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QUANTITATIVE ANALYSIS OF MONOSACCHARIDES, CYCLITOLS, SUCROSE, QUINIC AND SHIKIMIC ACIDS IN *PINUS RADIATA* EXTRACTS ON A GLASS SUPPORT-COATED OPEN TUBULAR CAPILLARY COLUMN BY AUTOMATED GAS CHROMATOGRAPHY

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SUMMARY

An automated gas chromatographic analytical procedure employing a glass support-coated open tubular capillary column was developed for the routine quantitative analysis of individual sugar-like components extracted from *Pinus radiata* tissues into 60% ethanol. Minimal clean-up procedures were required since the high resolution column permitted the separation of the monosaccharides, cyclitols and O-methyl cyclitols, sugar alcohols, sucrose, quinic and shikimic acids contained in the extracts.

This method was also applied to the analysis of similar extracts from other plant species.

INTRODUCTION

Analysis of monosaccharides by gas–liquid chromatography (GLC) has become a well-established technique since the initial analyses over a decade ago[1]. The method has been widely applied not only to the analysis of sugar-containing products, but also by physiologists and biochemists to the analysis of natural levels of "sugars" in living plants[2] and other organisms[3]. However, "normal" plant sugar extracts can, and frequently do contain substances other than monosaccharides. These other related compounds may be sugar alcohols, cyclitols, O-methyl cyclitols, polyhydroxy acids, etc., which can make separation of individual components very difficult. If identification only was required, then several GLC columns could have been used to completely separate unknown peaks, but for quantitative work this would have seriously increased analysis time and reduced throughput of samples. Thus a single-column method was desirable; this would also facilitate automation of the gas chromatographic analysis and calculation of the results. The latter became essential when large numbers of samples (up to 5000 per annum) were expected. This was the requirement in a project to quantify starch and "sugar extract" component levels in *Pinus radiata* tissues during growth.

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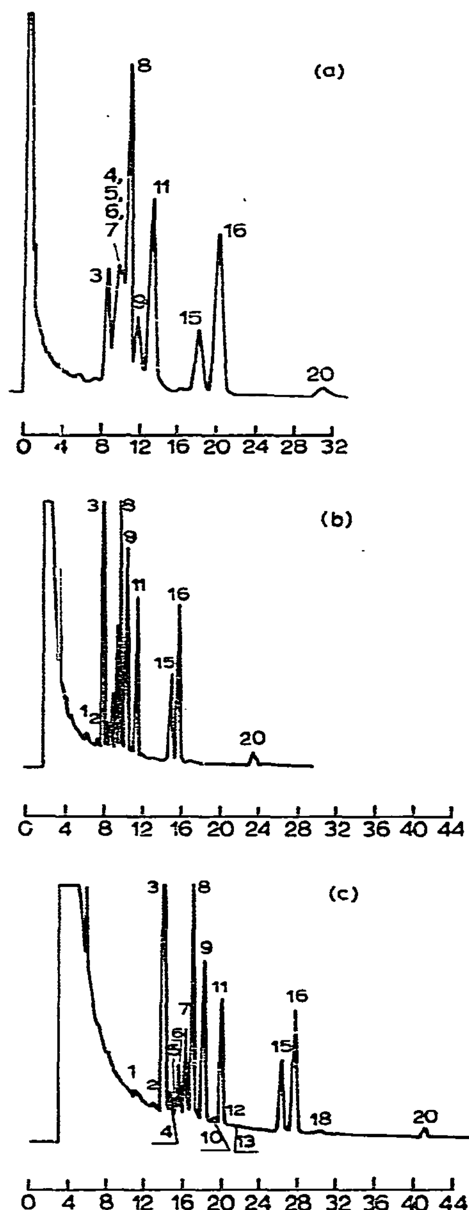

Fig. 1. Separation of sugar extract from *P. radiata* foliage on packed (a) and capillary (b) and (c) columns under different conditions. Conditions were: (a) 2 m × 4 mm I.D. glass column; 3% SE-30; oven temperature 175°; nitrogen carrier gas flow-rate 45 ml/min. (b) 60 m × 0.5 mm I.D. SE-30 glass SCOT capillary; oven temperature 200°; helium carrier gas flow-rate 7.5 ml/min; nitrogen make-up gas flow-rate 35 ml/min. (c) As for (b) except oven temperature 195° and helium flow-rate 5 ml/min. Peaks are as follows:

No.	*Compound*	*No.*	*Compound*
1	Unknown	10	Trace; unknown
2	Unknown	11	α-Glucose
3	Shikimic acid	12	Trace; MeO-cyclitol type?
4	Pinpollitol	13	Trace; MeO-cyclitol type?
5	Fructose I	15	Sequoyitol
6	Fructose II	16	β-Glucose
7	Fructose III	18	Trace; glucuronic acid
8	Pinitol	20	Myoinositol
9	Quinic acid		

Initial analysis of such "sugar extracts" on packed columns gave poor resolution of peaks (Fig. 1a) and showed the presence of unknown compounds[4]. Subsequent work led to the identification not only of the saccharide components (glucose, fructose, and sucrose), but also of the cyclitol myoinositol, O-methyl derivatives of cyclitols (pinitol, sequoyitol, and a new di-O-methyl cyclitol named pinpollitol[5,6]) and quinic and shikimic acids[7].

These findings were somewhat unexpected. It was known[8] that myoinositol was ubiquitous in plant tissues; pinitol was common in angiosperms and gymnosperms, and sequoyitol was also present in gymnosperms (which includes the *Pinus* spp.) but it was considered that with one or two exceptions[9] the levels would be minimal in living tissues. Instead these compounds and others were present in all *P. radiata* extracts in amounts equivalent to those of the monosaccharides. This appeared to be a more complex situation than had previously been described for other plant species.

As a consequence of this and the experience obtained from these earlier studies, the use of a capillary column for adequate resolution became essential. Furthermore, analysis of minimal tissue weight was required (approx. 100 mg dry weight), and the initial sample preparation and extraction had to be such that the same tissue (or extract) could be used not only for the sugar analysis, but also for starch, amino acids, and phenolics. The following describes the methods used and the typical results from the analysis of different *P. radiata* tissues for the monosaccharides, sucrose, cyclitols, and quinic and shikimic acids.

MATERIALS AND METHODS

Plant materials

Pinus radiata tissues were normally divided into buds, stem, roots, and foliage; older stem tissues were further divided into woody xylem, bark, and cortex. Pollen and callus tissue have also been analysed.

Plant material harvested in the field or from growth rooms was immediately frozen in liquid nitrogen and stored at —20° until required. It was then freeze-dried over 24–48 h, ground in a Wiley Mill to pass a 40-mesh sieve, and, if necessary, stored in screw-capped jars in a desiccator at 5°. Prior to extraction the samples were further dried *in vacuo* (at 14 mm/35° for 7 h) to remove any traces of moisture. Pollen was air-dried only.

Extraction

All extractions were duplicated. Dry ground tissue (100 mg) was weighed into a PTFE-lined screw-capped culture tube (16 × 125 mm) and 500 μl of [^{14}C]glucose (*ca.* 8 · 10^4 dpm) and 60% ethanol (5 ml) added. Tritiated glucose was used instead if working with ^{14}C-labelled plant tissue. The tubes were incubated in a water bath at 60° for 8 h, then centrifuged (1600 *g* for 5 min) and the supernatant plus washings (2 × 2 ml) carefully transferred to another test tube. The tissue residue was retained for subsequent starch analysis. The ethanolic extract was reduced to dryness in a vortex evaporator under vacuum (14 mm/40°), redissolved in water (5 ml), and washed with diethyl ether (3 × 5 ml for foliage extracts, 2 × 5 ml for all others). After centrifugation (1600 *g* for 10 min) and removal of the ether phase, 500-μl aliquots were transferred (in duplicate) to silanised 1-ml autosampler vials, and evaporated to dryness

(at 50°) in an air stream. The dry residues were stored at −20° until required for GLC analysis.

If phenolic compounds interfered with subsequent analysis the aqueous solution could be slurried with either charcoal (activated, acid washed) or polyclar AT (insoluble polyvinylpyrrolidone) prior to removing aliquots for GLC analysis.

Analysis of soluble sugars

Trimethylsilyl (TMS) derivatives of the sugar extracts were made by addition of pyridine (200 μl), hexamethyldisilazane (100 μl) and trimethylchlorosilane (50 μl) to the dried residue in the vials. The contents were mixed, the vials capped and left at 35° overnight. Reagents and samples must be kept dry throughout this sequence. Prior to GLC analysis the pyridine solution was taken to dryness (50°) under a current of dry nitrogen; sodium-dried hexane (250 μl, containing 1 μg/μl of *n*-docosane as internal standard) was added, the contents were mixed well, then centrifuged (100 *g* for 2 min). Duplicate aliquots (50 μl) were taken out for radioactivity determination (*i.e.*, placed in counting vials containing 10 ml of a solution of 4.5 g of "Permablend" scintillant per litre of AnalaR toluene). The hexane solutions of the sugar TMS derivatives were analysed as rapidly as possible to prevent derivative decomposition. However the addition of 1% trimethylsilylimidazole to the hexane solution considerably improved the stability of the sugar TMS derivatives.

GLC analysis of the soluble carbohydrate components was with a Pye 104 instrument equipped with a flame ionisation detector and a SGE (Scientific Glass Engineering, North Melbourne, Australia) splitless injector head connected to a 60 m × 0.5 mm I.D. SGE glass support-coated open tubular (SCOT) capillary column coated with SE-30 (number of effective plates 34,300 plates); oven temperature 200°, helium carrier gas flow-rate 7.5 ml/min; nitrogen make-up gas flow-rate 35 ml/min. The volume injected was 0.3 μl from a 1-μl syringe (SGE) using a Pye 104 S4 automatic injection unit. When analysing for sucrose only, the conditions were altered to helium flow-rate 8 ml/min and oven temperature 230°.

Calculation of results

A Hewlett-Packard 3380A reporting integrator was used for peak area integration and calculation of individual sugar mass concentrations in each sample. These were subsequently corrected for recovery losses as determined from the recovery of the [^{14}C]glucose. Alternatively, peak area values were utilised directly in a more comprehensive computation on an ICL 2903 computer, based on the following relationship:

$$\text{milligrams sugar per gram dry weight tissue} = \frac{\text{peak area sugar} \times \text{response of sugar (a)}}{\text{peak area standard} \times \text{response of standard (b)}} \times \text{standard mass (c)} \times \text{DF} \times \text{RF} \times \frac{1}{\text{wt}}$$

where

a = mass (μg)/area response for sugar standard;

b = mass (μg)/area response for *n*-docosane;

c = mass (μg) of *n*-docosane added to sample;

DF = dilution factor calculated from the volumes and dilutions used;
RF = recovery factor calculated from the recovery of radioactive glucose;
wt = weight of tissue extracted (in grams).

Final results were presented as mean values. The computing facility was developed because of the large number of samples needing to be analysed, with the object of using the instruments directly on-line to the computer in the future. Nevertheless, all necessary computations can be done equally well with the HP 3380A and a simple programmable calculator.

Starch analysis

The tissue residue from the soluble sugar extraction was gelatinised (4 ml water for 1 h at 100° in a water-bath) and hydrolysed by incubation with amyloglucosidase; the starch-glucose was quantified after oxidation with glucose oxidase/peroxidase reagent and reaction with 9 *N* sulphuric acid[10,11].

RESULTS AND DISCUSSION

Since it was expected that most analyses would be of small amounts of tissue from a variety of sources, snap freezing of samples in liquid nitrogen immediately at harvest was found essential. It reduced the variability in soluble sugar concentrations compared with non-frozen tissue analyses, as well as being a convenient and logical operation prior to freeze drying of the plant material. Significant changes in level of the monosaccharides occurred even within 1 h after harvest, and if oven drying was used instead of freeze drying the quantities of all components except cyclitols were significantly different and much reduced overall (Table I). The initial rise in glucose and fructose amounts could be readily attributed to sucrose or starch hydrolysis; their subsequent decrease was also due to continued enzymic action.

TABLE I

EFFECT OF POST-HARVEST DELAY AND TREATMENT ON SUGAR AND STARCH LEVELS

Constituent identification: 1 = shikimic acid; 2 = pinpollitol; 3 = fructose; 4 = pinitol; 5 = quinic acid; 6 = sequoyitol; 7 = glucose; 8 = myoinositol; 9 = sucrose; 10 = starch. Foliage from 12-year *P. radiata* sampled in April, 1977. The tissue was freeze dried, and extracted with 60% ethanol as in Materials and methods section; the results were corrected for extraction losses using radioactivity recovery factors; a, b and c indicate differences at 95% confidence level as determined by the Newman–Keuls multiple comparisons test. Sucrose and starch values were not tested.

Post-harvest treatment	*Extract constituents (mg/g dry wt.)*									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Frozen at 0 h	66.48^{a}	3.07	9.54^{b}	16.44	28.48^{a}	0.88	16.91^{b}	4.14^{a}	63.06	20.64
Frozen at 1 h	67.68^{a}	3.31	15.60^{a}	14.40	29.32^{a}	1.04	22.40^{a}	4.20^{a}	45.26	17.52
Frozen at 3$^{1}/_{2}$ h	74.57^{a}	3.12	8.80^{b}	14.18	28.84^{a}	1.17	15.18^{b}	5.42^{b}	106.00	17.62
Oven dried at 3$^{1}/_{2}$ h	38.81^{b}	3.86	3.06^{c}	16.78	7.22^{b}	0.76	4.81^{c}	4.93^{ab}	1.89	11.64

Soluble sugars have been traditionally extracted into 80% aq. ethanol; alternatively, 62.5% methanol (15 min at 55°) has been used, together with a chloroform wash step to remove lipid materials prior to colorimetric estimation[11]. Since larger

amounts of non-monosaccharide components were found, it was originally considered that a greater proportion of water would enhance extraction of these and of the monosaccharides[12], as well as reduce evaporative losses during the extraction period (8 h; chosen for practical convenience). Hence 60% aq. ethanol was used. Subsequent work has shown that aq. methanol further improved the extraction of all three groups of soluble components found in *P. radiata* (Table II). Lipid materials were removed by a diethyl ether wash; chloroform could be used, but at times unidentified interference from this solvent was noticed in a pronounced enhancement of the radioactive counts from the [^{14}C]glucose. This made its use inconvenient where recoveries were estimated by the radiotracer method.

TABLE II

COMPARISON OF DIFFERENT SOLVENTS FOR INITIAL SOLUBLE SUGAR EXTRACTION

All results corrected for extraction losses; tissue extracted was foliage from 12-year *P. radiata* harvested in March, 1977.

Solvent	*Concentration of soluble sugars (mg/g dry wt.)*		
	Monosaccharides	*Cyclitols*	*Acids**
100% water	71.84	27.16	52.06
80% ethanol	63.18	27.72	54.70
60% ethanol	66.08	28.52	50.64
80% methanol	69.55	30.12	60.22
60% methanol	66.89	28.18	61.54

* Quinic plus shikimic acids.

Interfering phenolic or ionic substances may also have to be removed or reduced substantially prior to GLC analysis. Evaluation of the use of insoluble PVP (Polyclar AT), activated charcoal, or ion-exchange resins showed that a slurry of PVP was the most efficient at removing phenolic substances (as determined by thin-layer chromatography and selective spray reagents). However, a more selective effect was shown by the charcoal or ion-exchange resin (Amberlite MB 3); the former removed the shikimic acid component, while the latter removed both the shikimic and quinic acids. Hence it would be necessary to use both PVP and ion exchange if a pure sugar extract was required. The use of a capillary column allowed for a far less stringent clean-up procedure as acidic substances were readily separated from the monosaccharide and cyclitol compounds (Fig. 1c). The analytical procedures further reduced (by the derivatisation and separation conditions) interference from compounds such as amino acids.

A known amount of [^{14}C]- or [^{3}H]glucose was added to the tissues being extracted, and the radioactivity in the final hexane solution of the sugar TMS derivatives was determined. This gave a very accurate recovery factor, as it was based on a compound identical with one that was being extracted, and also from the actual solution analysed by GLC. However, though it was accurate for glucose and probably fructose, it was only a close approximation for the cyclitol and acid components.

In most samples, more than 80% of [^{14}C]glucose was recovered, but recoveries as low as 30% have been obtained owing to decomposition of the TMS derivatives

and losses during the clean-up procedure, *e.g.*, by charcoal or PVP treatment. Incorrect recovery factors could result also from the purification procedures. Water-soluble fluorescent compounds extracted from the PVP, and chloroform residues if chloroform–methanol partitioning was used, enhanced the radioactivity counts. The former has to be thoroughly washed in water and 60% ethanol before use. Where a greater margin of error can be tolerated, the procedure can be simplified by omitting the [^{14}C]glucose. An illustration of the need for correcting for extraction losses is shown in Table III where recoveries of glucose and pinitol (as examples from the typical mixture of compounds) range from 85% down to 55%.

TABLE III

COMPARISON OF A TYPICAL RANGE OF GLUCOSE AND PINITOL RECOVERIES FROM *P. RADIATA* DURING A DROUGHT STRESS STUDY, BEFORE AND AFTER CORRECTION FOR EXTRACTION LOSSES

RF = radioactivity recovery factor. Percentage recoveries were 55, 71, 77, 81 and 85, respectively.

Day	*Compound (mg/g dry wt.)*			
	Glucose		*Pinitol*	
	−RF	*+RF*	*−RF*	*+RF*
1	14.86	26.82	12.26	21.30
2	16.68	23.52	16.42	23.14
3	21.84	28.20	17.38	21.29
4	23.86	29.54	20.05	24.72
5	21.96	25.75	20.58	24.18

Analysis of the TMS derivatives of the *P. radiata* monosaccharide components on a packed SE-30 column gave inadequate resolution of component peaks (Fig. 1a); and with the large numbers of samples for analysis it was also necessary to decrease the time per analysis. Use of a SCOT capillary column reduced analysis time and increased resolution, especially of the earlier peaks (Fig. 1b). A more constant detector response was achieved by injecting the TMS ethers in hexane which decreased the solvent tailing and detector contamination produced by the pyridine solutions.

"Ideal" capillary column conditions resolved all component peaks (Fig. 1c), but analysis time was impractical for the routine procedure. The analytical conditions chosen were therefore a compromise between good resolution and minimum time, and in fact with an efficient integrator individual peak areas changed very little from those obtained under optimal conditions. Analysis time could be further reduced by temperature programming after the monosaccharides had eluted and by determining sucrose amounts in the same sample.

The procedure was complex, but if care was taken quantification was accurate, error 2–5%, and replication good. In a drought stress study[13] most monosaccharide analyses had a coefficient of variation of less than 5% (Table IV). The main causes of variation were injection error and sample losses during extraction. The internal standard, *n*-docosane, corrected for the former, but in the case of reduced injection volumes (such as from a partially blocked syringe) integration error was increased for all peaks, and the correction was less accurate. To correct for extraction losses with

TABLE IV

REPRODUCIBILITY OF ANALYSES FROM A SINGLE TREE DURING A DROUGHT STRESS STUDY

S.D. = standard deviation calculated from eight replicates.

Day	*Compound (mg/g dry wt.)*	
	Glucose (±S.D.)	*Pinitol (±S.D.)*
13	49.66 ± 1.94	44.43 ± 1.98
19	40.80 ± 2.60	41.52 ± 1.63
20	43.03 ± 1.02	42.35 ± 1.58
20	50.09 ± 1.67	44.43 ± 1.56
20	50.88 ± 1.64	44.71 ± 2.50

good replication, *i.e.*, within ±5%, it was necessary to have recoveries greater than 60%.

The TMS derivatives of the monosaccharides and cyclitols were reasonably stable, but those of other components, *e.g.*, the soluble acids, were less stable and decomposition contributed to the variation in results, if the solutions were not analysed fresh. Under refrigeration and in sealed vials the derivatised samples would keep for a week in hexane and even longer in HMDS–TMCS–pyridine, or in hexane plus 1% trimethylsilylimidazole. The use of Tri-Sil-Z (Pierce, Rockford, Ill., U.S.A.) instead of HMDS–TMCS–pyridine may be warranted for larger sample volumes or less stable derivatives, for it should be possible to directly inject reagent solution without adverse effects.

The reliability of the method as a whole is largely dependent upon consistent preventive measures. These include correct choice of syringe (SGE, 1 µl capacity, removable-needle type); choice of septa and replacement after 100 injections; all solvents need to be kept dry and preferably under nitrogen; reagents should be fresh and not premixed to avoid excessive ammonium chloride formation; regular cleaning and silylation of the injector head; regular removal of the silica deposits in the detector, together with injection of the smallest possible volumes so that the deposit is much reduced. Actual analytical conditions are finally set by slightly altering the oven temperature and carrier gas inlet pressure so that the resolution between pinpollitol and the fructose peak is maximised.

Previous workers[5,6,12] had shown that pinpollitol, pinitol, sequoyitol, and myoinositol were present in *P. radiata* extracts, as well as the monosaccharides glucose and fructose and the disaccharide sucrose, by a combination of GLC, GLC–mass spectrometry (MS) and ^{13}C nuclear magnetic resonance techniques. The individual relative retentions listed in Table V showed that there were more peaks resolved by this analysis than those previously identified. Conversely, the absence of mannose in the extract was established by calculation of the relative fructose peak areas, as the column and conditions chosen provided sufficient resolution of the isomers of pure mannose and fructose, which overlap in a mixture. It should be noted that soluble acids were also detected. Positive assignment of individual peaks was still difficult as the relative amounts of compounds varied according to tissue type and season. For example, mannitol (peak 14) was found in reasonable amounts only in root extracts.

TABLE V

P. RADIATA "SUGAR EXTRACT" COMPONENT IDENTIFICATIONS

Compounds listed were isolated by preparative GLC and identified by comparison with authentic standards on SE-30 and OV-17 capillary columns and by GLC–MS. All R_{RT} values for TMS derivatives at the following GLC conditions: 60 m × 0.5 mm I.D. SE-30 glass SCOT capillary; oven temperature 195°; helium carrier gas flow-rate 5 ml/min; nitrogen make-up gas flow-rate 35 ml/min.

Peak No.	R_{RT}	*Standard* R_{RT}	*Identification*
1	0.584	—	Unknown
2	0.669	—	Unknown
3	0.734	0.732	Shikimic acid
4	0.752	—	Pinpollitol
5	0.781	0.785	Fructose I
6	0.797	0.800	Fructose II
7	0.820	0.824	Fructose III
8	0.859	0.866	Pinitol
9	0.914	0.918	Quinic acid
10	0.960	—	Trace; unknown
11	1.000	1.000	α-Glucose
12	1.030	—	Trace; MeO-cyclitol type?**
13	1.060	—	Trace; MeO-cyclitol type?**
14	1.194	1.211	Mannitol*,**
15	1.278	1.279	Sequoyitol
16	1.390	1.390	β-Glucose
17	1.500	1.518	Trace; gluconic acid*
18	1.578	1.563	Trace; glucuronic acid*
19	1.641	—	Trace; cyclitol?**
20	1.996	2.002	Myoinositol

* Identification by GLC only.

** Identification by GLC–MS only.

These conclusions were confirmed by similar analysis on an OV-17 capillary column and GLC–MS.

The procedures described have been in continuous use in our laboratory for the past 3 years, with a throughput of up to 5000 sample analyses per annum. The method is suitable for the analysis of soluble sugars from all pine tissues, as well as from other plant material such as pollen, tissue culture callus, grasses, cereals, and tobacco. A great range of soluble sugar levels, *e.g.*, from 1 to 50 mg glucose per gram dry wt. tissue (equivalent to 0.025% to 1.25% per gram fresh wt.) have been successfully quantified; other major components had similar concentration ranges.

Although this method of analysis of soluble sugars by automated GLC is more complex than traditional colorimetric procedures, it can still be used to analyse large numbers of samples in a relatively short time. Furthermore, each component sugar is individually quantified. It is the only accurate routine method for extracts such as those from *Pinus* spp., for the colorimetric procedures make no distinction between the different monosaccharides and do not respond to the other components present. It is anticipated that the selectivity and precision of this analytical procedure will be used to elucidate the relative roles of monosaccharides, soluble acids, and cyclitols in *P. radiata* metabolism.

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