arabinose as an internal standard, since initial chromatograms had indicated that all of the plant extracts were essentially free of this sugar. Aliquots (0·10 ml) of the plant extracts were dehydrated as before and 10 mg of arabinose was added before silylation. Peak areas were compared by weighing the chromatograms to determine the amounts of sugars contained in the extracts. Small correction factors were determined for the hexoses and for sucrose by comparison of the peaks produced by the derivatives from a mixture of equal weights of arabinose, glucose and sucrose. It appeared unnecessary to do this with other sugars since isomeric compounds of similar functionality are known to give identical responses with flame ionization detectors.[15] In most cases the results from two chromatograms were averaged to determine the sugar content of the plant extracts. The average deviation of the major components was generally within 5 per cent. With the minor components the deviations were sometimes higher when the accuracy with which the peak areas could be determined became a limiting factor.

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