CHROM. 8720

ANALYSIS OF PHENOLIC ACIDS AND FLAVONOIDS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY *

L. W. WULF and C. W. NAGEL

Department of Food Science and Technology, Washington State University, Pullman, Wash. 99163 (U.S.A.)

(Received August 6th, 1975)

SUMMARY

A high-pressure liquid chromatographic (HPLC) technique for the separation and quantitation of three classes of naturally occurring phenolic compounds has been developed. Investigated were a series of phenolic acids, both cinnamic and benzoic, and a variety of flavone compounds including flavones, flavonols, and flavanones as well as glycosylated flavone derivatives with mono- and disaccharide substitution. The effects of structural variations within the phenolic compounds upon elution order and retention were elaborated. The technique offers selectivity, resolution, speed, and sensitivity (minimum detectable amounts below 50 ng) far superior to classical techniques such as paper chromatography. Time of analysis by the HPLC technique is measured in minutes while an equivalent analysis by classical techniques would require several days, if not weeks.

INTRODUCTION

The phenolic constituents of grapes are responsible for the color, astringency, and bitterness of wine. Because the time of storage is long, many phenolic compounds undergo vast chemical changes during aging that modify a wine's character and determine the quality of the final product. For example, the red anthocyanin pigments (mol. wt. 500) of grapes are difficult to demonstrate in old wines. Instead, as shown by Somers[1,2], the pigments exhibit brick-red and tawny colors and show molecular weights as high as 50,000.

The phenolic compounds in grapes and wine are a heterogenous group of substances consisting of several classes of compounds. Among the classes present are phenolic benzoic acids, phenolic cinnamic acids and their quinic acid esters (*e.g.*, chorogenic acid), flavan-3-ols (catechins), flavan-3,4-diols (leucoanthocyanidins), flavonols and flavonol glycosides, high-molecular-weight "tannins", and in red types anthocyanins[3]. Furthermore, each class contains many compounds, thus making 40 phenolics in a white wine and 50 in a red wine not exaggerated figures. Such a large

heterogenous group of substances imposes demands upon the techniques that are used for studying a single component, a class of compounds or the totality of the phenolic constitutents in a grape or wine sample.

The use of paper chromatography (PC) is responsible for most of the knowledge of the phenolic constituents of grapes and wine; indeed, this is true for the entire plant kingdom. Selected texts on this subject are by Harborne[4] and by Ribereau-Gayon[5]. PC lacks the resolution, speed, and the quantitative accuracy needed for fast, reliable analysis of complex mixtures. Thin-layer chromatography[6,7] offers greater resolution and speed than does PC, but also suffers in quantitative accuracy. Gas chromatography (GC) is a fast, efficient, and accurate technique for analysis of complex mixtures and has been applied to separate phenolic compounds[8,9]. However, GC requires a derivatization step, thermal degradation may occur and higher-molecular-weight compounds cannot be analyzed.

The recent introduction of columns packed with efficient, small-diameter (to 5μm) particles is responsible for the development of high-pressure liquid chromatography (HPLC). Coupled with high-pressure pumping systems, and sensitive, accurate detectors the technique rivals GC. In addition, derivatization is not necessary, thermal degradation is not a problem and no limit is imposed upon the molecular size of compounds that can be chromatographed by HPLC.

In order to use the advantages of HPLC, development of a technique capable of separation of wine phenolics was desired. The purpose of this study was to examine the chromatographic behavior of several members of three classes of phenolic compounds. A recent example of HPLC separation of the phenolic compounds in beer has been published by Charalambous et al.[10].

EXPERIMENTAL

Apparatus

The liquid chromatograph used was a Chromatronix Model 3100 equipped with a dual wavelength (254 and 280 nm) ultraviolet detector and a 20-μl valve loop injector. Pressure supply was from a 3,000 p.s.i. nitrogen gas cylinder. Pressure delivery control was by means of a gas regulator valve rated for 4,000 p.s.i. input and 2,500 p.s.i. output. The chromatographic column, 30 cm × 4 mm I.D., was prepacked with μBondapak/C$_{18}$ (Waters Assoc., Milford, Mass., U.S.A.).

Chemicals

The benzoic and cinnamic acids were obtained from J. T. Baker (Phillipsburgh, N.J., U.S.A.). The cinnamic acids were of the *trans* configuration. All flavone compounds except quercetin and quercitrin were kindly donated by Dr. Bob Horowitz (Fruit and Vegetable Laboratory, USDA, Pasadena, Calif., U.S.A.). Quercetin was purchased from J. T. Baker while quercitrin and d-catechin were products of K & K Labs (Plainview, N.Y., U.S.A.). All compounds demonstrated a high purity and so were used without further purification. For HPLC analysis, solutions of the phenolic acids were prepared in acetonitrile–water (4:1, v/v) at a concentration of 25.0 mg per 100 ml. Similar solutions were made of the flavones at a concentration of 5 mg per 25 ml.

Procedure for phenolic acids and d-catechin

The phenolic benzoic and phenolic cinnamic acids, as well as *d*-catechin were chromatographed alone or in various mixtures on the μBondapak/C$_{18}$ column at pressures of 2,300–2,500 p.s.i. which gave a flow-rate of approx. 3.0–3.5 ml/min. Eluting solvents tested were water–acetic acid (95:5, v/v), water–acetic acid–methanol (90:5:5), water–acetic acid–methanol (83:5:12), water–acetic acid–methanol (80:5:15), and water–acetic acid–methanol (75:5:20). Acetic acid was used in the solvent to suppress ionization of the acid group. A detector sensitivity of 0.04 a.u.f.s. was normally used which required a dilution of the standard solutions by a factor of 10 or more in order to keep the eluted peaks on scale. Recorder chart speed was 6 in./h for all samples. Retention times were measured by a stopwatch. The retention times were measured to calculate two chromatographic parameters, the capacity factor, k', and the relative retention, α. These parameters were calculated by the equations[11]

$$k' = \frac{t_R - t_0}{t_0} \quad \text{and} \quad \alpha = \frac{k'_2}{k'_1}$$

where t_R = retention time of compound, t_0 = the time of zero retention measured as the time of the non-retained solvent peak, k'_2 = capacity factor of component 2, and k'_1 = capacity factor of component 1.

Procedure for flavone compounds

All conditions except for eluting solvents were the same as those used for the phenolic acids. The solvent used with the flavone compounds was water–acetic acid–methanol (65:5:30, v/v). Capacity factors and relative retentions were calculated for these compounds based on the measured retention times.

RESULTS AND DISCUSSION

Separation of phenolic acids and d-catechin

Fig. 1 illustrates the separation of 8 phenolic acids, *d*-catechin and cinnamic acid on μBondapak/C$_{18}$ using water–acetic acid (95:5) as eluent. Retention data for these compounds and chlorogenic acid are listed in Table I. The flow-rate of approx. 2.6 ml/min resulted in an elution time for this ten-component mixture of 33 min.

Elution time could be reduced by using a higher flow-rate, or by employing gradient elution. The chromatogram (Fig. 1) shows the elution order typical of reversed-phase systems, that is, polar components eluting before non-polar components.

Effect of structure on retention

The packing used exhibits remarkable selectivity between like compounds in the two series of phenolic acids investigated, as well as between similar compounds in each series. Selectivity is measured in terms of α. Calculation of α values for benzoic and cinnamic acids with the same ring substitution gives a quantitative evaluation of the selectivity of the packing between these two classes of compounds. For example: caffeic acid–protocatechuic acid, $\alpha = 4.45$; *p*-coumaric acid–*p*-hydroxybenzoic acid, $\alpha = 4.24$; and *o*-coumaric acid–salicylic acid, $\alpha = 1.98$.
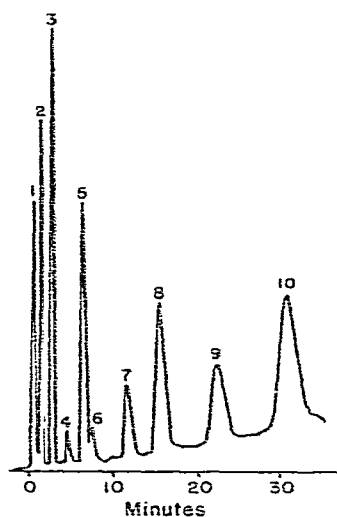
Fig. 1. Separation of phenolic acids, d-catechin and cinnamic acid on μBondapak/C₁₈ with water–acetic acid (95:5) as eluent. 1 = gallic acid, 2 = protocatechuic acid, 3 = p-hydroxybenzoic acid, 4 = d-catechin, 5 = caffeic acid, 6 = salicylic acid, 7 = p-coumaric acid, 8 = o-coumaric acid, 9 = ferulic acid, 10 = cinnamic acid.

TABLE I

RETENTION TIMES, CAPACITY FACTORS AND RELATIVE RETENTIONS OF PHENOLIC ACIDS, CINNAMIC ACID AND d-CATECHIN ON μBONDAPAK/C₁₈ USING WATER–ACETIC ACID (95:5) AS ELUENT

Flow-rate, approx. 2.6 ml/min. t_0, 85 sec.

Compound		t_R (min:sec)	k'	α
Gallic acid	3,4,5-trihydroxybenzoic acid	2:02	0.49	
Protocatechuic acid	3,4-dihydroxybenzoic acid	2:52	1.10	2.24
p-Hydroxybenzoic acid	4-hydroxybenzoic acid	4:12	2.07	1.88
d-Catechin	3,3',4',5,7-pentahydroxyflavanone	6:12	3.54	1.71
Caffeic acid	3,4-dihydroxy-trans-cinnamic acid	8:03	4.89	1.38
Salicylic acid	2-hydroxybenzoic acid	9:20	5.83	1.19
Chlorogenic acid	3-caffesylquinate	11:38	7.51	1.29
p-Coumaric acid	4-hydroxy-trans-cinnamic acid	13:22	8.78	1.17
o-Coumaric acid	2-hydroxy-trans-cinnamic acid	17:10	11.56	1.32
Ferulic acid	4-hydroxy-3-methoxy-trans-cinnamic acid	24:14	16.73	1.45
Cinnamic acid	trans-cinnamic acid	33:03	23.20	1.39

As a rough guide α values greater than 1.15 are required in order to get a resolution of 1 (98 % separation) between two components on this packing or on any other packing demonstrating a similar efficiency of approx. 1,500 plates per 30-cm column[12]. On this basis the α values of 4.45 and 4.24 represent excellent selectivity and resolution of the similar phenolic, cinnamic and benzoic acids. The structural difference that results in the high α values between these two groups of compounds is the presence of an extra ethylenic chain in the cinnamic acids. Because the selectivity for a two-

carbon chain is so high, the selectivity for phenolic acids with a one-carbon chain should be very good. Thus, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, and caffeic acid should be easily separated on μBondapak/C$_{18}$ in that elution order.

The separation between o-coumaric acid and salicylic acid is less than with the aforementioned pairs of compounds, α being less than half the previous values at 1.98. Such behavior indicates that one or both of the *ortho* hydroxylated compounds are atypical. Both salicylic and o-coumaric acid must be special cases, otherwise neither compound would be separable from its *para* isomer, while in fact the following α values were obtained: salicylic acid–p-hydroxybenzoic acid, α = 2.82; and o-coumaric acid–p-coumaric acid, α = 1.32.

Salicylic acid is retained longer than its *para* isomer because both polar functionalities in salicylic acid are tied up intramolecularly by hydrogen bonding. This would decrease the solvation of the polar groupings in salicylic acid with the aqueous solvent in comparison to the solvation of these groupings in p-hydroxybenzoic acid. Other data support this conclusion. The carboxyl group of salicylic acid readily ionizes (pK_a = 2.97) while that of p-hydroxybenzoic acid (pK_a = 4.48) does not. The increased ionization of salicylic acid results from the ability of the conjugate base to use its anionic charge to form a very stable intramolecular hydrogen bond. The second ionization constant (pK_a = 13.44) of salicylic acid as compared with that of p-hydroxybenzoic acid (pK_a = 9.40) indicates the strength by which the phenolic hydrogen is intramolecularly bound[13].

A similar intramolecular hydrogen bond is not possible in o-coumaric acid as the side-chain double-bond configuration is *trans*. This dissimilarity accounts for the separation being less between o-coumaric acid and salicylic acid (α = 1.98) than between the other pairs of phenolic cinnamic and phenolic benzoic acids. The separation of o-coumaric acid and p-coumaric acid is difficult to explain.

Within the cinnamic acid series of compounds the expected elution pattern in reversed-phase chromatography is observed. Ferulic acid elutes well after p-coumaric acid (α = 1.91) which indicates that a methoxy substituent is non-polar as it increases retention. This is in contrast to a decrease in retention produced by addition of a hydroxyl group.

Unexpectedly, chlorogenic acid eluted after caffeic acid. This behavior was unexpected because chlorogenic acid is considered to be a more polar compound than caffeic acid[14].

Effect of solvent strength on retention

The stronger solvents containing methanol showed several effects upon the retention of the phenolic acids and d-catechin. First, the general trend was a logarithmic decrease in k' with increasing concentrations of methanol. This means a linear increase in eluent strength during gradient elution can only be achieved by increasing the concentration of methanol in a logarithmic manner.

Surprisingly, the relative retention between compounds in one class that differ only in hydroxylation is independent of the methanol content of the solvent. This is reflected in Fig. 2 where the logarithm of k' is plotted *versus* the methanol content of the solvent. The nearly parallel lines among members of the hydroxylated benzoic acid class and among the hydroxylated cinnamic acids indicate that the separation factor between compounds in each class is independent of the methanol content of
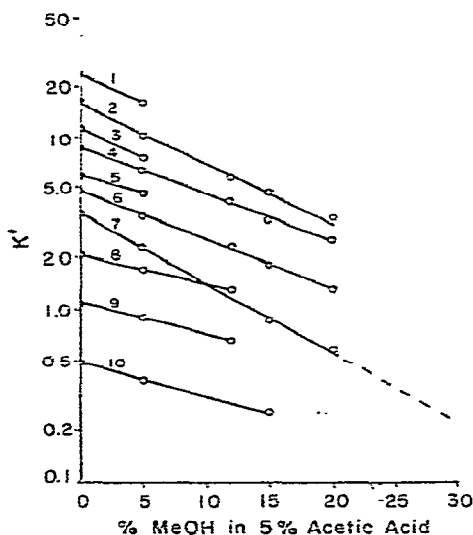
Fig. 2. Effect of methanol content of the solvent on the capacity factor k', of phenolic acids, d-catechin and cinnamic acid. 1 = Cinnamic acid, 2 = ferulic acid, 3 = o-coumaric acid, 4 = p-coumaric acid, 5 = salicylic acid, 6 = caffeic acid, 7 = d-catechin, 8 = p-hydroxybenzoic acid, 9 = protocatechuic acid, 10 = gallic acid.

the solvent, at least for the concentrations studied. Calculated slopes of log k' *versus* methanol concentration are: hydroxylated benzoic acids —0.0195, caffeic, p-coumaric, and cinnamic acids —0.0291, d-catechin —0.0397, ferulic acid —0.0372, and o-coumaric acid —0.0364. The cinnamic acids decrease much faster in retention time than the benzoic acids indicating that the separation factor between any cinnamic and any benzoic acid decreases with increasingly methanolic solvents. The extra non-polar ethylenic linkage in cinnamic acids results in a greater solubilizing action of the methanol towards this compound class than for the benzoic acids. Ferulic acid, with its methoxy group, is affected more by a methanolic solvent than is a simple hydroxylated cinnamic acid.

o-Coumaric acid does not behave as a typical cinnamic acid in its response to a methanol containing solvent. Some interaction, either steric or electronic, with the adjacent side chain makes the phenolic group less polar as methanol affects o-coumaric acid more than other hydroxylated cinnamic acids while salicylic acid exhibits no such behavior and responds as a typical hydroxylated benzoic acid to methanolic solvents.

The most dramatic effect of adding methanol to the eluting solvent is in the separation between d-catechin and p-hydroxybenzoic acid. As Fig. 2 illustrates the elution order can be reversed at methanol concentrations greater than about 11%. Evidently, d-catechin is much more soluble in methanol than p-hydroxybenzoic acid while the opposite is true in water.

Separation of flavone-type compounds

Substitution of a 4-keto group into the flavonoid nucleus has a very drastic effect upon elution behavior. Whereas d-catechin elutes early with water–acetic acid

(95:5), a methanol content of 30% is required for early elution of similar flavone aglycones. Calculation of α values between d-catechin and similar flavone compounds with methanol–acetic acid–water (30:5:65) reveals the magnitude of the effect of a 4-keto substituent. Between the flavone eriodictyol and d-catechin, α is greater than 12 while between quercetin, a flavonol and d-catechin, it is near 33 (assuming a k' of 0.22 for d-catechin in the 30% methanol solvent in Fig. 2 and using the k' for flavones in Table II). Very strong hydrogen bonding between the 4-keto group and the *peri*-hydroxyl group at position 5, with the formation of a planar, non-polar six-membered ring would explain this behavior. The non-polarity of 4-keto compounds is indicated by their almost total insolubility in water and the fact that the 5-hydroxyl group is totally inert to methylation by diazomethane.

TABLE II

RETENTION TIMES, CAPACITY FACTORS AND RELATIVE RETENTIONS OF 4-KETO-FLAVONOID DERIVATIVES ON μBONDAPAK/C$_{18}$ USING WATER–METHANOL–ACETIC ACID (65:30:5) AS ELUENT

Flow-rate approx. 3.1 ml/min. t_0, 75 sec.

Compound		t_R (min:sec)	k'	α
Naringin	Narigenin-7-neohesperoside	3:15	1.60	1.22
Hesperidin	Hesperetin-7-rutinoside	3:42	1.96	1.37
Eriodictyol	3′,4′,5,7-tetrahydroxyflavanone	4:36	2.68	1.12
Rutin	Quercetin-3-rutinoside	4:59	3.00	1.39
Myricetin	3,3′,4′,5,5′,7-hexahydroxyflavone	6:20	4.07	1.06
Quercitrin	Quercetin-3-rhamnoside	6:40	4.33	1.09
Narigenin	4′,5,7-trihydroxyflavanone	7:10	4.73	1.06
Morin	2′,3,4′,5,7-pentahydroxyflavone	7:30	5.00	1.45
Quercetin	3,3′,4′,5,7-pentahydroxyflavone	10:18	7.24	1.52
Luteolin	3′,4′,5,7-tetrahydroxyflavone	15:00	11.00	1.15
Kaempferol	3,4′,5,7-tetrahydroxyflavone	17:03	12.64	1.62
Rhamnetin	3,3′,4′,5-tetrahydroxy-7-methoxy flavone	26:30	20.20	

Within the flavonol class of compounds the elution order is the same as in the two classes of phenolic acids. That is, myricetin with trihydroxy substitution elutes before quercetin with two hydroxyl groups, and this compound, in turn, elutes before kaempferol which elutes before one of quercetin's monomethyl ether derivatives, rhamnetin. Separation factors between different pairs of flavonol aglycones are less than those for similar pairs of compounds in the phenolic cinnamic or benzoic acid classes: quercetin–myricetin, $\alpha = 1.78$; protocatechuic acid–gallic acid, $\alpha = 2.24$; kaempferol–quercetin, $\alpha = 1.75$; p-coumaric acid–caffeic acid, $\alpha = 1.80$; narigenin–eriodictyol, $\alpha = 1.76$; and p-hydroxybenzoic acid–protocatechuic acid, $\alpha = 1.88$.

Of course, a comparison such as this is valid only if α remains constant between the compounds in each pair in different solvents. This is certainly true between the phenolic acids in each pair listed above as already discussed, and by inference should be true for the flavone pairs.

Comparison of the flavone, luteolin with its flavanone counterpart eriodictyol reveals the effect of unsaturation between positions 2 and 3 upon elution behavior.

Luteolin elutes much later than eriodictyol, with $\alpha = 4.10$. One factor operating to make flavones much less polar than flavanones is a larger electron density on the oxygen atom of the 4-keto group of flavones resulting from resonance structures where the keto oxygen assumes a negative charge.

The larger electron density will make the hydrogen bond between the 5-hydroxyl group and the 4-keto group stronger and make both functional groups appear less polar to the solvent. However, this may not be the only possible explanation for the greater polarity of flavanones over flavones. In flavanones, the 4-keto group may be out of the plane of the adjacent phloroglucinol ring, thus making a hydrogen bond with the *peri*-hydroxyl group weaker and exposing both functionalities to stronger interactions with the solvent. Flavones, because of their total planarity, simply may be more difficult to solvate than the partially planar flavanones. The planar flavones would require a more ordered solvent structure and thus a larger entropy term in solvation than would flavanones. Most likely a combination of all of the above three mechanisms operate in making flavones less polar than flavanones.

With flavonols, the 3-hydroxyl group must be less polar than a similar substituent located in the B ring at position 3', otherwise kaempferol and luteolin would not be separable. Most likely the 3-hydroxyl group in flavonols participates in hydrogen bonding with the 4-keto group, thus decreasing its polarity.

Flavonoid glycosides are more polar than the parent phenolic aglycone, as expected. However, the separation between glycoside and aglycone is not dramatic.
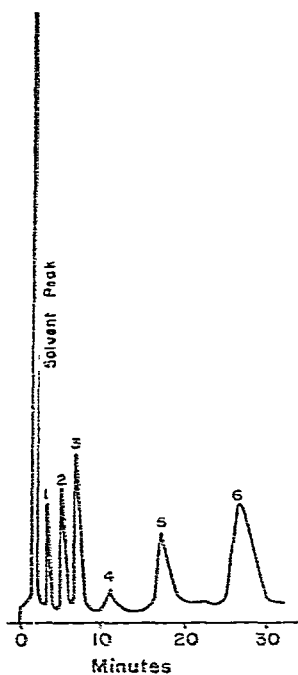

Fig. 3. Separation of 4-ketoflavonoid derivatives on μBondapak/C$_{18}$ with methanol–acetic acid–water (30:5:65) as eluent. 1 = Naringin, 2 = rutin, 3 = quercitrin, 4 = quercetin, 5 = kaempferol, 6 = rhamnetin.

Thus, glycosylation of quercetin with rhamnose introduces a separation factor of only 1.67 between the two compounds. Noting that quercitrin and myricetin elute very close together, it is fair to say that glycosylation at position 3 with rhamnose results in a polarity increase no more than can be achieved by addition of a phenolic hydroxyl group. Rutin, containing a disaccharide linkage is separated less from its monosaccharide relative quercitrin ($\alpha = 1.44$), than is quercitrin from quercetin ($\alpha = 1.67$).

The separations between biosides, monosides, and aglycones is not adequate for the resolution of a complex mixture containing many compounds of each group, mostly because all members of one group (*e.g.*, monosides) are not completely separated from all members of another group (*e.g.*, aglycones). However, within one group of substances such as aglycones the separations are good. This is also seen in the separation of the three biosides containing glucose and rhamnose where rutin, hesperidin and naringin are adequately separated from one another. Finally, most flavone-type compounds exhibit slightly tailing peaks, especially when larger amounts are injected. The separation of several flavone type compounds is illustrated in Fig. 3.

REFERENCES

1 T. C. Somers, *Nature (London)*, 209 (1966) 368.
2 T. C. Somers, *Phytochemistry*, 10 (1971) 2175.
3 V. L. Singleton and P. Esau, *Phenolic Substances in Grapes and Wine and their Significance*, Academic Press, New York, 1969, Ch. 2.
4 J. B. Harborne, *Comparative Biochemistry of the Flavonoids*, Academic Press, New York, 1967, Ch. 3 and 4.
5 P. Ribereau-Gayon, *Plant Phenolics*, Hafner, New York, 1972, Ch. 2 and 3.
6 N. Nybom, *Physiol. Plant.*, 17 (1964) 157.
7 K. Egger, in E. Stahl (Editor), *Thin Layer Chromatography*, Springer, New York, 2nd ed., 1969, pp. 655–672.
8 E. N. Christensen and A. Caputi, Jr., *Amer. J. Enol. Viticult.*, 19 (1968) 238.
9 E. S. Keith and J. J. Powers, *J. Food Sci.*, 31 (1966) 971.
10 G. Charalambous, K. J. Bruckner, W. A. Hardwick and A. Linneback, *Tech. Quart.*, *Master Brew. Ass. Amer.*, 19 (1973) 6.
11 B. L. Karger, in J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971, pp. 10 and 14.
12 B. L. Karger, in J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971, p. 19.
13 J. B. Hendrickson, D. J. Cram and G. S. Hammond, *Organic Chemistry*, McGraw-Hill, New York, 1970, p. 324.
14 V. L. Singleton and P. Esau, *Phenolic Substances in Grapes and Wine and their Significance*, Academic Press, New York, 1969, p. 71.