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Note

Identification of inositols and their mono-O-methyl ethers by gas–liquid chromatography

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Inositols and their O-methyl ethers are generally widespread in plants[1], particularly in dicotyledons, where they vary qualitatively and quantitatively. They are thus considered important phytochemically, taxonomically and physiologically. O-methyl inositols are of particular interest in grain and forage legumes where they are often the major low-molecular-weight carbohydrate component present in the plant tops[2,3]. They are also present at lower concentrations in legume seeds[4–6] where D-pinitol (1D-3-O-methyl-*chiro*-inositol), the most frequently encountered methyl inositol, also occurs as an α-galactoside[5–7]. D-Ononitol (1D-4-O-methyl-*myo*-inositol) and O-methyl-*scyllo*-inositol are found in a narrower range of species, the latter so far only having been reported in two species of mung bean[2,4]. Inositol methyl ethers thus comprise a significant portion of the diet of animals grazing legume-based pastures and of humans relying on legume grains as a protein source. Little is known of the metabolic fate of these compounds when eaten, but in the living plant it has been reported that their concentrations can increase greatly in response to water stress[2,3], and to a lesser degree salt stress[8,9]. It is therefore important when performing metabolic studies to be able to identify such major dietary components, and accurately determine their concentrations. Gas–liquid chromatography (GLC) is suitable for analysis of small amounts of tissue from large numbers of samples, and has been used to identify selected inositols and inositol methyl ethers[10–12].

This report describes the identification by GLC of the three inositol methyl ethers, and their parent inositols, which occur in grain and forage legumes[3], and includes comparisons of relative retention times of other naturally occurring cyclitols. Simple separations of ononitol from sequoyitol (5-O-methyl-*myo*-inositol) and O-methyl-*muco*-inositol from pinitol, previously unreported, are also demonstrated.

EXPERIMENTAL

GLC analysis was carried out on a Varian Aerograph 1740 instrument, fitted with a hydrogen flame ionisation detector and coupled to a Hewlett-Packard 3380A integrator-calculator.

Trimethylsilyl (TMS)ethers were separated in stainless-steel columns packed with either (a) 3% GE-SE-30 or (b) 10% Carbowax 20M, on 80–100 mesh Gas Chrom Q. Column oven temperature was initially 130°C, programmed to rise linearly

at 2°C min^{-1}. Acetates were separated at 180°C in a nickel column packed with 0.4% OV-225 on surface modified Chromosorb[13]. Column dimensions were 1800 × 3 mm O.D. The flow-rates of nitrogen and hydrogen were both 25 ml min^{-1}. Range on the gas chromatograph was set at 10^{-11} and 10^{-12} A/mV for analysis of TMS ethers and acetates respectively. Attenuator was set at 1 on the gas chromatograph and at 128 on the integrator.

Various mixtures containing *ca.* 0.2 mg of each inositol in aqueous solution were air-dried. The residues were trimethylsilylated with TMS-imidazole in pyridine (0.1 ml)[14], and 1–1.5 μl of the silylation solution was injected directly into the gas chromatograph. For acetylation, aliquots containing *ca.* 0.4 mg inositol were dried in a vacuum over phosphoric oxide, and then heated at 100°C with 0.5 ml of a acetic anhydride–pyridine (1:1) mixture for 15 min. The reaction solutions were air-dried, and the acetylated residues dissolved in dichloromethane (0.1 ml) before injection (0.2–0.3 μl) into the gas chromatograph.

RESULTS AND DISCUSSION

Previous results indicated that a non-polar methyl silicone might be a generally useful liquid phase for separation of TMS ethers of inositols and their O-methyl ethers[12]. That this was largely correct was demonstrated here using SE-30 (Table I). Of the six cyclitols of primary interest[3], only D-*chiro*-inositol and O-methyl-*scyllo*-inositol could not be distinguished from one another (fig. 1). In addition, *muco*-inositol did not separate from dambonitol (1,3-di-O-methyl-*myo*-inositol), nor did O-methyl-*muco*-inositol from pinitol, or ononitol from sequoyitol. However, when the same TMS inositols were run on polar Carbowax, many of the compounds which overlapped in SE-30 could now be separated (Table I). Exceptions were O-methyl-*muco*-inositol which still overlapped with pinitol, and ononitol with sequoyitol. In this phase *muco*-inositol overlapped with pinitol, as did D-*chiro*-inositol with L-que-brachitol (1L-2-O-methyl-*chiro*-inositol), and *myo*-inositol with O-methyl-*scyllo*-inositol. It was notable that on Carbowax the elution times of O-methylethers relative to their parent inositols were more variable than on SE-30. The presence of an O-

TABLE I

RELATIVE RETENTION TIMES OF SOME INOSITOLS AND THEIR O-METHYL ETHERS

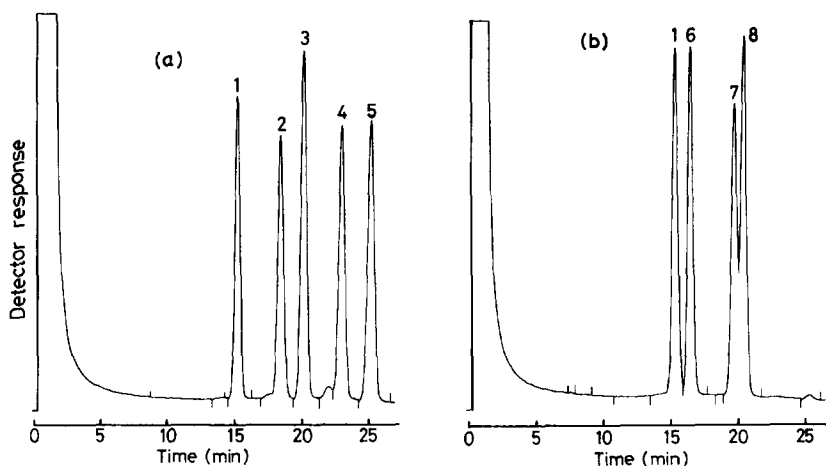| Inositol	SE-30	Carbowax	OV-225
Muco	0.76	0.45	1.27
D-*chiro*	0.82	0.53	0.77
myo	1.00	1.00	1.00
scyllo	0.92	0.87	1.30
O-Methyl-*muco*	0.59	0.46	0.72
D-Pinitol	0.61	0.45	0.61
D-Ononitol	0.78	0.83	0.85
Sequoyitol	0.79	0.84	0.78
O-Methyl-*scyllo*	0.82	0.99	0.97
L-Quebrachitol	0.67	0.53	0.59
D-Bornesitol	0.86	1.07	0.62
Dambonitol	0.74	1.21	0.55

Fig. 1. Gas–liquid chromatograms of TMS ethers on 3% SE-30. (a) Pinitol and free inositols. (b) O-methyl inositols. Peak identities: 1 = D-pinitol; 2 = *muco*-inositol; 3 = D-*chiro*-inositol; 4 = *scyllo*-inositol; 5 = *myo*-inositol; 6 = quebrachitol; 7 = ononitol; 8 = O-methyl-*scyllo*-inositol.

methyl group increased the retention time relative to the free inositol, and O-methyl-*scyllo*-inositol and bornesitol actually eluted after the parent inositol. The effect of methoxyl substitution was further illustrated by the relatively long retention time found for dambonitol. The relative retention times for free inositols on Carbowax were generally similar to a previous report[11], but no information on O-methyl ethers was presented.

It is clear from the results presented here that, with the exception of ononitol and sequoyitol, and O-methyl-*muco*-inositol and pinitol, the cyclitols in Table I can be readily identified from their relative retention times when compared on the two
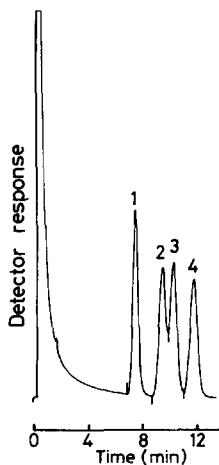

Fig. 2. Gas–liquid chromatographic separation on 0.4% OV-225 of acetate derivatives of sequoyitol and three O-methyl-inositols frequently encountered in pasture and grain legumes. Peak identities: 1 = pinitol; 2 = sequoyitol; 3 = ononitol; 4 = O-methyl-*scyllo*-inositol.

liquid phases of differing polarity as described. Although sequoyitol is considered to be confined to the gymnosperms[1], mention has been made of its occurrence in the leguminosae[15]. It was therefore relevant to attempt to find a convenient method for its separation from ononitol. Acetate derivatives, run in a low percentage coating of OV-225 on a surface modified support[13], proved to be successful (Fig. 2). One of the few reports of GLC analysis of inositol acetates[10] used a polyester column at 215°C. Temperatures of this order degrade the liquid phase quickly and cause much column bleed. The method described in the present work operated at a relatively low temperature (180°C), and because of the low percentage coating of liquid phase, the elution times were comparatively fast. For the usual coating of 3% OV-225 and a column temperature of 200°C, sequoyitol and ononitol were found to elute as broad peaks at *ca.* 33 and 35 min respectively. Pinitol and O-methyl-*muco*-inositol also separated as their acetates. Although the latter compound has not been reported in the legumionsae, it occurs frequently with pinitol in many gymnosperm species[16]. The acetate derivatives of inositols may not be of such general usefulness as TMS ethers, but because they make special separations possible as described, and are eluted in a different order, they offer further confirmatory evidence for the identity of inositols and their O-methyl ethers.

ACKNOWLEDGEMENTS

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