Food Structure

Volume 7 | Number 2 Article 10

1988

The Effect of Storage, Processing and Enzyme Treatment on the Microstructure of Cloudy Spartan Apple Juice Particulate

D. L. McKenzie

T. Beveridge

FOOD MICROSTRUCTURE, Vol. 7 (1988), pp. 195-203
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0730-5419/88$3.00+.00

THE EFFECT OF STORAGE, PROCESSING AND ENZYME TREATMENT ON THE MICROSTRUCTURE OF CLOUDY SPARTAN APPLE JUICE PARTICULATE

D.-L. McKenzie and T. Beveridge

Agriculture Canada Research Station
Summerland, British Columbia, Canada
VOH 1ZO

Abstract

The effect of blanching, post-harvest refrigerated (4°C) storage and enzyme treatment with polygalacturonase on the microstructure of Spartan apple juice was examined by thin sectioning and negative staining transmission electron microscopy. Particles were categorized as granules (3-54 nm), spheres (20-368 nm) and aggregates (12-2519 nm). Enzyme treatment with polygalacturonase significantly decreased granule size ($p \leq 0.01$). Storage of apples significantly decreased both granule size ($p \leq 0.01$) and aggregate length ($p \leq 0.05$) and also resulted in a web-like aspect in the microscopic appearance of juice particulate. The web-like aspect of the particulate was removed either through enzyme treatment with polygalacturonase or by blanching. Blanching of puree significantly increased granule ($p \leq 0.05$) and sphere size ($p \leq 0.01$), while significantly decreasing aggregate length ($p \leq 0.01$). In addition, blanching stabilized suspended particulate by what appeared to be the formation of a protective colloid which prevented particle aggregation through electrostatic repulsion.

Initial paper received May 27, 1988
Manuscript received August 19, 1988
Direct inquiries to T. Beveridge
Telephone number: 604 494 7711

Key words: Apple juice, Spartan Apples, enzyme treatment, storage, processing, post-harvet refrigeration, blanchung, polygalacturinase, microstructure, juice particles.

Introduction

Production of apple juice in Canada has centered mainly around the clarified, amber type of juice (Atkinson and Strachan, 1949a; Beveridge et al., 1986). In Japan, however, the majority of fruit juices are sold in the cloudy, unoxidized, 'natural' state (I. Yamashita, personal communication, 1987). A 'natural' apple juice can be produced through the inactivation of polyphenol oxidase by blanching the apple puree at 90°C in combination with an ascorbic acid or sulfite pretreatment (Holgate et al., 1948; Atkinson and Strachan, 1949b; Beveridge et al., 1986). Blanching successfully inhibits the development of the brown colour and cider-like flavour characteristic of oxidized juice, producing a naturally coloured, opalescent juice with a fresh apple flavour characteristic of the variety processed (Bauernfeind, 1958). The opalescence or cloud formed as a result of blanching is very stable with only a slight sediment being deposited during storage. On the other hand, the cloud formed in oxidized juice is very unstable, readily flocculating to form an undesirable thick layer of sediment at the bottom of the container (Atkinson and Strachan, 1949b).

The thermal stabilization of the juice cloud by blanching offers the possibility of marketing a 'natural', unoxidized juice in either the opalescent form or, upon cloud destabilization, in the clarified form allowing for further expansion of the apple juice market (Carpenter and Walsh, 1932; Atkinson and Strachan, 1949b). An understanding of the factors contributing to stabilization or destabilization of the cloud formed in apple juice is required to enable the manufacturer to efficiently produce either a cloudy or clarified unoxidized 'natural' apple juice. The objective of the present study was to use electron microscopy to gain a better understanding of the effects of blanching, refrigerated (4°C) storage and enzyme treatment with polygalacturonase on the nature of the cloud particulate present in juice from Spartan apples.

Materials and Methods

Apple Juice preparation

This study examined unoxidized apple juice processed with a blanching step, and oxidized juice processed without a blanching step. Oxidized juice was obtained from Spartan apples harvested at the Summerland Research Station in 1985. Both fresh and stored apples were examined, where 'fresh'

apples were stored at 4°C for two weeks after harvest and then processed, while 'stored' apples were stored for nine months at 4°C before processing. Apple maturity was measured by testing firmness and starch content. Fresh Spartan apples had an average starch rating of five (Lau, 1985), and an average firmness of 69.3 N as tested by a Magness-Taylor pressure tester with a 7.8 mm probe. Stored Spartan apples had an average starch rating of nine and an average firmness of 44.4 N as tested by a Magness-Taylor pressure tester with a 7.8 mm probe. The starch test involved transversely bisecting 10 apples perpendicular to the core and immersing the freshly cut surface of the top half of the fruit in a dilute iodine solution for one minute. The starch test gave a measure of apple maturity based on a nine-point scale where a rating of one represented an immature apple with the whole cut surface reacting to turn blue, while a rating of nine corresponded to an overmature apple in which none of the cut surface turned blue (Lau 1985).

A smooth puree was produced from 10 kilograms of apple by blending batches of 500 g of destemmed apple cut into two centimeter cubes with 200 ml of a 200-500 ppm sulfite solution (sulfite as potassium metabisulfite). The concentration of sulfite was adjusted within the range stated so that browning was only just inhibited prior to blanching. Juice was expressed from the puree by centrifugation at 7700 x g for 10 min with a Sorvall RC-5 centrifuge equipped with an SS-34 (10.7 cm) rotor. Enzyme-treated juice was prepared by incubation of the puree with 0.1% Irgazyme 100 (CIBA-GEIGY Corp.), a polygalacturonase with lyase and pectinesterase side activities, for 1 hr at 45°C. Juice was expressed from the enzyme-treated puree as described above. Juice and puree were frozen and stored at -18°C until required.

Unoxidized juice was produced as above with batches of the apple puree blanched as described by Beveridge et al (1986). The puree was heated to over 90°C for at least 25 sec, which was sufficient to destroy apple polyphenoloxidase (Beveridge and Harrison, 1986). After processing, the separate batches of puree were mixed together in a Hobart H 600 mixer, frozen and stored at -18°C.

Transmission Electron Microscopy (TEM)

All samples were examined with a Philips EM 300 transmission electron microscope operating at 60 kV. With the exception of thin sectioning, each treatment was performed twice; duplicate samples were prepared within treatments, then representative sections were photographed. Unless otherwise stated, measurements of particle dimensions were taken of 10 randomly selected particles within each particle category. The range and mean of these measurements were recorded along with descriptions of particle stain density.

Thin Sectioning

Duplicate 2 mL aliquots of juice were dialyzed overnight against 4 L distilled water at room temperature. The water was changed after the first hour of dialysis and again before the last hour of dialysis. Dialyzed juice was stored refrigerated (4°C) until used. Pellets of juice cloud were obtained by centrifugation of dialyzed juice at 343,000 x g for 30 min with a Beckman L8-M ultracentrifuge. The pellets were fixed in a 2% osmium tetroxide - 0.01M cacodylate buffer solution at pH 7.0 for 1 h, dehydrated in 50%, 70%, 95%, absolute ethanol and propylene oxide (two times each for 10 min), embedded in Epon 812 and cured at 60°C. Thin sections [60-90 nm (Hunter, 1984)] were obtained using a Reichert OM U2 ultramicrotome. The sections were stained first with 5% uranyl acetate for 20 min, washed in distilled water and then stained with Reynold's lead citrate in combination with 0.01 N NaOH (Sjostrand, 1967) for 10 min, washed with distilled water and placed on filter paper in a covered petri dish to dry.

Negative Staining

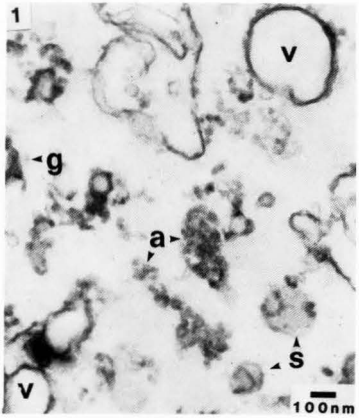
Copper grids (400 mesh) were prepared with a collodion support film (0.5% collodion in amyl acetate) and coated with carbon in an Edwards E306A high vacuum coating unit. The grid was placed on a drop of dialyzed juice for 5 min, transferred to a drop of distilled water for 1 min and washed with 10 drops 2% uranyl acetate. Excess stain was removed by touching the edge of the grid with a piece of filter paper. Grids were then air dried in a covered petri dish.

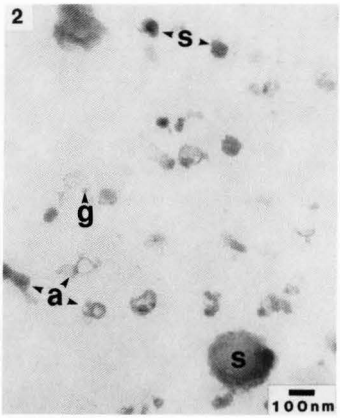
Results and Discussion

Thin Sectioning

Thin sections from pellets of cloud material from oxidized juice of fresh Spartan apples exhibited a high concentration of particles with a variety of structural characteristics and varying affinities for electron dense stains (Fig. 1). The structures were categorized as granules (g), spheres (s), aggregates (a) and vesicles (v) as tabulated in Table 1. Particle classification was complicated by the possible introduction of sectioning artifacts which could affect particle appearance and distribution (Hayat, 1981). Large vesicles with electron dense membranes were the most prevalent structures in sections of oxidized juice cloud (Fig. 1). Smaller structures such as spheres and granules were found not only individually, but also attached to the surface and within the interior of vesicles (Fig. 1). Spheres and granules also appeared to combine to form larger electron dense aggregates (Fig. 1). These structures were likely derived from fragments of cell walls and other cellular debris created during processing.

The varying affinity of vesicles, aggregates, spheres and granules for the electron dense stains was either an artifact of sectioning or indicated these structures were compositionally different. Since structures maintained these relative stain densities from section to section and from block to block, the differences were considered to be primarily compositional. The more electron dense aggregates and vesicular membranes in Figure 1, may have contained a greater number of exposed heavy metal binding sites than the less densely stained spheres and granules. Of the stains used, osmium reacts with proteins, lipids and membranes whereas lead reacts with hydroxyl groups of carbohydrates and sulfhydryl groups of protein, and uranium is bound by carboxyl and phosphoryl groups (Hayat, 1972; 1981). Studies have also shown that 2% uranyl acetate followed by lead citrate effectively stains cellulose (Hayat, 1981). Considering the composition of apple tissue, the most probable binding sites in the juice cloud would be hydroxyl and sulfhydryl groups of proteins from cell cytoplasm and membranes, with hydroxyl groups of polyanionic carbohydrates such as pectin from the middle lamella and phosphoryl groups of phospholipids from membranes





Figs. 1 and 2. Thin Section of pellets of cloud material from oxidized (Fig. 1) and unoxidized (Fig. 2) juice of fresh Spartan apples. Aggregate (a); granule (g); sphere (s); vesicle (v).

Table 1. Characterization of particles in thin sections of cloud material from the juice of fresh Spartan apples

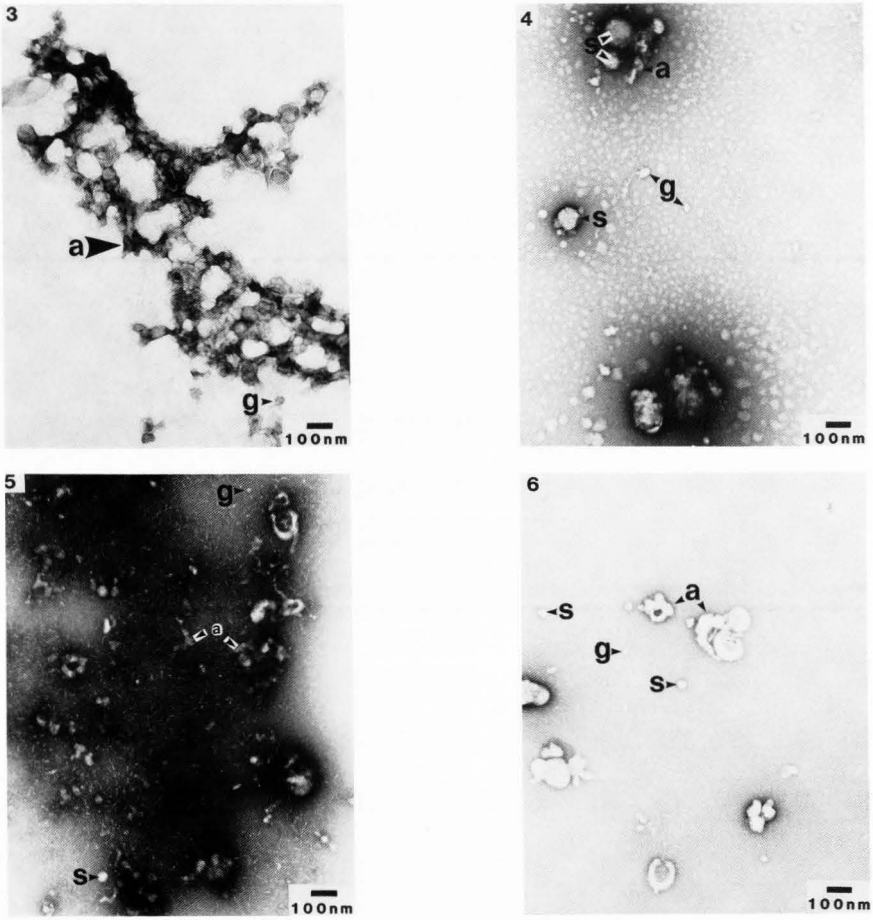
Sample	Particle	Dimension (nm)		Stain density*
		Range	Mean	
Oxidized juice cloud				
	granule	18 - 32	25 diam.	slight-moderate
	sphere	56 - 28	152 diam.	slight-moderate
	aggregate	70 - 679	367 length+	moderate-extreme
		35 - 275	157 width	
	vesicle	96 - 410	273 diam.	
		6 - 39	20 wall	extreme
Unoxidized juice cloud				
	granule	15 - 37	22 diam.	slight-moderate
	sphere	50 - 335	117 diam.	moderate-heavy
	aggregate	12 - 342	180 length+	moderate-heavy
		50 - 157	77 width	

* Extreme = black; slight = just discernible over background.

+ Using the least significant difference test, aggregate particles in cloud from oxidized juice were significantly longer ($p \leq 0.05$) than similar particles in cloud from unoxidized juice.

accounting for a smaller number of the binding sites (Hayat, 1981). Although the formation of aggregates could be an artifact of the dehydration procedure, the presence of heavy metal binding sites suggests aggregation could also be caused by hydrogen bonding between exposed groups on the surfaces of the spheres and granules.

Thin sections of cloud material from unoxidized juice of fresh Spartan apples (Fig. 2) had fewer particles with less variety of structural

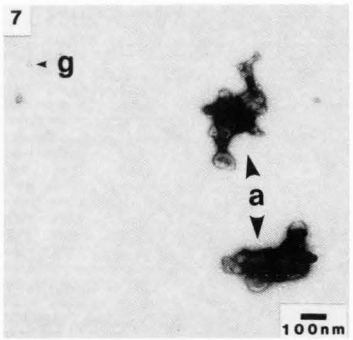


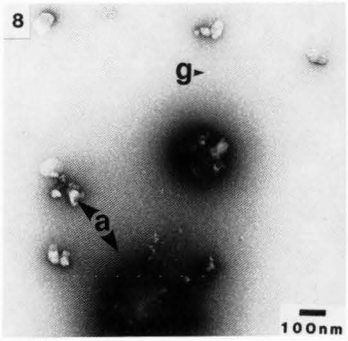
Figs. 3 - 6. Negatively stained particles from oxidized (Figs. 3 and 5) and unoxidized (Figs. 4 and 6) juice of fresh (Figs. 3 and 4) and stored (Figs. 5 and 6) Spartan apples not treated with enzyme. Aggregate (a); granule (g); sphere (s).

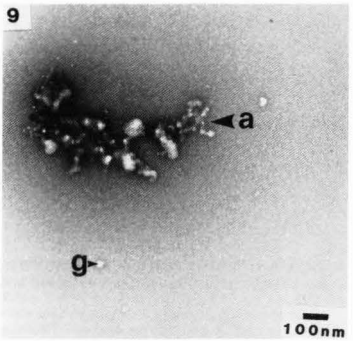
characteristics and a more uniform affinity for the electron dense stains than the structures of oxidized juice (Fig. 1 vs 2). Vesicles were not found in cloud from unoxidized juice and a comparison of other structures presented in Table 1 indicated that aggregates in unoxidized juice were significantly smaller ($p \leq 0.05$) and less distinct than those in oxidized juice. Blanching appeared to cause the disintegration and solubilization of aggregates observed in oxidized juice to form relatively smaller structures and more electron dense background in cloud from unoxidized juice (Fig. 1 vs 2).

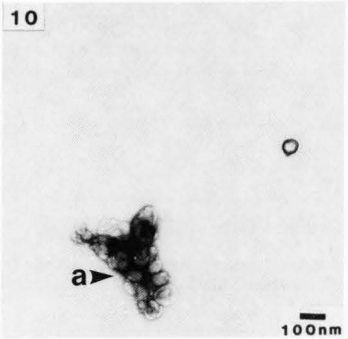
Negative Staining

The dimensions of particles from negatively









Figs. 7 - 10. Negatively stained particles from oxidized (Figs. 7 and 9) and unoxidized (Figs. 8 and 10) juice of fresh (Figs. 7 and 8) and stored (Figs. 9 and 10) Spartan apples treated with enzyme. Aggregate (a); granule (g).

stained juice samples (Figs. 3-10) are presented in Table 2. The structures were again categorized as granules (g), spheres (s) and aggregates (a) based on dimensions and stain density; no vesicular structures were present. Since vesicles were found only in thin sections of oxidized juice, it is likely that these structures were formed either as artifacts of centrifugation and alcohol dehydration or were cross-sections of spheres. Granules, spheres and aggregates on the other hand, were observed in each preparation of juice, although the relative proportions of the particles varied dramatically between preparations (Fig. 3 vs 4, 7 vs 8). Statistical analyses showed that treatment with enzyme significantly decreased granule size ($p \leq 0.01$), storage of apples also significantly decreased granule size ($p \leq 0.01$) and aggregate length ($p \leq 0.05$), and processing significantly increased granule ($p \leq 0.05$) and sphere size ($p \leq 0.01$) while significantly decreasing aggregate length ($p \leq 0.01$; Table 2).

Examination of Figs. 3-10 revealed several trends. Unoxidized juice from blanched puree not treated with enzyme had a larger number of smaller particles in the form of granules and spheres, whereas similarly prepared samples of oxidized juice consisted of fewer particles mainly in the form of aggregates (Figs. 3 vs 4). Blanching also significantly decreased aggregate length ($p \leq 0.01$), while significantly increasing granule size ($p \leq 0.05$) and sphere diameter ($p \leq 0.01$; Table 2). The aggregates appeared to be agglomerations of spheres and granules and could be an artifact of drying. However, aggregates were formed mainly in oxidized juice not treated with enzyme (Figs. 3, 5) and not in similarly prepared samples of unoxidized juice (Figs.4, 6). Since all grids were prepared in the same manner, the formation of aggregates in certain juice preparations and not in others suggests forces other than surface tension effects were involved in particle aggregation. More likely, the increased aggregation of

Table 2 - Characterization of particles on negatively stained grids of juice from Spartan apples.

Particle	Significant Treatment Effects		Dimensions (nm) Range	Dimensions (nm) Mean	Stain Density+
Granule (diameter)	i) Enzyme**	No enzyme	5 - 54	19	transparent
		Enzyme	3 - 20	12	
	ii) Storage**	Fresh	5 - 54	19	
		Stored	3 - 20	19	
	iii) Processing*	Unoxidized	3 - 54	17	
		Oxidized	5 - 20	14	
Sphere (diameter)	i) Processing**	Unoxidized	35 - 368	110	Slight-moderate with heavy envelope
		Oxidized	20 - 73	47	
Aggregate (length)	i) Processing**	Oxidized	35 - 2519	623	Slight-heavy with extreme envelope
		Unoxidized	60 - 1346	364	
	ii) Storage*	Fresh	35 - 2519	568	
		Stored	59 - 1346	400	
Aggregate (width)	not significantly different		17 - 611	155	

+ Extreme = black; slight = just discernible over background.

** Means significantly different at $p \leq 0.01$; * Means significantly different at $p \leq 0.05$.

particulate in oxidized juice results from the action of endogenous pectin methylesterase. This enzyme converts pectin to pectic acid which can react with calcium and other divalent ions to cause aggregation through the formation of calcium pectate bridges (JA Klavons, personal communication, 1988).

Compositional differences also existed between the particles forming the aggregates as indicated by the variation in stain density of the components within the aggregates (Fig. 3). Although negative staining with uranyl acetate was carried out, it appeared that some particles were negatively stained while other particles were positively stained (Fig. 3). Again, this suggested the presence of binding sites for uranium, e.g., protein carboxyl groups, pectin-like polyanionic carbohydrates which could be involved in hydrogen bonding and particle aggregation. Overall differences in staining were also observed between unoxidized and oxidized juices (Fig. 3 vs 4, 5 vs 6, 7 vs 8, 9 vs 10). Particles in oxidized juice were more heavily stained than similar particles in unoxidized juice which had only slight or moderately stained interiors surrounded by a diffuse layer of densely stained material (Fig. 3 vs 4). The presence of a negatively charged, heavy metal attracting envelope would stabilize the cloud particles in unoxidized juice not treated with enzyme by preventing the aggregation of particles through charge repulsion.

Storage of apples and no enzyme treatment during processing produced cloudy oxidized juice in which a small number of particles were embedded in a densely stained web-like matrix (Fig. 5). The particles also appeared to become less compact and less distinct than their counterparts in fresh juice (Fig. 3 vs 5) probably as a result of the gradual disintegration of cell structure during storage (Hulme, 1958; Hulme and Rhodes, 1971). The affinity of the web-like material for the uranyl acetate stain provided further evidence for the presence of uranium binding sites with potential for hydrogen bonding. The formation of a web-like matrix suggested the establishment of a gel network perhaps due to protein-protein or protein-pectin interactions (Fig. 5). The existence of a gel-like network which allowed for entrapment and imbibition of water, might account for the low yields of juice obtained when pressing stored fruit (Powrie and Tung, 1976; Glunk, 1981). Treatment with heat during blanching or Irgazyme 100 appeared to greatly reduce or eliminate the formation of a web-like matrix (Fig. 5 vs 6, 5 vs 9). The possibility therefore exists for improving juice yields by employing either a blanching step (Beveridge and Harrison, 1986) or an enzyme treatment when processing stored apples. Storage of apples resulted in a significant decrease in granule size ($p \leq 0.01$) and aggregate length ($p \leq 0.05$) in the juice (Table 2). The decrease in aggregate size was evidence for destructive processes occurring during storage.

The treatment of apple puree with Irgazyme 100 resulted in a significant decrease in granule size ($p \leq 0.01$, Table 2) as well as a decrease in the number of

particles present in the juice (Fig. 3 vs 7, 4 vs 8, 5 vs 9, 6 vs 10). The majority of particles present in oxidized juice treated with enzyme were granules most likely formed from the disintegration of larger aggregates (Figs. 7, 9). In contrast, treatment of unoxidized juice with enzyme resulted mainly in the formation of aggregates (Fig. 8). Aggregate formation in unoxidized juice after treatment with enzyme may have resulted from the enzymatic degradation of the intensely stained material surrounding the particles observed in the juice from untreated puree (Fig. 4 vs 8). Degradation of the enveloping material would reduce interparticle repulsion. The resulting interaction of particles would lead to the formation of a larger number aggregates than in untreated juice (Fig. 4 vs 8). Enzyme treatment of unoxidized juice seemed to follow several stages of degradation before reaching the same level of clarification observed in oxidized juice, thus accounting for the longer incubation times or larger amounts of enzyme required to clarify unoxidized juice (Beveridge et al., 1986). In oxidized juice, the enzymes appeared to directly reduce particle size and number (Figs. 3 vs 7). However, in unoxidized juice the enzymes first reduced the thickness of the enveloping material, followed by the formation of numerous aggregates (Fig. 8), before a decrease in particle number and degradation of aggregates became apparent (Fig. 10).

Enzyme treatment of stored apples appeared to be more effective for reducing cloud than enzyme treatment of fresh apples. Juice from stored apples treated with Irgazyme 100 generally had fewer particles than similar preparations of juice from fresh apples (Fig. 8 vs 10).

Conclusions

Particle dimensions as measured in thin sectioned and negatively stained apple juice showed that no significant difference existed with respect to aggregate dimensions ($p \leq 0.05$). However, the dimensions of the spheres and granules in thin sections of apple juice cloud were significantly larger ($p \leq 0.05$) than similar particles in negatively stained preparations (Table 1 vs Table 2). The fixation procedure for thin sectioning is more severe than for negative staining with a greater potential for introduction of artifacts and distortion of particles, possibly accounting for the differences in particle dimensions observed by these two techniques. Although statistical differences were noted, overall the appearance (Fig. 2 vs 4) and dimensions of the particles (Table 1 vs Table 2) observed by these two different techniques were very similar. Of the two techniques, thin sectioning was more time-consuming and therefore impractical for a large number of samples, while negative staining was simple and rapid, supplying substantial structural information as well as compositional information based on particle-stain interactions.

For the apple juice manufacturer, production of a cloudy unoxidized juice appears possible from either fresh or stored apples since stored apples produce juice with more particulate, while unoxidized juice from fresh apples is more resistant to enzyme clarification indicating a more stable suspension. Clarified unoxidized juice could also be made either by filtration of juice from fresh apples or by enzyme treatment of juice from stored apples, allowing the processor to produce several different products from a single raw material.

Acknowledgements

The authors gratefully acknowledge the generous donation of microscope time and associated preparatory and photographic materials by the Agriculture Canada Research Station, Vancouver, B.C. The authors thank Mr. F. Skelton and Dr. F. Leggett for their technical advice and guidance throughout this investigation.

Contribution number 691.

References

1. Atkinson FE, Strachan CC (1949a). Natural Apple Juice. Food Industries of South Africa, June: 37-39.

2. Atkinson FE, Strachan CC (1949b). Production of Juices. Agriculture Canada Technical Bulletin No. 68. 16-32.

3. Bauernfeind JC (1958). The Role of Ascorbic Acid in the Browning Phenomenon of Fruit Juice. F. Hoffmann-La Roche and Company, Ltd. Basil, Switzerland. p. 172.

4. Beveridge T, Harrison J (1986). Pear Juice Production from Heated Pear Mashes. Can. Inst. Food Sci. Technol. J. 19(1): 12-16.

5. Beveridge T, Franz K, Harrison J (1986). Clarified Natural Apple Juice: Production and Storage Stability of Juice and Concentrate. J. Food Sci. 51(2): 411-414, 433.

6. Carpenter DC, Walsh WF (1932). The Commercial Processing of Apple Juice. New York State Experimental Station Technical Bulletin No. 202. 3-21.

7. Glunk U (1981). Increasing the Juice Yield of Pressing with Special Remarks on the Warm Extraction of Apples. Flussiges Obst. 8: 248-255.

8. Hayat MA (1972). Principles and Techniques of Electron Microscopy: Biological Applications. Vol. 2. Van Norstrand Reinhold Co. New York. p. 30-151.

9. Hayat MA (1981). Principles and Techniques of Electron Microscopy: Biological Applications. Aspen Publishers, Inc. Rockville, MD. 301-316.

10. Holgate KC, Moyer JC, Pederson CS (1948). The Use of Ascorbic Acid in Preventing Oxidative Changes in Apple Juice. The Fruit Products J. and American Food Manufacturer 28: 100-112.

11. Hulme AC (1958). Some Aspects of the Biochemistry of Apple and Pear Fruits. Advances in Food Research 8: 297-413.

12. Hulme AC, Rhodes JC (1971). Pome Fruits. In: The Biochemistry of Fruits and Their Products. AC Hulme (Ed.). Academic Press. London. 333-373.

13. Hunter EE (1984). Practical Electron Microscopy: A Beginner's Illustrated Guide. Praeger Publishers. New York. p. 65.

14. Lau OL (1985). Harvest Indices for British Columbia Apples. British Columbia Orchardist July: 7-13.

15. Powrie WD, Tung MA (1976). Food Dispersions. In: Principles of Food Science Part I: Food Chemistry. OR Fennema, (ed.). Marcel Dekker, Inc. New York. p. 554.

16. Sjostrand FS (1967). Electron Microscopy of Cells and Tissues. Vol. 1. Instrumentation and Techniques. Academic Press. New York. p. 300.

Discussion with Reviewers

J.A. Klavons: In citrus juices, "cloud" is defined as particles that scatter visible light, (0.4-0.8 μm). It is generally isolated via centrifugation and therefore contains particles up to approximately 50 μm. Particles larger than 50 μm are considered " pulp". This paper deals with particles (or aggregates) up to approximately 2.5 mm. In the case of citrus juices, the cloud (particles up to 50 μm) have a different composition than do the larger "pulp" particles. As the particle size increases, the ratio of pectin to protein increases. Large "pulp" particles contain much pectin. The physical and chemical properties of pectins are complex and the authors mention them to some degree. However, a very important aspect of cloud stability in citrus juices is the heat inactivation of pectin methylesterase. In unpasteurized (unheated) citrus juices pectin methylesterase converts methoxy pectin to pectic acid. Pectic acid reacts with calcium, or other divalent ions in the juice to form calcium pectate, which destabilizes the cloud and results in cloud loss. Probably, apple juice (and apple puree) that has not been heat inactivated also contains pectin methylesterase (in addition to the pectinase that the authors mention). It would appear that in the cases of non-heat treated apple juice, this phenomenon could account for the aggregation of the particles.
Authors: The particles observed in apple juice ranged from 0.003 μm to 2.5 μm (scales were in nm not μm dimensions). Based on these measurements, the particles can be classified as "cloud" rather than "pulp". A discussion of pectin methylesterase and its possible role in the formation of aggregates through calcium bridging was considered important and so included in the text.

J.A. Klavons: I question the use of such high g-force (343,000 x g) for the isolation of such large particles. In the case of citrus cloud (as defined above) centrifugation at 27,000 x g for 15 minutes is sufficient to reduce the turbidity by 99%. The authors mention that some of the aggregation they are observing could be due to this treatment. I feel that this is a very real possibility.
Authors: We agree that some of the aggregation observed is probably due to this treatment, however, these centrifugal forces are required to completely sediment all cloud particles in the juice.

J.F. Chabot: What was the reason for the dialysis step?
Authors: The dialysis step was used to remove sugars which otherwise caused the collodion support film to split in the presence of the electron beam.

J.F. Chabot: Why not add fixative directly to the juice?
Authors: On negatively stained preparations, addition of fixative directly to juice prior to staining did not improve the quality of the grids so stain alone was used.

J.F. Chabot: Why fix first in osmium tetroxide, instead of glutaraldehyde?
Authors: Since only one fixation step was used, osmium tetroxide (rather than glutaraldehyde) was chosen as a fixative due to its ability to increase contrast.

J.F. Chabot: Do you think there would be a difference in size distribution if you fixed first, and then centrifuged, versus fixing the precipitate after centrifugation?
Authors: There would probably not be a difference, since in negatively stained preparations use of fixative prior to staining did not appear to alter the size distribution of particles present, however, this was not explicitly tested with the centrifuged particles.

J.F. Chabot: There seemed to be no definitive structures that could be related to normal cells in apples. Were no wall found? These usually have a substructure which is characteristic. It was impossible to relate granules and spheres to normal cytoplasm. Were the spheres lipid in nature?
Authors: No walls were found; however, based on the severity of the processing treatments this was not unexpected. Considering the low levels of lipid material present in apple juice, the spheres are more likely to be proteinaceous or pectinaceous in nature.

J.F. Chabot: Did blanching result in a loss of vesicles because of the effect on membranes?
Authors: Blanching could possibly have resulted in a loss of vesicles through heat-induced solubilization of the middle lamella with resulting dispersion of cell wall material.

J.F. Chabot: If all treatments were processed for transmission electron microscopy in the same manner, why would you attribute measured differences between treatments to artifact?
Authors: The fact that measurable differences existed was, on the contrary, used to suggest that the differences were real and not artifact.

J.F. Chabot: Did you examine any sections with different staining procedures, i.e., without lead, or without uranium salts?
Authors: No; however, phosphotungstic acid (PTA) was used with similar results to uranyl acetate in negatively stained preparations.

J.F. Chabot: Why would blanching change staining properties?
Authors: Blanching could lead to conformational changes in the components which might appear as changes in the staining properties of the particulate.

J.F. Chabot: Given the lack of structure in particles in juice, attributing a change as a result of storage to disintegration of cell structure seems unjustified on the basis of the data presented.
Authors: Storage is known to result in degradative changes in the middle lamella binding cells together through the cell walls. What we think we are seeing is a result of these changes in the pectinaceous material of the middle lamella.

J.F. Chabot: Attributing low yield to the formation of a water retaining matrix cannot be done from these pictures. No examination of the apple pulp has been presented.
Authors: This is true, however, it was clear from the centrifuged sediment resulting during yield measurement (3000 x g, 20 minutes) that changes had

occurred which resulted in increased water binding by the material in the pellet.

J.F. Chabot: In Fig. 1, how do you know the "v" is a membrane fragment? Could this structure and the one on the left be fragments of cell wall?
Authors: Yes, it is possible that vesicles could be either fragments of membranes or cell walls.

J.F. Chabot: In Fig.6, how do you distinguish between minor imperfections in the negative stain background from particles?
Authors: Similar structures were seen in other negatively stained grids of this sample as well as in similar samples examined by shadow casting.

K.G. Lapsley: Have other researchers also used the terms granules, spheres, and aggregates to differentiate the structural matter present in apple juice?
Authors: There appears to have been no work done prior to this study on the structure of particulate in apple juice as viewed by electron microscopy. However, electron microscopic investigations of orange juice particulate have used similar descriptors.

K.G. Lapsley: Have you tried any other techniques for compositional analysis?
Authors: Composition of cloud material has been investigated using HPLC and automated nitrogen analysis which suggest that apple juice "cloud" consists mainly of a combination of protein, pectin and cellulose.

G.G. Jewell: Does the quantity of appearance of the particles from the enzyme-treated juice change with either the pH of treatment, or does the presence of enzyme influence the negative stain?
Authors: The effect of treatment at different pH levels was not examined. The presence of enzyme did not appear to alter the effect of the negative stain since particles of similar shapes and dimensions were also obtained by shadow casting.

G.G. Jewell: What effect does incomplete pasteurization have on the structure of the cloud?
Authors: The effect of incomplete pasteurization on the structure of the cloud was not examined, but it would be expected that a gradual shift from a few large aggregate particles to increased numbers of spheres and granules would occur as the severity of the heat treatment increased.