

Oxalic Acid in Commercial Pectins Inhibits Browning of Raw Apple Juice

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Various commercial pectin preparations were tested for their ability to prevent enzymatic browning of fresh, raw, Granny Smith apple juice. Six of eight commercial pectin samples inhibited browning to some degree, and two of the pectin preparations completely prevented fresh, unrefrigerated, apple juice from browning for 24 h when added to juice at a concentration of 0.5% (w/v). Fractionation and testing of one pectin preparation showed that the antibrowning activity was not due to pectin, per se, but to the presence of low levels of oxalic acid (<0.5%) in the pectin preparations. When oxalic acid was removed from the commercial pectins, antibrowning activity was lost or greatly reduced. Pure oxalic acid was also found to inhibit browning reactions in fresh juice. Pure oxalic acid is not an approved food additive, but it does occur naturally in fruits and vegetables such as rhubarb and spinach.

Keywords: *Enzymatic browning; inhibition; apple juice; oxalic acid; pectin*

INTRODUCTION

Browning of raw apple juice is due mainly to the action of the enzyme polyphenol oxidase, which causes the oxidation of phenolic compounds that subsequently condense into colored polymers (see review by Vámos-Vigyázó, 1981). Although natural browning is acceptable in some segments of the apple juice industry, the color of pasteurized juice must frequently be adjusted to certain specifications. Presently, there are no cost-effective methods to produce a fresh (raw), nonbrowning juice without addition of sulfites. It is anticipated that a nonbrowning, fresh product would possess unique flavor and nutritive properties and be highly desirable to consumers.

Various methods have been described to control or prevent discoloration of fruit and fruit juices. These include the use of ascorbic acid and ascorbic acid derivatives (Ponting et al., 1972; Sapers et al., 1989; Janovitz-Klapp et al., 1990), cinnamic acids (Walker, 1976), citric acid, cysteine and cysteine derivatives (Walker and Reddish, 1964; Molnar-Perl and Friedman, 1990), honey (Oszmianski and Lee, 1990), cyclodextrins (Hicks et al., 1991), 4-hexylresorcinol (McEvily et al., 1991), and, until recently, sulfiting agents (see Joslyn and Braverman, 1954). The use of sulfites on fresh fruits and vegetables is limited (Langdon, 1987; Sapers, 1993). Because none of the currently used compounds are as efficacious and inexpensive as sulfites, much research is being done to find alternatives. Recently, we demonstrated that anionic polysaccharides such as carrageenan, xylan sulfate, and amylose sulfate were capable of inhibiting enzymatic browning in fresh juices (Tong and Hicks, 1991, 1993). To our knowledge, no one has examined pectin, another naturally occurring

anionic polysaccharide, as a browning inhibitor. Because pectin contains carboxyl groups that are known to bind various metal ions, we postulated that it may be able to chelate the copper cofactor of polyphenol oxidase, inactivate the enzyme, and prevent browning of fruit and vegetable juices. In this paper, we report that various commercial pectins do prevent browning of raw apple juice. We also describe the isolation and characterization of the antibrowning component.

MATERIALS AND METHODS

Pectins and Oxalic Acid. Various pectins were obtained from Bulmers Pectin (through Pectagel, Great Neck, NY), Hercules Inc. (Middletown, NY), and courtesy of Dr. Marshall Fishman of the Eastern Regional Research Center, Philadelphia, PA. Oxalic acid dihydrate was obtained from Sigma Chemical Co.

Apple Juice. Apples (*Malus domestica* Borkh cv. Granny Smith) were purchased from a local supermarket, sliced, and juiced with an Acme Supreme Juicerator Model 6001 lined with Whatman No. 1 filter paper. Granny Smith apples were used since they undergo browning at rates suitable for these studies (Sapers et al., 1989). Apples were not peeled before juicing, but defective (bruised or physiologically damaged) mesocarp was cut out and discarded prior to juicing. Juice was always collected in a beaker containing enough ascorbic acid to produce a final concentration of 50 ppm ascorbic acid. The ascorbic acid was used (Sapers et al., 1989) to delay the onset of browning until test material (pectins) could be mixed thoroughly with the juice. Aliquots of juice were added to beakers containing known quantities of pectin (dry powder). The final concentration of added pectin in juice was usually 0.5% (w/v). The pectin-juice mixtures were then stirred until all of the pectin was solubilized. Control juice samples contained only 50 ppm ascorbic acid.

Reflectance Measurements. A Byk-Gardner color machine spectrophotometer (Pacific Scientific, Silver Spring, MD) was used to measure reflectance (*R*) of the juice samples at 440 nm. Reflectance measurements were made immediately after the pectin was dissolved in the juice and usually after 6 and 24 h of incubation at room temperature. The following equation (Sapers et al., 1989) was used to determine percent

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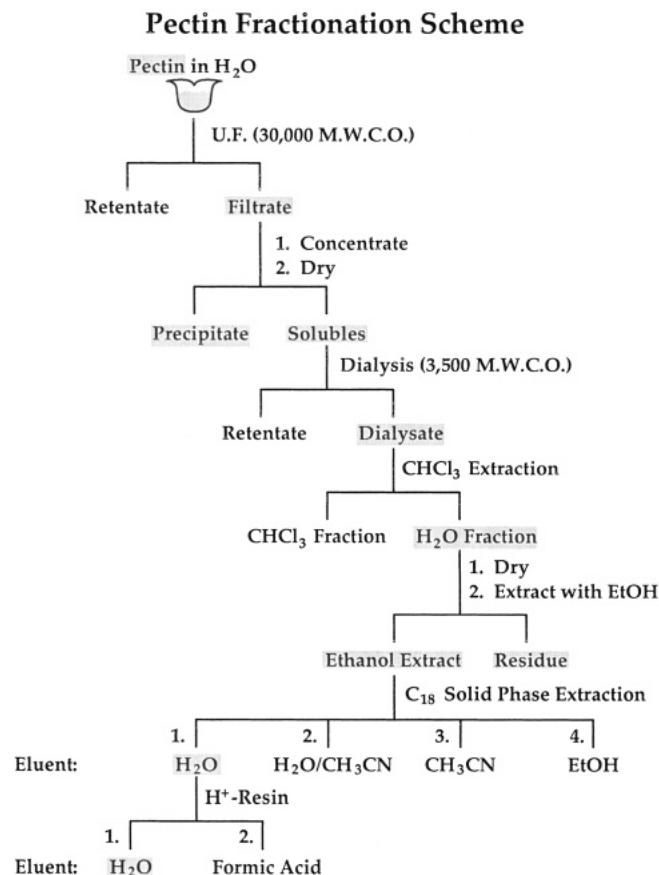


Figure 1. Scheme for fractionation of commercial pectin. Shaded fractions were active (inhibited browning in juice assay).

inhibition of browning:

$$\% \text{ inhibition} = \frac{(R_{\text{sample}_{24\text{h}}} - R_{\text{sample}_{0\text{h}}}) - (R_{\text{control}_{24\text{h}}} - R_{\text{control}_{0\text{h}}})}{(R_{\text{control}_{24\text{h}}} - R_{\text{control}_{0\text{h}}})} \times 100$$

A percent inhibition value of 100% indicates complete inhibition of browning, whereas a value of 0% indicates no inhibition.

For assessing the antibrowning activity of fractionated pectic material, the color of juice was usually determined visually because results were "all-or-nothing" (green vs brown). Initially, Granny Smith apple juice was green because the peel was green and imparted this color to the juice, which otherwise would be relatively colorless. After 24 h, untreated juice was always dark brown. All fractions from pectic materials were mixed with fresh apple juice so as to produce a final concentration of 0.5% (w/v), unless otherwise indicated. Juice color was observed immediately after addition of the pectic fraction and after 24 h incubation at room temperature.

Acidity and pH Determinations. Acidity of samples was monitored using Hydriion pH test paper and an Orion Model 611 pH meter and combination electrode.

Elemental Analysis. Inductively coupled plasma (ICP) analysis (performed by Galbraith Laboratories, Inc., Knoxville, TN) was used to determine amounts of metals in samples. Elemental analyses of carbon, hydrogen, nitrogen, and oxygen were also conducted by the same testing laboratory.

Fractionation. Pectin samples were fractionated according to Figure 1 to isolate and identify specific browning inhibitors. Pectin was solubilized in distilled water (15 g of pectin in 1.5 L of water) by stirring for 3 h at room temperature. The pectin solution was then fractionated using a Milipore Minitan ultrafiltration unit containing 30 000 MWCO cellulose filter plates. Retentate was stored at 4 °C until tested for antibrowning activity. Ultrafiltrate was evaporated with

a rotary evaporator to a few milliliters and held overnight at room temperature to allow material to precipitate. Precipitated material was collected on a Büchner funnel with Whatman No. 1 filter paper, dried under a stream of nitrogen, and stored in a desiccator. The remaining filtrate was dialyzed (3500 MWCO) for 3 days at 4 °C against distilled water. Dialysate and retentate were collected, evaporated with a rotary evaporator to a volume of less than 5 mL, lyophilized, and tested for activity. Active dialysate was solubilized in water (40–65 mg of material/mL of water), and an equal volume of chloroform was added. The water and chloroform fractions were mixed and allowed to separate, and the chloroform was removed. This extraction was repeated three times. All chloroform fractions were combined. A small amount of anhydrous magnesium sulfate was added to the chloroform extract to remove any traces of water, and the chloroform was transferred to a clean vial. Both water and chloroform fractions were then evaporated under N₂ at room temperature. The water fraction was evaporated to a thick syrup, which was then extracted two to three times with 1 mL portions of 95% ethanol. All ethanol fractions were combined and evaporated under N₂. The remaining residue from the ethanol-extracted water fraction was also dried under N₂. All dried fractions were tested for antibrowning activity in apple juice (approximately 0.25 mg of material/mL of juice).

Active material from the ethanol extraction step was solubilized in 1 mL of water and fractionated with a C₁₈ SepPak solid phase extraction column, pretreated with 20 mL of 100% ethanol, 2 mL of acetonitrile, and 10 mL of distilled water. After the sample was loaded onto the SepPak column, the column was washed with 8 mL of distilled water and then eluted with 8 mL of water:acetonitrile (17:3 v:v), 4 mL of acetonitrile, and 4 mL of absolute ethanol. All wash and eluted fractions were collected, evaporated under N₂, and tested for activity.

Active material from the C₁₈ SepPak column fractionation was solubilized in 1–5 mL of water and loaded onto a 5 mL bed volume Amberlite IR-120 (H⁺ form, cation-exchange) column. The column was washed with 20 mL of water, which was collected and dried by rotary evaporation at 35 °C and under N₂. The column was eluted with 10 mL of 5 N formic acid, and the eluent was also collected and dried under N₂.

HPLC. Reverse phase HPLC was performed using a Hewlett Packard model 1090 liquid chromatograph equipped with an Econosil C₁₈ column (Alltech), diode array UV/vis detector, and a Rheodyne sample injector containing a 50 µL loop. The column was run at 35 °C at 0.5 mL/min with the following step gradient: 0–20 min, 100% water:formic acid (95:5 v:v); 20–30 min, 80% methanol; 30–40 min, 100% water:formic acid (95:5 v:v). Elution of material was monitored at 280 nm. Fractions were collected on the basis of elution time, dried under N₂, and tested for activity.

After reversed-phase HPLC, active material was fractionated again on an HPX-87H⁺ (Bio-Rad Laboratories) column at 85 °C, with 0.06 N acetic acid as the mobile phase at 0.6 mL/min. Elution of material was monitored using an ERMA Model 7510 refractive index detector. Fractions were manually collected, dried under N₂, and tested for activity.

The separation of oxalate from other anions by high-performance anion-exchange chromatography (HPAEC) and its chemically suppressed conductivity detection were performed in our laboratory according to Rocklin et al. (1987). A Dionex series 4000i system was used which included a quaternary gradient pump, a 50 µL injection loop, an IonPac AS5A column, an IonPac AG5A guard column, an anion micro membrane suppressor (AMMS), and a conductivity detector. The AMMS regenerant used was 25 mM H₂SO₄. The mobile phase (1 mL/min) was a linear gradient that incorporated three eluents (1 = 10 mM NaOH, 2 = 100 mM NaOH, and 3 = water), which changed in a linear fashion from an initial setting of 7% 1, 93% 3 (first min) to 86% 2, and 14% 3 at 45 min. Galacturonic acid monohydrate (Sigma), sodium sulfate (Mallinckrodt), and calcium oxalate (Aldrich) were used as standards.

Oxalate Quantitation. Total oxalate was determined by Galbraith Laboratories, Knoxville, TN. Fifty milligrams of

Table 1. Inhibition of Apple Juice Browning by and Content of Oxalic Acid in Different Pectins and Fractions Thereof

pectin	% inhibition ^a	% oxalic acid in pectin ^b
P-1	35 (23, 11)	nd ^c
P-2	51 (33, 8)	0.49
P-3	90 (17, 8)	0.31
P-4	55 (11, 5)	0.44
P-5	59 (28, 11)	0.13
P-6	100 (9, 5)	0.36
P-7	5 (11, 5)	nd
P-8	3 (6, 5)	nd
precipitate from P-3	inhibited; % not determined	
HPX-87H ⁺ active fraction from P-3	inhibited; % not determined	

^a Percent inhibition of browning at 24 h at room temperature for 0.5% pectin in fresh juice. Values in parentheses are standard deviations and number of replicate experiments, respectively.

^b Determined by commercial laboratory (see Materials and Methods). ^c nd = none detected.

pectin sample was mixed with 5 mL of 10% HCl, boiled for 15 min, cooled to room temperature, and then diluted to 25 mL final volume with distilled water. The sample was further diluted $\frac{1}{10}$, filtered, and then separated using HPAEC as previously described except that the system was equipped with an AG4A guard column and an AS4A separator column. The mobile phase was an isocratic eluant of 2.0 mM sodium carbonate and 0.75 mM bicarbonate. Calcium oxalate and oxalic acid dihydrate were used as standards.

RESULTS AND DISCUSSION

Antibrowning Activity of Pectins. The pectins that were tested for antibrowning activity were Hercules slow set pectin, Hercules AV/LM, Bulmers low-methoxy, Bulmers slow set, and Bulmers pectin types 104, 106, 137, and 566. These pectins are referred to throughout the rest of this report by the coded designations of P-1–P-8. All of these pectins were extracted from citrus. Several pectins gave greater than 50% inhibition of apple juice browning (Table 1), and two pectins, P-3 and P-6, inhibited browning 90% and 100%, respectively, when dissolved in fresh juice at a 0.5% (w/v) concentration. Because we had limited quantities of pectin P-6, we focused our fractionation efforts on citrus pectin P-3 in order to identify the active component(s).

Physical and Chemical Fractionation. Pectin P-3 was fractionated as shown in Figure 1. Ultrafiltration using 30 000 MWCO filter plates was used first to fractionate P-3. The original intent in using ultrafiltration was to remove any diatomaceous earth which is sometimes used during the production of commercial pectin (Pfeffer et al., 1981). When the retentate and filtrate were retested for antibrowning activity, it was found that the retentate was unable to inhibit browning while the ultrafiltrate inhibited browning 100%. Although the crude pectin had an off-white color, the concentrated ultrafiltrate was brown. This ultrafiltrate was then dialyzed with 3500 MWCO tubing for 3 days against distilled water, with at least one change of water per day. Both the dialysate and retentate were recovered. After lyophilization, the dialysate was brown and friable, while the retentate was white and fluffy. ¹³C NMR spectra of the dialysate contained none of the resonances expected from pectic–sugar residues (data not shown). At a concentration of 0.5% (w/v), however, this dialysate inhibited apple juice browning 100% while the retentate did not inhibit browning. To determine if the active components in P-1, P-2, P-5, and P-6 (other

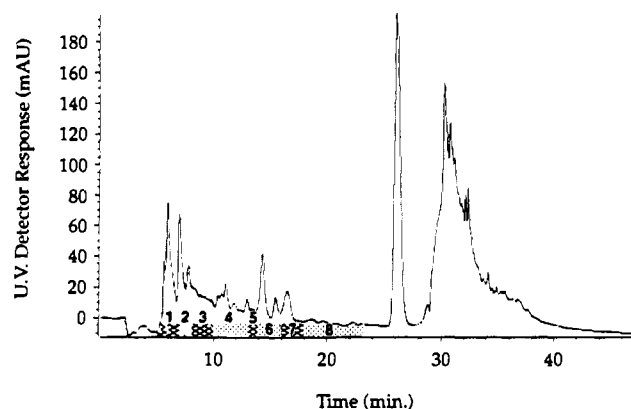


Figure 2. Fractionation of pectic material on Econosil C₁₈ reversed-phase column. Chromatographic conditions are described under Materials and Methods. Fraction numbers are given above cross-hatched (collected fraction) areas.

active commercial pectins in Table 1) were also dialyzable, samples were dialyzed (10 000 MWCO membrane) and retested for browning inhibition. In all cases but one, dialyzed pectins had negligible antibrowning activity (data not shown). Only with P-5 was there a relatively small (18%) decrease in activity after dialysis.

An aqueous solution of the dialysate from P-3 was then further fractionated by extraction with chloroform. Both water and chloroform fractions were brown colored, but the water fraction was active while the chloroform fraction was not. The water fraction was dried and extracted with 95% ethanol. Less than 10% of the water fraction was soluble in 95% ethanol. However, the ethanol soluble material was able to inhibit apple juice browning. The water fraction remaining after ethanol extraction was also active. For further fractionation, however, the ethanol soluble material was used because it was thought to have a smaller ratio of inactive to active material than the water fraction. Both the ethanol and water fractions were brown.

The ethanol soluble material was dried, resolubilized in water, and separated on a C₁₈ SepPak previously conditioned with ethanol, acetonitrile, and water. The SepPak was eluted with water, water:acetonitrile (17:3 v:v), 100% acetonitrile, and, finally, 95% ethanol. The water and ethanol fractions were clear, while the fractions containing acetonitrile were yellow. When tested for activity, only the water fraction was able to inhibit apple juice browning.

The SepPak water soluble material was then put through a column containing IR-120 (H⁺ form) cation-exchange resin. This column was washed with water and eluted with 5 N formic acid. Material that was eluted with formic acid did not inhibit apple juice browning, but the initial fraction eluted with water did.

HPLC. Fractionation of the active IR-120H⁺-treated material from P-3 on a C₁₈ reversed-phase column resulted in the chromatogram shown in Figure 2. The following fractions were collected on the basis of retention time: (1) 5–7 min, (2) 7–8 min, (3) 8–10 min, (4) 10–13 min, (5) 13–14 min, (6) 14–16 min, (7) 16–18 min, (8) 18–23 min, and (9) 23–28 min. Fractions were dried under N₂, stored in a vacuum oven (30 °C) overnight, weighed, and solubilized in 400 μ L of water. A 50 μ L aliquot of each solubilized fraction was added to 1 mL of juice for the browning assay. Only material eluted between 5 and 7 min (fraction 1) was able to inhibit browning.

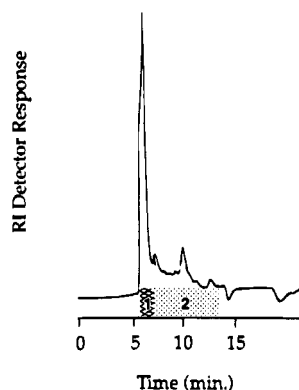


Figure 3. Fractionation of active fraction from Figure 2 on an HPX-87H⁺ column. Conditions are given under Materials and Methods. Fractions collected are shown by cross-hatched areas.

Active material from fraction 1 was then injected onto an HPX-87H⁺ column and elution was followed using refractive index detection. A typical resulting chromatogram is shown in Figure 3. Multiple injections were made, and eluting material was collected in two fractions (see Figure 3). These fractions were dried under N₂ and, when sufficient material was collected, tested for activity using 0.025% test material (w/v) in apple juice. Fraction 1 was active while fraction 2 was not.

Elemental Analysis. Early in the fractionation of P-3, prior to dialysis, the ultrafiltrate was concentrated on a rotary evaporator. If stored overnight, white material precipitated from the concentrate (Figure 1). This material was filtered from the liquid, dried in a desiccator, weighed, and tested for antibrowning activity. When added to fresh apple juice so that its final concentration was 0.5% (w/v), this material inhibited browning 100%. This precipitate was white and relatively insoluble in room-temperature water, ethanol, chloroform, 1 N NaOH, or 2 or 12 N HCl. When ashed in a bunsen burner flame, a white ash was left. To determine if the precipitate was diatomaceous earth that was not removed during ultrafiltration or dialysis, it was analyzed by ICP spectroscopy. The ICP spectrum indicated that the major metal present was calcium. The material contained only traces of other metals (approximately 0.3% silicon, 0.2% aluminum, 0.2% strontium, and less than 0.2% iron and zinc). The percentages of carbon, hydrogen, and oxygen found were 18.43%, 1.99%, and 39.67%, respectively. Theoretical percentages for calcium oxalate are C, 18.75%; H, 0.0%; O, 49.96%. On the basis of these data, we conclude that the white precipitate was crude calcium oxalate containing small amounts of other insoluble salts.

To summarize the data to this point, we found that the active, water soluble material fractionated from P-3 had a low molecular weight (readily migrated through 30 000 and 3500 MWCO dialysis tubing), was very polar (from elution profiles or solid phase and C₁₈ media), was acidic (for example, pH of ethanol extract was 3.3), and eluted from the HPX-87H⁺ ion-exclusion column where organic acids typically elute (Hicks et al., 1985). We therefore speculated that the active, fractionated P-3 material contained oxalic acid and that the P-3 precipitate was a crude form of the insoluble salt, calcium oxalate.

Determination of Oxalate in Pectins and Experimental Juice Samples. A commercial laboratory confirmed the presence of oxalate species in all of the

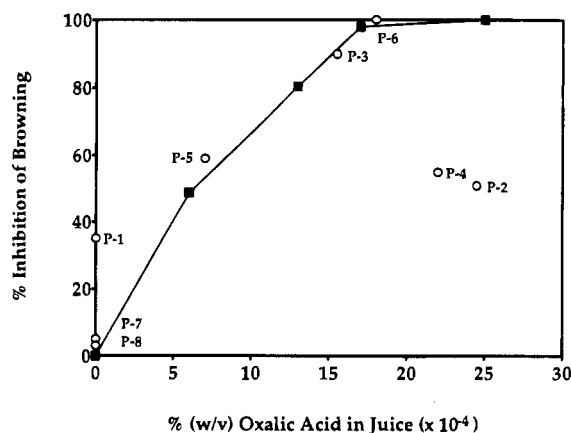


Figure 4. Dose-response curves showing inhibition of browning as a function of oxalic acid concentration in standards (closed squares) or citrus pectins (open circles).

pectins that had provided >50% browning inhibition in the apple juice assay (Table 1). From these data, we calculated the amount of oxalate (expressed in % oxalic acid, Table 1) present in the apple juice samples that contained 0.5% of these crude pectins. These values were plotted versus the percent browning inhibition values that were experimentally determined (Figure 4, open circles).

Juice Assays with Oxalic Acid and Calcium Oxalate. To determine whether oxalic acid alone could inhibit browning of apple juice, we added varying amounts of oxalic acid to fresh apple juice. Figure 4 (closed squares) shows that oxalic acid was effective in preventing browning at concentrations as low as 0.001% (w/v juice). The response in Figure 4 for the calculated oxalic acid content of crude pectins in general overlaps that of pure oxalic acid. The exceptional points are for P-1, P-2, and P-4. Sample P-1 may contain other inhibitors of enzymatic browning. Samples P-2 and P-4 were the only "standardized" pectins used. Standardized pectins are diluted with sugars by the manufacturers. Because calcium oxalate is relatively insoluble, it was not possible to do an accurate dose-response curve as in Figure 4. Calcium oxalate, suspended in fresh apple juice at a concentration of 0.5%, however, did inhibit browning in the apple juice system for 2 h, at which time the control was brown. By 24 h, the calcium oxalate sample had undergone browning to the same extent as the control.

Chromatographic Identification of Oxalic Acid. Injections of oxalic acid alone, the active peak collected from the HPX-87H⁺ column (described above), and a 1:1 mixture of these two samples were made onto the HPX-87H⁺ column (Figure 5). Oxalic acid and the active peak coeluted on this system.

To verify the results from the HPX-87H⁺ column chromatography, we also injected the same samples on a Dionex HPAEC system as described by Rocklin et al. (1987). In this system, oxalic acid also coeluted with the isolated active peak (Figure 6). Analysis of the P-3 sample on this system showed that P-3 contained 0.3% oxalic acid. This value was later confirmed by the commercial laboratory. Upon the basis of the fractionation schemes described, the qualitative and quantitative chromatographic analyses, and the inhibition assays, we conclude that the principal components in these commercial pectins that inhibit enzymatic browning in apple juice are oxalic acid and oxalate salts. Of course, the presence of additional inhibitors in these pectins, especially in P-1 (See Figure 4), cannot be dismissed.

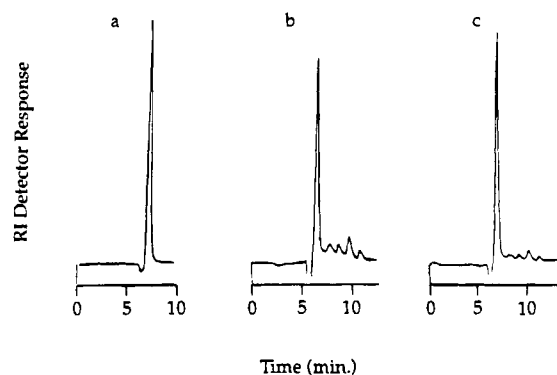


Figure 5. Chromatogram of (a) oxalic acid standard, (b) active fraction collected from Figure 3, and (c) samples from panels a and b mixed 1:1 on an HPX-87H⁺ column using conditions described under Materials and Methods.

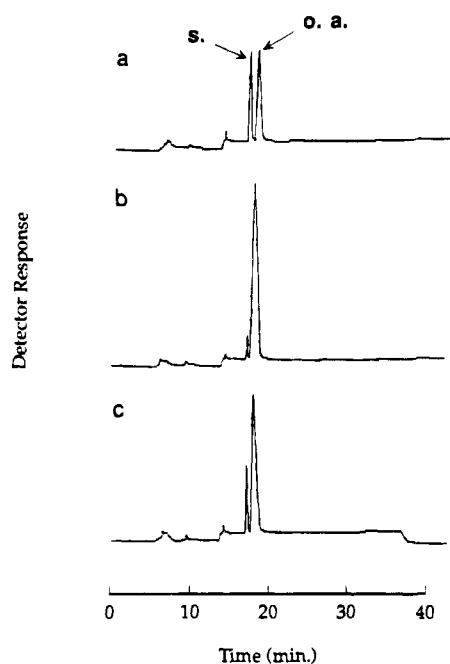


Figure 6. Chromatogram of (a) active fraction collected from Figure 3, (b) oxalic acid standard, and (c) samples from panels a and b mixed 1:1 on a IonPac AS5A anion-exchange column, using the conditions described under Materials and Methods. s = solvent peak. oa = oxalic acid peak.

The concentration of oxalic acid in the commercial pectins ranged from about 0% to 0.5%. We speculate that this oxalic acid is a degradation product of the ascorbic acid that was originally present in the citrus-processing wastes used as the source of these pectins. Oxalic acid is a well-known (Shin and Feather, 1990) ascorbic acid degradation product, and the procedures used to extract and precipitate the pectin may also have concentrated oxalic acid and its salts. It is also noteworthy that ammonium oxalate has been used for small-scale extraction of pectins from citrus peel, but we do not know if it is used for commercial scale pectin extraction. Garleb et al. (1991) described the use of ammonium oxalate to extract pectin and recommended using ultrafiltration with 3000 Da MWCO hollow fiber filters to remove any oxalic acid that may remain with the pectin. Upon the basis of the results in this report, pectins dialyzed in this way would not contain oxalic acid and would not be expected to prevent browning of apple juice. Whatever the origin of the oxalic acid, it appears that it may not be unique to the commercial suppliers of the products studied here, since an undia-

lyzed sample from a third supplier (not Hercules or Bulmers) also completely inhibited browning of apple juice for 24 h, at 0.5% concentration. It should also be noted that all investigations conducted on sample P-3 were done on a single sample from one lot. However, a second sample with a different lot number was similarly effective at preventing browning.

There have been few previous reports on the inhibition of polyphenol oxidases by oxalic acid. Marciano et al. (1983) reported that oxalic acid inhibited an *o*-diphenol oxidase from sunflower. Ferrar and Walker (1993) noted that the phytopathogen *Sclerotinia sclerotiorum* secreted oxalic acid which inhibited the *o*-diphenol oxidase activity in infected apples and dwarf bean pods. They reported that in the presence of oxalic acid, apple *o*-diphenol oxidase displayed mixed, competitive type inhibition with an inhibition constant, K_i of 0.55 mM. Interestingly, Pifferi et al. (1974) claimed that oxalic acid did not inhibit the activity of an *o*-diphenol oxidase (EC 1.10.3.1) extracted from cherry fruit. However, the amount of oxalic acid tested was not reported. Amounts of oxalic acid in P-3 (0.30%) are in the range of the amounts found in rhubarb, 0.21% (Hodgkinson, 1977) or 0.26–0.62% (Prenen et al., 1984), and spinach, 0.32% (Hodgkinson, 1977), 0.36–0.78% (Prenen et al., 1984), and 1.76% (Yamanaka et al., 1983). Naturally occurring amounts of oxalic acid in citrus pulp are very low, about 0.006% in orange (Hodgkinson, 1977). Although oxalic acid is not absorbed by the colon, and most of it is precipitated as calcium oxalate in the small bowel (Prenen et al., 1984), it can be toxic to humans when ingested in the range of 2–30 g. The amounts of pectin we used in apple juice to prevent browning should result in levels of oxalic acid well below those levels.

Although the mechanism of browning inhibition by oxalic acid is unknown, it can be speculated that oxalic acid may chelate the critical copper prosthetic group of PPO. Inhibition of PPO by this mechanism has previously been reported for a number of other chelating agents (Mayer and Harel, 1979; Vámos-Vigyázó, 1981). Although enzymatic browning can be reduced by simple reduction of pH values, the levels of pectin and oxalic acid used here were so low that they resulted in no measurable changes in pH values of the juice samples.

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