Identification of Methyl β-D-Glucopyranoside in White Clover Foliage

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Gas–liquid chromatographic (GLC) analyses of carbohydrates in white clover (*Trifolium repens* L.) tissue indicated the presence of an unidentified component in the 80% ethanolic extract of the plant material. The purpose of this research was to isolate and identify the component and determine the distribution of this component in forage legume species. The component was isolated from the 80% ethanolic extract of plant tissue by column chromatography and identified as methyl D-glucopyranoside by gas chromatography–mass spectrometry. Subsequent chemical tests indicated the compound to be methyl β-D-glucopyranoside (methyl glucoside). Methyl glucoside was found to be present in the foliage of 13 cultivars of white clover. However, methyl glucoside was not found to be present in the foliage of 5 cultivars of alfalfa (*Medicago sativa* L.), 11 cultivars of red clover (*Trifolium pratense* L.), and 9 cultivars of bird's-foot trefoil (*Lotus corniculatus* L.) grown in small field plots. Methyl glucoside was found only in foliage material, being highest in stems and petioles (11.4 mg/g of tissue dry weight). Leaflet tissue contained only 2.5 mg/g of dry weight.

Carbohydrates in forage legume plants have been extensively studied by researchers concerned with photosynthesis, translocation of organic materials, and forage quality (Greub and Wedin, 1969; Grateluesc hen and Smith, 1967; Raguse and Smith, 1965; Smith et al., 1964). Generally, clover and alfalfa are characterized by sucrose and starch accumulation (Smith, 1969). Starch is the primary nonstructural polysaccharide accumulated in species of the Leguminosae, and sucrose is the major carbohydrate component in the 80% ethanol extract from legume foliage material. Smaller quantities of glucose, fructose, melibiose, raffinose, and stachyose have been found in the ethanolic extract of certain legumes (Hirst et al., 1959).

We recently reported a rapid method for gas chromatographic analysis of mono- and disaccharides extracted from plant material (Phillips and Smith, 1973). In the development of this method, several compounds, in addition to the commonly occurring carbohydrates, were detected in the ethanolic extract from legumes. Some of these compounds have been isolated and identified (Phillips and Smith, 1974). This paper describes the identification of methyl β-D-glucopyranoside in the 80% ethanol extract from white clover.

MATERIALS AND METHODS

Plant tissue was collected from plants grown in the greenhouse and field plantings of forage cultivars established at the Georgia Mountain Experiment Station located near Blairsville, GA. A detailed description of plant culture techniques have been previoulsy reported (Smith and Phillips, 1980). Following harvest, the dried plant tissue was ground to pass a 40-mesh screen in a Wiley mill prior to extraction.

The dried ground tissue was extracted with 80% aqueous ethanol and the extracts were cleared as previusly described (Smith and Phillips, 1980). The cleared extracts were either (1) frozen and freeze-dried before preparation for GLC analysis or (2) cleared of mono- and disaccharides by repeated passage through columns of Dowex 21K (OH^-) (Roseman et al., 1952) prior to GLC analysis. The eluant from the Dowex 21K column contained primarily pinitol, methyl glucoside, and some *myo*-inositol. Subsequent studies have indicated methyl glucoside recovery rates of greater than 87% following passage of the plant extract through the Dowex 21K column. This is in agreement with recovery data reported for methyl α-D-glucopyranoside (Roseman et al., 1952). The methyl glucoside and cyclohexanehexols (cyclitols) were further separated by cellulose-powder chromatography using Whatman cellulose powder (CC31) as the column packing (2 cm i.d. × 12 cm high) and acetone–water (4:1 v/v) as the mobile phase. The acetone–water eluate was dried to an aqueous phase at room temperatures and the aqueous solution was freeze-dried prior to preparation for GLC and mass spectrometry. The isolated methyl glucoside was identified by mass spectrometry and GLC of derivatives before and following hydrolysis by autoclaving (15 psi) for 15 min in 2 N H_2SO_4.

All GLC flow rates, temperatures, and program conditions were as described previously (Phillips and Smith, 1973). Methyl nonadecanoate was used as the internal standard. Mono- and disaccharides were identified by cochromatography with derivatives of known carbohydrates. Pinitol was identified by cochromatography with pinitol isolated from soybean (Phillips and Smith, 1974). Methyl glucoside was quantified by comparing the molar response with authentic methyl β-D-glucopyranoside (Sigma Chemical Co., St. Louis, MO).

The mass spectra were recorded with an AEI Model MS-30 (spectra obtained by Shrader Analytical & Consulting Laboratories, Inc., Detroit, Mi 48210) and an LKB 9000 spectrometer. All samples were trimethylsilyl derivatives (Phillips and Smith, 1973) dissolved in dimethylformamide and were admitted through a gas chromatograph equipped with a column packed with Chromosorb W coated with 5% SE-30. All spectra were obtained at 70-eV ionizing potential. Molecular identification was by comparison of the spectra with the standard spectra for methyl 2,3,4,6-tetra-*O*-(trimethylsilyl)-β-D-glucopyranoside.

RESULTS AND DISCUSSION

The major benefit in determining sugar mixtures by GLC of silylated derivatives at mutarotation equilibrium is the need to resolve only GLC peaks for single isomers of each monosaccharide. The predictability of the concentration of other isomers, due to ratio distribution, makes it unnecessary to resolve all GLC peaks. We have recently published the relative retention times and percentage of total sugar concentration represented by each GLC peak (isomer) of individual sugars silylated at mutarotation equilibrium (Phillips and Smith, 1973). When using this method to analyze the carbohydrates in the

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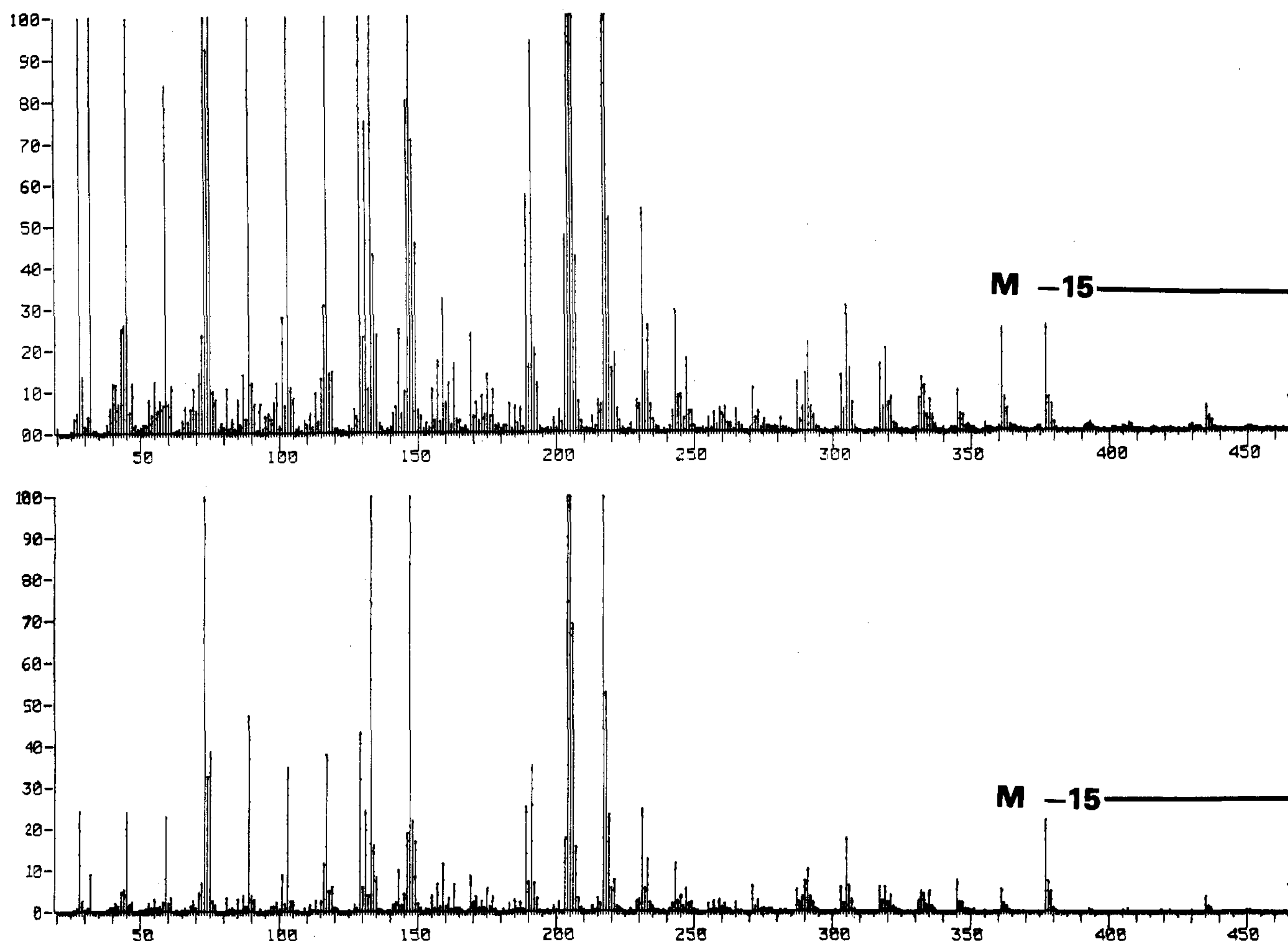

Figure 1. Electron impact mass spectra of the trimethylsilyl ether of methyl D-glucopyranoside. (Top) Purified compound from white clover foliage tissue. (Bottom) Authentic methyl D-glucopyranoside. Molecular mass − 15 (M − 15) is 467.

ethanolic extract of white clover tissue, it was noted that an unidentified component was interfering with the α-glucose peak, giving an α-glucose/β-glucose concentration ratio much higher than that expected at mutarotation equilibrium. The interfering component was found to be stable in Dowex 21K (OH^-) columns and elutriate with the cyclitols (*myo*- and *chiro*-inositol and pinitol) when column chromatographing the tissue extract. This component was easily separated from the cyclitols on columns of cellulose powder. The mass spectrum (Figure 1) for this component indicated the compound to be methyl D-glucopyranoside. The important ions for comparison are at m/e 231, 377, 435, and 467. The later ion is equal to the molecular mass [methyl 2,3,4,6-tetra-*O*-(trimethylsilyl)-D-glucopyranoside] less 15 (M − 15). Subsequent cochromatography of the isolated component (clover X) with the commericially obtained methyl β-D-glucopyranoside (standard) and the hydrolyzed component and standard confirmed the component to be methyl D-glucopyranoside (Figure 2). The two peaks in the hydrolyzed fractions of both samples were identified as α- and β-glucose by cochromatography with standared glucose (Sigma Chemical Co., St. Louis, MO). Also, the concentration ratios of peak 1/peak 2 (0.68) was comparable to the expected ratio for α-glucose/β-glucose (0.70) at mutarotation equilibrium. The methyl glucoside, isolated from white clover, was readily hydrolyzed by β-D-glucoside glucohydrolase (Sigma Chemical Co., St. Louis, MO) and cochromatographed with authentic methyl β-D-glucopyranoside. The methyl nonadecanoic based relative retention time was slightly higher for methyl β-D-glucopyranoside (0.66) compared to the relative retention time

Table I. Carbohydrate and Methyl Glucoside Concentrations in Regal Ladino Clover Leaflet, Stem, and Root Tissue Harvested from Plants Cultured in the Greenhouse

	mg/g of tissue (dry wt)		
compound	leaflet	stem	root
fructose	1.7b[a]	7.6a	3.1b
glucose	2.4b	16.5a	0.9b
sucrose	20.6b	31.6a	23.8b
methyl glucoside	2.5b	11.4a	0c

[a] Numbers in a row followed by the same letter are not significantly different at the 5% level by using Duncan's multiple range test.

for authentic methyl α-D-glucopyranoside (Sigma Chemical Co.) (0.63). These data indicate the methyl glucoside isolated from white clover to be methyl β-D-glucopyranoside.

The concentration of methyl glucoside was found to be highest in the stem tissue of greenhouse-cultured clover plants (Table I), fairly low in leaflet tissue, and not present in root tissue. The concentration of methyl glucoside was not significantly different from glucose concentration in leaflet and stem tissue. Sucrose was considerably higher than methyl glucoside in all three tissues.

The methyl glucoside was found to be present in the foliage of 13 cultivars of white clover with the concentrations ranging from 16.5 to 6.3 mg/g of dry tissue (Table II). However, methyl glucoside was not found in the foliage of 5 cultivars of alfalfa, 11 cultivars of red clover, and 9 cultivars of bird's-foot trefoil. It has not been found

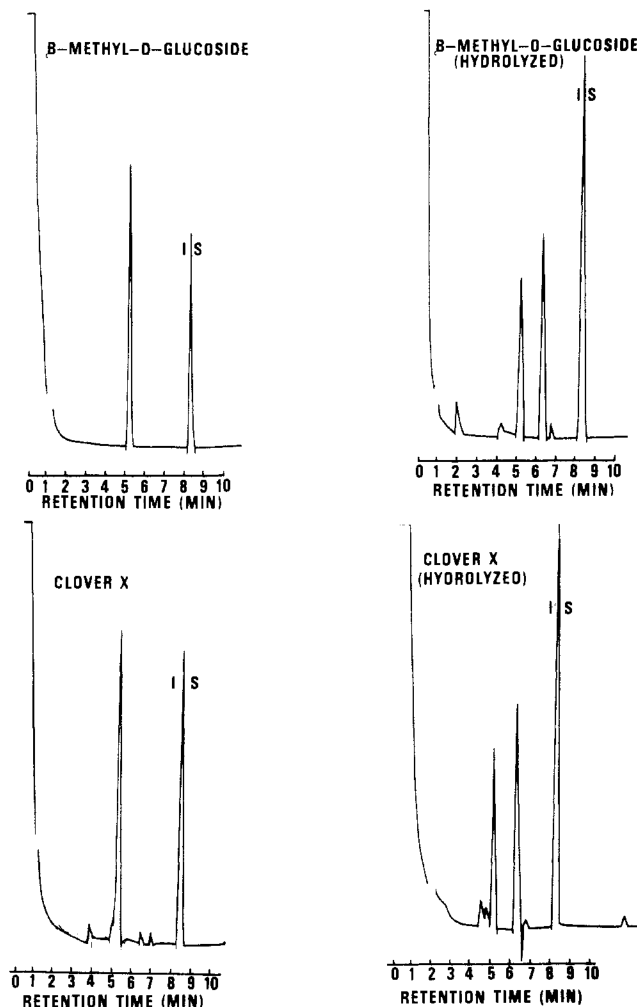

Figure 2. Chromatograms of authenic methyl D-glucopyranoside before (top left) and following (top right) hydrolysis and of methyl glucoside isolated from clover (clover X) before (bottom left) and following (bottom right) hydrolysis. Methyl nonadecanoate was used as the internal standard (IS).

in tissue of any other plant species analyzed in our laboratory and appears to be unique to white clover. Methyl glucoside was found to be present in white clover foliage tissue regardless of the presence of root nodules. The greenhouse experiments (Table I) were conducted on plants without nodules, whereas the field-grown plants (Table II) were nodulated.

To our knowledge, this is the first report of the natural occurrence of methyl glucoside in higher plants. The *O*-methyl monosaccharides reported in nature are mostly found as constituents of polysaccharides from bacteria (Lornitzo and Goldman, 1968; Morrison et al., 1967; Kenndy, 1978) and fungi (Turvey and Griffiths, 1973; Fareed and Percival, 1977; Turvey and Williams, 1976). The most frequently encountered compounds are 3-O-methylmannose and -rhamnose, followed by 6- and 3-*O*-methyl galactose, 3-*O*-methylxylose, and 2-*O*-methylgalactose, 3-*O*-methloxylose, and 2-*O*-methygalactose.

Table II. Occurrence of Methyl Glucoside in the Foliage of White Clover Cultivars Grown in Field Plots

cultivar	mg/g (dry wt)	cultivar	mg/g (dry wt)
Regal Ladino	16.5a[a]	Sacramento	9.0c
S.C. med. flower	14.9a	California Ladino	8.8c
Nolin's Improved	13.2a	Arcadia	8.6c
Lucky Ladino	12.8ab	Nematode Tolerant	8.2cd
K-6-8	12.4ab	Ladino KO-176	7.1cd
Tillman Ladino	11.3b	Florida XP1	6.3d
La-5-1	9.5c		

[a] Numbers followed by the same letter are not significantly different at the 5% level by using Duncan's multiple range test.

Due to the limited research, reported, pertaining to the physiological importance and metabolic functions of methyl glucoside in higher plants, the significance of this compound is unknown. However, this compound has been, unknowingly, extracted in the ethanolic fraction by other researchers and probably quantified as glucose by most GLC methods and as sucrose when analyzed by colorimetric methods. Methyl glucoside could be relevant in chemical taxonomy and would serve as an important trait to be used in determining white clover composition in a forage mixture by chemical methods.

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