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Note

Separation of pinitol and some other cyclitols by high-performance liquid chromatography

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Pinitol (1D-3-O-chiro-inositol) was recognized to be a major cyclitol in foliage and stems of many legume species only a few years ago[1], although it has been known to exist at low levels in certain other plants. Generally, separation of pinitol and various other cyclitols from plants has been a difficult problem. Separation of inositols and polyhydric alcohols has been attempted by partition chromatography with ion-exchange resins[2–4], and by thin-layer chromatography. Some examples of the later method include two-dimensional chromatography of carbohydrates on silica gel impregnated with boric acid[5], sodium borate[6], by 1-butanol–aqueous boric acid mixtures[7], and by use of lead(II) on silica gel[8]. More recently, gas–liquid chromatography (GLC) has been successfully utilized for separation and quantitation of these compounds. Phillips and Smith[9] reviewed this technique comprehensively and evolved a faster method for separation of pinitol from plant extracts. During the past few years high-performance liquid chromatography (HPLC) has been used for the separation of sugars in various foods and food products[10–13]. Data on the use of this technique for separation of plant cyclitols is limited although separation of some polyhydric alcohols in fruits[14] and of phytic acid in rice bran[15] has been reported.

Research on the metabolism of pinitol required a fast and accurate method to isolate and purify radioactively labelled pinitol from legume plant extracts treated with [^{14}C]myo-inositol. This was of importance because radioactively labelled pinitol was not available from commercial sources. The more widely used GLC methods, although very precise, were useless since these methods required cyclitol derivatization prior to chromatography, making the cyclitols unavailable for further metabolic studies. This report describes an HPLC method developed in our laboratory for separation, purification and concentration of [^{14}C]pinitol from soybean plant extracts.

MATERIALS AND METHODS

Apparatus

The analysis was carried out using a Micromeritics (Norcross, GA, U.S.A.) solvent delivery system (Model 750) connected to a universal syringe loading sample injector (Model 730, equipped with a 100-μl loop) and a refractive index (RI) detector

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(Model 771) set at $0.05 \cdot 10^{-3}$ RI unit sensitivity. Chromatograms were recorded on a Heath Schlumberger (Benton Harbor, MI, U.S.A.) recorder (2100 series, 10 mV, full scale range) set at a chart speed of 0.4 cm/min. The chromatographic column was a pre-packed silica-bonded stainless-steel column (Microsil) purchased from Micromeritics. A 5 cm × 0.46 cm stainless-steel precolumn, packed with 10-μm silica gel (Whatman, Clifton, NJ, U.S.A.) using a column packer (Model 705, Micromeritics) was connected in series to protect the main column. The void volume of the system was about 5.8 ml.

Reagents

The following reagents were used: (a) HPLC-grade acetonitrile (Fisher Scientific, Fairlawn, NJ, U.S.A.); (b) water, glass redistilled and degassed for 5 min; (c) myo-inositol was purchased from Sigma (St. Louis, MO, U.S.A.). Pinitol and chiro-inositol were prepared in our laboratory and the purity was checked by gas chromatography–mass spectrometry. Sequoyitol was kindly supplied by Dr. Laurens Anderson of the Department of Biochemistry, University of Wisconsin, Madison, WI, U.S.A. [^{14}C]Myo-inositol (specific activity 278 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.).

Standard solutions were prepared by dissolving 50 mg of each compound in 5 ml of the solvent except sequoyitol which, due to a limited quantity, was used at a lower concentration (15 mg/5 ml).

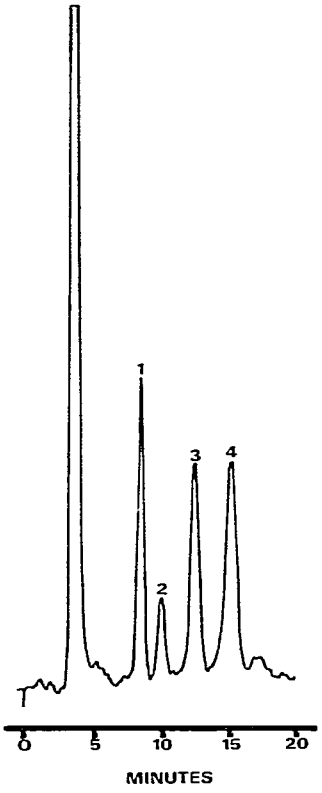
Liquid chromatography

The mobile phase employed acetonitrile–water (78:22). The solvent was degassed under vacuum for 5 min and filtered through a 0.22-μm Millipore filter. The column was equilibrated for about 45 min with the mobile phase. A 25-μl aliquot of each standard solution or mixture was chromatographed at a flow-rate of 2 ml/min. The identity of standard peaks was checked by GLC according to the method of Phillips and Smith[9].

RESULTS AND DISCUSSION

Fig. 1 shows the separation of various cyclitol standards using a mobile phase of acetonitrile–water (78:22) at a flow-rate of 2 ml/min. The elution times of pinitol, sequoyitol, chiro-inositol and myo-inositol were 8 min 38 sec, 10 min 8 sec, 12 min 45 sec, and 15 min 23 sec, respectively. Each peak was collected and identified by GLC[9]. This system provided a rapid and baseline resolution of all cyclitols. Increasing the acetonitrile concentration in the solvent beyond 78% reduced the retention time but the peak widths were increased while decreasing concentration of acetonitrile or the flow-rate delayed the elution time unnecessarily.

Fig. 2 shows the chromatographic profile of the [^{14}C]myo-inositol-treated soybean plant extract which had been cleared of the sugars by running through a set of ion-exchange columns before injecting on the HPLC column. There was a very good separation between [^{14}C]pinitol (peak 2) and myo-inositol (peak 6); consequently, it was quite easy to collect the radioactively labelled pure pinitol from the rest of the intermediates of cyclitol metabolism. These samples contained small quantities of methanol, which eluted immediately after the solvent front, resulting in a



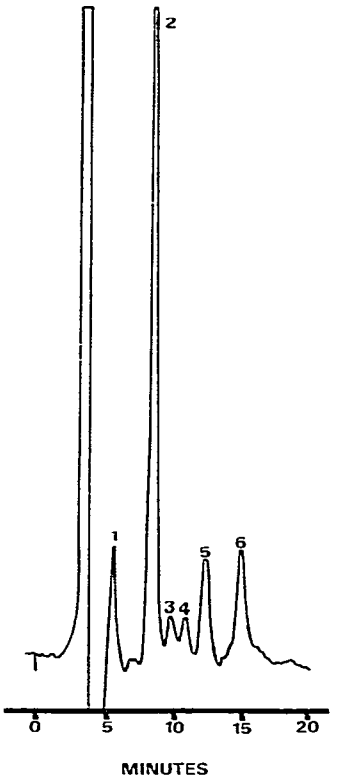


Fig. 1. Chromatogram of cyclitol standards [concentration 10 μg/μl except for sequoyitol (3 μg/μl)] on a Microsil column at room temperature, with mobile phase acetonitrile–water (78:22) at a flow-rate of 2 ml/min, chart speed 0.4 cm/min. Peaks: 1 = pinitol; 2 = sequoyitol; 3 = chiro-inositol; 4 = myo-inositol.

Fig. 2. Separation of pinitol and related cyclitols from soybean plant extract which had been run through the ion-exchange columns. Mobile phase acetonitrile–water (78:22) at a flow-rate of 2 ml/min, chart speed 0.4 cm/min. Peaks: 1 = unknown; 2 = pinitol; 3 = sequoyitol; 4 = unknown; 5 = chiro-inositol; 6 = myo-inositol.

negative peak. Peaks 1 and 4 are currently unidentified, although it should be possible to collect and identify these peaks by co-chromatography with known standards or by gas chromatography–mass spectrometry.

A problem encountered during these analyses was that glucose, if present in the plant extract, eluted with sequoyitol since these compounds had the same capacity ratio. Therefore it is absolutely necessary to remove sugars before sequoyitol determinations could be accomplished.

No attempt was made to quantify the amount of each cyclitol present in these samples. However, it is possible to quantify the compounds by comparing the peak height of the sample to the corresponding linear standard curves constructed by running different concentrations of the standard solutions through the system. The proposed HPLC method is relatively rapid and is quite useful for the separation and

quantitation of pinitol and other cyclitols in situations where these compounds have to be saved for further biochemical work.

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