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IDENTIFICATION OF THREE FLAVAN-3-OLS FROM GRAPES

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Abstract—Three phenolic substances present in sizable amount in grapes, particularly in underripe grape seeds, are relatively easily extracted into ether and were isolated and unequivocally identified as (+)-catechin, (+)-(2R:3S)-5,7,3′,4′-tetrahydroxyflavan-3-ol; (−)-epicatechin, (−)-(2R:3R)-5,7,3′,4′-tetrahydroxyflavan-3-ol; and (−)-epicatechin-3-gallate. These identifications clear up previous conflicting and incomplete reports and show that the grapevine synthesizes the catechin isomers to be expected from work with other plants.

INTRODUCTION

THE PHENOLIC substances of the grape berry have considerable importance as food constituents and reaction substrates.[1] The berry's total extractable phenols are present in only about 10 per cent or less in the pulp with the remainder about two-thirds in the seeds and one-third in the skin. The seeds are an important source of phenols in grape products, particularly red wines.[2] The catechin fraction extractable in ether is an important part of the phenols of grape seeds, red wines, and some white wines, and appears to be an important source of pigment in browned musts and white wines.[3] It also appears to be a significant source of bitter flavor.[4] Specific identification of the phenols in this fraction is therefore of considerable interest, particularly since the structural configuration of flavan-3-ols affects their rate of oxidation and other properties important in foods.[5]

Some workers consider that sufficient examples have been examined to conclude that only the 2R configuration occurs naturally in the flavanols of plants; that is, (+)-catechin, (−)-epicatechin, and the corresponding gallocatechins.[6] However, many reported identifications depended on paper chromatography and did not involve actual study of the optical activity. While it has been demonstrated that catechin epimers can be separated by adsorptive paper chromatography, the differences are small and identification by this means alone is considered uncertain, particularly in complex mixtures.[7]

In studies which did involve isolation of various fractions and measurement of their optical rotations,[8,9] the catechins of grapes or wine have been reported to be (+)-catechin,

[1] V. L. SINGLETON and P. ESAU, *Advances in Food Research*, in press.
[2] V. L. SINGLETON and D. E. DRAPER, *Am. J. Enol. Viticult.* **15**, 34 (1964).
[3] J. A. ROSSI, JR., and V. L. SINGLETON, *Am. J. Enol. Viticult.* **17**, 231 (1966).
[4] J. A. ROSSI, JR., and V. L. SINGLETON, *Am. J. Enol. Viticult.* **17**, 240 (1966).
[5] J. W. CLARK-LEWIS, *Current Trends in Heterocyclic Chemistry*, p. 40, Butterworth's, London (1958).
[6] K. WEINGES, *Phytochem.* **3**, 263 (1964).
[7] E. HASLAM, *Chemistry of Vegetable Tannins*, 179 p., Academic Press, New York, (1966).
[8] S. V. DURMISHIDZE, *Dubil'nye Veshchestva i Antotsiany Vinogradnoi Lozi i Vina*, 323 p., Izdatel. Akad. Nauk. S.S.S.R., Moscow (1955).
[9] S. V. DURMISHIDZE, *Trudy Acad. Nauk. Gruz. S.S.R., Lab. Biokhim. Rastenii* 252 (1966).

(±)-catechin, (+)-epicatechin gallate, (−)-gallocatechin, (+)-gallocatechin, and (±)-gallocatechin. Other investigations of these components of grapes have been largely made using paper chromatography. These reports are conflicting, some indicating the expected isomers others indicated the isomers not found in other plants or epimerized products.[1] Some appear confused (for example suggesting three separate paper chromatographic spots for (+)-, (−), and (±)-isomers) and proof of the true situation is considered insufficient.[1] It therefore seemed necessary to investigate more rigorously the specific identity of the major catechins naturally present in grapes.

RESULTS AND DISCUSSION

It was previously shown that the ether-extractable fraction from fresh grape seeds consisted of only three phenolic substances in appreciable amounts designated as E_1, E_2, and E_3.[10] Based upon paper chromatography, these appeared to be unspecified isomers of catechin (E_1), epicatechin (E_2), and epicatechin gallate (E_3). The latter substance tended to disappear from seeds of ripe grapes, so unripe grapes were the source chosen for further study.

Conditions designed to minimize epimerization or other reaction were used to isolate these three phenols in chromatographically pure form. Solvent extraction, partition chromatography, lyophilization, and other procedures were conducted rapidly, without heating, and with limited exposure to light or oxygen plus interim storage at low temperature. Recovery from underripe *Vitis vinifera* wine grapes (juice about 15 per cent sugar) was about 56 g of fresh seeds per kg of grape clusters and about 9·9 g of lyophilized extract containing all the extractable phenols per 100 g of fresh seeds. This dry solid extract was equivalent by Folin–Ciocalteu phenol assay[11] to about 610 mg of gallic acid per g of extract.

When this solid was subjected to partition column chromatography with mobile ether

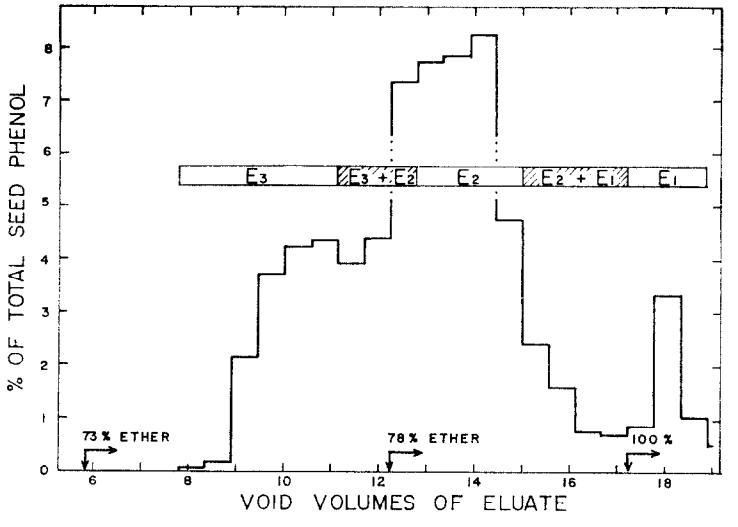

FIG. 1. SEPARATION BY COLUMN PARTITION CHROMATOGRAPHY OF E_3, E_2, AND E_1 IN PURE FORM. THE HORIZONTAL BAR INDICATES THE PAPER-CHROMATOGRAPHICALLY PURE FRACTIONS AND THE OVERLAPPING FRACTIONS.

[10] V. L. SINGLETON, D. E. DRAPER and J. A. ROSSI, JR., *Am. J. Enol. Viticult.* **17**, 206 (1966).
[11] V. L. SINGLETON and J. A. ROSSI, JR., *Am. J. Enol. Viticult.* **16**, 144 (1965).

and aqueous stationary phases, the three phenols of interest moved as a group near the ether front. The combined ether eluate containing all three putative catechins typically assayed about 30 per cent of the total phenol extractable from grape seeds with a range of about 15 to 70 per cent in the limited number of diverse samples examined. By linear gradient methods it was shown that a relatively high ether content was required in ether–heptane mixtures for satisfactory chromatographic separation. Based on these studies a program of sequential development with 60, 73, or 78 per cent ether in heptane and 100 per cent ether was chosen and satisfactory recovery of a major portion of each of the three phenols in pure form was obtained (Fig. 1). The fractions found to contain similar components by paper chromatography were combined as shown by the horizontal bar in Fig. 1, and the respective combined fractions were E_3, 21·1 per cent; E_3+E_2, 22·4 per cent; E_2, 40·9 per cent; E_2+E_1, 7·9 per cent; and E_1, 7·6 per cent of the total ether-extractable phenol present in the original unfractionated "Zinfandel" seed extract.

A number of comparisons between the substances isolated from grapes, authentic catechin samples and values from the literature[12–14] are shown in Table 1. Chromatographically pure crystalline E_2 had the same melting point (Table 1), undepressed mixed melting point, and characteristic sintering at 145–155° as anhydrous (+)-catechin. Other properties of E_2 are essentially identical to those of (+)-catechin, notably specific optical rotation, acetate properties, and u.v. absorption (Table 1), as well as i.r. absorption spectra.[14] Further paper chromatographic comparisons also agreed with previous findings[10] that E_2 was indistinguishable from (+)-catechin.

Similarly, data from Table 1 and i.r. spectra plus paper chromatography show that E_1 is (−)-(2R:3R)-5,7,3′,4′-tetrahydroxyflavan-3-ol, ((−)-epicatechin).

Although precipitation of E_3 under conditions which had given crystalline products[13] gave nonbirefringent granules which fused near the reported melting point for (−)-epicatechin-3-gallate from tea, the acetate was nicely crystalline. Comparisons between E_3, E_3 acetate, (−)-epicatechin-3-gallate isolated from green tea and literature values for the latter[12, 13, 15] (Table 1) show essential identity between values for E_3 and for (−)-epicatechin-3-gallate.

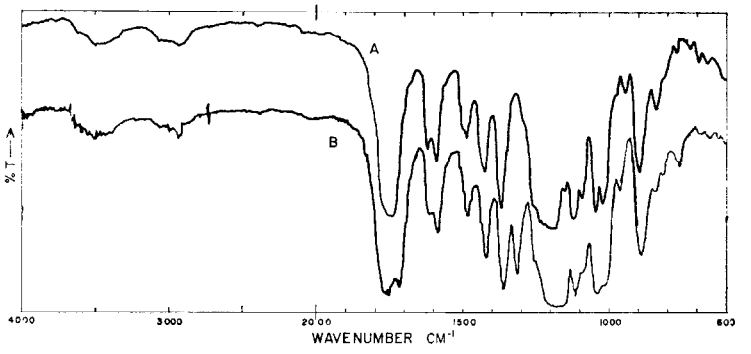

FIG. 2. INFRARED SPECTRA OF E_1 ACETATE (A) AND E_3 ACETATE (B). (RESP., (−)-EPICATECHIN PENTAACETATE AND (−)-EPICATECHIN-3-GALLATE HEPTAACETATE.)

[12] K. HERRMANN, *Z. Lebensm. Untersuch. Forsch.* **109**, 487 (1959).
[13] L. VUATAZ, H. BRANDENBERGER and R. H. EGLI, *J. Chromatogr.* **2**, 173 (1959).
[14] H. L. HERGERT and E. F. KURTH, *J. Org. Chem.* **18**, 521 (1953).
[15] T. K. CHUMBALOV and M. M. MUKHAMED'YAROVA, *Khim. i Khim. Teknol., Alma-ata, Sb* **2**, 209 (1964); *Chem. Abstr.* **64**, 1011g.

TABLE 1. PROPERTIES OF ISOLATED AND AUTHENTIC SAMPLES OF FLAVAN-3-OLS

Substance	M.p. °		$[\alpha]_D$		λ_{max} nm		ϵ_{max}	
	Found	Lit.[12–14]	Found	Lit.[12–14]	Found	Lit.[12–14]	Found	Lit.[12–14]
E_1	236–237		−58·3° (EtOH)		280		3,450	
(−)-Epicatechin		235–239		−69° (EtOH)	280	280		3,580
E_1 acetate	150–151		−13·2° ($C_2H_2Cl_4$)					
(−)-Epicatechin pentaacetate		151–152		−12° ($C_2H_2Cl_4$)				
E_2	173–175		0° (EtOH) +16·1° (Me_2CO)		279		3,730	
(+)-Catechin	174	176–177	0° (EtOH) +16·0° (Me_2CO)	0° (EtOH) +17·1° (Me_2CO)	280	280	3,890	4,060
E_2-acetate	134–135		+40·8° ($C_2H_2Cl_4$)					
(+)-Catechin pentaacetate	134–135	132–133		+40·6° ($C_2H_2Cl_4$)				
E_3			−188° (EtOH)		279		13,595	
(−)-Epicatechin-3-gallate	236	253		−190° (EtOH)	280	279		14,000
E_3-acetate	122		−117° (Me_2CO)					
(−)-Epicatechin-3-gallate heptaacetate		119·5–120·5		−93·7 (benzene)[15]				

Mild hydrolysis of E_3 produced equal amounts of E_1 ((−)-epicatechin) and gallic acid as shown by paper chromatography. The i.r. spectra of the acetates of E_3 and of E_1 are very similar, indicating a close relationship (Fig. 2). Maxima at 1685 and 1315 cm^{-1} in the E_3 spectrum absent in that of (−)-epicatechin indicate that the gallic acid is ester-linked. The sharp maximum at 1315 cm^{-1} in the spectrum of E_3 acetate absent in that of E_1 acetate also strongly suggests E_3 is a gallate ester of E_1.

Authentic (−)-epicatechin-3-gallate from tea was compared with pure E_3 from grapes by one-dimensional paper chromatography in six systems. The respective R_f values (average of four replicates) were: 2% acetic acid 0·35, 0·34; 15% acetic acid 0·45, 0·44; 30% acetic acid 0·61, 0·59; butanol–acetic acid–water (4:1:5) 0·83, 0·83; butanol–acetic acid–water (4:1:2·2) 0·87, 0·87; and 2-propanol–water (3:2) 0·77, 0·77. In no case, including two-dimensional chromatograms, was any separation of mixed samples of the two substances suggested by the results. It therefore appears that E_3 is (−)-epicatechin-3-gallate.

EXPERIMENTAL

Grape Seeds and Their Extraction

"Emerald Riesling" white grapes (275·8 kg) from the University vineyard, Davis, harvested at a juice Brix of 15·8° were passed through a small winery-type stemmer and roller crusher. The rollers were so spaced that each berry was broken open but the seeds were not damaged. The crushed grapes were shaken in small portions in hardware-cloth baskets (¼-in. mesh) beneath the surface of a tank of water so that the dense seeds escaped and settled to the bottom of the tank while the less-dense skins and most of the pulp was removed with the basket and discarded. The seeds obtained free of all other tissue by rinsing and rapid settling in water were drained and weighed 16·9 kg. The seeds were coarsely ground by passing them through a hand-operated wet mill (A. W. Straub & Co.), packed loosely into glass percolators and extracted exhaustively chromatographically with first 95% EtOH then 50% EtOH–water.[2] The combined extracts were freed from EtOH at low temperature *in vacuo* and lyophilized yielding 1830 g of dry extracted solids equivalent in phenol content[11] to 669·8 mg of gallic acid per g.

As another example, 192·9 kg of "Ruby Cabernet" red grapes at 15·2° Brix gave 9·6 kg of seeds and 874 g of extracted solids at 547·2 mg/g gallic acid equivalent. Since an unusually high proportion of E_3 was present in it, much of the work was done on a similar but much smaller sample from "Zinfandel" grapes, assaying 655 mg of gallic acid equivalent phenols per g.

Chromatography

A glass column 5 cm wide was packed with 5 g of dry Celite 545 diatomaceous earth, then 100 g of Celite 545 (wetted with 0·5 ml of H_2O per g containing 0·1% $K_2S_2O_5$ and 1% HOAc), and finally 12 g of Celite 545 (wetted with 6 ml of the same solution containing 3 g of the phenolic extract from grape seeds). The column was developed successively (each saturated with aqueous 0·1% $K_2S_2O_5$ and 1% HOAc) with 500 ml of heptane, 500 ml of 60% (V/V) Et_2O in heptane, 1300 ml of 73% Et_2O, 1000 ml of 78% Et_2O, and finally 300 ml of Et_2O free of heptane. Fractions (100 ml) were collected and analyzed by two-dimensional paper chromatography and for total phenol content.[11] Fractions qualitatively similar in composition were combined, concentrated *in vacuo* to a small volume of aqueous solution free of organic solvent and lyophilized.

Paper chromatography was conducted as previously described.[10] Fast blue V.B. salt, 0·05% in water, as a spray reagent gave rose color for E_1 and (−)-epicatechin, red for E_2 and (+)-catechin, and purple for E_3 without color changes when oversprayed with 20% Na_2CO_3 solution. Gallic acid gave purple becoming green with Na_2CO_3.

Crystallizations

E_1 was dissolved in MeOH, filtered, concentrated *in vacuo* until nearly saturated, and H_2O was added dropwise to induce crystallization. The crystals were filtered and dried *in vacuo* (P_2O_5) at room temperature for at least 1 week. E_2 was similarly crystallized as was a commercial sample of (+)-catechin. E_3 was precipitated from a concentrated MeOH solution by dropwise addition of CH_2Cl_2.

Acetates

Each known or isolated phenol (300 mg) was acetylated (K_2CO_3/Ac_2O) at 100° for 5 hr.

Instrumental Procedures

M.ps were determined in a capillary tube. Optical rotations were measured with sodium lamp, 1 dm micro cell, at 24°. Solvents and concentrations for polarimetry were: E_1, ethanol, 3·6 g/100 ml; E_2, acetone, 13·4 g/100 ml; E_3, 95% ethanol, 1·6 g/100 ml; E_1 acetate, $C_2H_2Cl_4$, 4·2 g/100 ml; E_2 acetate, $C_2H_2Cl_4$, 6·1 g/100 ml; E_3 acetate, acetone, 1·1 g/100 ml. U.v. spectra were determined in EtOH. I.r. spectra were determined in KBr wafers.

E_3 Hydrolysis

E_3 (20 mg) in 10 ml of 2 N HCl was refluxed for 5 hr. The flavanols precipitated as phlobaphenes, the solution was filtered, and the gallic acid was separated by paper chromatography. The amount of gallic acid produced was determined with a densitometer by comparing spot area and intensity from known amounts of gallic acid treated identically with $FeCl_3$–$K_3Fe(CN)_6$.[10] The gallic acid recovered was 0·65–0·91 moles/mole of E_3, averaging 0·75. If gallic acid alone was refluxed 5 hr in 2 N HCl, 10% of the gallic acid converted to degraded compounds immobile on paper in 2% acetic acid.

If hydrolysis of E_3 was carried out under milder conditions which avoided appreciable phlobaphene formation, E_1 was a major product as shown by paper chromatography and the apparent yield of E_1 was about equal to that of gallic acid.

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