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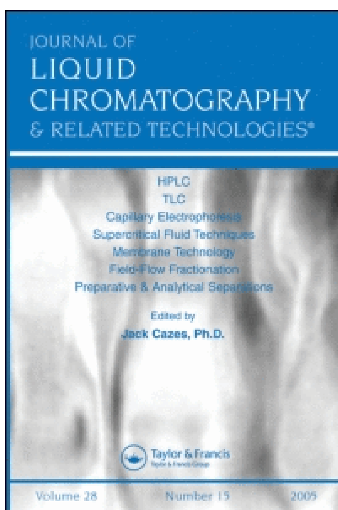

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DESALTING AND FREEZE-DRYING TECHNIQUES TO PREPARE PLANT DERIVED NEUTRAL SUGAR EXTRACTS FOR QUANTITATIVE HPLC

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ABSTRACT

Desalting and sample concentration are frequent prerequisites for the quantitative HPLC analysis of sugars and polyhydric alcohols in extracts of biological material. However, desalting using Dowex mixed-bed resins and sample concentration by conventional freeze-drying adversely affect the recovery of the carbohydrates being analysed, making quantitation almost impossible. In the present paper we report modified sample preparation procedures which overcome this technical problem. Efficient desalting was achieved by batchwise deionisation with an Amberlite mixed-bed deionisation resin, incorporating thymolphthalein to provide a sensitive indicator of resin exhaustion. Complete recovery of neutral sugars was possible provided the resin was subsequently washed with water. Concentration of dilute extracts with full recovery of neutral sugars was achieved by freeze-concentration using a Savant

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Speed-Vac centrifugal freeze-concentrator. The combination of these desalting and freeze-concentration procedures gave excellent recoveries of the neutral sugars together with dramatic improvements in HPLC column life and chromatographic resolution.

INTRODUCTION

High-performance reverse-phase partition chromatography using cation-exchange resins is an ideal method of separating a variety of mono and oligosaccharides. However, the application of these resins in the quantitative HPLC analysis of sugars present in biological extracts is severely compromised by the presence of protein, lipid and cationic contaminants (1). Since these contaminants can adversely affect chromatographic resolution and drastically shorten column life, sample preparation procedures must ensure their efficient removal. As described by other workers (2) partial deproteinisation can be accomplished with solvent and heat treatments, but complete deproteinisation requires the addition of Zn^{++} and Ba^{++} salts, resulting in further contamination by divalent cations. Similarly, other effective deproteinisation procedures such as perchloric acid precipitation also increase the levels of contaminating salts. Contaminating salts can be removed by sample pretreatment with mixed-bed resins. However, the resins investigated to date (eg Dowex AG-501-X8) have been shown to be inappropriate in the preparative clean-up of biological samples (2) because of differential binding of sugars and polyhydric alcohols. Other deionisation procedures such as the use of in-line precolumns packed with anion- and cation-exchange resins (3) are only useful when the level of contamination with inorganic salts is low.

Another common problem with the application of quantitative HPLC to the analysis of biological extracts is the low concentration of the metabolites to be analysed. Sample

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concentration procedures involving freeze-drying are frequently used, but this can result in the removal of low and intermediate molecular weight compounds from the concentrates by the high vacuum (4).

In our laboratory we have experienced similar problems with deionisation and freeze-drying techniques, making routine quantitative HPLC analysis of neutral sugar extracts of plant material almost impossible. However, in the present paper we report modifications to these techniques which render them highly appropriate for the preparative clean-up of biological samples.

METHODS

Materials

All chemicals used were obtained from either BDH Chemicals Ltd (Poole, Dorset, UK) or Sigma Chemical Co Ltd (Poole, Dorset, UK).

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Extraction of Neutrals Sugars

Endosperm tissue (about 100 mgs wet weight) from developing wheat grain sampled at mid grain filling (about 20 days post anthesis) was homogenised in 3ml ice-cold 1M Perchloric Acid (PCA) using a Polytron vortex homogeniser. The tissue homogenate was centrifuged at 3,000x g for 15 minutes at 4°C using an MSE Chilspin bench centrifuge. The supernatant (PCA soluble fraction) was decanted off and neutralised with 5M KOH and 1M KH_2PO_4. Aliquots (0.1μCi each) of radioactively labelled [U-^{14}C] sucrose (>350mCi/mmol, >13GBq/mmol), D-[U-^{14}C] glucose (>230mCi/mmol,>8.5GBq/mmol), or D-[U-^{14}C] fructose (150-300mCi/mmol, 5.5-11GBq/mmol), obtained from Amersham International PLC, (Bucks, UK) were added to the neutralised

soluble extracts (typically 7-8ml volume) and used to assess recoveries following deionisation and freeze-drying. Deionisation was achieved by batchwise treatment of the samples with Amberlite mixed-bed resin containing thymolphthalein indicator (Type MB-1A, obtained from Sigma Chemical Co, Poole, Dorset, UK). The resin treatment involved adding the resin until the thymolphthalein indicator just retained its initial colour (typically requiring 3-5g resin for 7-8ml neutralised PCA extract).

Samples of deionised biological extracts or buffered (10mM Morpholino-ethane sulphonic acid (MES) buffer pH 6.5) standards containing ^{14}C- labelled sugars were concentrated by freeze-drying using a Savant Speed-Vac centrifugal freeze-concentrator (Model SVC 100H obtained from Savant Instruments Inc, Hicksville, NY, USA) fitted with an Edwards two-stage high vacuum pump (Model E2M2 obtained from Edwards High Vacuum, Crawley, Sussex, UK). The vacuum achieved was between 50 and 100 millitorr. Radioactivity was measured with a Beckman 8086 liquid scintillation counter using external standards to correct for quenching by the H# principle.

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Chromatographic Conditions

A Waters Sugar Analyser 1 (Waters Associates Ltd, Northwich, Cheshire, UK) fitted with a Bio-Rad HPX-87C carbohydrate analysis column (300 x 7.8mm column; column temperature 85°C) and a Bio-Rad Micro-Guard carbohydrate refill cartridge (Bio-Rad Laboratories Ltd, Watford, Hertfordshire, UK) was used for HPLC analyses. The sugars were eluted with water as the mobile phase at a flow-rate of 0.7ml/min, and detected using a Waters R401 Refractive Index monitor coupled to a Trivector Trilab 2,000 chromatography data system (Trivector Scientific Ltd, Sandy, Bedfordshire, UK).

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatographic profile of a neutralised PCA-soluble extract of the carbohydrates present in endosperm tissue of developing wheat grain. In our experience, only very dilute samples can be analysed (eg 20μl injection from 8ml of a neutralised PCA extract of 100mg wet weight of endosperm tissue) because the high levels of contaminating salts adversely affect chromatographic resolution (Figure 1) and can irrevocably damage a new column. Thus in order to routinely use HPLC to separate neutral sugars, the samples must first be deionised and then concentrated before analysis. Efficient desalting was achieved with an Amberlite mixed-bed resin and full recovery of the sugars was possible provided the resin was washed with a small volume of water (Figure 2). Sample concentration was achieved using a Savant Speed-Vac centrifugal freeze-concentrator with recoveries of neutral sugars approaching 100% (Table 1).

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TABLE 1

% Recoveries of Neutral Sugars after Freeze-Drying to Dryness Results are expressed as Mean ± S.E., n=4 except * where n=2. Samples were freeze-dried in a Savant Speed-Vac centrifugal freeze-concentrator.

	% Recovery	
1ml 10mM Sucrose in MES buffer (pH 6.5)	98.6%	±0.8
100mls ^{14}C Sucrose in MES Buffer (pH 6.5)	95.1%*	
1ml ^{14}C Sucrose in neutralized/desalted PCA extract of wheat endosperm	97.7%	±0.8
10mls ^{14}C Sucrose/Glucose/Fructose in neutralized/desalted PCA extract of wheat endosperm	99.5% *	

FIGURE 1: Chromatographic Profile of Neutral Sugar Extract of Developing Wheat Grain. (Refractometer attenuation x 1) 20μl injection from 8ml of a neutralised PCA extract of 100mg wet weight of endosperm tissue. Approximate amounts of Sucrose, Glucose and Fructose were 5.7 nmoles, 0.9 nmoles and 3.8 nmoles respectively.

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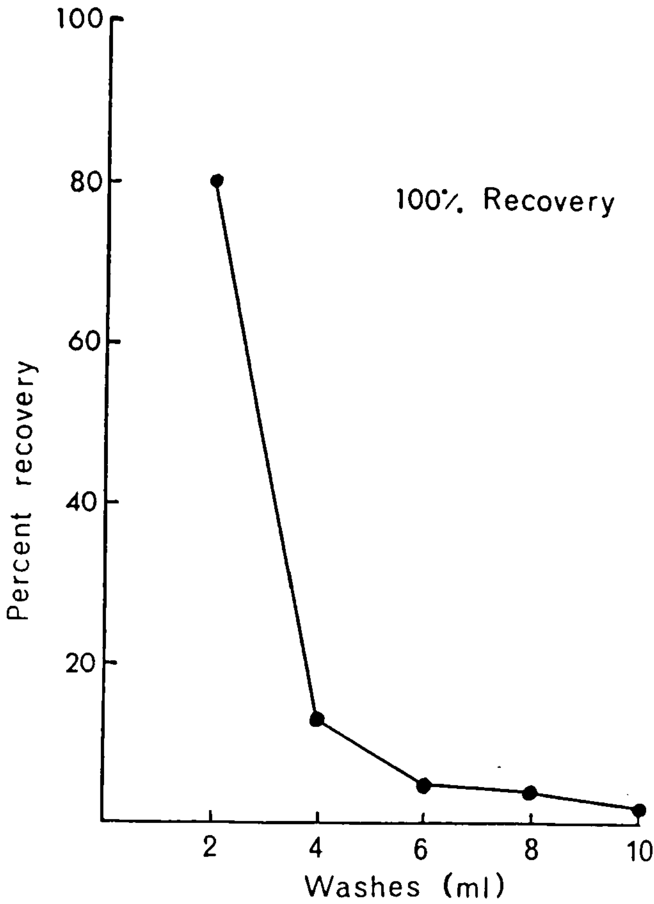

FIGURE 2: Recovery of ^{14}C Sucrose, Glucose and Fructose. Repeated 2ml washes of amberlite mixed-bed resin following deionisation treatment of a PCA-soluble neutral sugar extract of developing wheat grain.

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The combination of these effective sample deionisation and concentration procedures allowed analyses of more concentrated extracts (eg 40 times more concentrated - Figure 3), with dramatic improvements in chromatographic resolution and column life. In other experiments we have successfully analysed samples which were upto 4,000 times more concentrated than that shown in Figure 1.

Our finding that neutral sugar extracts can be desalted with an Amberlite mixed-bed resin (OH^- and H^+ forms) contrasts with the report (2) that neutral sugars bind to other mixed-bed resins such as Dowex AG-501-X8 (OH^- and H^+ forms). Thus, although the sugar-binding capacity of Dowex resins is due to an interaction with only the anionic (OH^- form) exchanger (2) this binding does not appear to be a general feature of all OH^- form anion exchangers. Similarly, our finding that centrifugal freeze-concentration gives good recoveries of neutral sugars contrasts with the report (4) that conventional freeze-drying techniques give variable recoveries of neutral sugars. Furthermore, although the data presented in this paper relate only to the advantages of centrifugal freeze-drying for neutral sugar extracts of plant material, we have also observed full recovery of other plant cell metabolites including hexose phosphates and nucleotide sugars (data not presented). Thus, whilst we would agree with Van Sumere _et al_ (4) that conventional freeze-drying of biological material should be avoided, centrifugal freeze-drying may have general applicability for the concentration of many low or intermediate molecular weight compounds.

In conclusion, the combination of effective desalting and freeze-concentrating techniques have enabled us to prepare plant-derived neutral sugar extracts in a highly concentrated

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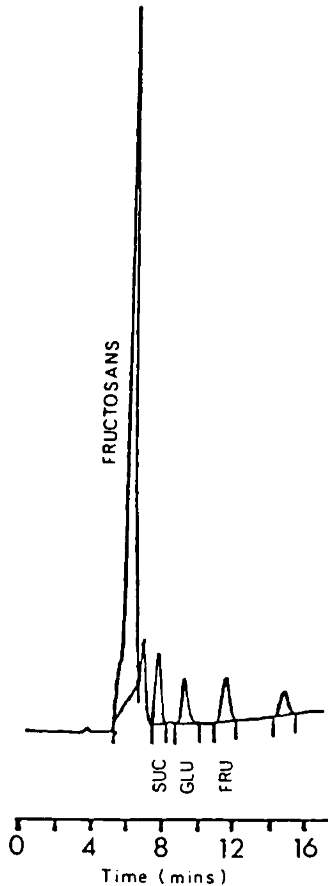

FIGURE 3: Chromatographic Profile of Desalted and Freeze-Concentrated Neutral Sugar Extract of Developing Wheat Grain. (Refractometer attenuation x 64). 50μl injection from 500μl of a freeze-concentrated neutralised and desalted PCA extract of 100mg wet weight of endosperm tissue. Approximate amounts of Sucrose, Glucose and Fructose were 230 nmoles, 40 nmoles and 150 nmoles respectively.

form suitable for routine and quantitative HPLC analysis. These procedures have dramatically improved chromatographic resolution and significantly increased HPLC column life.

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