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Registry No. Ascorbic acid, 50-81-7; 3-hydroxy-2-pyranone, 496-64-0; nitrite, 14797-65-0.

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Characterization of 2-*S*-Glutathionylcaftaric Acid and Its Hydrolysis in Relation to Grape Wines

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The major phenolic product formed in grape juice or must by enzymic oxidation is shown to be 2-*S*-glutathionylcaftaric acid by proton NMR studies and fractional hydrolysis. Acidic hydrolysis yields a total of seven new products, the last of which is identical with 2-*S*-cysteinylcaffeic acid synthesized enzymically. All but one of these hydrolysis products were separated by HPLC and identified by comparisons with prepared knowns and by hydrolysis sequence. Aged bottled wines had an increased content of caffeic acid from hydrolysis of caftaric acid and 2-*S*-glutathionylcaffeic acid from hydrolysis of glutathionylcaftaric acid with time. Additional hydrolysis products also appeared to be present in older wines, but uncertainty about the original content, losses to other reactions, and more complicated chromatograms make conclusions uncertain.

Caffeoyl-L-(+)-tartaric (caftaric) acid has considerable interest as the major phenolic substance of grape juice prepared with little oxidation and minimal extraction from skins or seeds. It is also a major nonflavonoid phenol of all freshly fermented grape wines from musts not excessively oxidized (Singleton et al., 1978). In the grape and a few other plants hydroxycinnamoyltartaric acid derivatives apparently replace the chlorogenic (quinic) acid analogues common in most plants. In protected fresh juice from wine grapes about 100 mg/L of *trans*-caftaric acid is considered typical, although there is appreciable varietal variation. About one-fifth as much coutaric (*p*-coumaroyltartaric) acid and a few milligrams/liter of fertaric acid (the ferulic analogue) usually are found (Ong and Nagel, 1978ab; Nagel et al., 1979). Smaller amounts of the cis isomers usually accompany the predominant trans forms of the compounds, and if exposed to light after separation, either isomer is readily returned to an equilibrium mixture of the two (Singleton et al., 1977, 1978). The cis forms have been confused as glucose derivatives, but that has been rectified (Ong and Nagel, 1978a; Baranowski and Nagel, 1981).

In the course of normal commercial juice or must preparation and laboratory sampling without thorough protection from oxidation, a portion of the caftaric and coutaric acids is converted to a new derivative. This derivative is much more polar (in terms of extraction from juice) than the original caftaric acid. The proportion of the new product to the caftaric acid increases, as the exposure to oxygen increases (Singleton et al., 1984). It was shown that this derivative was the result of a four-component reaction involving caftaric (or coutaric) acid, phenoloxidase, oxygen, and glutathione (Singleton et al.,

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1985a). The product behaves in an electrochemical detector as a regenerated vicinal diphenol. Similar (but separable by HPLC and paper chromatography) derivatives were produced with cysteine and other sulfhydryl compounds and when caffeic acid was substituted for caftaric acid (Singleton et al., 1985a). The glutathione adduct was no longer a substrate for grape polyphenoloxidase. This reaction limits enzymic browning of musts and, since it does not occur during raisining, may explain why sun-dried raisins become so brown compared to enzymically oxidized must (Singleton et al., 1985b).

The purpose of the research to be reported here was to prove the specific structure of the glutathione–caftaric acid reaction product and to investigate the occurrence of its hydrolysis products in wine.

EXPERIMENTAL SECTION

Chromatographically pure caftaric acid and crude grape phenoloxidase in the form of an acetone powder were prepared from grape juice as described previously (Singleton et al., 1978, 1985a). Glutathione, caffeic acid, cysteine, and other reagents not separately specified were purchased and used without further purification. Cysteinyl, glutathionyl, and cysteinylglycine derivatives of caffeic and caftaric acids were prepared by aerating 1 mM solutions of the phenolic acid with 2 mol equiv of the sulfhydryl compound in the presence of 2 g/L of crude grape phenolase in aqueous 3 g/L of potassium bitartrate at room temperature. Cysteinylglycine was prepared by acid hydrolysis of glutathione according to Gutcho and Laufer (1954) and used without isolation to prepare caffeic and caftaric acid thioether adducts.

Samples for HPLC were filtered through a 0.45-μm membrane filter immediately before use and were prepared at concentrations similar to grape juice and adequate for direct injection of 10–50 μL. The HPLC apparatus was a Waters Chromatography Division (Millipore Corp., Milford, MA) system including a 720 controller, 730 data module, M6000A pumps, and a 710B autoinjector and a Perkin-Elmer (Norwalk, CT) LC-55B variable-wavelength detector. The effluent was monitored at 320 nm, and known dilutions of *t*-caftaric acid were used to determine the response factor (mass/unit peak area) and thus to calculate the amount of caftaric acid or derivatives in each peak. The column was reversed phase, C18 (5-μm packing), 4.6 × 250 mm, protected with a guard column of the same material (Brownlee Labs., Inc., Santa Clara, CA). Two solvent systems were applied, both based upon 0.01 M ammonium dihydrogen phosphate made to pH 2.6 with phosphoric acid. In the one case, isocratic development followed addition to the above solvent of 14% by volume of 80% acetonitrile in water (developer A). Developer B involved addition of 17% by volume of 80% methanol in water and, after 15 min of isocratic development, a linear gradient from 17% to 50% of the 80% methanol in 15 min. Flow rate was 1.5 mL/min, and the temperature was ambient, about 21 °C.

Hydrolyses were conducted in aqueous pH 1 sulfuric acid, refluxing under nitrogen. Intermediate samples of 200 μL were collected at frequent intervals, immediately cooled, and refrigerated until analysis was completed.

Paper chromatograms were prepared immediately after samples were taken. Each (30 μL) was spotted on Whatman No. 1 chromatographic paper along with phenolic, amino acid, and peptide standards. Ascending development (16 h) was with the upper phase of butanol–acetic acid–water (2:1:1). Chromatograms were examined for fluorescence under UV followed by ninhydrin spray (Gutcho and Laufer, 1954; Pataki, 1969).

Table I. Chromatographic Properties of Caffeic Acid, Caftaric Acid, and Their Thioether Adducts in the Glutathione Series

			HPLC: R_t, min	
no.[a]	substance	paper chrom, R_f, BAW	A: CH_3CN	B: MeOH
	caffeic acid	0.95	12.15	26.00
	caftaric acid	0.65	4.68	8.05
1	glutathionylcaftaric	0.40	3.57	9.58
2	Gly-CyS caftaric	0.43	4.11	11.04
3	Glu-CyS caftaric	0.40	3.57	8.66
4	glutathionylcaffeic	0.60	9.42	28.62
5	cysteinylcaftaric	0.45	3.24	7.20
6	Gly-CyS caffeic	0.65	13.65	31.87
7	Glu-CyS caffeic	(unavail)	(with 4)	(with 4)
8	CyS-caffeic (synth)	0.64	7.95	26.00
8	CyS-caffeic (hydrol of 1)	0.64	7.95	26.00

[a] See Figure 2.

The NMR data were obtained in Me_2SO-d_6 using Me_4Si as the reference. Spectrometers, 360 MHz, were used in the U.S. from Nicolet Magnetics Corp. (Fremont, CA) and in France from Bruker Analytische Messtechnik GmbH (Silberstreifen, D-7512 Rheinstetten 4, West Germany). Electronic spectra were obtained with a Perkin-Elmer (Norwalk, CT) Model 3840 diode array spectrophotometer.

RESULTS AND DISCUSSION

It has been proposed that the reaction involves production of the *o*-quinone from either caftaric or caffeic acid by autoxidation catalyzed by polyphenoloxidase (PPO) followed by 1,4-addition of the sulfhydryl to make a thioether with the ring (Singleton et al., 1985a). This reaction is well-known in organic chemistry and has been invoked to explain the "inhibition of browning enzymes" by cysteine and similar sulfhydryl compounds. It does not seem to have been properly recognized that the antibrowning effect results from conversion of the phenolic substrate via enzymic oxidation to a product neither brown nor a substrate for the enzyme and not from inhibition of the enzyme per se (Singleton et al., 1985ab). Under appropriate conditions including adequate oxygen, the reaction will continue in the presence of relatively high concentrations of a sulfhydryl compound with increasing proportions of the substrate being converted. In grapes such as Thompson Seedless, which apparently have an adequate content of glutathione, essentially quantitative conversion of *trans*-caftaric acid to the glutathione derivative can be achieved (based upon HPLC and 320-nm absorbance).

It was considered important to eliminate the possibility that the glutathione reaction product involved further cyclizations or other reactions that would lead to nonhydrolyzable linkages. If the product of the reaction between caftaric acid and glutathione was the expected "simple" thioether, it should be possible to hydrolyze it to the analogous product synthesized from caffeic acid and cysteine. This was accomplished. Via retention characteristics and cochromatography in three systems, a major product of acidic hydrolysis of glutathionylcaftaric acid was identical with cysteinylcaffeic acid (Table I). This showed not only that the thioether linkage was the same in both the caffeic and caftaric derivatives but also that there were no further cyclizations or other nonhydrolyzable substitution reactions between the other functional groups of the amino acid portion and the caffeic acid moiety. In no instance was any indication obtained for the breaking of the thioether bond to liberate caffeic acid or its derivatives.

Table II. Proton NMR Spectra of Aromatic and Vinyl Protons of Caffeic Acid Derivatives

compd	aromatic/vinyl proton position,[a] δ				
	2	5	6	α	β
t-caffeic acid	7.03 (d, J = 1.8)	6.76 (dd, $J_{5,6}$ = 8.1, $J_{5,2}$ = 1.8)	6.97 (dd, $J_{6,5}$ = 8.1, $J_{6,2}$ = 1.8)	7.42 (d, J = 16.2)	6.17 (d, J = 16.1)
t-caftaric acid	7.03 (d, J = 1.8)	6.78 (dd, $J_{5,6}$ = 7.9, $J_{5,2}$ = 1.8)	7.05 (dd, $J_{6,5}$ = 7.9, $J_{6,2}$ = 1.8)	7.57 (d, J = 16.2)	6.24 (d, J = 16.2)
t-*S*-cysteinyl-caffeic		6.93 (d, J = 8.4)	7.23 (d, J = 8.3)	8.17 (d, J = 15.9)	6.28 (d, J = 15.9)
S-glutathionyl-caftaric		6.83 (d, J = 8.3)	7.23 (d, J = 8.3)	8.27 (d, J = 16)	6.28 (d, J = 16)
S-Cys Et caffeoate		6.94 (d, J = 8.4)	7.27 (d, J = 8.4)	8.24 (d, J = 15.9)	6.36 (d, J = 15.9)

[a] Key: d = doublet, dd = double doublet. J values in Hertz.

Table III. Proton NMR Spectra of Saturated Protons of Substituted Caffeic Acid Derivatives[a]

compd	tartaric		cysteine	
	2	3	α_{Cys}	β_{Cys}
caftaric	5.33 (d, J = 2.5)	4.57 (d, J = 2.5)		
S-cysteinylcaffeic			2.94 (t)	2.23, 2.24
S-glutathionylcaftaric	5.35 (d, J = 3)	4.38 (d, J = 3)	4.07	2.89, 3.31
S-CyS Et caffeoate			3.94 (br)	3.25, 3.24

[a] Key: d = doublet, t = triplet, br = broad. J values in Hertz.

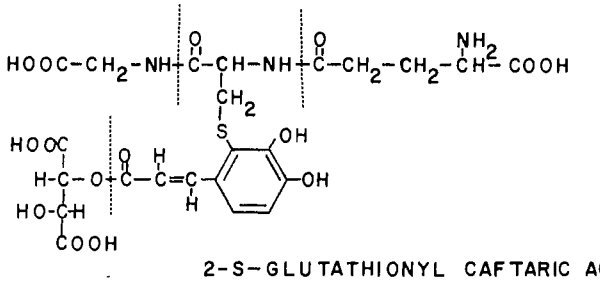

Figure 1. Structure and hydrolyzable linkages (dashed lines) of 2-*S*-glutathionylcaftaric acid.

The site of substitution of the thioether function was shown to be the 2-position of the aromatic ring by means of proton NMR studies (Figure 1), the American and French authors independently reaching the same conclusions. Data are shown in Tables II and III. The basic ring-substitution pattern of 2-*S*-glutathionylcaftaric acid is similar to that of naturally occurring caftaric acid (Table II), with a pair of AB doublets at 6.83 and 7.23 ppm (J = 8.3 Hz) indicating an ortho orientation and assignment to protons on carbons 5 and 6. The loss of long-range, secondary splitting as observed in both caffeic and caftaric acids plus no signal at about 7 ppm with a coupling constant of 1.8 Hz confirm the absence of an ABX meta-coupled proton at the 2-position. These assignments show substitution of the glutathionyl moiety at the 2-position of the ring.

Substitution is through the cysteine sulfur since the single α proton is seen as a broad triplet at 2.94 ppm and the two nonequivalent β protons are singlets at 2.23 and 2.24 ppm in 2-*S*-cysteinylcaffeic acid (Table II). In the glutathionyl derivative the cysteinyl protons are observed at 4.07 ppm (α-Cys) and 2.89 plus 3.31 ppm (β-Cys). Also, substitution at the 2-position causes a downfield shift of the vinyl α proton of 0.6–0.7 ppm but has negligible effect on the vinyl β proton (Table II). This contrasts with the small effect on the α proton caused by esterification of the caffeic carboxyl in the formation of caffeic esters. The trans configuration is maintained as shown by the coupling constant (J = 16 Hz) for the *S*-glutathionylcaftaric acid.

The tartaric moiety appears to be unaffected by glutathione substitution and differs little from the chemical shift values obtained for caftaric acid (Table III). Hence, there is no heterocyclic ring formation or substitution through secondary ester or amide formation between free acid or amine groups of the glutathione portion with the hydroxy or carboxy groups of the tartaric acid. Additionally, the integrity of the glutathione moiety in the reaction product is confirmed by the assignment of the glutamic and glycine protons. The glutamic α proton gives a double doublet at 3.56 ppm (J = 7.00 Hz), with two nonequivalent β protons giving a broad multiplet at 1.88 ppm and the two nonequivalent γ protons giving a broad multiplet at 2.33 ppm. The two protons α to the glycine amino group show a doublet at 3.62 ppm (J = 5.81 Hz). The cysteine amino group gives a doublet at 8.38 ppm (J = 8.00 Hz), while the glycine amino group gives a broad multiplet signal at 8.23 ppm.

In addition to the data shown in Tables II and III, the protons of the phenolic hydroxyls are indicated by two broad signals at 9.22 and 10.36 ppm assigned, respectively, to the 3- and 4-hydroxyls of cysteinylcaffeic acid. This indicates, as noted previously by oxidation–reduction potential, that the enzymically oxidized caffeic and caftaric acids have been regenerated into hydroquinone form by the addition of the thio compound. Thus, these products should be capable of further nonenzymic oxidation in the course of wine storage. This is the first concrete demonstration of "regenerative polymerization" postulated as a source of the high oxygen capacity of wines (Singleton et al., 1982, 1985).

The exclusive substitution of the thio group at the 2-position was at first unexpected, considering the possibilities of competitive or multiple substitutions involving the 5- or 6-positions as well. The fact that, after initial substitution, the 2-*S*-glutathionylcaftaric acid is no longer a substrate for polyphenoloxidase explains the lack of multiple substitution (Singleton et al., 1985a). Models indicate the accessibilities of the 2- and 6-positions are equivalent and the 5-position slightly more open. The controlling factor as pointed out to us by Prof. Edwin Haslam (Chemistry Department, University of Sheffield, U.K.) evidently is that the 2-position is doubly activated for nucleophilic substitution through conjugative delocalization of an electron to the side-chain carbonyl as well as to an oxygen on the ring. Only single activation by electron migration to the ring oxygens is available to positions 5 or 6.

If the glutamic acid, glycine, and tartaric acid ester and amide linkages are individually hydrolyzed, glutathionylcaftaric acid (a five-component unit) should yield (Figure 2) three four-component hydrolysis products (**2**–**4**),

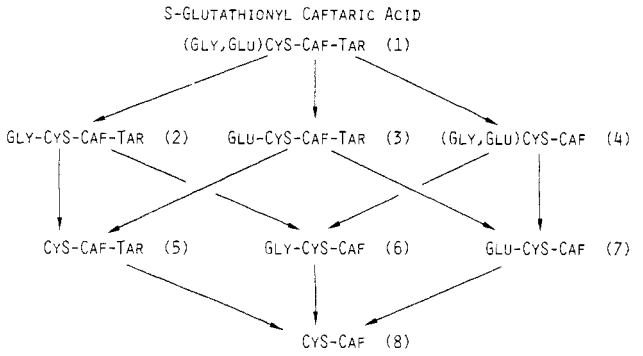

Figure 2. Hydrolysis products of 2-*S*-glutathionylcaftaric acid.

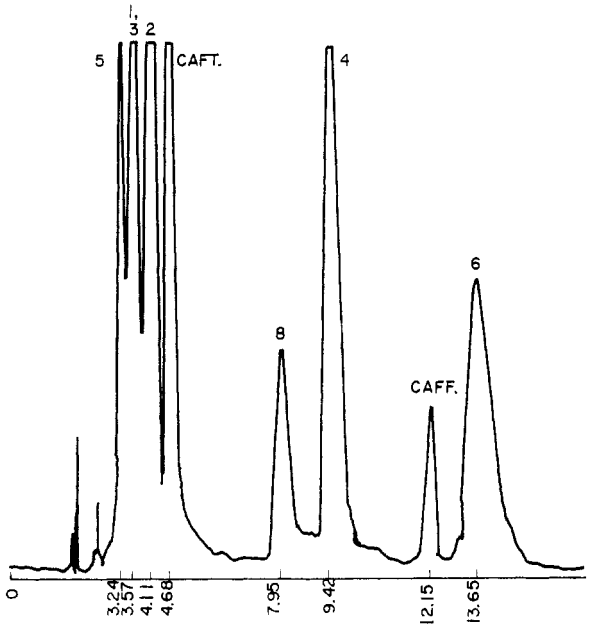

Figure 3. HPLC chromatogram (developer A, acetonitrile) at 320 nm of caffeic acid, caftaric acid, 2-*S*-glutathionylcaftaric acid (1), and hydrolysis products of the latter (2–6, 8). Retention times are in minutes.

three three-component products (5–7), and one two-component product (8). Thus, a partial hydrolysate may contain a total of eight of these compounds including the original glutathionylcaftaric acid. Of these, six (1,2,4–6,8) were available for direct comparison. The remaining two, those retaining Glu and without Gly (3,7), were deduced by reaction sequence, chromatographic behavior, and collection of the desired HPLC peaks followed by further hydrolysis and chromatographic study. Figures 3 and 4 show chomatograms with the two HPLC developers. Note that by using both solvents all hydrolysis products but 7 were separated. The source of confusion remaining is the possibility of cis–trans isomers and products of other reactions such as oxidation. It is true that small unidentified peaks were found in hydrolysates that evidently come from such sources. Acidically hydrolyzed 2-*S*-glutathionylcaftaric acid rapidly gave six new peaks by HPLC. Paper chromatography of the hydrolysates showed free glutamic acid and glycine plus, depending on completeness of hydrolysis, additional fluorescent and ninhydrin positive spots. Figure 5 shows a summary plot of peak area at 320 nm for samples taken every few minutes for a total of 6 h during refluxing at pH 1. Note that products 2–4 increase to a maximum near 90 min as glutathionylcaftaric acid (1) is hydrolyzed in acid. This is consistent with their being intermediates before generation of hydrolysis products 5–7. Product 5 reaches a lower maximum content and peaks considerably earlier than product 6. One would

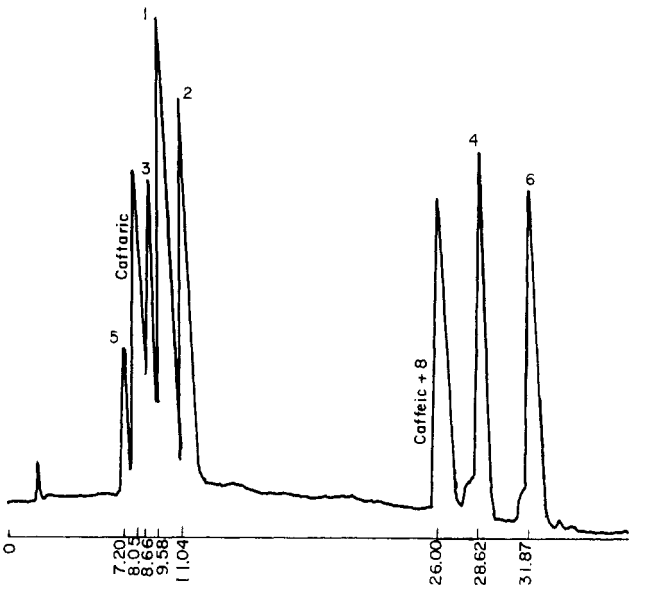

Figure 4. HPLC chromatogram (developer B, methanol) at 320 nm of caffeic acid, caftaric acid, 2-*S*-glutathionylcaftaric acid (1), and hydrolysis products of the latter (2–6, 8). Retention times are in minutes.

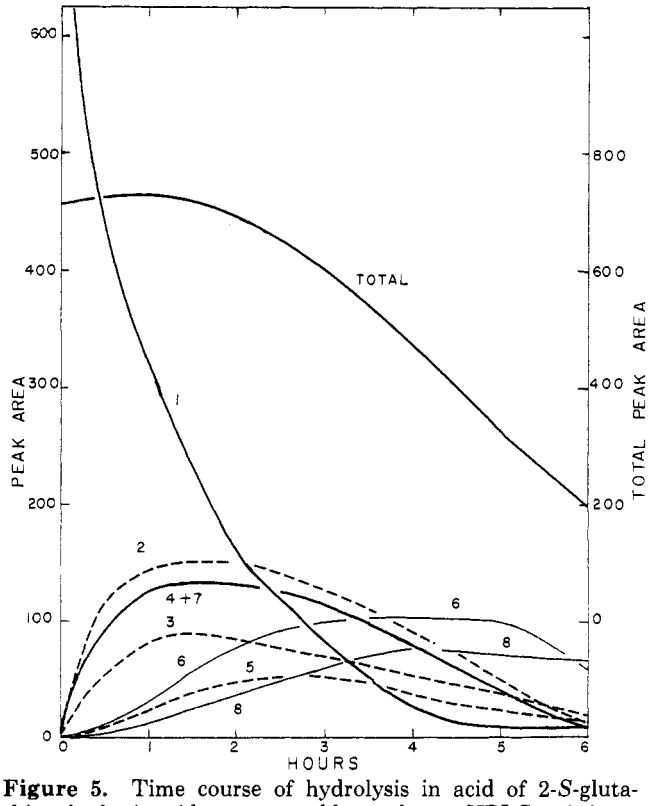

Figure 5. Time course of hydrolysis in acid of 2-*S*-glutathionylcaftaric acid as measured by peak area HPLC at 320 nm and the loss in total peak area.

expect the tartaric acid unit (ester linkage) to be hydrolyzed off more readily than the glycine moiety (amide linkage). Product 6 is highest in concentration and most resistant to hydrolysis of the three-component products, consistent with its identity with Gly-CyS-Caf (6) produced by enzymic synthesis. Product 7 was not identified as a separate peak in hydrolysates by the HPLC systems employed. The amount formed is unknown, but when product 3 was separately hydrolyzed, it gave 5 and a product coeluting with 4, evidently 7.

Product 8 is latest to develop and is the highest at the longest period of hydrolysis tested. This agrees with its

Table IV. Content (mg/L as *trans*-Caftaric Acid) by HPLC at 320 nm of Selected Caftaric Acid Derivatives in Wines of Different Vintages

vintage	*t*-caftaric	caffeic	*S*-glutathionyl-caftaric	*S*-glutathionyl-caffeic	*S*-cysteinyl-caffeic
1984	22.3	4.0	49.2	0.9	2.3
1983	7.8	0.4	19.5	1.0	0.9
1982	21.1	0.5	15.	1.1	1.4
1981	9.7	1.3	16.4	2.2	0.6
1980	3.3	3.0	15.5	1.4	0.9
1979	1.2	1.9	16.4	2.6	0.9
1973			13.5	3.5	0.3
1969	39.6	3.1		1.1	1.0
1964	1.4		9.7	5.5	0.6
1962			8.1	7.3	0.4

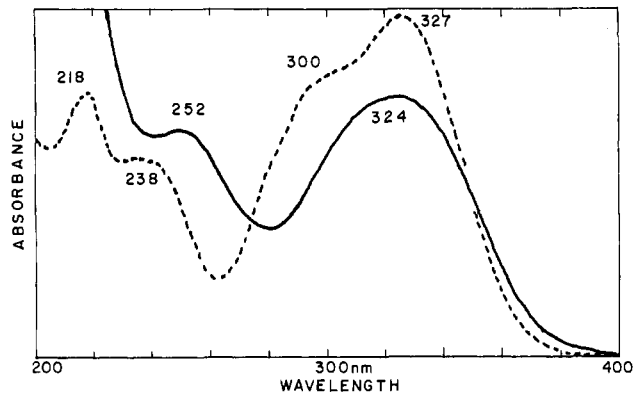

Figure 6. Ultraviolet absorbance spectra of *trans*-caftaric acid (dashed line) and 2-*S*-glutathionylcaftaric acid in water.

identity with synthesized 2-*S*-cysteinylcaffeic acid (8). There is considerable apparent loss of the total 320-nm absorbing material (Figure 4) with longer hydrolysis times. Two possible sources of this apparent loss are changes in spectral characteristics in the 320-nm region and additional reactions leading to other products. Some of the latter occurred because discoloration of the solution developed as hydrolysis proceeded. Cis isomers have a considerably lower extinction, and solvent can play a considerable role (Singleton et al., 1978). However, the absorption maximum for glutathionylcaftaric acid was very little different from that of caftaric acid in water (Figure 6). It has not yet been possible to accurately determine the molar extinction of *trans*-2-*S*-glutathionylcaftaric acid, but it appears near that reported for *trans*-caftaric acid (16000 in water, 20600 in ethanol).

Limited data from a series of wines made in our cellar from white vinifera grape juice clarified without the use of pectinases are shown in Table IV. Coelution in two solvents served for the HPLC identifications. These wines cover a 22-year period and show, with the exception of the 1969 sample, comparable results to acid hydrolysis just discussed. Glutathionylcaffeic acid appears to accumulate in cellar-stored wine (13 °C) to a considerable extent, indicating again the relative lability of the tartrate ester bond. The caftaric acid decreases with time, and less clearly the caffeic acid increases. Similarly, glutathionylcaftaric decreases as glutathionylcaffeic increases. Note also, as with acidic hydrolysis with sulfuric acid, there appear to be losses over and above hydrolysis. Except for *S*-cysteinylcaffeic acid (8) the other hydrolysis products are not shown because the amounts were negligible or were rendered questionable by the more complicated chromatograms from wines. The 1969 sample appears analogous to a commercial wine previously reported (Singleton et al., 1985a) in having evidently been made so as to retain most of its caftaric without conversion to glutathionylcaftaric acid. It therefore does not fit the more typical pattern as shown by the other wines.

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Registry No. 1, 100045-65-6; 2, 100045-66-7; 3, 100045-67-8; 4, 100045-68-9; 5, 100045-69-0; 6, 100045-70-3; 7, 100045-71-4; 8, 100045-72-5; caffeic acid, 331-39-5; caftaric acid, 67879-58-7.

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