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Nature of the Protein Constituent of Commercial Orange Juice Cloud

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The cloud of five commercial orange juice concentrates contained an average of 52.4% protein. Most of the cloud protein was soluble in the chaotropic reagent 10 M urea–6% citric acid, pH 2.5. The cloud of two ready-to-serve pasteurized orange juices showed much the same behavior as the concentrates, although the total cloud weights and cloud protein contents were less. Some of the cloud appears to be formed during juice processing. The primary causes of the protein insolubility in the juice are due to inherently insoluble protein, complex formation with low molecular weight organic cloud constituents, and covalent bonding to other cloud constituents. Protein–pectin complex formation is believed to play only a minor role.

INTRODUCTION

Research on the composition and the chemical and physical properties of citrus cloud contributes to an understanding of quality factors in citrus juices and their products. Orange juice is the largest volume citrus product with a worldwide market. It is of prime importance to the citrus industry to maintain and improve citrus product quality to remain competitive and to meet consumer demands.

The turbidity of citrus juices is due to a fine suspension of particulate matter known as "cloud". These particles range in size from roughly 0.4 to 5.0 μm. Quality factors such as flavor, color, texture, and aroma are partly attributable to the cloud. If the cloud is mechanically removed from the juice, the resulting "serum" is a bland, watery material.

Maintaining and improving the quality of citrus juices and citrus-based beverages is of ongoing concern to the citrus industry. The major role that cloud plays in the quality of these products necessitates a comprehensive study of all of its components and their interaction with one another.

Citrus cloud is a complex and dynamic system whose properties are best understood through a comprehensive study of all of its components. Previous work on citrus cloud has focused on the causes of its instability in orange juice (Baker and Bruemmer, 1969; Krop, 1974), a problem that is due primarily to soluble factors in the juice and which can usually be prevented by appropriate heat treatment during processing. Previous work on the chemical and physical properties of citrus cloud have included studies by Baker and Bruemmer (1969), Scott et al. (1965), Mizrahi and Berk (1970), Venolia et al. (1974), Venolia and Peak (1976), and Kanner et al. (1982). Recent work on the chemical and physical properties of citrus cloud has focused on commercial lemon juice (Klavons and Bennett, 1985, 1987). Protein is the single most abundant constituent of citrus cloud and contributes significantly to its behavior. Dissimilarities in pH, ionic strength, and processing conditions of lemon and orange juices suggested differences in the cloud of the two juices. To better understand the chemical and physical nature of citrus cloud, we have undertaken a study of the protein constituent of orange juice cloud isolated from commercial juices.

To study the cloud protein from commercial orange juice, we have first solubilized it using the chaotropic reagent urea and subsequently determined to what extent the solubilization can be reversed, via extensive dialysis, to yield the original insoluble cloud protein.

EXPERIMENTAL PROCEDURES

Sources of Juice Samples. Five commercial frozen orange juice concentrates were purchased at local supermarkets and were used to make single-strength samples A–E. Two ready-to-serve pasteurized orange juices (not from concentrates) were purchased at local supermarkets and were used as is (samples F and G).

Preparation of Single-Strength Juice Samples. Samples A–E were prepared by reconstitution of the concentrates with 3 volumes of distilled water. The Brix values of the single-strength juices were determined by refraction as 11.6, 11.5, 11.3, 11.3, and

11.4 °Brix, respectively. The Brix values of the ready-to-serve pasteurized juices, samples F and G, were 11.5 and 12.0 °Brix, respectively. All subsequent protein determinations and cloud weights of the juice samples were corrected to a Brix value of 11.8 (a standard value for single-strength orange juice). The correction was made for each juice by multiplying actual protein or cloud weight values by 11.8/Brix value of the juice. This was done to standardize the values for easier comparison.

Isolation and Extraction of Cloud. Pulp was removed from all samples (A–G) by a low-speed centrifugation: 360*g* for 10 min. The pulpless juice (10 mL) was centrifuged at 27000*g* for 15 min. This produced a supernatant whose optical density (OD) at 600 nm was less than 0.05 (about 1% of the original turbidity of the juice). The supernatant was decanted, and the cloud pellet was redispersed in 10 mL of deionized water by vortexing. The suspension was centrifuged as before and the supernatant decanted. This washing process was repeated. Total cloud weights of samples A–G were obtained at this stage by freeze-drying them to constant weight. Additional cloud samples were further extracted for 1 h with 10 mL of methanol with occasional vortexing and centrifuged as before, and the supernatant was decanted. Methanol-insoluble cloud weights of samples C and E–G were obtained at this stage by freeze-drying them to constant weight. This extraction removed liposomes and most low molecular weight organic constituents and also dehydrated the sample. The remaining methanol-insoluble cloud contains primarily macromolecular components such as protein, pectin, hemicellulose, and cellulose. Additional cloud samples from A–G were dried under a stream of nitrogen. The dried cloud was further extracted for a least 30 min with 0.5 mL of dimethyl sulfoxide (DMSO), followed by addition of 20 mL of isopropyl alcohol to the DMSO–cloud mixture. Precipitation of macromolecular components was allowed to occur for at least 1 h, the suspension was centrifuged, and the pellet was dried as done previously. The DMSO extraction was necessary to remove the flavanone glycoside hesperidin, which, along with other small molecular weight organic constituents, tends to interfere with subsequent analyses. Protein was determined on samples of washed, extracted, and dried cloud. All reagents used were of the highest purity obtainable.

Solubilization of Cloud Protein in 10 M Urea–6% Citric Acid, pH 2.5, and Subsequent Reversal to Initial Juice Conditions. Water-washed cloud samples of A, B, and D (Table I) and samples C and D (Table II) were extracted with methanol followed by DMSO–isopropyl alcohol as described previously. Water-washed cloud samples C and E (Table I), sample E (Table II), and samples F and G (Table III) were washed with water only, with no organic extractions. Cloud samples were then treated with 10 M urea–6% citric acid, pH 2.5 (urea–citrate), overnight at 4 °C, with stirring. Urea, combined with 6% citric acid, pH 2.5, has proven to be a more effective reagent for the solubilization of cloud protein than urea alone (Klavons and Bennett, 1985); thus, it was utilized in this study. The soluble and insoluble fractions of each sample were separated via centrifugation (at 20 °C, to avoid precipitation of urea). The insoluble fraction of cloud samples C and E–G were extracted with DMSO–isopropyl alcohol as described previously (as these samples had not yet received this treatment). All samples were washed twice with deionized water and dried under a stream of nitrogen. Protein was then determined on all of the urea–citrate-insoluble fractions. The soluble fractions were extensively dialyzed vs 1% citric acid, pH 3.7, in Spectrapor membrane tubing of molecular weight cutoff 2000, obtained from Spectrum Medical Industries, Los Angeles, CA. Protein was determined on the reprecipitate (insoluble fraction).

Isoelectric Focusing of Urea–Citrate Soluble Protein. Isoelectric focusing (IEF) was conducted on a sample of orange juice cloud protein to establish its putative heterogeneity. Cloud protein from sample E was solubilized in 10 M urea, with no citric acid, otherwise as described previously. IEF was performed at 4 °C for 75 min on the solubilized orange cloud protein on a 2117 Multiphor unit using an LKB Ampholine PAGplate, pH 3.5–9.5, both from Pharmacia LKB Biotechnology, Uppsala, Sweden. IEF protein standards ranging from p*I* 3.5 to p*I* 9.3 were obtained from Sigma Chemical Co., St. Louis, MO. IEF initial running conditions were 50 mA and 240 V, and the final conditions were 29 mA and 1500 V. The resulting PAGplate was stained with Coomassie Blue R 250 (Sigma).

Treatment of Urea–Citrate-Soluble Cloud Remaining Soluble after Dialysis with Cloud Methanol Extract. Cloud from sample E was washed, extracted with methanol, and solubilized in urea–citrate as described previously. The solubilized cloud was extensively dialyzed to remove the urea, and the resulting protein separated into the soluble and insoluble fractions as described previously. The original methanol extract was evaporated to dryness with a stream of nitrogen. The soluble protein fraction remaining after dialysis was added to the dried methanol extract and gently magnetically stirred overnight vs a control containing no methanol extract.

Simulation of Lemon Juice Conditions in Orange Juices. The pH and ionic strength of the orange juice sera of samples D and E were altered to simulate those conditions encountered in lemon juice to determine what effect they have on the orange juice cloud protein. Samples D and E were reconstituted with distilled water, and enough solid citric acid was added to bring the final citric acid concentration in the diluted juice to 6%. The pH was adjusted to 2.5 with potassium hydroxide and the volume adjusted to that of single-strength juice as described previously. The resulting juices were stored at 4 °C overnight. Cloud from sample D was extracted with methanol followed by DMSO–isopropyl alcohol as described previously. Cloud from sample E was water-washed only.

Protein Analysis of Cloud and Cloud Fractions. The washed, extracted, and dried cloud fractions were solubilized in 0.05 M potassium hydroxide. Protein was determined according to the trichloroacetic acid precipitation method of Schaffner and Weissmann (1973), as modified by Klavons and Bennett (1985), by correlating the protein determined colorimetrically with that obtained via Kjeldahl nitrogen. Kjeldahl nitrogen analysis was performed on four replicates of sample A by Truesdail Laboratories, Inc., Tustin, CA. A conversion factor was obtained for cloud sample A, which correlated the Kjeldahl protein (N × 6.25) value to the TCA protein determination. This conversion factor was used to determine the protein of all subsequent samples according to the TCA protein procedure. Thus, TCA protein value of unknown cloud sample times 1.728 equals the Kjeldahl protein value of the unknown cloud sample.

RESULTS AND DISCUSSION

The total cloud weight and the total cloud protein present in five commercial orange juice concentrates, after adjustment to equal Brix values, were quite similar (Table I). The total weight of commercial orange juice cloud is also quite similar to the total weight of commercial lemon juice cloud, after adjustment to their respective single-strength Brix values (Klavons and Bennett, 1985). The cloud of commercial orange juice concentrates contained an average of 52.4% protein (Table I). This result is similar to that of fresh orange juice cloud obtained from hand-squeezed Valencia orange reported by Baker and Bruemmer (1969). However, the cloud of commercial lemon juice concentrates contained an average of only 29.8% protein (Klavons and Bennett, 1985). The extracted cloud weight amounted to 67.5% of the total cloud weight (Table I). This represents the macromolecular, polymeric fraction of the cloud, including the protein. The remaining 32.5% represents low molecular weight organic components, some of which may be complexed to the polymeric fraction. Commercial lemon juice cloud contained approximately 50% polymeric components and 50% low molecular weight organic components (Klavons, unpublished results).

The proteins from the three methanol-insoluble cloud fractions (samples A, B, and D) showed an average of 82.7% solubilization in urea–citrate, while the proteins from the two water-washed cloud (samples C and E) showed an average of 97.7% solubilization in urea–citrate (Table I). It is not certain whether this difference is meaningful or is due to differences in the composition of the juice

Table I. Total Cloud and Protein Weights of Commercial Orange Juices from Concentrates[a]

	sample				
	A[b]	B[b]	C[c]	D[b]	E[c]
total cloud weight	223 ± 4	211 ± 6	210 ± 3	205 ± 3	254 ± 3
extracted cloud weight			143 ± 5		170 ± 5
total cloud protein	108.9 ± 1.1	112.5 ± 2.2	113.7 ± 2.3	116.3 ± 0.9	123.7 ± 2.3
total cloud protein soluble in 10 M urea–6% citric acid, pH 2.5	88.6 ± 2.9	84.6 ± 3.5	111.2 ± 2.3	106.3 ± 1.1	120.7 ± 2.3
total cloud protein soluble in 10 M urea–6% citric acid, pH 2.5, that reprecipitates when dialyzed vs 1% citric acid, pH 3.7 (orange juice conditions)	51.0 ± 4.6	56.3 ± 1.9	86.1 ± 3.5	72.4 ± 6.2	98.6 ± 2.8

[a] Values given in milligrams per 100 mL of single-strength juice, 11.8 °Brix, ±SEM ($P = 0.05$). [b] Cloud samples washed with water and extracted with methanol followed by DMSO–isopropyl alcohol prior to urea–citrate solubilization. [c] Cloud samples washed with water only prior to urea–citrate solubilization.

concentrates. It is also possible that the methanol extraction of samples A, B, and D sufficiently dehydrated the cloud samples such that a contact problem existed when aqueous urea–citrate was added that resulted in inefficient solubilization. Previous studies on methanol-insoluble lemon juice cloud from concentrate, however, showed 100% solubilization of the cloud proteins in urea–citrate (Klavons and Bennett, 1985). The most dramatic difference in the clouds of commercial orange and lemon juice concentrates occurred in the attempted reversal of the urea–citrate solubilization to the original orange juice conditions (1% citric acid, pH 3.7). In the methanol-extracted orange juice cloud an average of 53.0% of the original cloud (or 64.1% of the urea–citrate-soluble) protein reprecipitated, and in the water-washed cloud 77.7% of the original (or 79.6% of the urea–citrate-soluble) protein reprecipitated (Table I). Only 24.1% of the total (or of the urea–citrate-soluble) lemon juice cloud reprecipitated (Klavons and Bennett, 1985). The difference in protein reprecipitates among the methanol-extracted and water-washed orange juice cloud samples suggests the presence of low molecular weight constituents of the cloud that are responsible for some of the protein's insolubility. When these low molecular weight constituents are missing from the cloud due to organic extraction, much less of the cloud protein returns to its original insoluble state.

The methanol extract that was added back to the protein that remained soluble after dialysis caused all of this protein to reprecipitate (sample E, methanol extracted). This confirms the presence of low molecular weight organic constituents in the cloud that complex with otherwise soluble proteins in the original cloud and insolubilize them. In the case of the water-washed cloud, an unknown amount of these low molecular weight organic constituents would be available to insolubilize protein, as some of this material is likely lost upon dialysis. The organic extracted cloud samples indicate that 29.7% of the orange juice cloud protein was rendered insoluble due to a complex with low molecular weight constituents (N.B. 100% − 17.3% − 53.0% = 29.7%). Commercial lemon juice cloud (which was also methanol extracted) contained 75.9% of this material (Klavons and Bennett, 1985). The influence of these low molecular weight constituents on cloud formation and retention is currently being investigated.

The 17.3% of the orange cloud protein insoluble in urea–citrate represents protein which could be covalently linked to other constituents, such as hemicellulose or other polysaccharides. Commercial lemon juice cloud contained none of this material (Klavons and Bennett, 1985). One possible explanation for this would be that the harsher conditions of lemon juice concentrate, which has a pH of less than 2, could hydrolyze covalent protein–polysaccharide linkages.

Via extensive dialysis the urea–citrate-solubilized cloud was returned to its initial conditions in the orange juice (1% citric acid, pH 3.7). Accordingly, an average of 53.0% of the original cloud protein reprecipitated (Table I). This represents the maximum amount of cloud protein in the original juice present as inherently insoluble protein. Alternatively, it could represent protein that has been rendered insoluble due to ionic complexation with another macromolecular component, such as pectin. Commercial lemon juice cloud contained only 24.1% of this material (Klavons and Bennett, 1985).

Table II. Cloud Protein Weights of Commercial Orange Juices from Concentrates under Lemon Conditions[a]

	sample	
	D[b]	E[c]
total cloud protein	98.5 ± 4.1	117.2 ± 2.9
total cloud protein soluble in 10 M urea–6% citric acid, pH 2.5	74.7 ± 4.9	114.3 ± 3.0
total cloud protein soluble in 10 M urea–6% citric acid, pH 2.5, that repricipitates when dialyzed vs 6% citric acid, pH 2.5 (lemon juice conditions)	14.9 ± 2.6	40.3 ± 3.2

[a] Values given in milligrams per 100 mL of single-strength juice, 11.8 °Brix, ±SEM ($P = 0.05$). [b] Cloud samples washed with water and extracted with methanol followed by DMSO–isopropyl alcohol prior to urea–citrate solubilization. [c] Cloud samples washed with water only prior to urea–citrate solubilization.

When orange juice cloud samples were subjected to lemon juice conditions (6% citric acid, pH 2.5), a small portion of the cloud protein dissolved, as determined by comparing the protein values of Table I to those of Table II. This amounted to 15.3% in the case of the methanol-extracted cloud (sample D) and 5.3% of the water-washed cloud (sample E). The orange juice cloud protein solubilized under lemon juice conditions could represent the maximum portion of the original orange cloud protein present as a protein–pectin complex, as this would be expected to dissociate in the high ionic strength environment. When the orange juice cloud subjected to lemon juice conditions was treated with urea–citrate, 75.8% of the methanol-extracted cloud protein (sample D) and 97.5% of the water-washed cloud protein (sample E) were solubilized in urea–citrate (Table II). These results were much the same as that under the original orange juice conditions for the similarly treated samples (Table I). The attempted reversal of the dialysis on the organic extracted juice cloud (sample D, Table II) was dramatically less effective than on the water-washed cloud (sample E, Table II), with 19.9% vs 35.3% of the urea–citrate-soluble protein reprecipitating, respectively. These results follow the same trend as the methanol-extracted and water-washed samples of Table I and further establish the presence of low molecular weight cloud components that affect the protein solubility. The overall lower amount of reprecipitated orange cloud protein under lemon conditions is undoubtedly due to the high ionic strength of the medium and is

Table III. Total Cloud and Protein Weights of Ready-to-Serve Pasteurized Orange Juice[a]

	sample	
	F[b]	G[b]
total cloud weight	152 ± 2	159 ± 6
extracted cloud weight	102 ± 3	106 ± 4
total cloud protein	79.8 ± 1.7	78.6 ± 1.7
total cloud protein soluble in 10 M urea–6% citric acid, pH 2.5	75.1 ± 2.9	72.6 ± 2.0
total cloud protein soluble in 10 M urea–6% citric acid, pH 2.5, that reprecipitates when dialyzed vs 1% citric acid, pH 3.7 (orange juice conditions)	52.4 ± 4.5	57.2 ± 6.2

[a] Values given in milligrarms per 100 mL of single-strength juice, 11.8 °Brix, ±SEM ($P = 0.05$). [b] Cloud samples washed with water only prior to urea–citrate solubilization.

similar to the results obtained previously for lemon cloud protein (Klavons and Bennett, 1985). These results indicate some similarity in the nature of the protein constituents of commercial orange and lemon juice clouds, although their environments (pH and ionic strength) play a role in establishing relative amounts of constituent proteins.

The average total cloud weight of the ready-to-serve pasteurized orange juices (Table III) was 70.5% of the average total cloud weight of the juices prepared from concentrates (Table I). The percentage of methanol-extractable cloud components and the percentage of cloud protein were very similar to those of the concentrates (Tables II and III). The cloud from the ready-to-serve juices was only water-washed prior to urea–citrate solubilization and shows the same high degree of reversal as that of the cloud samples from concentrates, 69.2% of the total cloud protein (Table III). Although the basic protein character of the ready-to-serve juices is essentially the same as that of the concentrates, the lower amount of total cloud indicated that juice concentration conditions may well play a key role in cloud formation.

The IEF performed on the orange juice cloud protein (from sample E) showed a continuous streak containing approximately a dozen distinct bands. All of these proteins had acidic isoelectric points below pH 5.5. This result indicates the extreme heterogeneity of the orange cloud proteins.

Commercial orange juice concentrates are usually blends of different varieties of oranges selected for consistency of product quality. Although the data presented do show consistent trends, unknown variables related to the composition of the juice concentrates may affect empirical percentages of the types of insoluble proteins presented in this study.

We conclude that commercial orange juice cloud protein is a complex, heterogeneous material comprising over half of the total cloud weight. Some of the cloud protein particles appear to be formed during juice processing, considering the lower amounts of total cloud and cloud protein in ready-to-serve pasteurized juice. The chemical and physical properties of the proteins are influenced by their environment (the pH and ionic strength of the juice serum). The insolubility of approximately 53% of the orange cloud protein is due to inherently insoluble protein and perhaps a small amount of protein–pectin complex. Approximately 30% of the cloud protein is present as a complex with low molecular weight cloud constituents. The remaining 17% is likely due to the establishment of covalent bonds, such as with hemicellulose.

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