

Factors Influencing the Formation of Precipitates and Hazes by Gelatin and Condensed and Hydrolyzable Tannins

Peter Calderon,¹ Jerome Van Buren, and Willard B. Robinson

A comparison of tannic acid and quebracho tannin coprecipitation with gelatin showed that the principal difference between the two types of tannin was their behavior under different pH conditions. Tannic acid-gelatin precipitation was much lower at pH 3.5 than at pH 5.0, while quebracho tannin-gelatin precipitation was similar at both pH levels. The effect of alcohol was to decrease the affinity of

tannin for gelatin and to decrease the solubility of the tannin-gelatin complexes. Aggregation of complexes to sedimentable size apparently takes place through the formation of tannin links between smaller complexes. Formation of aggregates was reversible. The amount of the tannin-gelatin precipitates and their composition were influenced by the relative concentrations of the reactants.

The reaction between proteins and tannins produces hazes and precipitates in natural beverages such as fruit juices, wines, and beer. Under some circumstances a protein, such as gelatin, is added to a hazy or potentially hazy tannin-containing beverage to entrap haze particles in the resulting coagulum and to decrease the tannin in the beverage. The interaction between proteins and tannins appears dependent upon the formation of hydrogen bonds between the hydroxyl groups of polyphenolic tannins and the carbonyl groups of the protein peptide bonds (Gustavson, 1954). Covalent or ionic bonds also may be involved, but there is general agreement that they play a minor role in the primary protein-polyphenol reaction.

The ability of polyphenols to form complexes with proteins increases with the number of phenolic hydroxyl groups (Endres and Hörmann, 1963) and with the degree of polymerization. Tannins are complex polyphenols showing molecular weight of the order 1000 to 3000. Proteins as different as egg albumin and gelatin readily combine with tannins to form precipitates under similar experimental conditions. Zitko and Rosik (1962) have suggested that one tannin molecule binds with two or more peptide groups and thus can form crosslinks between protein chains. The degree of crosslinking would be expected to be dependent on the number and accessibility of the peptide carbonyl groups on the protein molecules as well as the relative concentrations of tannins and proteins. Formation of hazes and, eventually, of precipitates appears dependent on crosslinking and increases in the size of the complexes.

In view of these considerations, the present work was undertaken to explore the influence of various factors on the quantity and composition of precipitates resulting from the interaction of proteins and tannins. Factors studied were relative amounts of tannins and protein, pH, alcohol,

and salt. Comparison was also made between tannic acid, a hydrolyzable tannin, and quebracho tannin, a condensed tannin (Gustavson, 1956). Haze formation was investigated as a function of pH and tannin and protein concentrations.

PROCEDURE

Tannic acid (Mallinckrodt analytical reagent) was purified with 2-butanone following the procedure of Filachione *et al.* (1955) to yield a material with an osmotically determined molecular weight of 1100. Quebracho tannin (J. S. Young Co.) was used as received. Gelatin (Difco) was derived from calfskin and had an isoelectric point of 5.0. Throughout this work, pH was adjusted through the use of HCl or NaOH.

Tannins were measured using the Folin-Ciocalteu reagent (Singleton and Rossi, 1965). Protein was calculated by multiplying Kjeldahl nitrogen by 6.25. Haze readings were made with the Gardner Hazemeter ($U \times 10$). This instrument yields a measure of low angle scattering by a suspension.

In carrying out the experiments, the gelatin solutions at the appropriate pH were added to 100-ml. volumetric flasks, followed by distilled water, salt, and ethanol as required. Tannin was added with constant stirring. Volume was then about 90 ml. The solutions were adjusted to volume and allowed to stand at room temperature for 1 hour. A portion of the suspension was centrifuged at $68,500 \times G$ for 30 minutes. Nitrogen and tannin determinations were made on the clear supernatants.

RESULTS AND DISCUSSION

A comparison of the gelatin precipitated at the two pH levels (Table I) shows that at a given tannic acid level, less was carried down as the pH was lowered. At pH 5.0, almost all of the gelatin was removed from solution when the tannic acid was 180 mg. per 100 ml., while at pH 3.5, at the same tannic acid level, 37% came down. Even at 720 mg. per 100 ml. of tannic acid, 23% remained in solution at pH 3.5. The presence of ethanol increased the precipitation of gelatin, particularly at pH 3.5. NaCl had little influence on gelatin precipitation.

The per cent of tannic acid appearing in the pH 5.0 pre-

New York State Agricultural Experiment Station, Department of Food Science and Technology, Cornell University, Geneva, N. Y. 14456

¹ Present address, Winton Hill Technical Center, Procter and Gamble Co., Cincinnati, Ohio

precipitate decreased as the amount of added tannic acid increased. However, at pH 3.5, for plain gelatin and in the presence of 0.001M NaCl, the per cent of the added tannic acid precipitated increased as the tannic acid was raised. As the tannic acid was increased further, the per cent in the precipitate decreased.

The presence of 15% ethanol resulted in a decline in the per cent tannic acid precipitated at 5.0 and at most tannic acid levels at pH 3.5. At low tannic acid, the ethanol caused a considerable rise in the amount precipitated.

The per cent gelatin precipitated at a given quebracho tannin level (Table II) was not markedly affected by pH or other conditions, except at low levels of tannin where a lowered pH decreased the amount of gelatin precipitated. Most of the gelatin was precipitated when the quebracho addition reach 150 mg. per 100 ml.

At pH 5.0, as the amount of quebracho was increased, the per cent of added quebracho appearing in the precipitate decreased. At pH 3.5, as the quebracho was raised initially, the percent quebracho appearing in the precipitate increased, but at higher quebracho levels the per cent in the precipitate decreased. The use of ethanol caused a decrease in the quebracho precipitated. The NaCl depressed the precipitation of quebracho only at the low quebracho level.

The effects of conditions on the ratio of tannin to gelatin (*T/P* ratio) in the precipitate are presented in Table III. As the level of tannin in the reaction mixture increased, the *T/P* ratio of the precipitate increased. Ethanol caused a decrease in the precipitate *T/P* ratio. Lowering the pH at the low tannic acid levels caused a decrease in the *T/P* ratio for the gelatin with or without ethanol but caused an increase in the precipitate *T/P* ratio in the presence of NaCl. The low pH decreased the binding of tannic acid in the absence of salt. At high tannic acid levels the precipitate formed at pH 3.5 had a higher *T/P* ratio. At pH 3.5 the inclusion of NaCl gave a precipitate with a higher *T/P* ratio than at pH 5.0. The highest *T/P* ratio found was 2.59 at pH 3.5 in 0.001M NaCl.

There were many similarities between tannic acid and quebracho tannin with regard to their interaction and precipitation with gelatin. They were as follows.

Per Cent Tannin Precipitated. At pH 5.0, as the tannin increased, the per cent of added tannin appearing in the precipitate decreased. At pH 3.5, as the amount of tannin went from the lowest level to the next higher level, the per cent tannin appearing in the precipitate increased. On further raising the tannin level, the per cent precipitated decreased. At pH 5.0 the presence of ethanol reduced the per cent precipitated.

Table I. Gelatin and Tannic Acid Precipitated at Various Initial Concentrations of Tannic Acid, Mg. per 100 ML.

Conditions ^a	% of Gelatin Precipitated					% of Tannic Acid Precipitated				
	45	90	180	360	720	45	90	180	360	720
pH 5.0	64	88	99	93	94	77	76	55	29	17
pH 3.5	13	24	37	49	52	7	20	22	16	10
pH 5.0, 15% ethanol	76	95	96	100	98	65	63	39	22	13
pH 3.5, 15% ethanol	56	59	61	65	72	27	22	15	15	11
pH 5.0, 0.001M NaCl	72	90	100	100	100	77	75	54	32	24
pH 3.5, 0.001M NaCl	10	27	30	43	73	21	30	25	22	21

^a 80 mg. gelatin per 100 ml.

Table II. Gelatin and Tannin Precipitated at Various Initial Levels of Quebracho Tannin, Mg. per 100 ML.

Conditions ^a	% of Gelatin Precipitated					% of Quebracho Tannin Precipitated				
	38	75	150	300	600	38	75	150	300	600
pH 5.0	56	79	87	85	91	66	52	36	20	18
pH 3.5	50	88	91	93	96	64	70	50	29	18
pH 5.0, 15% ethanol	66	81	90	100	95	41	40	35	30	17
pH 3.5, 15% ethanol	36	81	93	100	100	30	47	35	29	26
pH 5.0, 0.001M NaCl	67	87	93	94	94	57	50	36	21	20
pH 3.5, 0.001M NaCl	46	88	91	96	100	53	68	49	29	28

^a 80 mg. gelatin per 100 ml.

Table III. Ratio of Tannin to Gelatin (*T/P*) in the Precipitate at Various Initial Levels of Tannins

Conditions ^a	Initial Concn. of Tannic Acid, Mg./100 ML.					Initial Concn. of Quebracho Tannin, Mg./100 ML.				
	45	90	180	360	720	38	75	150	300	600
pH 5.0	0.68	0.95	1.25	1.40	1.63	0.56	0.61	0.78	0.88	1.48
pH 3.5	0.30	0.94	1.34	1.47	1.73	0.61	0.74	1.03	1.17	1.41
pH 5.0, 15% ethanol	0.48	0.75	0.91	0.99	1.18	0.30	0.46	0.73	1.13	1.34
pH 3.5, 15% ethanol	0.27	0.42	0.55	1.04	1.38	0.40	0.54	0.71	1.09	1.95
pH 5.0, 0.001M NaCl	0.53	0.94	1.21	1.44	2.16	0.40	0.53	0.73	0.84	1.60
pH 3.5, 0.001M NaCl	1.18	1.25	1.87	2.30	2.59	0.55	0.72	1.02	1.12	2.10

^a 80 mg. gelatin per 100 ml.

Per Cent Gelatin Precipitated. Most of the gelatin was precipitated at pH 5.0 when the tannin reached the 150 to 180 mg. per 100 ml. level. At this pH, similar changes were seen when ethanol or NaCl was present.

The differences in the behavior of the tannic acid and quebracho tannin systems also should be noted.

Per Cent Tannin Precipitated. More quebracho tannin was precipitated when the pH was lowered. With tannic acid, more was precipitated at pH 5.0. At comparable tannin levels, there was a greater amount of tannic acid precipitated at pH 5.0 and a greater amount of quebracho tannin precipitated at pH 3.5. At low tannic levels, pH 3.5, NaCl depressed the amount of quebracho tannin precipitated, while it increased tannic acid precipitation.

Per Cent Protein Precipitated. More protein was precipitated at pH 5.0 by tannic acid than at pH 3.5, but quebracho tannin precipitated protein about equally as well at both pH levels. On a weight basis, comparable amounts of gelatin were precipitated by both tannins at pH 5.0, but at pH 3.5 about twice as much gelatin was precipitated by the quebracho tannin.

T/P Ratios of Precipitates. With plain gelatin there was little change in the T/P ratios when the quebracho tannin system pH was lowered, but the tannic acid system showed decreased T/P ratios with such a shift. With ethanol, this pH change caused increased T/P ratios for quebracho tannin and decreased ratios for tannic acid. NaCl increased the T/P ratios at pH 3.5 with tannic acid, while with quebracho the salt produced a lower T/P ratio at 5.0. At comparable amounts of protein precipitated, quebracho tannin was associated with lower T/P ratios.

A major difference in the behaviors of tannic acid and quebracho tannin lies in the influence of pH on their combination with gelatin. A lowering of the pH to 3.5 caused a marked decrease in the strength of tannic acid binding but little or no change in quebracho tannin binding. This was best seen at low tannin levels. Zitko and Rosik (1962) report a similar conclusion for tannic acid, stating that as the pH was lowered the equilibrium binding constant decreased. In addition, there was less effect of pH on aggregation to sedimentable size with quebracho tannin-gelatin complexes. The net result was that greater percentages of both gelatin and tannin would be sedimentable at low pH and tannin levels in quebracho tannin systems.

The general effect of ethanol was to increase gelatin precipitation and decrease tannin precipitation and, therefore, result in a decreased T/P ratio in the precipitate. Ethanol may have increased gelatin crosslinking through small crystallites (Boedtke and Doty, 1954). NaCl had relatively little effect on tannin precipitation but tended to increase gelatin precipitation at pH 5.0, especially when quebracho tannin was used.

The influence of the relative amounts of gelatin and quebracho tannin on complexes formation can also be observed through the amount of haze produced. Figure 1 shows a haze increase as the quebracho tannin was raised, and Figure 2 shows a haze decrease as the gelatin concentration was increased. Excess of gelatin reduced the amount of haze. Also of interest was the decrease in haze when the pH was lowered. In view of the relatively small effect of such a pH shift on the precipitation of gelatin seen earlier (Table II), the amount of haze material probably was

similar, but the particle size was smaller at pH 3.5. Such a decrease may have been due to electrostatic inhibition of large aggregate formation at pH 3.5.

An increase in the amount of gelatin reduces the number of tannin molecules bound on each molecule of gelatin, decreasing the tendency of small complexes to bind with each other. Comparable results have been observed with tannin-polyvinylpyrrolidone mixtures (Chapon *et al.*, 1961).

Similar decreases in haze were seen when gelatin was added to a hazy gelatin-tannin mixture or when the pH of a hazy suspension was lowered from pH 5.0 to 3.5. The build-up of aggregates is reversible but is difficult to observe if the aggregate particles attain a large size—large enough to precipitate from suspension.

The presence of both tannin and gelatin in the supernatants indicates that soluble complexes of tannin and protein are possible under appropriate conditions. Such soluble complexes might involve aggregates containing a number of gelatin molecules. To test for the presence of such complexes, a few light-scattering observations were

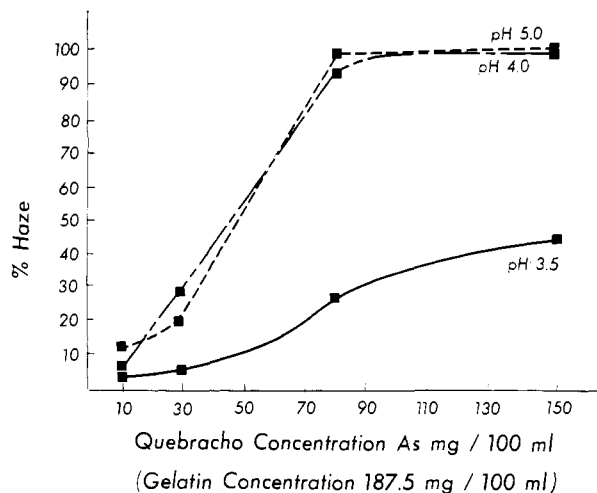


Figure 1. Effect of tannin concentration on haze formation

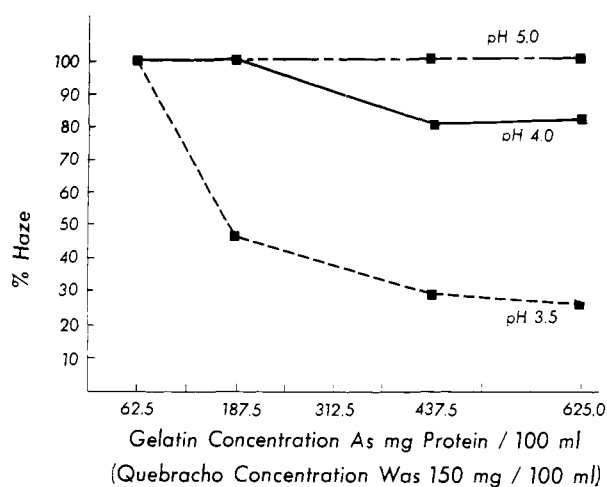


Figure 2. Effect of gelatin concentration on haze formation

made. An intense, well collimated light beam was passed through solutions, and the light scattered at 90° to the path of the incident light was observed. Gelatin or tannin solutions scattered very little light. Addition of 1 mg. of quebracho tannin to 50 ml. of a 0.1% gelatin solution caused a large increase in 90° scattered light, which was also polarized perpendicular to the path of the incident light. No precipitate formed in this quebracho-gelatin mixture. Supernatants obtained from other mixtures of quebracho and gelatin also showed pronounced light scattering ability. Thus, complexes form between tannin and protein of a relatively small size, not readily sedimented by centrifugation, yet which are large enough to enhance the light-scattering ability of their solutions.

The interaction between tannin and gelatin might be represented by the equation



(M = average number of tannin molecules bound per gelatin molecule, and N = average number of gelatin molecules in an aggregate) with both M and N increasing as the T/P ratio of the reactants increased. N would be expected to increase as a function of M up to a point where the number of tannin molecules bound on most of the gelatin molecules interfered with crosslinking between gelatin molecules by means of tannin bridges. In actual practice, this limit has not been observed even when the T/P ratio of the reactants was 80 (Page, 1942), although it may be approached under the reaction conditions described by Mejbaum-Katzenellenbogen (1955) for the turbidometric estimation of protein. As N increases the light scattering ability of the system increases, and as N goes to rather high levels precipitation and coagulation occur.

Precipitate T/P was influenced by the pH of the mixtures, and at the pH levels used here the influence was certainly due to the charge carried on the gelatin molecules. A solution of such complexes experiences both electrostatic and the London-van der Waals effects (van Olphen, 1963), and the balance of these determines the ease with which the complexes can approach sufficiently close for crosslinking through the formation of stable hydrogen bond configurations. The expected result would be the reduction of N as the pH was lowered from 5.0, in the region of the isoelectric point, to pH 3.5. The T/P ratio in the precipitate should then increase when pH was lowered assuming an unchanged distribution of M values. Since these were the

results observed, the aggregation to sedimentable size in tannin-gelatin systems was probably achieved through tannin bridges. A similar conclusion can be drawn from the binding of tannin-protein complexes onto insoluble polyamides as observed by Harris (1965).

The T/P ratios of the precipitates are expected to have a high degree of correlation with M (Equation 1). Hence, lowering the pH from 5.0 to 3.5 decreased M in low tannic acid systems lacking salt. Apparently some electrostatic effects are operative in the primary combination of protein and tannin. This is compatible with the view of hydrogen bonds possessing some electrostatic aspects (Cannon, 1955). Such effects on M would be most important at low reactant levels. At high tannin levels, the presence of excess tannin and the accessibility of sites become the predominating influences on M or T/P values. Since lowering of pH enhanced the accessibility of sites by increasing the distance between protein chains, the T/P ratios of the precipitates were higher.

The effect of alcohol might be described as a decreased affinity of tannins for proteins in the presence of ethanol. There have been many reports of the greater ability of alcohol-water mixtures, as compared with water, to remove polyphenols from polyamides (Endres and Hilal, 1963).

LITERATURE CITED

- Boedtker, H., Doty, P., *J. Phys. Chem.* **58**, 968 (1954).
 Chapon, L., Chollott, B., Urion, E., *Bull. Soc. Chim. Biol.* **43**, 429 (1961).
 Cannon, C. G., *Mikrochim. Acta* **1955**, p. 555.
 Endres, H., Hilal, M., *Phytochem.* **2**, 151 (1963).
 Endres, H., Hörmann, H., *Angew. Chem.* **75**, 288 (1963).
 Filachione, E. M., Harris, E. H., Luvisi, F. P., *J. Am. Leather Chem. Assoc.* **50**, 301 (1955).
 Gustavson, K. H., *J. Polymer Sci.* **12**, 317 (1954).
 Gustavson, K. H., "Chemistry of the Tanning Process," p. 142, Academic Press, New York, 1956.
 Harris, G., *J. Inst. Brew.* **71**, 292 (1965).
 Mejbaum-Katzenellenbogen, W., *Acta Biochem. Polon.* **2**, 279 (1955).
 Page, R. O., *J. Soc. Leather Trades Chem.* **26**, 71 (1942).
 Singleton, V. L., Rossi, J. A., *Am. J. Enol. Viticult.* **16**, 144 (1965).
 van Olphen, H., "An Introduction to Clay Colloid Chemistry," p. 37, Interscience Publishers, New York, 1963.
 Zitko, V., Rosik, J., *Collection Czech. Chem. Commun.* **27**, 2058 (1962).

Received for review November 15, 1967. Accepted February 8, 1968. This work was supported by USDA Contract No. 12-14-100-8173 (74) from the Agricultural Research Service. Approved by the Director of the New York State Agricultural Experiment Station, Geneva, N. Y., as Journal Paper No. 1604.