

Identification of Ellagic Acid as a Precipitate from Loganberry Wine

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A light-colored crystalline precipitate sometimes forms slowly in loganberry wine and may reduce the wine's acceptability after bottling. The significant constituent of this precipitate has been isolated and rigorously identified as free ellagic acid by ultraviolet and infrared absorption spectra, derivative preparation, and paper chromatography in comparison with synthesized ellagic acid. The ultraviolet absorption of ellagic acid in ethanol shifts gradually with the addition of small portions of anhydrous sodium acetate from an intense maximum at 255 to 256 $m\mu$ to an equally intense maximum at 286 to 288 $m\mu$ and the loss of a secondary maximum at 366 to 367 $m\mu$. Isobestic points near 267, 348, and 376 $m\mu$ were found. Although ellagic acid has been reported in several fruits, including grapes and raspberries (a parent of loganberries), it apparently has not been previously identified as a source of troublesome turbidity in wine.

SOME lots of commercial loganberry wine have deposited a fine, light-colored precipitate with time. This precipitate may continue to form during bottle aging and merchandizing of initially clear wine and result in poor appearance and decreased acceptability of the wine. Loganberry wine is often sold in colorless, flat-bottomed bottles and this precipitate is a light purplish gray which contrasts with the red wine and is very noticeable on the bottom of the bottle. When disturbed, the precipitate is readily suspended in the wine and the wine remains turbid for several hours before it again can be decanted relatively clear.

Because of high natural acidity, loganberries are usually relatively highly ameliorated with water and sugar, up to 60% of the total volume being permitted by federal regulation, in contrast to the 35% maximum permitted with many other fruits and the typical zero additions with ripe wine grapes. This precipitate therefore appeared to be a substance with unusually low solubility. The usual tests for the identification of precipitates in wine indicated that this was not any of the substances previously encountered. It therefore was of interest to study this condition and to identify the responsible substance.

Materials and Methods

The wines were commercial lots prepared from frozen loganberries by methods essentially typical for berry wines. The berries were allowed to thaw, 125 p.p.m. of SO_2 and yeast inoculum were added, and the fermentation was conducted at 65° to 75° F. for about 7 days. The free-run fluid was drawn off through the false bottom of the fermentor, and the residual pomace was leached with the ameliorating sugar solution in three portions. The com-

bination of separated wine and ameliorant adjusted to be equivalent to an original Brix of 23° was fermented further to about 14% alcohol and then treated with SO_2 to arrest the fermentation. The wine was fined with bentonite and Sparkolloid (trade-mark) at 2 pounds each per 1000 gallons and racked after 2 weeks. After about 1 month of aging the wine should be ready for final filtration and bottling, but the unstable lots continued to deposit the precipitate even if filtered and bottled.

The amount of this precipitate present in a single bottle of unstable wine was estimated by filtration of portions of a 4-year-old sample presumed to have completed precipitation. The supernatant was used to correct for filterable, suspended amorphous material, and the precipitate was dried and weighed after collection on a membrane filter of 0.45-micron pore diameter and washing twice with 5 ml. of cold water. A larger sample (about 10 grams) of precipitate was collected from a tank of the unstable loganberry wine, washed with 12% ethanol plus 1% acetic acid in water, and dried in a desiccator.

The precipitate was studied visually with a polarizing microscope. Paper chromatograms were prepared by spotting about 10 $\mu g.$ of the solid to be studied from pyridine solution onto Whatman No. 1 paper and developing (after $1/2$ to 2 hours of equilibration in the vapor of the solvent) at 25° \pm 2° C., descending mode, with the appropriate solvents. The developed chromatograms were studied under ultraviolet light, and ultraviolet light in the presence of ammonia vapor. Chromatograms were sprayed with a freshly prepared aqueous solution of equal volumes of 1% $FeCl_3$ and 1% $K_3Fe(CN)_6$ or 0.05% Fast Black K salt (72) in water followed by 20% Na_2CO_3 .

Ellagic acid was synthesized from gallic acid by autoxidation of methyl gallate in aqueous ammonia (4, 7).

The synthetic ellagic acid and the wine precipitate were purified by decoloration with activated carbon and recrystallization several times from hot pyridine. The tetracarboxy derivative of ellagic acid and the similar derivative of the isolated substance were prepared from the sodium phenolates with ethyl chloroformate (2, 73).

The purified samples used for spectral analysis were treated with 10% sulfuric acid to remove residual pyridine, washed with water and acetone, and dried at 80° C. Ultraviolet spectra were recorded in absolute ethanol (8, 9) with a Bausch and Lomb 505 spectrophotometer. Infrared spectra were determined by the KBr disk technique. Other details of the methods employed were standard procedures or are not considered critical to reproducing the work.

Results and Discussion

Appearance and Amount of Precipitate. The precipitate collected from bottles of unstable loganberry wine was composed of a high proportion of very small birefringent crystals. They appeared colorless in reflected or non-polarized transmitted light under the microscope, but the noncrystalline portion of the precipitate was generally red or amber. The crystals appeared to be rectangular or blunt-ended needles. They occurred individually rather than as rosettes or large masses. Although a few slightly larger crystals were present in the sediment from large tanks, the crystals were mostly about 0.5 micron wide and 1 to 10 microns long.

The amount of the dry crystalline precipitate collectable from an unstable bottled wine was 86.3 mg. per liter. This amount was sufficient to produce the ill effects on appearance already described, but additional precipitate probably had been removed by treatments prior to bottling.

Table I. Ultraviolet Absorptivity of Ellagic Acid and Wine Precipitate

Substance	EtOH				EtOH + NaOAc		Isosbestic Points, $M\mu$	
	λ_{max} , $m\mu$	$\log \epsilon$	λ_{max} , $m\mu$	$\log \epsilon$	Shoulders, $m\mu$	λ_{max} , $m\mu$		$\log \epsilon$
Ellagic acid (synthesized)	255	4.70	366	4.00		277	4.54	267, 347, 376
Ellagic acid (8, 9)	255	4.77	366	4.13	293, 353	287	4.78	
Isolate from wine precipitate	256	4.70	366	4.02	292, 354	288	4.68	267, 348, 377
Wine precipitate	256	4.66	367	4.00	289, 354	286	4.65	

Isolation of Crystalline Substance.

The wine precipitate was not completely soluble in hot pyridine. The insoluble residue was 6.3 and 8.6% of the original weight in two experiments. The solid which crystallized from the cooled pyridine, when dried to constant weight at 80° C., represented 52.3 and 52.6% of the amount of solid originally dissolved. Another experiment conducted by dissolving a further portion of the precipitate from loganberry wine in the dark red-brown pyridine mother liquor recovered from one of the above experiments gave 8.2% insolubles and an 84.2% recovery of recrystallized solid. Thus it appears that the major part of the wine precipitate can be recovered by recrystallization from pyridine.

The crystals, particularly after treatment in hot pyridine with decolorizing carbon and further recrystallization, were pale yellow needles, which upon standing in air or heating lost pyridine of crystallization and became more intensely yellow. The ellagic acid synthesized from gallic acid behaved similarly, except that removal of extraneous colored material was much less difficult. Neither the isolated substance nor ellagic acid melted at temperatures at the limit of the available apparatus (ellagic acid melting point > 360° C.).

The recrystallized substance and the crude wine precipitate itself as well as synthetic ellagic acid could be sublimed as a bright yellow crystalline solid, but high vacuum and high temperature were required (an oil bath at 250° C. was insufficient, but a micro gas burner was effective). Jurd (9) has reported that ellagic acid can be so sublimed directly from an ellagitannin without preliminary hydrolysis.

Qualitative and Color Reactions.

The isolated crystalline material and synthetic ellagic acid gave identical reactions in a series of qualitative tests. Sodium bicarbonate solution had no immediate effect, but 10% sodium carbonate or sodium hydroxide gave a slow reaction to produce a yellow solution indicating weakly acidic (phenolic) groups without free carboxyls. The Greissmeyer test for ellagic acid (9)—a bright, transient red with fuming nitric acid and dilution with water—was positive. Ferric chloride solution produced a blue-black phenol color test. These tests were also positive with the crystals present in the original wine precipitate.

Spots on paper gave a dull purple on a

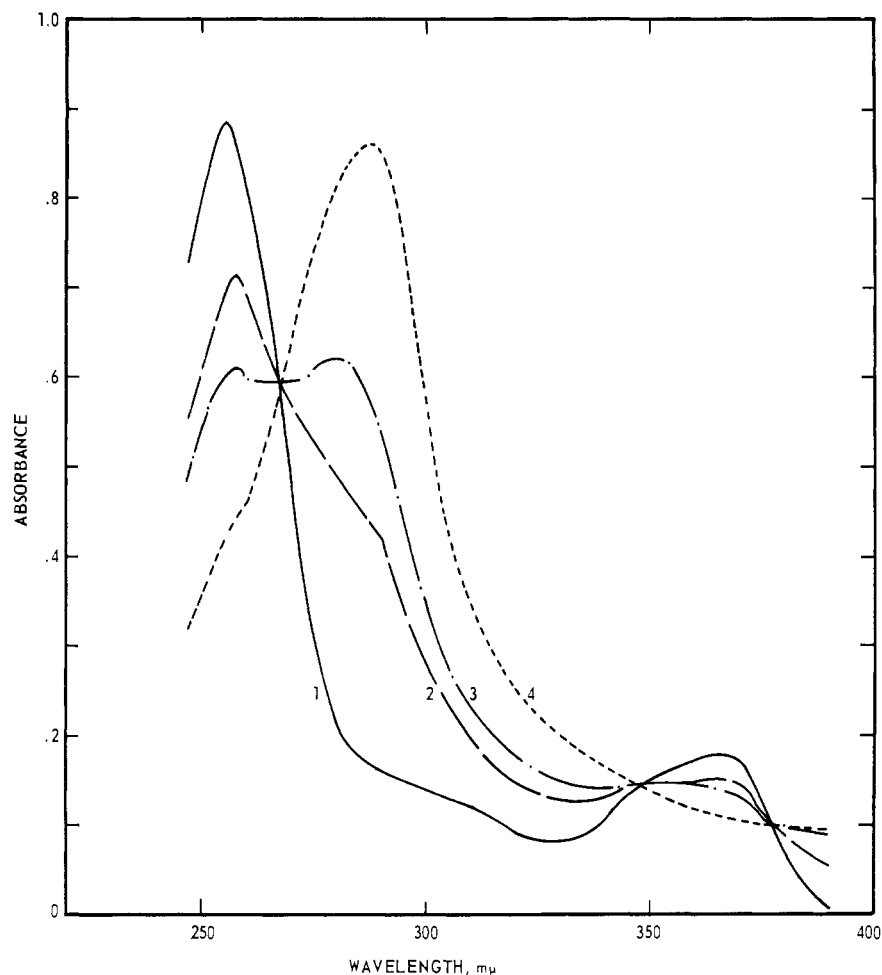


Figure 1. Ultraviolet absorption spectra of crystalline substance isolated from loganberry wine precipitate

- 1. In absolute ethanol
- 2, 3, 4. Absolute alcohol with increasing additions of very small amounts of anhydrous sodium acetate

pink background with Fast Black K followed by sodium carbonate. Blue spots were produced with ferric chloride-ferricyanide spray. Ultraviolet light gave a faint blue-white fluorescence converted to bright yellow when the paper was fumed with ammonia.

Spectra. The ultraviolet absorption spectrum of the synthetic ellagic acid in absolute ethanol agreed very well with that reported by Jurd (8, 9) and that of the isolated crystalline substance was essentially identical (Table I, curve 1 in Figure 1). The solution of the impure wine precipitate directly in ethanol without any heating gave a very similar absorption spectrum with only small amounts of impurities indicated by very slight shifts and decreases in absorbance at the maxima (Table I) or increases in absorbance at other wavelengths.

The saturation of these solutions by addition of anhydrous sodium acetate to the cuvettes, however, produced a bright yellow gelatinous precipitate. Addition of anhydrous sodium acetate in very small amounts to a fresh portion of each solution produced a sequence of changes, an intermediate stage of which was similar to that reported by Jurd (8-10) (Table I, Figure 1). Addition of sufficient (about 0.3 mg. per ml.) sodium acetate to produce apparent completion of the shift of the 256- $m\mu$ maximum to one at 288 $m\mu$ of essentially identical molar absorbance probably corresponds to the conversion of the two more acidic phenolic groups para to the carbonyl function into the sodium salts (10, 14) without sufficient conversion of the other two to produce insolubility. The apparent discrepancy between these re-

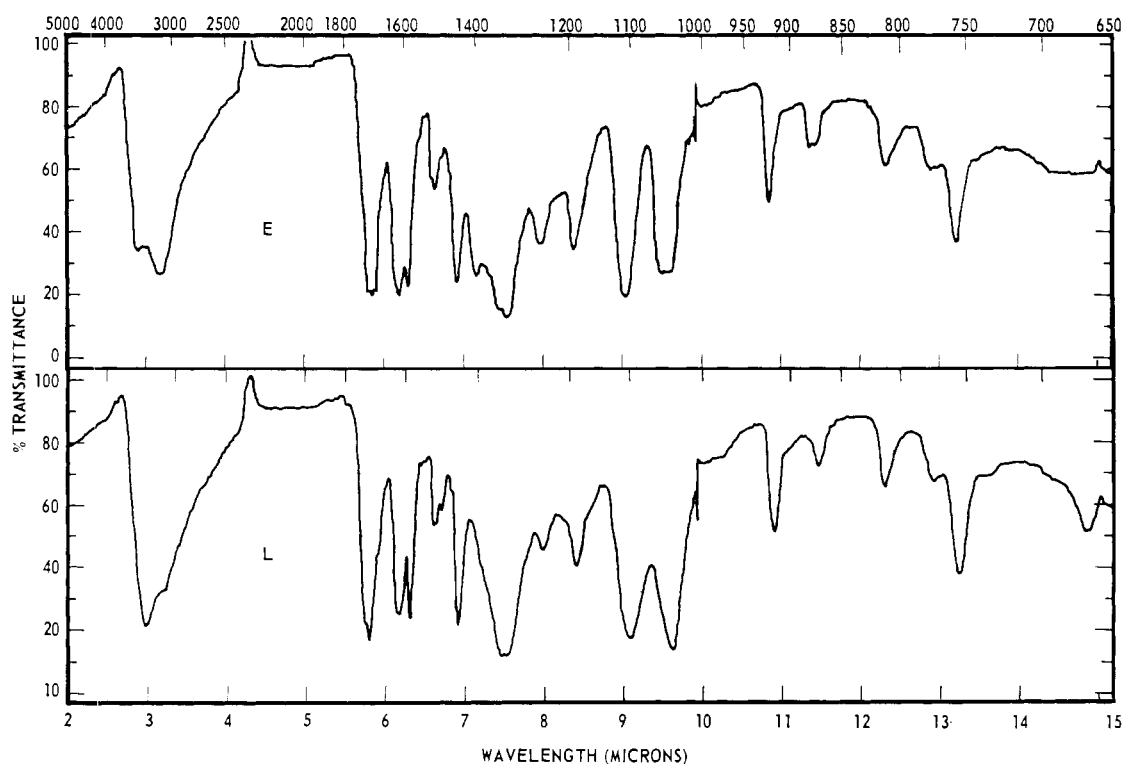


Figure 2. Infrared absorption spectra

E. Synthetic ellagic acid
 L. Crystalline substance isolated from loganberry wine precipitate
 0.5% in KBr disks

sults and the earlier reports (3, 8-10, 14) may depend upon the condition of the solvent used. Ours was freshly opened commercial absolute ethanol and no attempt was made to dry it specially.

In any case, the value of the shift produced by sodium acetate recommended by Jurd as a means of identification of ellagic acid is verified by our data and the isosbestic points disclosed by sequential small additions should serve as additional useful features for such identification. The identity of ellagic acid and the crystalline substance present in and isolated from loganberry wine precipitates seems highly probable from these spectra.

The infrared spectra of the isolated substance and that of synthesized ellagic acid are shown in Figure 2. There is very close agreement between them, since at least 15 features are nearly identical in absorbance and frequency. Differences do appear, notably at frequencies of about 3300, 1400, and 680 cm^{-1} . These are attributed to differences in crystal form, hydrogen bonding, or possibly small compositional differences such as presence of different amounts of residual solvent of crystallization. Ellagic acid and similar compounds are notorious for crystallizing in more than one form and as solvates and hydrates. These infrared spectra are considered to be confirmatory evidence of the identity of the isolate with ellagic acid.

Table II. Paper Chromatography of Ellagic Acid and Wine Precipitate

	$(R_f \text{ values})$			
	Solvent A	Solvent B	Solvent C	Solvent D
1. Ellagic acid (synthesized)	0.39	0.74	0.00-0.03	0.83
2. Isolate from wine precipitate	0.38	0.74	0.00-0.03	0.83
3. Mixture of 1 and 2	0.39	0.74	0.00-0.03	0.80
4. Wine precipitate	0.40, 0.48, 0.69	0.75	0.00-0.03	0.83

All spots detectable with $\text{FeCl}_3\text{-K}_3\text{Fe}(\text{CN})_6$. Solvent A = concd. $\text{HCl-HOAc-H}_2\text{O}$ (3:30:10); B = dimethylformamide adjusted to pH 3.5 with formic acid; C = 6% HOAc in H_2O ; D = pyridine-water (40:60).

Paper Chromatography. Ellagic acid gives discrete, nontailing spots on paper with a satisfactory R_f in relatively few solvents. Our results are shown in Table II. Ellagic acid and the substance recrystallized from the wine precipitate were not separable or distinguishable in any of the four developing solvents. The crude wine precipitate contained smaller amounts of two additional substances detectable by phenolic-type reactions, particularly on chromatograms developed by solvent A. With this solvent discrete spots were formed without tailing or residue at the origin. The fastest moving spot was very faint and the intermediate spot was considerably weaker than the spot corresponding to ellagic acid. These additional substances were not identified.

Derivative Comparison. The tetracarboxyellagate and the analogous derivative of the isolated substance gave a relatively sharp capillary melting point

of 246-47° C. observed without magnification [literature 244°, 247° C. (2, 8, 13)]. Microscopic observation with a hot-stage, polarizing apparatus revealed that some melt formed at 240° C. and the last crystal disappeared at 249° C. Cooling the melt to 245° C. produced partial crystallization and upon reheating the last crystal again disappeared at 249° C. Identical melting behavior occurred with synthetic ellagic acid, the isolated substance, and an equal mixture of the two ground together.

Conclusions

The data presented are considered to prove that ellagic acid is the predominant and significant substance in the light-colored crystalline precipitate which forms slowly in some lots of loganberry wine.

Since the paper chromatographic, ultraviolet spectral, and other studies upon the crude precipitate prepared and

observed under very mild conditions show the characteristics expected from free ellagic acid, it is considered proved that the precipitate is predominantly free ellagic acid rather than an ellagitannin, free hexahydroxydiphenic acid, or other possible precursors of ellagic acid.

Ellagic acid has a very low solubility in aqueous solutions and is readily removed from solution or suspension by most fining, filtration, or adsorptive treatments. It therefore appears that the ellagic acid precipitate results from the slow generation of ellagic acid. In preliminary experiments with canned loganberries, free ellagic acid was not found. It appears most probable that the source of the ellagic acid is an ellagitannin present in the loganberries which is relatively soluble in wine and is produced or extracted in variable amounts to account for the different degree of instability of different lots of loganberry wine. The slow rate of formation of the crystalline precipitate presumably reflects the slow hydrolysis of the more soluble ellagitannin and perhaps delayed lactonization of the resultant hexahydroxydiphenic acid. It is possible, of course, that enzymes present in the berry or even "tannase" produced in an occasional moldy berry are involved and synthesis from simpler

substances such as gallates could occur.

These considerations are strengthened by the fact that improved, but not always completely stable, clarity of the unstable loganberry wine has been achieved by heating or treatment with gelatin or activated carbon. It is probably significant that although we were unable to find a report of the presence of ellagates in loganberries, ellagic acid has been reported from raspberries (7). The loganberry is reported to be a cross between European red raspberries and California blackberries. A precipitate very similar to that described here has been noted in a commercial red raspberry wine (7). We have been able to locate no previous report of wine turbidity or sediment produced by ellagic acid, which is interesting in view of reports of its presence in grapes and grape wines (5, 6).

Acknowledgment

Very able technical assistance was supplied by Diana E. Draper. Infrared spectra were supplied through the courtesy of Gunter Zweig and George Mallett, Agricultural Toxicology Department, University of California, Davis, Calif.

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Received for review June 21, 1965. Accepted October 4, 1965.

AMINO ACIDS OF HYBRIDS

Alien Genome Combinations and Influence on Amino Acid Composition of Cereal Protein Fractions

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Comparisons were made of the amino acid composition of comparable protein fractions of rye (*Secale cereale*-RR), durum (*Triticum durum*-AABB), *Triticale*, the synthetic species comprised of AABBRR, and *Tritipyron*, a synthetic species composed of genomes AABBEE. Proteins of a common wheat (*Triticum vulgare*) which has the genomic constitution of AABBDD were also investigated. In most cases, the amino acid composition of the "whole" proteins of *Triticale* was intermediate between that of the two parents, rye (RR) and durum (AABB). Differences in amino acid composition of protein fractions of *Triticale*, however, reflected a dominant effect of either parental genome (RR or AABB) or were intermediate between the two parents. A decrease in the individual amino acid of one protein fraction of *Triticale* was, in general, compensated for by an increase in the same amino acid in another fraction. In the case of the synthetic species *Tritipyron* (AABBEE) and common wheat (AABBDD), data for the parental genomes EE and DD are not available and any differences in the observed amino acid patterns from that of durum (AABB) are tentatively ascribed to the influence of the EE and DD genomes.

STARCH-gel electrophoresis studies of proteins (10) using aluminum lactate-urea buffer (4, 9) indicated some variable effects attributable to conse-

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quences of alien genome combinations. In the investigations on some protein fractions derived from a synthetic rye-wheat hybrid, *Triticale* (10), it was found that although a general additive effect pervaded, a number of what were believed to be new or different hybrid sub-

stances were elaborated. The general additive effect of alien genome combinations had been noted (6, 7) early in the development of new synthetic cereal species. Some differences were later detected by immunologic techniques (7) and by starch-gel electrophoresis (10) due