Caftaric Acid Disappearance and Conversion to Products of Enzymic Oxidation in Grape Must and Wine

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Enzymic oxidation during crushing and must preparation causes major losses of caftaric and coutaric acids in large part by conversion to a single specific reaction product quantifiable by HPLC. Similar reaction products are produced by oxidation in the presence of cysteine and other sulfhydryl compounds, and the identical one is produced by the interaction of the four components: glutathione, caftaric (or coutaric) acid, active polyphenoloxidase, and oxygen. This is believed to be the first proof of rather high levels (typically about 160 mg/L) of free glutathione in crushed grapes. While it is a product of oxidation, S-glutathionyl caftaric acid is itself oxidizable, but is not a substrate for grape polyphenoloxidase. It is not brown, but has an absorption maximum at about 325 nm sufficiently similar to that of caftaric acid that it appears satisfactorily quantitated by the same relative extinction. Its production is important in limiting the browning of musts. It survives into commercial wines and is important in explaining the nonflavonoid phenol content of wines and the oxidation of white wines.

Caftaric (caffeoyl tartaric) acid and other hydroxycinnamate derivatives are the major phenols other than flavonoids in grapes and wines (2,5,6,8,9,11). These compounds are predominant in easily expressed juice as opposed to the flavonoids which are almost confined to the firmer tissues and are more slowly released to musts or wines (2,8,9,10). White wines made from juice have a relatively low total phenol content, but a high proportion of that total is caftaric acid and related compounds. Red wines made by pomace fermentation or hot pressing, on the other hand, have contents of caftaric acid and relatives similar to those of white wines, but much higher contents of flavonoids and total phenols. Clearly, understanding of amounts and reactions of hydroxycinnamate is crucial to understanding cold-pressed juice, musts and white wines and is also important with red wines.

There can be large losses of caftaric acid during normal crushing operations, and a large but variable portion of these losses results from conversion to a specific reaction product recently discovered in our laboratory (13). This report will present further details on this disappearance of caftaric acid and the formation of this and related products.

Extraordinary precautions are necessary to ensure that none of the caftaric acid of grapes is lost and the true content is determined. When such precautions were taken (air excluded, high SO_2, high ascorbic acid), the caftaric acid content averaged 106 mg/L of juice (8 grape varieties, 37 samples) and coutaric acid averaged 10 mg/L (13). Experimental winery crushing conditions caused the loss of 35% to 100% of the original caftaric acid, and 12% to 73% was accounted for in this newly identified "grape reaction product" (GRP). The resultant amounts of caftaric acid or this reaction product in the must were retained relatively unchanged through wine fermentation. Nagel and coworkers (6) reported variable but similarly high values for caftaric acid in musts with 1000 ppm SO_2 and great losses in conversion to wine. The findings (13) also explained lower values for caftaric acid reported for less well protected musts and wines (10). Such studies also illustrate the value of HPLC in determining a specific entity as opposed to analyses by less specific methods such as total nonflavonoids by colorimetric gallic acid equivalence.

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The authors thank the Wine Institute and the American Vineyard Foundation for financial support toward this research and the Ecole Nationale Superieure Agronomique for the participation of M. Salgues while on leave.

Manuscript submitted for publication 6 June 1984.

Materials and Methods

Trans-caftaric acid was isolated from vinifera grape juice in chromatographically pure form by scaling up essentially the same procedure used for wine (11,12). Grapes (Napa Gamay, 400 kg) were crushed and pressed under a flowing stream of CO_2 (to exclude as much air as possible) in the presence of 900 g of potassium metabisulfite (to inhibit phenol oxidase) and 900 g of ascorbic acid (to reduce any quinone formed). The caftaric acid and other phenols were adsorbed on Darco G-60 activated carbon, eluted with methanol and concentrated at low temperature *in vacuo*. After final chromatography on Sephadex LH-20, and verification of identity and purity by HPLC, the appropriate fractions containing only *trans*-caftaric or *trans*-coutaric acids were combined and evaporated to dryness at 37°C or below in a rotary vacuum evaporator. The dry material was stored until needed under N_2, in the dark and in a refrigerator.

Grape polyphenoloxidase (PPO) was prepared as a crude acetone powder from Grenache grapes harvested at 21°Brix from the University's Davis vineyard. The grapes (11.3 kg) had been chosen to be free of any visible mold or rot. They were destemmed by hand, 6 g of ascorbic acid was added, and the berries passed through a small motorized screw press with a fine (about 1 mm) screen. The juice was strained by gentle squeezing through two layers of cheesecloth and an equal volume of acetone (cooled by dry ice) added. The grapes were refrigerated in advance, and the processing was as cold, rapid, and protected from air as practicable. With periodic additions of dry ice to keep the mixture cold and blanketed, the precipitate was settled, decanted, and filtered on Whatman #1 paper under vacuum. The cake was resuspended in 500 mL of an ice-cold aqueous solution containing 6 g/L of Na_2HPO_4 adjusted to pH 7.3 with phosphoric acid. After

thorough mixing, 1 L of cold (dry ice) acetone was added, the precipitate recovered by filtration, washed with acetone containing dry ice and then with cold, peroxide-free ether. The cake was dried in a hood at room temperature overnight, ground in a mortar to pass 65 mesh, and stored in a freezer (about −20°C) until needed. About 60 g or 5.3 g/kg grapes or 6.6 g/L juice was obtained. It was an active crude catecholase causing rapid browning of solutions of appropriate substrates such as catechol. All but faint traces of phenolic substrates were absent as indicated by sensitive qualitative tests such as spot-testing an ethanolic extract with fresh 1% ferric chloride-potassium ferricyanide solution.

Wines were made in our experimental cellars except the commercial samples which were purchased on the local retail market.

For the most part it was possible to carry out HPLC by direct injection of samples after filtration through 0.45 μ membrane filters. For a few samples too dilute for this, partial concentration *in vacuo* was necessary. The HPLC apparatus was a Waters Associates system including a 720 controller, 730 data module, M6000 A pumps, a 710B autoinjector and a Perkin-Elmer LC-55B variable wavelength detector. The effluent was monitored at 320 nm and known dilutions of *trans*-caftaric acid were used to determine the response factor (mass per unit peak area) and thus to calculate the amount of caftaric acid or derivatives in each peak. The column was reversed phase, C18 (5 μ packing), 4.6 × 250 mm, protected with a guard cartridge of the same packing (Brownlee Labs., Inc. Santa Clara, CA). Isocratic development was with 0.05 *M* ammonium dihydrogen phosphate made to pH 2.6 with phosphoric acid and 16% methanol at 1.5 mL/min and at ambient temperature. Direct injections, 50 μL, were made of the filtered sample along with comparable injections, 30 μL, of a standard solution of known caftaric acid. Quantitation was based on similar absorbance of caftaric and GRP at 320 nm with caftaric acid serving as the standard.

Enzymic reactions were carried out by incubating about 0.5 mg/mL or less of caftaric acid in 3 g/L potassium bitartrate solution at room temperature for 10 minutes or more with the appropriate enzymic preparation at recommended effective concentrations (see results).

Paper chromatography was on Whatman #1 paper with *n*-butanol:acetic acid:water (4:1:5 by volume) freshly made supernatant or with 6% acetic acid. Detection conditions on the dried chromatograms were UV at 254 nm, UV during NH_3 exposure, ninhydrin spray, or ferric chloride-ferricyanide spray under usual conditions (8).

Table 1. Caftaric acid and reaction product in commercial wines by HPLC.

California wine	Caftaric acid	GRP*
	mg/L	mg/L**
Chablis A (Central Valley)	15	6
Chablis B (Central Valley)	7	9
Rosé (Central Coast)	42	29
Chardonnay A	14	12
Chardonnay (Central Valley)	16	12
Sauvignon blanc (Central Valley)	15	12
White Riesling (Napa, 1979)	66	0
Pinot blanc (Central Coast, 1979)	66	32

* Grape Reaction Product.
** Calculated from the HPLC response factor (mass units/peak area) determined with caftaric acid.

Results and Discussion

The newly recognized "grape reaction product" (GRP) appears in must as caftaric acid disappears following exposure to oxygen in the presence of active enzymes and the absence of agents capable of reducing quinones (13). If its appearance is prevented by inhibiting these reactions, caftaric acid is retained, and there is a roughly reciprocal relationship in the relative amounts of the two compounds. GRP is evidently a derivative of caftaric acid. We first wanted to be sure this reaction and GRP occurred commercially in wines and were not artifacts confined to our conditions.

GRP in commercial and experimental wines: Commercial bottled wines (8 different labels, 6 different producers) were analyzed for caftaric acid and GRP by HPLC with the results shown (Table 1). GRP was found in all but one wine and amounted in these seven instances to from half as much as to more than the amount of caftaric acid retained. Some wines retained considerable caftaric acid, and both substances were present in wines four years old at analysis.

Table 2 gives comparable data on the original, fully protected juice and the wines made experimentally from the same grapes. Note that there was considerable loss of caftaric acid in converting juice to wine. These data combined with those previously reported (13) and compared with Table 1, show that this loss occurs primarily in the course of crushing and must preparation with relatively little further change in either caftaric acid or

Table 2. Caftaric acid (C) and GRP content (mg/L) of fully protected original juice (*i.e.*, no oxidation) and experimental dry table wines.

Variety	Juice (protected)		Wines: Free run (6 mo analysis)		12-Hour pomace contact (6 mo analysis)		Fermented 5-day on pomace (Analysis at pressing)		(Analysis after 6 mo)	
	C	GRP	C	GRP	C	GRP	C	GRP	C	GRP
Chardonnay	191	0	86	18	73	23	124	12	113	11
Chenin blanc	100	0	40	7	16	8	46	7	41	3
French Colombard	76	0	16	12	13	14	—	—	29	4
Sémillon	142	0	0	36	0	41	0	28	3	32
Thompson Seedless	64	0	9	22	10	24	46	18	40	20
Carignane	50	0	—	—	—	—	—	—	54	15
Ruby Cabernet	67	0	—	—	—	—	—	—	35	28

GRP during fermentation and good table wine storage practices.

There appears to be (Table 2) a slight additional loss of caftaric acid and gain of GRP in the 12-hour pomace wines compared to the wines from free run juice. This is attributed to more oxidation during the 12-hour holding on pomace. There appears also to be a slight loss of both caftaric acid and GRP between pressing from the skins and analysis about six months later. The wines fermented on the pomace for five days retained more caftaric acid than the other two wines. Note that there is no indication of appreciable extraction of caftaric acid from the pomace, as expected (2). The two red wines are in the range of the whites. Semillon showed higher disappearance of caftaric acid overall and more into products other than GRP than any other variety. Almost all the original caftaric acid content was accounted for by the sum of that retained plus the GRP in Thompson Seedless and the two red varieties Carignane and Ruby Cabernet. These results have been correlated with earlier work (10) and peak number 55 in that work with a retention time of about 33 minutes under different chromatographic conditions is the same GRP. It was particularly prominent in Thompson Seedless wine.

From the data obtained, one can conclude the same GRP occurs in most commercial wines and wines from all grape varieties tested so far. Once the varietal and seasonal effects are better understood, the ratio of caftaric acid to GRP may prove useful as an index of exposure to enzymic oxidation during must preparation even after the wine is finished.

Enzyme effects on caftaric acid: Since prevention of air contact minimized the conversion of caftaric acid to the newly found GRP, oxidation appeared to be the mechanism of conversion. The speed of the reaction and the effect of SO_2 strongly indicated polyphenoloxidase (PPO) as the catalytic agent. Grape PPO and a series of pectinase preparations were compared as to their effects on caftaric acid. Some commercially available pectinase preparations were chosen to compare their oxidase effects, if any, and to see if hydrolysis of caftaric acid to caffeic acid was a factor in forming the GRP.

The results (Table 3) allowed several conclusions. Caftaric acid without fresh juice or other source of enzyme was stable under the conditions of exposure. A proportional amount of the caftaric acid of grape juice disappears as GRP appears. Grape PPO alone caused major losses (73%) in caftaric acid without producing GRP. This result clearly suggested that an additional component of grape must was necessary to form GRP. Hydrolysis of caftaric to caffeic acid was apparently not involved in forming GRP. At least hydrolysis alone was not sufficient, since caffeic acid was produced in large but variable amounts by all but one of the pectinase preparations and no GRP formed (Table 3). None of the pectinase preparations appeared to have much oxidase activity (PPO contamination), because the recovery as either caffeic acid or unchanged caftaric was generally high. At one time, this was a frequent complaint with commercial pectinases used in winemaking.

Table 3. Effects of different enzyme preparations on caftaric acid solutions.

	Recovery %*			
Enzyme added	GRP	Caftaric acid	Caffeic acid	Total
Fresh Juice (+ air)	present	decreased	absent	—
None	0	100	0	100
Grape PPO (6 mg/L)	0	27	0	27
Clarex L (0.05 mL/mL)	0	0	66	66
Rohapect VR (1.17 mg/mL)	0	7	74	81
Spark L (0.05 mL/mL)	0	20	78	108
Polygalacturonase (0.05 mL/mL)	0	111	0	111

* Based on HPLC analyses assuming the same molar absorbance at 320 nm for all three compounds. This, and other factors, may contribute to recovery greater than 100%.

The pectin methyl esterase was presumably sufficiently nonspecific to hydrolyze caftaric to caffeic acid. Polygalacturonase free of pectin esterase activity provided by Rohm Corp. of West Germany did not hydrolyze caftaric acid. This type of hydrolysis of hydroxycinnamate esters had been previously reported by Burkhardt (1), and others. When the Spark L pectinase (high esterase activity, no apparent oxidase) was applied under the same experimental conditions to GRP alone there was no change in amount or HPLC retention time of GRP. It thus appears that GRP is not hydrolyzable by pectin methyl esterase, even though it retains the tartaric moiety.

The nature of the fourth component forming GRP: It seemed probable that the reactant necessary to form GRP during enzymic oxidation of caftaric acid was a reagent capable of reacting with quinones. Amino groups and sulfhydryl compounds appeared likely candidates. The first test was to add a relatively high level of lysine as the grapes were crushed in air. The result was a high loss of caftaric acid and a slightly diminished production of GRP without production of a new product peak on the chromatogram at 320 nm. In order to simplify the system and the resultant HPLC patterns, the respective phenols, additives, and active grape PPO acetone powder at 6 mg/L were incubated about 10 minutes in the presence of air at 25°C with the results shown in Table 4.

None of the amino compounds that lacked SH groups gave a reaction product separated and recognized at 320 nm by HPLC. This does not mean that interaction was absent, however. Note that methionine and cystine appeared to protect caftaric acid to some degree from oxidative loss to unknown compounds (Table 4). The situation with adenine also suggested some interaction. Although disappearance of caftaric acid was complete whether or not adenine was added with active PPO, the brown color produced was considerably less in the presence of adenine. All sulfhydryl compounds except Clelands reagent (1,4-dithiothreitol) gave reaction products when caftaric acid was oxidized in their presence by grape PPO. The retention times in the reversed phase HPLC under the conditions employed were well separated and longer than the original caftaric acid except for the product with cysteine. Furthermore, the peaks from the sulfhydryl derivatives were well separated from each other.

The retention time for the enzymically synthesized

Table 4. Enzymic oxidation of caftaric, coutaric, or caffeic acids in the presence of added compounds.

Phenol oxidized	Compound added	% Phenol Retained	HPLC Retention time (minutes)	Product % of orig.	% Lost
Caftaric acid (36 mg/L)	None, no PPO	100	7.8	0	0
Caftaric acid (36 mg/L)	None	0	—	0	100
Caftaric acid (36 mg/L)	Proline	0	—	0	100
Caftaric acid (36 mg/L)	Lysine	0	—	0	100
Caftaric acid (36 mg/L)	Adenine	0	—	0	100
Caftaric acid (36 mg/L)	Guanosine	0	—	0	100
Caftaric acid (36 mg/L)	Xanthine	0	—	0	100
Caftaric acid (36 mg/L)	Methionine	47	7.8	0	53
Caftaric acid (36 mg/L)	Cystine	35	7.8	0	65
Caftaric acid (36 mg/L)	Cysteine	0	7.0	77	23
Caftaric acid (36 mg/L)	Glutathione	0	8.7	76	24
Caftaric acid (36 mg/L)	Sodium sulfide (H_2S)	0	9.3	20	80
Caftaric acid (36 mg/L)	1,4-Dithiothreitol	0	0	0	100
Caftaric acid (36 mg/L)	2-Mercaptoethanol	0	14.7	48	52
Caftaric acid (36 mg/L)	Mercaptoethanolamine	0	10.2	54	46
Caftaric acid (36 mg/L)	α-Mercaptopropionyl glycine	0	16.0	41	59
Caftaric acid (36 mg/L)	2-Amino-6-mercapto purine	0	18.3	43	57
Caftaric acid (36 mg/L)	α-Thioglycerol	0	11.7	28	
			12.2	33	39
Coutaric acid	Glutathione	—	8.7	—	—
Coutaric acid	Cysteine	—	7.0	—	—
Caffeic acid	Glutathione	0	37.2	—	—
Caffeic acid	Cysteine	0	29.3	—	—
Caffeic acid	None	0	—	0	100
Caffeic acid	None, no enzyme	100	24.8	0	0

caftaric-glutathione reaction product was identical to that for the natural GRP formed in juice, and when the synthesized product was added to the natural in oxidized grape juice, they behaved as a single peak in HPLC and a single spot on paper chromatograms. These and other data such as identical behavior during electrochemical detection allow the conclusion they are identical. Caffeic acid enzymically oxidized with glutathione gives a different reaction product separable by HPLC from the GRP. Thus, hydrolysis of caftaric to caffeic acid is not involved in the formation of GRP.

A considerable portion (under the conditions employed) of the caftaric acid was lost, *i.e.*, neither retained nor demonstrated in the reaction products identified. This loss was least with cysteine and glutathione, indicating they were more efficient in producing the reaction products. Note (Table 4) that coutaric acid gave the same reaction product (GRP) as did caftaric. This is because cresolase activity of grape PPO was converting coutaric to caftaric acid, and then it was oxidized further. Paper and HPLC co-chromatography demonstrated the conversion of coutaric to caftaric acid by the same conditions in the absence of sulfhydryl compounds and verified that the products with glutathione behaved identically. Similarly, the cysteine reaction product in the presence of PPO with coutaric was identical to that with caftaric acid.

Some concentration relationships: In order to estimate the "natural" and the best conditions to produce GRP and related products, and to estimate the competitive significance of amino compounds, quantitative studies were undertaken. Some of the data are shown in Tables 5 and 6. Table 5 shows that there was no detectable reaction product with cysteine until more than one mole of cysteine per mole of caftaric acid was present and the amount of reaction product increased with more cysteine. It approached about 80% conversion to reaction product and 20% apparently lost to unknown products. This 80% yield was achieved at 6 moles cysteine per mole of caftaric acid and appeared to represent nearly the asymptotic limit. Table 6 shows similar data in the presence of two levels of β-alanine as an example of an amino competitor.

Table 5. Enzymic (6 mg/mL PPO) oxidation of caftaric acid (35.7 mg/L) in the presence of cysteine at increasing molar ratios.

PPO	Cysteine (moles/M caftaric)	Percentage after reaction: Caftaric unchanged	Reaction product	Lost
0	3.0	100	0	0
+	0.5	15	0	85
+	1.0	2	0	98
+	1.5	0	65	35
+	3.0	0	76	24
+	6.0	0	79	21

Several conclusions and tentative conclusions can be made from these data. Cysteine and glutathione do not differ much as effective reactants with the quinone form of caftaric acid. This is reasonable, since glutathione is a tripeptide with cysteine the middle unit and the only SH group. In fact, glutathione appears slightly more effective and perhaps this is appropriate since it is evidently the natural reactant. Amino groups in the form of β-alanine

Table 6. Enzymic oxidation of caftaric acid in the presence of cysteine or glutathione and beta-alanine.

SH compound	Moles/mole caftaric	% After Reaction: 1.5 M/M alanine Unchanged	Reaction product	Lost	3.0 M/M alanine Unchanged	Reaction product	Lost
Cysteine	0.5	12	0	88	13	0	87
	1.5	0	60	40	0	67	33
	3.0	0	70	30	0	75	25
Glutathione	0.5	12	0	88	17	0	83
	1.5	0	70	30	0	71	29
	3.0	0	75	25	0	75	25

at relatively high levels appear to encourage slightly the production of either the cysteinyl or glutathionyl caftaric acid and appeared to do this by minimizing the loss of caftaric acid to unrecognized products (Table 6). The main conclusion, however, is that amino groups, at least in β- alanine, were not major competitors with sulfhydryl groups in this system.

No reaction product with either cysteine or glutathione was detected at or below 1 mole of sulfhydryl per mole of caftaric acid, but a high level was found at 1.5 mole/mole. This strongly indicates the reaction products are monosubstituted thioether derivatives. This is predicted from the proposed mechanism (Fig. 1) since disubstitution would require a second oxidation and an intermediate and presumably result in two peaks by HPLC. Both HPLC and paper chromatography indicated only the one product in significant amount, and it remained major from the earliest it was detectable during the rapid reaction.

For the reaction to occur in grape musts as it does, it appears that glutathione must be free in the must immediately upon crushing at about 1.5 moles or more per mole of caftaric acid. Since an average value (13) for the grape samples so far carefully determined was 106 mg/L of caftaric acid, this would calculate to be about 160 mg/L of glutathione. The apparent high conversion and quantitative recovery found with Thompson Seedless and two red varieties (Table 2) suggests both high glutathione contents in these varieties and good equivalence between peak area response factors for caftaric acid and GRP (S-glutathionyl caftaric acid). Although considered widespread in plants, there are few analytical data specifically on glutathione content, and as far as we are aware, this is one of few specific detections and quantitative estimates in plants and the first in grapes (7).

Table 7. Paper chromatographic Rf values (one dimensional, ascending).

Enzymic reaction product of	BAW	6% HOAc
Caftaric + Glutathione	0.25	0.98
Caftaric + Cysteine	0.29	0.92
Caffeic + Glutathione	0.49	0.75
Caffeic + Cysteine	0.60	0.64

Some observations on the nature of GRP as S-glutathionyl caftaric acid: The reaction is believed to be enzymic oxidation to caftaric quinone followed by spontaneous chemical (nonenzymic) reaction with glutathione to substitute the ring to produce the thioether (Fig. 1). This substitution regenerates the hydroquinone form of the caffeic moiety. An electrochemical detector gave a very strong HPLC peak for GRP (S-glutathionyl caftaric acid) and similar products between caffeic and cysteine or glutathione under conditions such that quinones and monophenols did not react, but vicinal dihydroxyphenols did.

Paper chromatography gave intensely fluorescent yellow spots (enhanced by ammonia) separate from and more polar than the respective starting caftaric or caffeic acids for the cysteinyl and glutathionyl reaction products. Representative Rfs are shown in Table 7. The reaction product spots, but not the original phenolic acid spots, reacted with ninhydrin; thus, the incorporation of the amino acid derivatives and the link through the thio group are substantiated. The new products reacted with the ferricyanide spray as readily oxidized phenols.

The conclusion that the GRP of crushed grapes is the reaction product between glutathione and caftaric acid is based upon identical behavior in three different chroma-

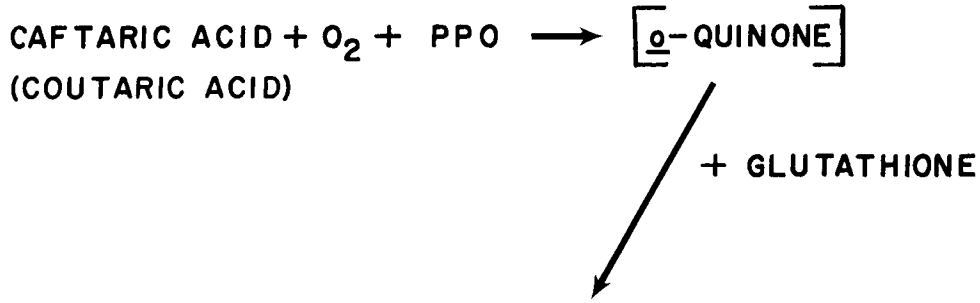
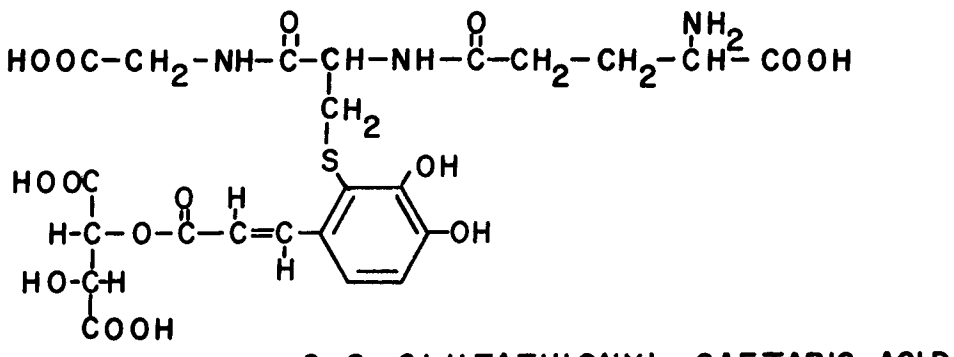


Fig. 1. The proposed reaction sequence leading to the formation of S-glutathionyl caftaric acid in grape must. At this stage the position of ring substitution is unknown.



2-S-GLUTATHIONYL CAFTARIC ACID

tographic systems and in every reaction so far tested (including some not mentioned here, such as very high polarity and low extraction from aqueous solution with organic solvents (13)). That the product of grape PPO oxidation of caftaric acid in the presence of glutathione is an S-glutathionyl caftaric acid (Fig. 1) is concluded from the evidence cited. It shows that tartaric acid remains attached to the caftaric unit, and glutathione is incorporated. Suppression of quinone formation (lack of oxygen, PPO inhibition with SO_2) or rapid reduction (excess ascorbic acid) prevents formation of GRP and the glutathione-caftaric reaction product. Other free sulfhydryl compounds in the same system give related but different products, whereas other functional groups (amine, alcohol, disulfide, carboxyl) give no such products in identifiable amounts. The formation of the thioether with caftaric acid is the result of the glutathione mercapto group serving as an electron-rich nucleophilic center to substitute into the electrophilic quinone ring. By proton transfer and the equivalent of an enol shift, the vicinal dihydroxy ring of the caffeic moiety is regenerated. Pending further structural and property studies, the working hypothesis is justified that the GRP is S-glutathionyl caftaric acid of the type (but perhaps not the position substituted) indicated in Figure 1.

S-glutathionyl caftaric acid in relation to browning: A solution of enzymically synthesized S-glutathionyl caftaric acid was treated with active PPO for one hour at room temperature without significant reduction of the original content as monitored by HPLC. No oxygen was consumed as measured by an oxygen electrode. At the end of the period, caffeic acid was added to the mixture and was rapidly oxidized (disappeared) with the consumption of dissolved oxygen. These data allow conclusions that S-glutathionyl caftaric acid (GRP) is not a substrate for grape PPO and is also not a strong inhibitor of PPO action on active substrates. This is certainly reasonable since this reaction product accumulates in the normal must preparation as caftaric and coutaric acids are oxidized.

In effect, grape must has a built in system to resist browning. The glutathione traps the quinone almost completely in a colorless form and prevents it from proceeding directly to brown polymers. Caftaric acid trapped as GRP (the glutathionyl thioether) is inert or nearly so to further PPO oxidation, and the reaction rapidly reaches completion with caftaric acid gone but browning minimal. If PPO is inhibited by SO_2, caftaric acid is retained and browning is minimized. If it is not, glutathione limits the reaction following the initial effects of browning. Similar effects related to cysteine were previously postulated (5), and cysteine has long been known as an inhibitor of PPO (3,4). It has not always been recognized that the sulfhydryl inhibition was not of the enzyme itself, but rather of browning caused by continuation of the reaction. This is the first detailed demonstration of a specific reaction of this type in natural unmodified grape juice, and we are unaware of previous results of similar significance in any other product without additions. The effects of adding cysteine and glutathione to chlorogenic acid being oxidized by apple polyphenoloxidase as studied by Walker (14) are most pertinent and complement this study very well.

Conclusions

1. A specific reaction product is produced during crushing of all grape varieties so far tested.

2. This product is the result of interaction of four components: active grape polyphenoloxidase, caftaric (or coutaric) acid, oxygen, and glutathione.

3. Evidence is presented that this product is S-glutathionyl caftaric acid.

4. The reaction sequence is believed to be enzymic production of caftaric quinone followed by spontaneous coupling of the thiol group to give the ring-substituted thioether with regeneration of the *o*-hydroquinone group.

5. Caffeic acid and probably other vicinal dihydroxyphenols in the presence of cysteine and other sulfhydryl compounds in addition to glutathione give similar but different products.

6. Amino compounds such as lysine, adenine, and β-alanine appear to have small but not zero influence on the course of the reaction and associated browning.

7. Because the S-glutathionyl caftaric acid has similar spectral characteristics to caftaric acid and is not itself a substrate for grape polyphenoloxidase, the reaction helps limit browning during must preparation.

8. Caftaric acid and S-glutathionyl caftaric acid which survive must preparation tend to also survive fermentation and wine storage for a period of several years. It appears that S-glutathionyl caftaric to caftaric acid ratios may become a useful index of previous must oxidation even when applied to finished white table wines.

9. S-glutathionyl caftaric as well as caftaric acids appear autoxidizable nonenzymically and need study as important portions of the oxidizable phenols in wines, especially white wines.

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