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Note

Differentiation between glucose, mannose, allose and galactose in plant glycosides by high-performance liquid chromatographic analysis

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Allose, the 3-isomer of glucose (Fig. 1), is a rare natural sugar, first reported in vascular plants in 1971 as the sugar moiety in two phenylpropanoids isolated from *Protea rubropilosa* Beard (Protaceae).[1,2] Since then more than twenty allose-containing compounds have been described including 10 irridoids[3,4] 1-chroman-type[5] compound and 14 flavonoids.[6–13]

The identification of sugars in plant glycosides traditionally is performed following acid hydrolysis by paper chromatography (PC) or by thin-layer chromatography (TLC) on cellulose.[14] However glucose, allose and mannose have very similar R_F values in the commonly used solvent systems making a differentiation between them difficult. Of the more than twenty compounds mentioned above allose was therefore either identified by the classical method of osazone and *p*-bromophenylhydrazone derivatives or by gas chromatography as trimethylsilyl and methyl derivatives. Both technique are time consuming. Identification by NMR spectroscopy is possible, but large samples are necessary: a signal at *ca.* 67.4 ppm in the ^{13}C NMR

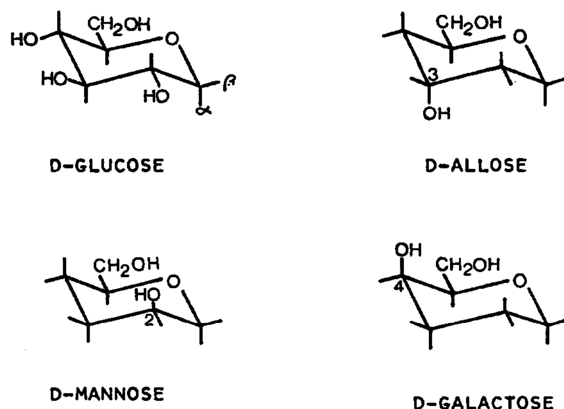

Fig. 1. Structures of the 4 isomer hexoses; for mannose, allose and galactose only the hydroxyl group distinguishing them from glucose is shown.

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is characteristic for the C-4 of either allose or mannose, but not for glucose, whereas a large coupling constant of more than 7 Hz for the anomeric proton in the ^{1}H NMR may be assigned to glucose or allose, but not to mannose.

In continuation of our research on allose-containing flavonoid glucosides in the genus *Stachys* (Labiatae)[11,12] we found a simple high-performance liquid chromatography (HPLC) method for easy differentiation between glucose, mannose, allose and galactose in plant glycosides. The analysis was performed with an Aminex HPX-87P monosaccharide column, commonly used for quality control in food industry[15,16] and for biomass processing[17]. In a short analysis time (25 min) good separation was achieved with high reliability in retention times, using simple isocratic operation and degassed, deionized water as solvent.

EXPERIMENTAL

Materials

Reference compounds glucose, mannose and galactose were obtained from Sigma (St. Louis, MO, U.S.A.) and allose from Fluka (Buchs, Switzerland). The allose-containing flavonoid glucoside (Fig. 2) was isolated from *Stachys recta* L. (Labiatae) as previously reported[11].

Preparation of the glycoside sample

For hydrolysis of the flavonoid glycoside 20 mg were dissolved in 10 ml 0.1 *N* trifluoroacetic acid and refluxed on a steam bath for 50 min. After repeated evaporation *in vacuo* of the hydrolysis solution, the residue was taken up in water and the flavonoid aglycone extracted from the aqueous solution with ethyl acetate. To remove remaining traces of polyphenolic compounds the aqueous solution was further filtered through Sep-Pak Aluminium N cartridges (Waters Assoc., Milford, MA, U.S.A.) and then the filtrate was freeze-dried.

Aparatus

Chromatography was carried out using a Beckman 110A pump with a Labocord 100 refractometer (Labomatic, Allschwill, Switzerland) for monitoring the effluent. Peak areas were calculated with a Shimadzu Chromatopac C-R3A data processor. The sugars were separated with an Aminex HPX-87P heavy metal monosaccharide analysis column (Bio-Rad Labs., Richmond, CA, U.S.A.), packed with an 8% cross-linked sulfonic acid type cation-exchange resin in the lead form, particle

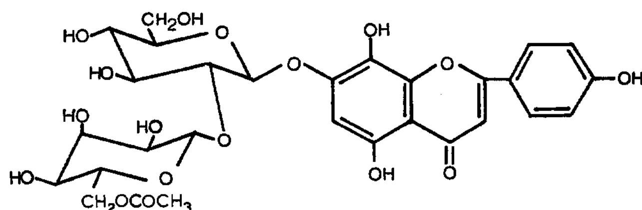

Fig. 2. Structure of the allose-containing flavonoid glucoside isoscutellarein 7-O-(2″-O-6‴-O-acetyl-β-D-allopyranosyl-β-D-glucopyranoside).

size, 9 μm; column size, 300 × 7.8 mm I.D. For column protection a Bio-Rad in-line carbohydrate deashing system was used, consisting of one cation and one anion exchange micro-guard cartridge.

Operating conditions

Milli-Q standard water (Millipore, Bedford, MA, U.S.A.), kept degassed during the analysis process by maintaining it at 85°C, was used as the mobile phase. The separations were performed at a temperature of 85°C, obtained by heating the column with a Bio-Rad column heater. The flow-rate was 0.5 ml/min.

RESULTS AND DISCUSSION

Chromatograms of the reference compounds and the glycoside sample are shown in Fig. 3. The reference compounds eluted in the sequence glucose, galactose, mannose, allose, with each compound clearly separated by a difference in retention

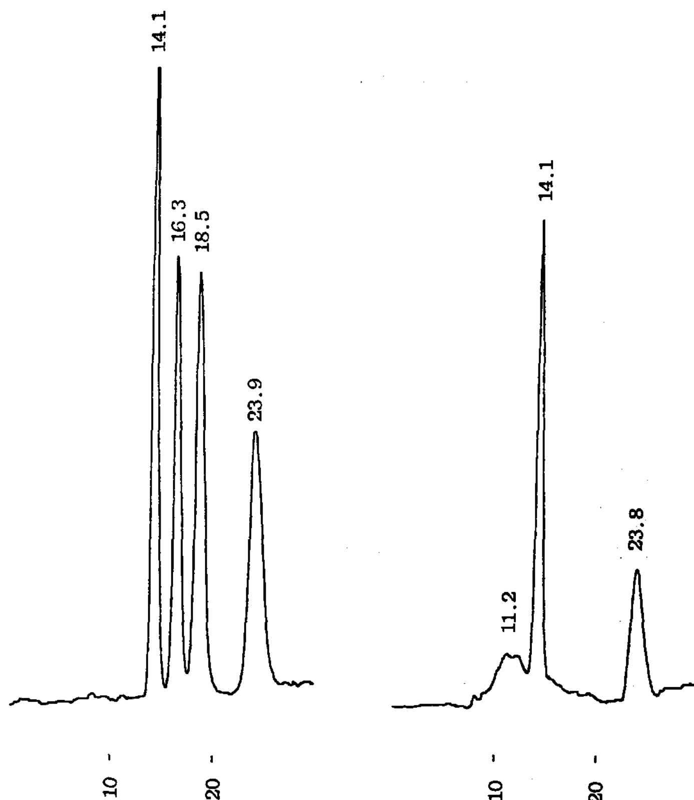

Fig. 3. Chromatograms of the reference compounds (left) and the sugars from the allose-containing flavonoid glucoside sample (right). The sugars eluted in the following order: glucose (t_R = 14.1 min), galactose (t_R = 16.3 min), mannose (t_R = 18.5 min) and allose (t_R = 23.9). Corresponding values were obtained for glucose (t_R = 14.1 min) and allose (t_R = 23.8 min.) in the hydrolyzed flavonoid glucoside sample.

time of more than 2 min. It is interesting that glucose and allose show the greatest separation since these sugars are the most difficult to separate by PC or TLC. A high reliability in retention time between the reference compounds and the glycoside sample can be observed thus allowing easy and accurate identification of these hexoses when obtained from plant glycosides.

The reference compounds were applied in equal quantities of *ca.* 2.5 mg each in 1 ml water; 20 μl of the solution were injected. All compounds gave about the same peak areas indicating essentially identical detectability by the refractive index (RI) detector. In order to obtain similar concentrations for the glycoside sample relative to the reference compounds, an amount of 5 mg/ml was used according to the diglycosidic character of the sample; 20 μl were injected. On the chromatogram of the glycoside sample in addition to peaks for allose and glucose a broad peak with t_R = 11.2 min was detected typical of disaccharides; thus, this peak is probably a trace of the disaccharide unit allosylglucose.

Detection limit

With the method described above (using an RI detector) reference compounds of the four isomer hexoses could be detected by injection of a minimum amount of 1 μg each (dissolved in 10 μl water). For plant glycosides however larger amounts may be required depending on the purity of the sample. The detection limit further depends considerably on the column quality and the disturbance caused by pulsation of the pumps.

ACKNOWLEDGEMENTS

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