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STRUCTURE AND COMPOSITION OF APPLE JUICE HAZE

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Abstract

Haze obtained from commercial apple juice over the 1991 season contained from 11.4 to 29.0% protein (w/w), trace quantities of both metal cations and polymeric carbohydrate, and gave strong responses to tests for phenolic compounds indicating a protein-phenol haze. SDS-PAGE of the 2-mercaptoethanol reduced, guanidinium hydrochloride dissaggregated haze particles gave a continuous smear indicating a population of molecules ranging from 29K to greater than 205K daltons. Transmission electron microscopy of negatively stained preparations revealed the presence of spherical bodies only partially penetrable by stain and possessing a subunit structure. These particles were embedded in a material presumably polymerized in such a way as to form chain-like aggregates. Protein-phenol haze particles consist of two structural components: spherical particles, probably protein, embedded in polymerized phenolics.

Key Words: Apple, juice, haze, particulates.

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Introduction

Development of haze in apple juice during storage is a common problem for juice manufacturers which appears sporadically, and often with no obvious cause. Typically, the haze contains 3-49% (weight/weight, w/w) protein and 3-60% (w/w) polyphenol with evidence of carbohydrate and cations such as calcium, magnesium, iron or copper (Letzig and Nurnberger, 1963; Johnson *et al.*, 1968; Heatherbell, 1976a). Carbohydrate hazes derived from starches (Heatherbell, 1976b) or arabans (Schmitt, 1985) are known but these appear to be much less common occurrences. In beer (Gramshaw, 1970) and probably apple juice (Johnson *et al.*, 1969), protein-polyphenol hazes arise through polymerization of simple polyphenols such as catechin or epicatechin to polymeric procyanidins which interact with protein to form insoluble complexes which appear as haze. Polymeric phenols (procyanidins) have greater affinity for protein than do simple phenolics (catechins) (Ansano *et al.*, 1984) and greater potential for immediate haze formation, at least in beer (Gramshaw, 1967). Further, some protein constituents are more susceptible to incorporation in hazes in both beer (Wainwright, 1974) and apple juice (Hsu *et al.*, 1989). The phenol-protein complexes aggregate perhaps through the dehydrating effects of the phenolic constituent (Gramshaw, 1970) resulting in growing particulate and ultimately haze formation.

Light microscopy of haze isolated from clarified apple juice revealed refractile particulate apparently imbedded within an amorphous matrix (Lea, 1990). Electron microscopy of beer chill hazes showed relatively uniform sized, spherical particles arranged in small aggregates. Permanent beer haze was an amorphous mass, possibly of closely aggregated spherical particles although the fine structure was unclear from the photographs (Claesson and Sandegren, 1963). Little comparable data is available for hazes derived from commercial fruit juices.

In 1991, a local apple juice company was struck with a quality control problem centering around storage haze formation. This offered the opportunity to examine the composition and structure of apple juice haze formed under conditions of normal commercial practice.

Material and Methods

Source of haze

Haze was obtained from a commercially packed apple juice donated by a local manufacturer. Six production batches were sampled between November 21 and December 5, 1991 at 3 to 5 day intervals. The haze was isolated by centrifugation at 10,000 g at 5°C in a Sorval refrigerated centrifuge. The resulting pellet was resuspended in distilled water and resedimented twice. Part of the pellet was resuspended in distilled water for electron microscopic examination, and the remainder freeze-dried for chemical analysis.

Rate of haze formation

One liter packages of commercially packed juice was held at 0, 10, 20, 30, and 40°C. At intervals these packages were withdrawn from storage, examined visually for haze production and the mixed juice scanned from 400 to 700 nm in a Varian DMS 100 spectrophotometer equipped with 1 cm cells.

Protein

Determined by the micro Kjeldahl procedure, the factor 6.25 was used to calculate protein (AOAC, 1975). Discontinuous, SDS-PAGE was performed according to the procedures of Laemmli (1970) using a Bio-Rad Mini-Protean II Dual Slab Cell (Bio-Rad Laboratories, Richmond, California). Prior to electrophoresis haze samples were dissolved in 5.3 M guanidine hydrochloride containing 3.3 M lithium chloride, 0.01 M 2-mercaptoethanol, 0.02 M boric acid, and 0.01 M sodium hydroxide. The mixture was left overnight at 4°C, dialysed overnight against distilled water, pelleted in an Eppendorf microfuge (1 hour, 14000 rpm) and the resulting pellet dissolved in 5 M urea containing 0.2% 2-mercaptoethanol prior to gel electrophoresis at pH 6.8 (tris buffer, 10% SDS).

Light microscopy

Performed on settled haze particles extracted directly from the bottom of commercial tetra-pak cartons with a pasteur pipette. The sample was covered with a coverslip prior to examination and photographed using a Zeiss standard microscope equipped with a MC 63 photographic system.

Transmission electron microscopy

Samples were prepared for microscopy by placing a drop of carefully resuspended haze on a carbon coated, copper grid for 1 minute. Excess sample was touched off to filter paper and the grid stained negatively with 2% uranyl acetate. The air dried grids were examined in a JOEL 1130 transmission electron microscope operated at 60 kV.

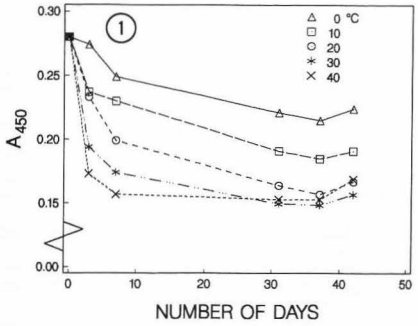
Results and Discussion

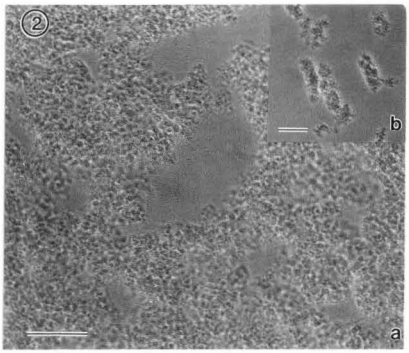
Qualitatively, the amount of haze present increased with increasing time and temperature of storage. This increase was reflected in a decrease in juice absorbance in the region around 450 nm as haze material formed, with no increase in absorbance in the spectral region around 700 nm. This is shown quantitatively in Figure 1 where the drop in absorbance at 450 nm is apparent as a function of both temperature and time. Clearly temperature has the greatest effect on both the rate and extent of haze development. The quantity of haze isolated from various manufacturing batches of juice varied between 0.3 and 1.7 mg dry weight/liter of juice depending on production time and tending to be greater toward the later production dates. Protein in the haze varied from 11.4 to 29.0% (w/w) in agreement with literature estimates. Atomic adsorption analysis of sulphuric acid digested haze detected only trace quantities of calcium and magnesium, and qualitative tests (van Buren, 1989) for iron and copper were negative. Paper and high pressure liquid chromatography of hydrochloric acid digests of haze provided no evidence for polymeric carbohydrate (Block *et al.*, 1952; Beveridge *et al.*, 1986). Semi-quantitative, colorimetric tests for polyphenol (van Buren, 1989) suggested that 45.7% to 75.8% (w/w) of the haze was polyphenol in nature and strongly positive qualitative tests for phenolics were also obtained. Quantitative aspects of these tests must be considered cautiously since the color yield depends upon the type of phenolic material making up the haze and its degree of polymerization (Gramshaw, 1970). However, it is a reasonable assumption that the bulk of the non-protein material is polyphenolic.

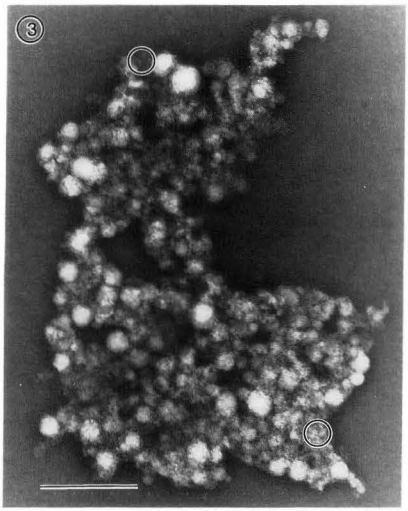
Light microscopy (Fig. 2) of the haze particles settled from the juice revealed an amorphous, granular material very similar to the haze particles shown in the photographs reproduced in Lea (1990). These large aggregates were easily disrupted into much smaller particles by agitation. On standing, however, the particles could be observed to aggregate once again by interparticle adherence during random collisions. This would suggest the presence of attractive forces between particles or the particles seem to be "sticky". The particles were readily stained by methylene blue or amido black 10B (1% in acetic acid), the latter dye indicating the presence of considerable protein consistent with a protein content of 11.4 to 29.0% determined previously.

Transmission electron microscopy (TEM) was performed on haze obtained from the same juice samples as used for light microscopy. Individual "particles" of haze as revealed by TEM showed the presence of large, spherical bodies only somewhat penetrable by the electron dense stain (Fig. 3). These bodies, some of which appear to have a subunit structure of their own, are imbedded within a matrix of other material. Some portions of the aggregated particle, particularly the circled regions, suggest this background material may have a complex substructure. The spherical particles probably represent the protein portion of the protein-polyphenol complex of the haze since spherical shapes such as this are typical of denatured proteins and this assignment would be consistent with the large protein content determined analytically. Examination of a thin portion on one edge of a particle revealed the existence of secondary structure, or perhaps the existence of a second type of structure in the haze particles (Fig. 4). The second structure appears to consist of material polymerized in such a way as to form chain-like aggregates. This material may form the background structure observed in Figure 3, and may consist of polymerized polyphenol, or polyphenol-protein complex. Further study is required to distinguish the chemical composition of the two structural types, or to determine if one structural type is a precursor of the other.

Electrophoresis (SDS-PAGE) of the 2-mercaptoethanol reduced, guanidine hydrochloride dissaggregated haze







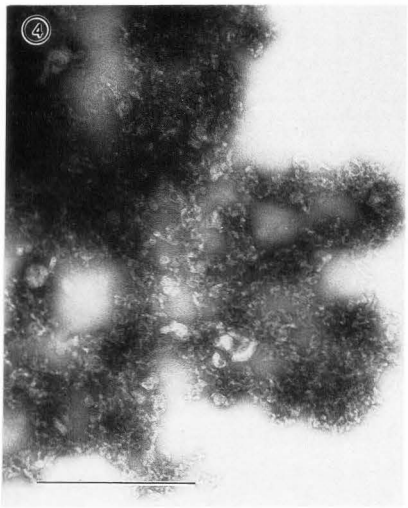


Figure 1. Absorbance at 450 nm of apple juice stored at 0, 10, 20, 30, and 40°C.

Figure 2. Light microscopy of haze particles formed in apple juice after 15 days at 40°C. Insert: Phase contrast microscopy of the same haze particles after agitation. Over time these smaller particles "stick" together as they come in contact to reform the aggregates seen in the larger photograph. Bar = 10 μm.

Figure 3. Negatively stained, transmission electron micrograph of a typical haze particle isolated from apple juice. The aggregation of spherical particles is clearly apparent. Circled areas represent regions of apparent background substructure discussed in the text. Bar = 0.5 μm

Figure 4. Thin edge of a negatively stained haze particle showing the second type of structure forming the haze particle. The chain like aggregates of polymerized material can either form the particle on their own or form the embedding material for spherical aggregates. Bar = 0.5 μm.

material gave no evidence of protein bands as expected for SDS-PAGE of intact protein molecules. Instead a continuous smear was obtained indicating a population of proteins ranging in molecular weight from less than 29,000 to more than 205,000 daltons. The protein exist as individual proteins covalently cross-linked, probably by polymerization of polyphenols as would be expected (Gramshaw, 1970). Further attempts to characterize the protein or to identify possible sources would be fruitless because of this extensive cross-linking.

Electron microscopy has revealed the existence of two internal substructures in protein-phenol haze particles. These structures result from a complex mixture of phenol oxidative, phenol-protein and protein-protein reactions and interactions. How these reactions and interactions result in these complex structures awaits further study, however it is probable that the structures reported here are characteristic of protein-phenol haze, at least in apple juice. Negative staining is a simple technique in electron microscopy, so that its application, in combination with light microscopy may provide a useful diagnostic test for the presence of these protein-phenol complex hazes.

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Discussion With Reviewers

J. Barnowski: It is unusual to not have an increase in absorbance at about 625 nm with haze formation. Measurement of NTU (turbidity) would have been helpful.

R.E. Wrolstad: Re: Figure 1: the authors state that haze increased with time and temperature of storage (apparently this is from visual observation). Figure 1 shows a decrease in A_{450} nm with increasing time and temperature, and in addition, the authors state that there was no increase in absorbance at 700 nm. If instruments designed for measuring haze (nephelometers, transmission colorimeters such as Hunter or Gardiner instruments) are unavailable, absorbance at long wavelengths (650-700 nm) is frequently used to measure haze (light scattering). Absorbance at 450 nm will be a measure of yellow or brown color. An interpretation of Figure 1 which fits the authors hypothesis is that yellow-brown colored melanoidin pigments (oxidized polymeric phenolics) are polymerising and condensing with other compounds, becoming insoluble and precipitating (as haze) from the system. As they come out of solution, a decrease in A_{450} nm is observed. Evidently absorbance at 700 nm is not sensitive enough to measure the observed visual increase in haze.

Authors: When the visible region scans came back as showing decreases in the spectral region around 400-450 nm and no increases in absorbance at 700 nm, we were surprised also. (We used the spectrophotometer since a nephelometer was not available). We had anticipated marked increases in the region around 700 nm. We cannot explain the reason for the results, however, the mixed juice became visually lighter in yellow or brown color as haze formed. The haze which formed in these cartons was of a very fine particulate that was difficult to see and required some practice for its regular, routine detection. Thus we find the results believable. It is also for this reason we chose to use the decrease in absorbance at 450 nm as an index of haze formation.

In answer to Dr. **Wrolstad**, we expected increases in absorbance at 700 nm and only scanned the samples because it was easily done on the instrument and "in case"! We concur with Dr. Wrolstad's interpretation of the events leading up to the results of Figure 1, but the lack of absorbance increase at 700 nm was unexpected.