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Isolation of *l*-Epicatechin and *d*-Catechin by Column Chromatography

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A simple method for the isolation of large quantities of *d*-catechin and *l*-epicatechin is given. Polyamide chromatography of a grape extract was found to separate these flavanols from other classes of phenolic metabolites. The flavanols themselves were separated by using Sephadex LH-20 chromatography, giving good enough resolution to allow for the crystallization of individual compounds. The combination of these two chromatographic separations is likely to be applicable to the isolation of large quantities of many other phenolic metabolites.

The flavanols *d*-catechin and *l*-epicatechin (3,3′,4′,5,7-pentahydroxyflavan) are common constituents of grapes and have been implicated in the aging of red wines (Timberlake and Bridle, 1976). In a series of ongoing experiments in our laboratory investigating the mechanism of the aging process, a need arose for a relatively inexpensive source of the pure compounds. This paper reports a procedure for the quick, large-scale isolation of *d*-catechin and *l*-epicatechin from grapes.

MATERIALS AND METHODS

Apparatus. A Waters Associates (Milford, MA) high-performance liquid chromatograph (LC) was used for the identification of the compounds and in the determination of their purity. This included a Model 6000A pump and a U6K injector. Detection at 280 nm for the catechins was accomplished by using a Micromeritrics Model 785 chronometer detector (Norcross, GA), coupled to an Omniscribe recorder (Houston Instruments, Austin, TX). The solvent used was acetonitrile–2% acetic acid (1:9), pumped at 2 mL/min. All solvents were distilled and filtered through a 0.45-μm Millipore filter. Solvents were degassed immediately prior to use. The column used was a Zorbax ODS (25 cm × 4.6 mm i.d.; Du Pont, Inc., Wilmington, DE) reverse-phase column preceded by a 4 cm × 4.6 mm i.d. precolumn packed with Lichrosorb ODS.

Sample Preparation. White grapes were chosen over red grapes for the catechin isolations to alleviate the problems encountered in the separation of the red grape anthocyanins from other phenolic compounds. In other fruits, it has been shown that the catechin levels are highest in the immature stages, decreasing during ripening, so that relatively immature fruit is favored as a phenolic source (Mosel and Herrmann, 1974; Stöhr and Hermann, 1975). Seibel 9110 (Verdelet) grapes were obtained at ~10 °Brix, from the Irrigated Agriculture Research and Extension Center, Prosser, WA. To 500 g of destemmed grapes in a Waring blender was added 200 mL of 95% ethanol adjusted to 1000 ppm of SO_2, added as $NaHSO_3$. After the mixture was blended for 2 min, the resultant slurry was filtered through four layers of cheese cloth. The filtrates from 5 kg of grapes were combined and centrifuged at 5000*g* for 15 min. The supernatant was decanted and evaporated under vacuum at 30 °C to ~1 L. This was extracted 3 times with an equal volume of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under vacuum. The resultant residue was resuspended in ~60 mL of distilled water prior to column separation.

Column Chromatography. Chromatographic separation of the catechins was accomplished by using two suc-

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Table I. High-Performance LC Analysis of Selected Fractions from LH-20 Separation of Catechins (Figure 1)

fraction	d-catechin[a]	l-epicatechin[a]
90	0.5	99.5
132	70.0	30.0
154	99.5	0.0

[a] Expressed as (absorbance of the compound of interest)/(total absorbance of sample) × 100. Absorbances at 280 nm.

cessive supports. Krause (1978) reported that polyamide CC-6 chromatography could be used for the class separation of the flavonoids, hydroxycinnamyl glucose esters and the hydroxycinnamyl quinate esters. It was found that this procedure was also applicable to the separation of the catechins from the hydroxycinnamic acid derivatives, these being the two major classes of phenolic compounds in white grapes.

The total extract was applied to a 19 × 3.5 cm column of CC-6 (Brinkmann Instruments, Westbury, NY) (200-mL packed volume). Elution was with 1 L of distilled water, followed by 1 L of methanol and finally by 1 L of 0.01% (v/v) NH_3 in methanol. Each eluting solvent was collected separately. The methanol fraction was evaporated to dryness, yielding a thick paste which was dissolved in a minimum (~40 mL) of hot water. The solution was then filtered through Whatman No. 1 paper to remove oils extracted from the seeds and divided between two centrifuge tubes. These were flushed with nitrogen, capped, and stored overnight at 4 °C. The tubes were then centrifuged at 10000*g* at 2 °C to pack down the large amounts of white crystals (*d*-catechin). The supernatant was removed and saved, and the crystals were resuspended in 5 mL of ice water and recentrifuged. This wash was repeated, and all washes and the original supernatant were combined. The pellets were lyophilized, yielding 2.06 g of the white crystals.

The combined supernatant and washes, shown to contain both *d*-catechin and *l*-epicatechin by high-performance LC, were separated by using Sephadex LH-20 chromatography, which has recently been shown to be useful for the separation of other phenolic compounds (Singleton et al., 1977). A 25 × 2 cm column was packed with previously swelled LH-20 and equilibrated with distilled water. Half of the combined supernatant and washings (~25 mL) were applied to the column. One liter of distilled water was used to wash off an early eluting high molecular weight fraction. Elution of the remaining compounds was with 2 L of 15% (v/v) ethanol at 3 mL/min. Fractions of 10 mL were collected, and their absorbance at 280 nm was determined (Figure 1). Fractions 62–136 contained appreciable amounts of *l*-epicatechin and were combined; fraction 137–200 were combined as *d*-catechin sources (Table I). Column cleanup was accomplished by washing the column with 1 L of 95% (v/v) ethanol, followed by reequilibration with water prior to applying the second half of the supernatant.

The *l*-epicatechin fractions from the two runs were combined and evaporated to dryness under a vacuum, yielding a yellowish paste. This was suspended in ~10 mL of hot water, transfered to a centrifuge tube, and allowed to cool, first at room temperature (~20°C) and than at 2 °C overnight to avoid a colloidial precipitate. The resultant suspension was centrifuged at 10000*g* for 10 min a 2 °C. The crystals were suspended in 2 mL of ice-cold distilled water and recentrifuged. This wash procedure was then repeated once more. The crystals were transferred to a vacuum desiccator and dried over phosphorus pentoxide for 3 days. This procedure was also used for the combined *d*-catechin fractions.

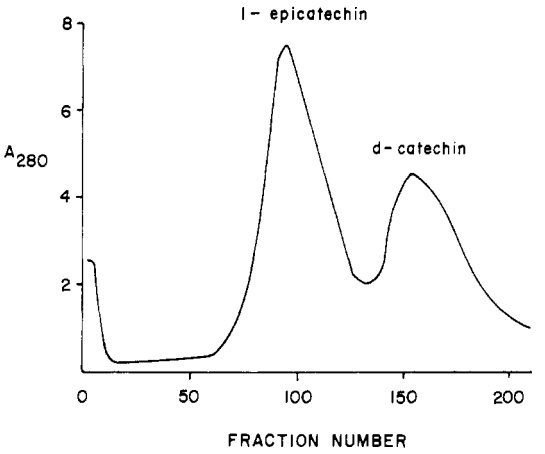

Figure 1. Elution of catechins from LH-20. Solvent: 15% (v/v) ethanol, 10-mL fractions.

RESULTS AND DISCUSSION

High-performance LC analysis of the three fractions from the CC-6 chromatographic separation showed the water fraction to be essentially free of any phenolic compounds. This fraction should have contained any hydroxycinnamyl glucose esters, none of which have been reported in grapes, so their absence is not surprising. But, this water wash was useful in removing small amounts of residual sugars and acids extracted by the ethyl acetate. The methanol fraction yielded the catechins and the methanol–ammonia fraction yielded the hydroxycinnamic acid–tartaric acid esters. This preliminary step was then very helpful in isolating the flavanols from the contaminating hydroxycinnamic acid derivatives.

The crystals isolated directly from the methanol fraction of CC-6 chromatography were shown to be 99.7% *d*-catechin [mp 177–179 °C obsd [mp 177 °C, reference from Weast (1970)]]. By removal of this large quantity of *d*-catechin, separation of the residual *d*-catechin and *l*-epicatechin in the supernatant was possible on the LH-20 column, yielding 296 mg of l-epicatechin [98.5% pure; mp 247 °C dec obsd [mp 245 °C dec, corrected reference from Weast (1970)]] and 590 mg of *d*-catechin (97% pure).

This procedure constitutes a simple method for the isolation of large quantities of *d*-catechin and *l*-epicatechin. Alhough originally described for the isolation of flavonoids, polyamide chromatography of the grape extract was also found to separate the flavanols from other classes of phenolic metabolites, notably the cinnamates. The flavanols themselves are separatable by using Sephadex LH-20 chromatography, giving good enough resolution to allow for the crystallization of individual compounds. The combination of these two chromatographic separations is likely to be applicable to the isolation of large quantities of many other phenolic metabolites.

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