Protein Coagulation Cloud in Citrus Fruit Extract. 1. Formation of Coagulates and Their Bound Pectin and Neutral Sugars†

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Protein coagulation was studied in a serum of citrus fruit aqueous peel extract with regard to its role in cloud stability. Heat coagulation in enzymatic pectin degraded serum occurred within the temperature range 40–100 °C. In the same serum, conversion of soluble proteins into insoluble ones occurred also during heat vacuum concentration. Heat-coagulated proteins were found in the flavedo extract, while they were not detected in the albedo extract. The coagulation intensity was affected by pH, the largest amount of coagulate being obtained around pH 4.5. Enzymatic pectin degradation markedly increased the coagulation due to reduction in the pectic polymer size. Coagulated protein bound pectin and associated neutral sugars were identified, and their amounts were found significantly lower in heat-inactivated extract, probably by inhibition of demethoxylation by pectin methyl esterase. The amounts of the coagulated protein bound neutral sugars (particularly rhamnose, arabinose, and glucose) were in accordance with the amount of the coagulate-bound pectin.

INTRODUCTION

Proteins have been identified in citrus fruit extracts of either the juice sacs or the peel and in their cloud particles (Klavons and Bennett, 1985, 1987; Shomer, 1988; Shomer et al., 1985; Sinclair, 1984). A significant portion of proteins in the peel extract was found to be soluble and coagulable (Shomer, 1988; Shomer et al., 1985). Klavons and Bennett (1985) suggested that the insolubility of protein in the juice could also be attributed to several causes such as heat denaturation, inherent insolubility, and complexes of protein with another constituent. Heat coagulation of proteins of peel extract was found to be involved with the cloud stability, due to binding of the colloidal constituents to the protein, mainly at pH 3.5–4, indicating its isoelectric range (Shomer, 1988). However, complexes of proteins with other constituents, such as polysaccharides and tannins, have been found either in model solutions (Imeson et al., 1977) or in fruit extracts (Krop and Pilnik, 1974; Van Buren and Robinson, 1969; Van Buren and Way, 1978). Hence, it is important to characterize the soluble proteins to understand their role in the cloud stability of turbid extracts. The occurrence of cloud components is an important factor in juices—in which the turbidity is desirable—and in clear beverages—in which the turbidity is not desirable.

The accepted approach to explain cloud instability in citrus fruit juices is based on the formation of pectate gels due to demethoxylation of the pectic polymer by pectin methyl esterase (Baker, 1980; Bruemmer, 1980; Sinclair, 1984). The effect of polysaccharides on the colloidal binding and coagulation of soluble proteins was studied in model solutions (Imeson et al., 1977) and in tissue extracts (Shomer, 1988; Shomer et al., 1982). Enzymatic pectin degradation (EPD) enables heat coagulation of proteins in the peel extract, where without EPD the heat coagulation was obstructed. It was concluded that the high molecular size of the pectic polymer is the factor which suppresses protein coagulation (Shomer, 1988). Furthermore, it was shown that addition of high molecular weight pectin into protein solution moderated markedly heat coagulation and resulted in delicate ultrastructure of the coagulate (Shomer et al., 1982). The mechanism of cloud flocculation in citrus fruit extracts was suggested to be based on encapsulation of insoluble colloidal constituents and emulsified oily droplets by coagulable polymers (Shomer, 1988). Pectin is "coagulated" due to its conversion into calcium pectate gel, and protein is coagulated by heat or acid denaturation.

Proteins in the cloud may be fractionated according to their coagulation temperatures, and each fraction has a different electrophoretic pattern and specific ultrastructure (Shomer, 1991). It was also shown that the presence of nondegraded pectin, from both the albedo and the flavedo, caused finer ultrastructure of the coagulated proteins.

The cloud consists of several fractions of particles such as oil microdroplets, membranes, intracellular organelles, pigment bodies (plastids), and hesperidin crystals and complexes of these colloidal bodies with proteins (Merin and Shomer, 1984; Shomer, 1988; Shomer et al., 1985). However, it is not yet known what the binding affinity is between the heat-coagulated proteins and the specific cloud particles, and this aspect is important for the understanding of the mechanism(s) of the cloud stability.

The present study examined the conditions under which protein coagulation takes place in flavedo and albedo extracts, as related to the coagulates' structure, including EPD, and identified the coagulated protein bound pectin and neutral sugars.

MATERIALS AND METHODS

Preparation of Peel Extract. Orange (*Citrus sinensis* L. Osbeck, cv. Valencia) peel was grated to yield pieces of approximately 2 × 5 mm. The flavedo was grated from the outer side of the peel and kept for further tests; the remaining albedo was then grated and kept separately. The grated matter was immersed in distilled water (1:3 w/v, respectively) and shaken vigorously for 30 min at room temperature. Inactivated extract was obtained by heating the peel at 100 °C for 10 min before grating. The essential oil that accumulated at the top of the liquid column was decanted by vacuum, and the aqueous peel extract was filtered through Whatman No. 1 paper and centrifuged at 100000*g* (Beckman Model L5-50 ultracentrifuge). The resulting clear serum of the aqueous peel extract (SPEX) was used for the experiments.

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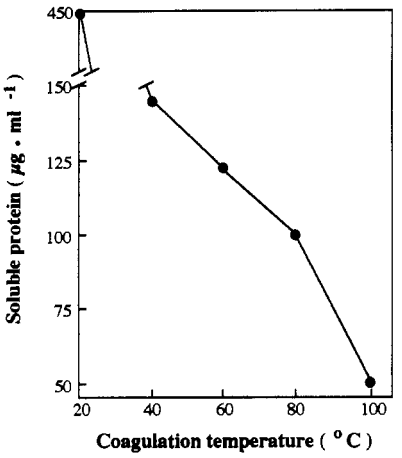

Figure 1. Effect of heating temperature on the amount of soluble proteins remaining in an enzymatic pectin degraded serum of peel extract after coagulation.

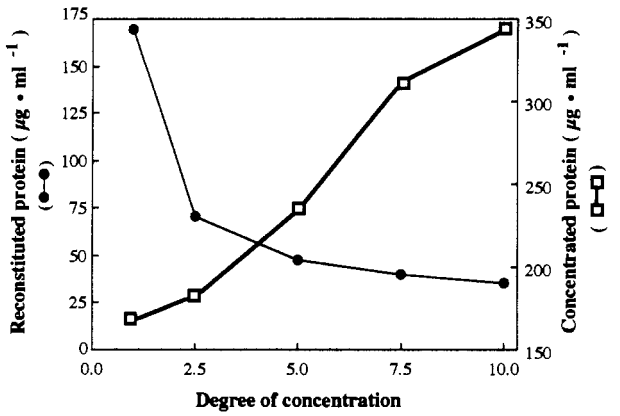

Figure 2. Effect of heat vacuum concentration on the amount of soluble proteins remaining in an enzymatic pectin degraded serum of aqueous peel extract. The soluble protein was determined either in the concentrates or in the recovered single-strength serum.

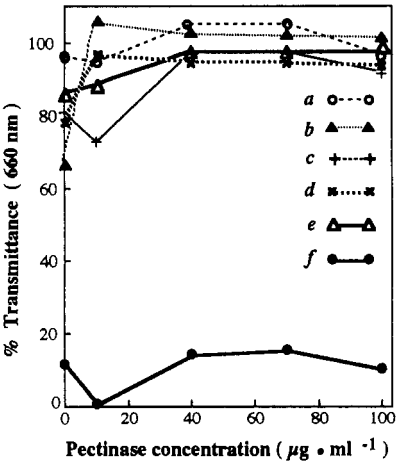

Figure 3. Protein heat coagulation at 100 °C, in a serum of aqueous extracts of flavedo and albedo after enzymatic pectin degradation (determined by the decrease in light transmittance due to the formation of suspended coagulates). (a) Serum of flavedo extract; (b) serum of albedo extract; (c) heat-inactivated and heat-coagulated albedo extract; (d) heat-coagulated albedo extract; (e) heat-inactivated albedo extract; (f) heat-coagulated flavedo extract.

Enzymatic Treatment. The enzymatic pectin degradation (EPD) was done by incubating the SPEX with pectinase Ultrazyme-100 G Novo Ferment Schweiz (consisting mainly of EC

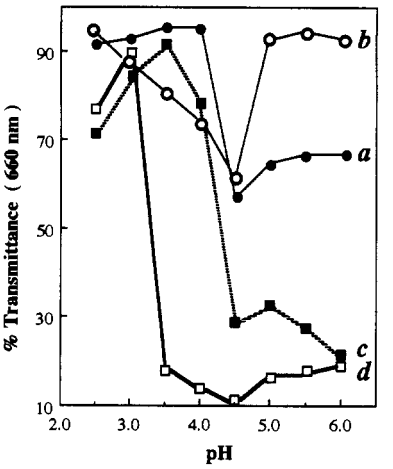

Figure 4. Effect of pH and enzymatic pectin degradation (EPD) on protein heat coagulation in a serum of flavedo extract (determined by decreasing transmittance, due to the formation of suspended coagulates). (a) Without EPD; (b) after EPD; (c) without EPD, but heat-coagulated; (d) after EPD and heat-coagulated.

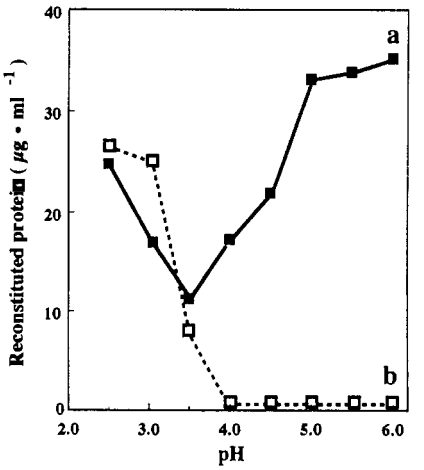

Figure 5. Effect of pH and enzymatic pectin degradation on protein heat coagulation in a serum of flavedo extract (determined by the amount of the soluble protein remaining after coagulation). (a) Without EPD and without heat coagulation; (b) after EPD and heat-coagulated.

4.2.2.2) for 30 min at 40 °C with shaking. This enzyme has been shown to cause pectin degradation (Shomer, 1988).

Protein Heat Coagulation. Heat coagulation of the serum proteins was done by heating to the desired temperature (in the range 40–100 °C), while stirring, and the coagulated proteins were precipitated from the serum by centrifugation at 1500 *g*. To enable the coagulation during heat vacuum concentration, the EPD was done by pectinase with 10 μg mL^{-1} serum. The serum was then concentrated in a rotaevaporator at 40 °C in vacuum to the desired concentration. It was then recovered to its original single-strength concentration and centrifuged at 1500*g* for 15 min to precipitate the coagulated protein.

Chemical Analyses. The amount of soluble protein was determined according to the dye binding method of Bradford (1976).

The sugar composition of the polysaccharides was analyzed in freeze-dried matter obtained from a known amount of SPEX of both the coagulated and the soluble matter. The latter was dialyzed against distilled water to remove low molecular weight solutes. Alditol acetates of sugars that were obtained from acid-hydrolyzed polysaccharides were analyzed according to the method of Sloneker (1971), using gas–liquid chromatography (Hewlett-Packard 5790A series gas chromatograph) with a flame ionization detector. The analyzed solution was injected into a column of 3% SP-2340 on Supelco Port 100/120 mesh, under the following conditions: the column temperature was increased from

Table I. Neutral Sugars and Pectin of High Molecular Weight Components of a Dialyzed Serum ($\mu g\ mL^{-1}$) of Citrus Fruit Aqueous Peel Extract following Enzymatic Pectin Degradation (EPD), before and after Heat Inactivation and/or Heat Coagulation[a]

sugar	control, non-EPD	control	inactivated	coagulated	inactivated and coagulated
rhamnose	11.6 ± 2.0ab	15.6 ± 2.0	7.6 ± 29b	15.5 ± 0.5a	8.9 ± 2.0ab
arabinose	40.2 ± 3.6	84.2 ± 35.2	66.1 ± 3.5	72.2 ± 6.8	91.0 ± 24.3
xylose	22.0 ± 2.4	30.1 ± 5.9	21.0 ● 4.4	28.1 ± 0.5	22.9 ± 4.4
mannose	22.3 ± 5.4a	12.1 ± 18b	8.5 ± 1.5b	13.3 ± 0.7b	11.1 ± 2.0b*
galactose	36.7 ± 4.3	46.2 ± 6.5	36.4 ± 12.2	48.6 ± 2.1	49.3 ± 7.0
glucose	258.3 ± 33.4	185.0 ± 27.6	168.9 ± 30.4	202.4 ● 10.0	218.7 ± 37.1
GA[b]	288.8 ± 84.8a	111.2 ± 31.4b	109.4 ± 30.0b	173.4 ± 21.2ab	125.4 ± 42.3b

[a] Means (of four replicates) marked by different letters are significantly different (*$p < 0.05$; **$p < 0.01$) according to Duncan's multiple range test. [b] GA, galacturonic acid.

120 to 220 °C at a rate of 4 °C/min; injection was at 250 °C and detection at 250 °C. Nitrogen was used as carrier gas at a rate of 30 mL min^{-1}. Oxygen and hydrogen were used for the combustion at rates of 250 and 25 mL min^{-1}, respectively.

Pectin, as uronic acid, was analyzed according to the method of Blumenkrantz and Asboe-Hansen (1973).

RESULTS AND DISCUSSION

Protein Coagulation. Heat coagulation of the proteins was observed at a relatively wide range of temperatures (Figure 1). A fraction of the proteins was already coagulated during the enzymatic pectin degradation at the temperature range 30–40 °C. Each increment of the treated temperatures led to the formation of an additional amount of coagulate. The sensivitity of the protein undergoing coagulation at the relatively low temperature of 40 °C may be one of the reasons for the presence of protein in the natural juice clouds. In other words, it is possible that some of the soluble juice proteins are so sensitive to coagulation that they might coagulate during squeezing.

Precipitated coagulates of proteins are distinguished by their physical properties. The proteins, which were characterized by electrophoresis, were electron dense, while the pectins were undetectable by transmission electron microscopy. Hence, the fine and moderate protein flocks, which appear as thready patterns, are suggested to represent the structure of the pectic lattice on which the electron-dense protein is bound (Shomer et al., 1991).

Since insoluble proteins were found in natural juice (Klavons and Bennett, 1985, 1987; Sinclair, 1984), it is possible that some of those proteins were coagulated during squeezing and therefore might affect cloud stability. Heat vacuum concentration (at 40 °C) of SPEX, after EPD, led to the formation of insoluble protein as was calculated from the reduced amount of the remaining soluble protein (Figure 2). These insoluble proteins are similar in nature to those obtained by heat denaturation at 100 °C. Although coagulate obtained by heating at 100 °C and by heat vacuum concentration resulted in similar electrophoretic patterns, their ultrastructures were significantly different (Shomer et al., 1991). These structural variabilities, as affected by coagulation conditions, might lead to differences in the suspendibility of particles of different structures and dimensions.

Since the orange peel consists of two main parts, the albedo and flavedo, the protein coagulation in extracts of both tissues was examined. Figure 3 shows the effect of protein heat coagulation on the turbidity of the flavedo and albedo SPEXes. There was no observed protein coagulation of albedo SPEX, either in the presence or in the absence of EPD. On the other hand, heat denaturation of the flavedo SPEX resulted in distinct coagulation, particularly after EPD. Some of the proteins were coagulated during EPD incubation, while most of the soluble proteins coagulated as a result of the heat denaturation. However, the soluble nondegraded flavedo pectin suppressed the decrease in transmittance due to the prevention of coagulation of the major part of the soluble protein. Only the EPD allowed the entire heat coagulation to take place at 100 °C, and as a result decreasing transmittance was observed (Figure 3).

The pH of the SPEX is in the range 3.5–4.5, depending on the sample of the examined fruit. In a defined flavedo SPEX sample, the turbidity was significantly affected by the pH level (Figure 4) as a result of protein heat coagulation. This was observed in either the presence or absence of EPD, where obvious turbidity appeared also in nonheated serum at the pH range 4.5–6.0. The changes in the amount of soluble proteins after removal of the coagulated ones are shown in Figure 5. These changes are ascribed to the electric charge of the proteins, since around the isoelectric point of most of them, coagulation is at its highest rate. Without EPD, the coagulation in the whole peel (flavedo and albedo) SPEX was suppressed (Shomer, 1988), while in the flavedo SPEX it was relatively intense (Figure 4).

These results indicate that the heat-coagulable proteins of the peel extract are derived mostly from the flavedo tissue and that the nondegraded albedo pectin suppresses the coagulation of the flavedo protein. Indications of the effect of pectin on the protein coagulation were also observed by differences in both electrophoretic patterns and ultrastructures between protein coagulates which were obtained under various conditions such as tissue origin (flavedo, albedo, and whole peel), coagulation temperature, pH, and EPD (Shomer et al., 1991).

Protein-Bound Polysaccharides. Since the structure of heat-coagulated protein was affected by the EPD, it is important to determine whether the pectin and the neutral sugars are bound to the coagulated proteins. Hence, the composition of the polysaccharides in the serum was determined, following removal of the low molecular weight solutes by dialysis. The pectin and the neutral sugars in the coagulates were analyzed after they were washed several times with distilled water.

No significant quantitative differences were found in the galacturonic acid or the neutral sugars between the control and the various treated sera (Table I). Contrary to the serum, significant quantitative differences in galacturonic acid and some of the neutral sugars were found in the coagulates of the different treatments (Table II). Following heat inactivation, the coagulate included significantly lower amounts of galacturonic acid, rhamnose, arabinose, and glucose, as compared with the noninactivated treatments. These results indicate that the amounts of the coagulated protein bound pectin and neutral sugars are dependent on endogenous enzymatic activity in the SPEX. It seems that this phenomenon is related to

Table II. Neutral Sugars and Pectin (μg mL^{-1} Serum) Linked to Heat-Coagulated Proteins Obtained in a Serum of Citrus Fruit Aqueous Peel Extract following Enzymatic Pectin Degradation (EPD), with and without Heat Inactivation and/or Heat Coagulation[a]

sugar	control EPD	inactivated	coagulated	inactivated and coagulated
rhamnose	4.4 ± 0.7a	tr[b]	5.3 ± 2.0a	tr**
arabinose	21.6 ± 5.4ab	3.6 ± 1.6b	43.3 ± 14.5a	5.1 ± 0.8b*
xylose	0.6 ± 0.3a	tr	0.7 ± 0.2a	0.6 ± 0.1a*
mannose	1.4 ± 0.3	2.0 ± 1.7	2.2 ± 0.3	2.1 ± 0.4
galactose	4.5 ± 0.7	3.0 ± 2.2	6.3 ± 0.2	3.9 ± 1.5
glucose	10.5 ± 1.5a	tr	10.8 ± 1.4a	2.2 ± 0.9b**
GA[c]	294.2 ± 17.6cb	47.7 ± 20.9c	382.4 ± 30.8a	82.8 ± 24.1c**

[a] Means (of four replicates) marked by different letters are significantly different (*$p < 0.05$; **$p < 0.01$) according to Duncan's multiple range test. [b] tr, trace. [c] GA, galacturonic acid.

demethoxylation by pectin methyl esterase, which exposes the R–COO$^-$ groups of the pectin to be bound to the protein.

The pectic polymer includes within its molecular structure also small portions of neutral sugars such as rhamnose, arabinose, and glucose (Fry, 1988; O'Beirne et al., 1982; Seymour et al., 1990). In this context, the presence of a larger amount of protein-bound galacturonic acid was in accordance with relatively higher amounts of rhamnose, arabinose, and glucose and vice versa. This indicates that some portion of these neutral sugars is a part of the pectic polymer. Even so, in all the treatments, the amounts of the neutral sugars—and particularly of the glucose—in the dialyzed serum (Table I) were higher than those found in the coagulates (Table II).

In noninactivated SPEX treatments, larger quantitites of pectin were found in the coagulates (Table II) than in the serum (Table I). These findings are due to the dialysis of the serum, which removed low molecular weight pectin before and especially after EPD; at the same time, the binding of nonmethoxylated pectin to coagulated protein [which is electrostatic in nature (Imeson et al., 1977)] exists also with low molecular weight galacturonic acids. However, EPD of noninactivated serum, heat inactivation, and heat treatment for protein coagulation prevent the formation of pectate gels. Therefore, it appears that galacturonic acid and the neutral sugars, which are found in coagulate, are protein-bound composites rather than pectate gel.

CONCLUSIONS

Protein coagulation exists in aqueous peel extract, and its degree is dependent on several factors such as coagulation temperature, concentration, pH, and degradation level of soluble pectic polymers. Most of the coagulable proteins originate in the flavedo. The albedo pectin and its molecular size affect the ability of the flavedo protein to be coagulated. Indications are that the increased amounts of protein-bound galacturonic acid and neutral sugars are dependent on pectin demethoxylation induced by pectin methyl esterase.

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LITERATURE CITED

Baker, R. A. The role of pectin in citrus quality and nutrition. In *Citrus Nutrition and Quality*; Nagy, S., Attaway, J. A., Eds.; ACS Symposium Series 143; American Chemical Society: Washington, DC, 1980; pp 109–128.

Blumenkranntz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484–489.

Bradford, M. M. A. rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

Bruemmer, J. H. Relationship of citrus enzymes to juice quality. In *Citrus Nutrition and Quality*; Nagy, S., Attaway, J. A., Eds.; ACS Symposium Series 143; American Chemical Society: Washington, DC, 1980; pp 151–166.

Fry, S. C. *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*; Logman Scientific and Technical, Biddle Ltd.: Guilford and King's Lynn, England, 1988.

Imeson, A. P.; Ledward, D. A.; Mitchell, J. R. On the nature of the interaction between some anionic polysaccharides and proteins. *J. Sci. Food Agric.* **1977**, *28*, 661–668.

Klavons, J. A.; Bennett, R. D. The nature of the protein constituent of commercial lemon juice cloud. *J. Agric. Food Chem.* **1985**, *33*, 708–712.

Klavons, J. A.; Bennett, R. D. Nature of the pectin constituents of commercial lemon juice cloud. *J. Agric. Food Chem.* **1987**, *35*, 159–161.

Krop, J. J. P.; Pilnik, W. Effect of protease preparation on cloud loss of citrus juice. *Lebensm. Wiss. Technol.* **1974**, *7*, 125–126.

Merin, U.; Shomer, I. Structural stability of fresh and frozen-thawed 'Valencia' (C. sinensis) orange juice. *J. Food Sci.* **1984**, *49*, 1489–1493.

O'Beirne, D.; Van Buren, J. P.; Mattick, L. R. Two distinct pectin fractions from senescent Idered apples extracted using non-degredative methods. *J. Food Sci.* **1981**, *47*, 173–176.

Seymour, G. B.; Colquhoun, M.; DuPont, S.; Paesley, K. R.; Selvendran, R. R. Composition and structural features of cell wall polysaccharides from tomato fruits. *Phytochemistry* **1990**, *29*, 725–731.

Shomer, I. Protein self-encapsulation: a mechanism involved with colloidal flocculation in citrus fruit extracts. *J. Sci. Food Agric.* **1988**, *42*, 55–66.

Shomer, I.; Lindner, P.; Ben-Gera, I.; Vasiliver, R. Ultrastructure of denaturated potato proteins. *J. Sci. Food Agric.* **1982**, *33*, 565–575.

Shomer, I.; Lindner, P.; Vasiliver, R.; Kanner, J.; Merin, U. *Colloidal* fractions of citrus fruit aqueous peel extract. *Lebensm. Wiss Technol.* **1985**, *18*, 357–365.

Shomer, I.; Vasiliver, R.; Salomon, R. Protein Coagulation Cloud in citrus fruit extracts. 2. Structural Characterization of Coagulates. *J. Agric. Food Chem.* **1991**, following paper in this issue.

Sinclair, W. B. *The Biochemistry and Physiology of The Lemon and Other Citrus Fruits*; University of California, Division of Agriculture and Natural Resources: Los Angeles, 1984; ISBN 0-931876-64-8.

Sloneker, J. H. Determination of cellulose and apparent hemicellulose in plant tissue by gas-liquid chromatography. *Anal. Biochem.* **1971**, *43*, 539–546.

Van Buren, J. P.; Robinson, W. B. Formation of complexes between protein and tannic acid. *J. Agric. Food Chem.* **1969**, *17*, 772–777.

Van Buren, J. P.; Way, R. D. Tannin hazes in apple juice. *J. Sci. Food Agric.* **1978**, *43*, 1235–1237.

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