esters using boron trifluoride butanol (14%/wt) HMF was converted to the butyl ester of levulinic acid. The stored prunes also contained furfural, which is the thermal degradation product of a pentose sugar (fructose → furfural → furoic acid). The presence of furfural was confirmed by GC–MS of an underivatized chloroform extract, concentrated by the method of Stafford et al. (1978). This is not seen in the gas chromatogram (Figure 1) because most of the furfural present in prunes would be removed from the prune extract in the two-step extraction procedure used for the GC method, and any remaining furfural would be lost when the extract was taken to dryness prior to the derivatization step.

The presence in stored dried prunes of furfural, HMF, and possibly levulinic and furoic acid, which are oxidation products of HMF and furfural, respectively, did not affect the quantitation of sorbic acid by the gas chromatographic procedure because of its specificity. The procedure involves the formation of the *n*-butyl derivative of sorbic acid, which has a definite, reproducible, gas chromatograph relative retention time (Figure 1), and uses decanoic acid as an internal standard, to aid quantitation. Other compounds could coelute with the butyl sorbate or butyl decanoate, but in the capillary GC–MS analyses of stored and unstored samples only spectra consistent with the presence of *n*-butyl sorbate and *n*-butyl decanoate were recorded. No mass spectral evidence was found for coelution of any interfering components in the GC peaks for these two compounds.

The visible absorption spectrophotometric methods (1 and 2) rely on the reaction of 2-thiobarbituric acid (TBA) and a product produced by the oxidation of sorbic acid, which gives a colored compound having an absorption maximum at 530 nm. However, as shown by Jennings et al. (1955), numerous aldehydes and other compounds (Sinnhuber et al., 1958) react with TBA to produce a highly pigmented product. Therefore, HMF and furfural could cause high results when these methods are used. Method 1 would tend to reduce the presence of these carbonyl compounds with the extraction steps. In method 2 any carbonyl compounds would be measured.

Quantitation based upon UV absorption measurements can also produce erroneous results, unless the sample is sufficiently pure or is purified to exclude other interfering UV-absorbing compounds, for example, compounds produced during storage. In method 3, levulinic and furoic acid and possibly small amounts of HMF and furfural would be extracted along with any sorbic acid present in the sample. In method 4 furfural would be steam distilled over to the distillate and be measured as sorbic acid. Any additional furfural produced during the distillation would also be distilled. The absorption maxima of these compounds, except sorbic acid, are in the 280-nm range, but the absorption band shoulder extends into the UV region of interest for the analysis of sorbic acid.

These results indicate that a more specific analytical procedure for determining sorbic acid, such as the previously discussed GC method, should be used to analyze prunes or other products containing sugars stored for prolonged periods of time. A high-performance liquid chromatography (HPLC) method similar to the one developed by McCalla et al. (1977) for use with wine would prove even more desirable, since the time-consuming derivatization step required for the GC method could be eliminated.

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Registry No. 5-(Hydroxymethyl)-2-furaldehyde, 67-47-0; 2-furaldehyde, 98-01-1; sorbic acid, 110-44-1; levulinic acid, 123-76-2.

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Cyclitols of Soybean Leaves

Some minor cyclitols of soybean [*Glycine max* (L) Merrill] leaves are identified as D-(+)-ononitol (D-4-*O*-methyl-*myo*-inositol), sequoyitol (5-*O*-methyl-*myo*-inositol), and bornesitol. They were characterized by GC–MS, ^{13}C NMR spectroscopy, and polarimetry.

Soybean cyclitols are interesting because they constitute the major soluble carbohydrates in soybean (Phillips and Smith, 1974) and because they may play a role in nitrogen fixation in the nodules (Streeter and Bosler, 1976; Streeter, 1980; Kawai and Kumazawa, 1982).

Pinitol (D-3-*O*-methyl-*chiro*-inositol) is the predominant soybean cyclitol. Plouvier (1950) discovered pinitol in soybean leaves wherein pinitol may be 0.1–0.7% of the fresh leaf weight (Phillips and Smith, 1974; Dougherty, 1976; Streeter, 1980; Binder and Kogan, 1981). In soybean

seeds, pinitol is found free, in two galactosylpinitols (Schweizer et al., 1978; Schweizer and Horman, 1981) and in a digalactoside (Quemener and Brillouet, 1983).

myo-Inositol and D-*chiro*-inositol have also been identified in leaves, stems, roots, nodules, and seeds (Dougherty, 1976; Schweizer et al., 1978; Streeter, 1980, 1981; Kawai and Kumazawa, 1982; Kouchi, 1982). A galactosyl derivative of D-*chiro*-inositol is found in seeds (Schweizer and Horman, 1981).

Recently, a minor soybean cyclitol was identified as sequoyitol (Streeter, 1980; Phillips et al., 1982; Kouchi, 1982). In unpublished work, we had identified a minor soybean cyclitol as ononitol. We reinvestigated and now report our results.

MATERIALS AND METHODS

Lyophilized soybean leaves were extracted with acetone and then with methanol. After removal of solvent from the methanol extract, an aqueous solution of the residue was heated to the boiling point while an amount of calcium oxide about 3 times the weight of the residue was being stirred into the solution. Solids were removed from the cooled solution by centrifuging. The solution was neutralized with 85% phosphoric acid, decolorized, and filtered. The filtrate was put through a cation-exchange resin column and then neutralized with anion-exchange resin in the hydroxide ion form. This solution was evaporated to dryness, and the residue was taken up in hot methanol, filtered, and evaporated. Most of the pinitol and *chiro*-insoitol present in this residue, after formation of isopropylidene derivatives (Hasegawa and Nakajima, 1973), was separable from the other cyclitols by partitioning between chloroform and water. Cyclitol derivatives in the water solution were hydrolyzed by making the solution acidic with HCl and warming. The free cyclitols were then chromatographed on a 5 cm × 36 cm column of Whatman standard-grade cellulose powder with 8:1 acetone–water as the eluant (Angyal et al., 1957) and rechromatographed on a 2 cm × 45 cm cellulose column with 6:1 acetone–water as the eluant. Fractions were monitored with a refractive index detector.

Reference samples of D-(+)-ononitol and sequoyitol were obtained from natural sources (Binder and Haddon, 1984). A sample of L-(+)-bornesitol was provided by Frank Loewus, Washington State University, Pullman, WA.

Trimethylsilyl (Me_3Si) ethers of cyclitols were prepared by reaction with 2:1 hexamethyldisilazane–trimethylchlorosilane in pyridine. After removal of pyridine on a rotary evaporator, the Me_3Si ether derivatives were dissolved in heptane and analyzed by gas chromatography on a 5.5 m × 3.18 mm 2% Silar 10C column at 180 °C and by GS–MS with a 15 m × 0.32 mm methyl silicone column (Binder and Haddon, 1984).

^{13}C NMR spectra were obtained with a PFT-100 NMR spectrometer (JEOL, Inc.) operated with a pulse width of 17.6 μs, a repetition rate of 2 s, and 32K data points to cover a spectral width of 10 kHz (to suppress spurious signals), giving a resolution of 0.6 Hz, and with acetonitrile as the reference (3.67 ppm in D_2O at room temperature).

RESULTS AND DISCUSSION

Cyclitols isolated after chromatography on cellulose were identified by GC–MS and their ^{13}C NMR spectra. Fractions that would contain cyclitols with R_f's between those of pinitol and *myo*-inositol (Angyal et al., 1957) were combined and solvent was removed. When a portion of the residue was trimethylsilylated and analyzed on the Silar 10C column, it showed three major peaks with areas 74.3%, 18.9%, and 2.9% of the total. By comparison with standards, these were tentatively identified as Me_3Si ethers of ononitol, sequoyitol, and bornesitol, respectively. GC–MS analysis confirmed these assignments (Binder and Haddon, 1984). Independent confirmation came from the ^{13}C NMR spectrum of the mixture. The most prominent peaks are attributable to the carbon atoms of ononitol (85.2, 76.4, 75.2, 75.0, 73.7, 73.3, and 62.6 ppm) while five lower intensity peaks reveal sequoyitol (86.9, 74.7, 74.4, 73.8, and 62.3 ppm). Although three peaks for bornesitol are obscured by the ononitol and sequoyitol peaks, small peaks showing bornesitol appear at 83.2, 77.1, 70.4, and 59.4 ppm.

The D line optical rotation observed for a 22.45 mg/mL aqueous solution of the ononitol–sequoyitol–bornesitol mixture was $\alpha = 0.128$. Sequoyitol is optically inactive so the rotation is mostly due to ononitol and indicates that it is (+)-ononitol (lit. $[\alpha]_D = 6.6 \pm 1°$) (Plouvier, 1955). No natural (−)-ononitol has been reported. Although calculations might indicate that the bornesitol present is also dextrorotatory (lit. $[\alpha] = 32°$) (Girard, 1871), the optical activity of 3–4% of impurities present is unknown and the bornesitol reported in Leguminosae (Plouvier, 1955) is the levorotatory form. Presumably, in soybean it is also D-(−)-bornesitol.

While the GC chromatographic properties of Me_3Si ethers of ononitol, sequoyitol, and *chiro*-inositol are quite similar (Loewus and Shah, 1972), these compounds are adequately separated on some columns and retention times give a good indication of identity. Where the possibility of misidentification exists, as with the Me_3Si ethers of mannitol and ononitol, confirmation of identity should come from GC–MS (Binder and Haddon, 1984).

The ononitol content in soybean leaves is variable. In third trifoliate leaves (counting from the apical tip) from four soybean cultivars sampled at four stages of vegetative growth, ononitol constituted 0.01–0.10% of the fresh leaf weight and was present in greater amounts than *myo*-inositol (Binder and Kogan, 1981).

Soybean is the first species in which the combination of pinitol, ononitol, sequoyitol, bornesitol, *chiro*-inositol, and *myo*-inositol is shown to be present. However, pinitol, ononitol, and bornesitol occur in alfalfa (*Medicago sativa*) (Rendig and McComb, 1962; McComb and Rendig, 1962; Kindl and Hoffmann-Ostenhof, 1966a), and pinitol, sequoyitol, bornesitol, and D-*chiro*-inositol occur in three species of Asclepiadaceae (Kindl and Hoffmann-Ostenhof, 1966b). *myo*-Inositol is ubiquitous in biological material, but the other cyclitols have taxonomic interest.

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Registry No. D-(+)-Ononitol, 6090-97-7; sequoyitol, 523-92-2; D-(−)-bornesitol, 484-71-9.

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Molluscicides from Olive *Olea europaea* and Their Efficient Isolation by Countercurrent Chromatographies

Two iridoid glycosides possessing a molluscicidal property, oleuropein and ligstroside, were isolated from fresh fruits of olive *Olea europaea* by using two efficient countercurrent chromatographies, rotation locular countercurrent chromatography (RLCC) and droplet countercurrent chromatography (DCCC). This is the first isolation of ligstroside from olive.

In our preliminary screening for naturally occurring molluscicides for the control of schistosomiasis (Henderson et al., 1983; Kloos and McCullough, 1983; Kubo et al., 1983b), we found that the crude methanol extract of fresh fruits of bitter olive *Olea europaea* (Oleaceae) exhibited molluscicidal property against the South American snail *Biomphalaria glabratus*. Although the chemical constituents in this plant have been extensively studied (Schneider and Kleinert, 1972; Inoue et al., 1974), it was not known which of the constituents were responsible for this observed biological activity. Separation of the crude extract into ether, ethyl acetate, and water-soluble portions indicated the active components were in the ethyl acetate portion. Biological activity was monitored as previously described (Nakanishi and Kubo, 1977). Due to its polar nature, the bioactive ethyl acetate extract seemed ideally suited for further separation by countercurrent chromatography as has been previously applied to the resolution of many polar mixtures (Hostettmann, 1980; Kubo et al., 1983a).

This communication describes the efficient isolation of two molluscicidal iridoid glycosides by two countercurrent chromatographies, rotation locular countercurrent chromatography (RLCC) and droplet countercurrent chromatography (DCCC).

MATERIALS AND METHODS

Materials. A methanol extract (162.4 g) was obtained from fresh fruits of *O. europaea* (2.1 kg), which were collected in the University of California Berkeley campus in Sept 1982. Then, for the further separation, the methanol extract was partitioned into ether (6.8 g), ethyl acetate (24.6 g), and water-soluble (131.0 g) portions.

RLCC. A RLCC separation was performed on a Model RLCC—A (Tokyo Rikakikai Co., Tokyo, Japan). The RLCC solvent system of chloroform–methanol–water (13:7:4 v/v) was chosen by prescreening the components of the ethyl acetate extract on a TLC plate (Macherey, Wagel and Co., Duren, GFR, Polygram Sil G/UV 254). The upper phase was chosen as the mobile phase in our RLCC system. The crude methanol extract (1.0 g) was dissolved in a (1:1 v/v) mixture of the mobile and stationary phases and injected into the RLCC apparatus by using a 3-mL sample chamber. The eluents were collected in 1.4-mL fractions. Fractions were monitored by TLC (Sil G/UV 254) with the organic layer of this solvent system. Visualization of the compounds on the TLC plate was accomplished by UV spectroscopy (Chromato–UVE Cabinet, Model CC-60, Ultra Violet Products, Inc, CA) and using vanilin–sulfuric acid–ethanol 3 g:1.5 mL:100 mL) as a spray reagent.

DCCC. A Model DCC-300-G2 (Tokyo Rikakikai Co., Tokyo, Japan) with 300 glass columns (400 mm × 2 mm i.d.) was used for the DCCC separation. The same solvent system as for RLCC was chosen, and the ethyl acetate extract (3.8 g) was dissolved in the solvent of the mobile phase and injected into the DCCC apparatus by using a 10-mL sample chamber. The eluents were collected in 1.5-mL fractions. Fractions were monitored with the same way as on the RLCC separation.

Biological Assay. A molluscicidal activity was monitored as described previously (Nakanishi and Kubo, 1977).

RESULTS AND DISCUSSION

On a preliminary examination of the molluscicidal activity of the crude methanol extract of olive fruits, the extract killed the South American snail *B. glabratus* within 2 h at 2000 ppm. For further screening, the methanol extract was separated into ether, ethyl acetate, and water-soluble portions. The ethyl acetate extract was found to have the molluscicidal activity.

The R_f values of the main components in the active ethyl acetate extract were determined to be 0.2–0.5 on a TLC plate by using the organic layer of chloroform–methanol–water (13:7:4 v/v). In order to isolate such polar compounds, the very simple RLCC method was employed. In order to prevent any of loss of the active principles, the crude methanol extract was used for the RLCC separation. Figure 1 provides the weights of eluted compounds vs. the fraction number. Even though RLCC requires only small

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