

Leakage of Anthocyanins from Skin of Thawed, Frozen Highbush Blueberries (*Vaccinium corymbosum* L.)

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ABSTRACT

Factors affecting the tendency of thawed blueberries to leak pigmented exudate were investigated. Drip and anthocyanin leakage rates (ALR) were determined spectrophotometrically. Leakage vs time curves were linear or two-phase linear, ALR varying with cultivar, ripeness, and berry condition. Dewaxing increased ALR with most cultivars. ALR did not correlate with berry anthocyanin content, surface area, or cuticle thickness. ALR and amount of drip were poorly correlated. ALR varied from berry-to-berry within samples. Leakage was observed to be nonuniform on berry surfaces, appearing at skin cracks and ruptures, the calyx area, and other point sources. An hypothesis relating leakage to skin condition, fluid accumulation, and anthocyanin content is presented.

INTRODUCTION

THE LEAKAGE of pigmented exudate through the skin of frozen blueberries during thawing (drip) may detract from product appearance. The extent of drip with frozen strawberries depends on cultivar (Ferry and Cabibel, 1973) and freezing rate (Kaloyereas, 1947). Crivelli and Rosati (1975) reported cultivar differences in drip loss from thawing raspberries and thornless blackberries. Previously, we reported differences in the tendency of highbush blueberries to leak during thawing, Elliott berries being especially prone to this behavior (Sapers et al., 1984b). Differences in SEM images of the epicuticular wax were seen between this cultivar and Burlington, a cultivar not prone to leakage.

With sirup-packed berries, solutes including pigments diffuse from the fruits into the sirup during frozen storage at temperatures above -18°C (Guadagni and Nimms, 1957). Moon et al. (1936) considered the color of frozen blueberries to be enhanced by the pigmented sirup resulting from this process. Guadagni et al. (1960) demonstrated that the diffusion of pigments from frozen raspberries to sirup follows first order kinetics, the rate constant obeying the Arrhenius equation. In a previous study of anthocyanin leakage from raw or cooked highbush blueberries, we obtained linear or two-phase linear leakage vs time curves, leakage rates varying among the cultivars compared and appearing to be associated with the incidence of skin rupturing and with berry pigment content (Sapers et al., 1985). Our objectives in the present study were to determine the mechanism of anthocyanin leakage from thawing blueberries and the basis of cultivar differences in the tendency to leak.

MATERIALS & METHODS

Sample preparation and measurement of leakage rates (dynamic system)

Samples of nine highbush blueberry cultivars (Berkeley, Bluecrop, Bluetta, Burlington, Coville, Earliblue, Elliott, Jersey, and Weymouth) were obtained from the USDA, Rutgers University Blueberry and Cranberry Research Center in Chatsworth, New Jer-

sey in 1982 (two harvests) and 1983. Cleaned, dry berries were packaged in 1/2-gallon polyethylene freezing containers and frozen at -13°C , the containers being lined up in a single row directly facing the blower to assure uniform and rapid freezing. After several days' equilibration, the containers were transferred to the freezer shelves and stored for as long as 18 months.

Anthocyanin leakage was determined with 50g portions of blueberry samples, sorted to exclude atypically large, small, or defective berries and weighed in the frozen state. The berries were thawed overnight at 3°C and then equilibrated at 25°C for 1 hr. Immediately prior to the determination, drip was removed from the thawed berries by rinsing with three successive 35-mL portions of distilled water. The rinsings were collected under suction, combined and retained for volume measurement and spectrophotometric analysis. After draining and rinsing, selected samples were dewaxed in CHCl_3 prior to the leakage measurements. Leakage was determined in a "dynamic" model system by stirring the rinsed or dewaxed, rinsed berries in 500 mL distilled water under standardized conditions; taking aliquots at 4-min intervals over 24 min; filtering the aliquots; diluting the aliquots with pH 3 McIlvaine's buffer; and measuring their absorbance at 519 nm, the absorption maximum of blueberry anthocyanins at this pH, with a Perkin-Elmer Model 552 UV-visible spectrophotometer. Leakage rates were obtained by plotting absorbance vs stirring time curves and measuring their slopes. Procedures for dewaxing and determining leakage rates were described in detail previously (Sapers et al., 1985). The combined rinsings from the thawed berries were diluted with an equal volume of pH 3 buffer and analyzed spectrophotometrically at 519 nm. Data on the condition and number of berries (used to estimate total surface area Sapers et al., 1985) in each portion evaluated for leakage were recorded. Leakage data were compared with values of the total anthocyanin content, titratable acidity, soluble solids content, and soluble solids-acidity ratio (SS/A) determined previously on each berry sample (Sapers et al., 1984a).

Berry-to-berry variation in leakage

To determine whether leakage from thawed samples was subject to berry-to-berry variation, the individual berries in a 50g sample that had been thawed and rinsed by the procedures described above were blotted on absorbent tissue and then distributed in 3 oz plastic cups, each cup containing 20 mL distilled water. After 24 min at approximately 20°C (without stirring), 20 mL pH 3 McIlvaine's buffer was added to each cup, and after mixing, the coloration of the liquid was evaluated subjectively.

To compare potential causes of leakage, sets of five matched berries that were found to be similar in leakage behavior after 15 min in the static system described above, were selected for study. The berries were treated by making a 5-mm slit in the skin with a razor blade, abrading the skin by scraping with the blade perpendicular to the skin, or removing a section of skin with the razor blade. Leakage rates were measured with a scaled-down version of the dynamic system used for 50g samples. Each matched set of berries was added to 100 mL distilled water in a 150 mL beaker and stirred with a 25 mm magnetic stirring bar under standardized conditions for 24 min. Aliquots were taken after 8, 16, and 24 min, diluted with an equal volume of pH 3 buffer, and analyzed spectrophotometrically.

Examination of individual berries

Individual berries from frozen samples were thawed and rinsed as described above. Cross sections of fresh, frozen, and thawed berries were compared visually to determine the extent of anthocyanin diffusion from the skin into the berry interior. After rinsing to remove adhering drip, thawed berries were oriented in different positions and blotted on absorbent tissue to locate sources of leak-

age on the berry surfaces. Leakage sites also were observed with a Bausch and Lomb StereoZoom microscope at a magnification of 14-60X.

Measurement of skin thickness

Cuticle and epidermal cell wall thickness were determined for Bluetta and Elliott blueberries by microscopic examination of embedded sections. The skin specimens (approx 1 mm cubes) were immersion-fixed with 4% glutaraldehyde in 0.01M Na cacodylate pH 7 for 4 hr at room temperature, postfixed with 1% OsO₄ in the same buffer for 2 hr, dehydrated in a graded ethanol series, and embedded in Spurr resin. Following polymerization, sections 1 μm thick were cut with an LKB IV Ultratome and mounted on glass slides, which were later cover-slipped. Measurements of skin thickness were taken with a 16X micrometer eyepiece mounted on an Olympus BH-2 light microscope with a 100X objective. For each cultivar, sections were prepared from five berries, and 40 thickness measurements were made per berry. Skin thickness was operationally defined as the distance from the outer berry surface to the cytoplasmic surface of the epidermal cell outer wall.

Statistical methods

Correlation and regression techniques were used to investigate relationships between leakage rates and other variables. Analysis of variance techniques were used to separate out sources of variability for leakage rates such as cultivar, harvest date and season, and the dewaxing treatment. Comparisons between means were made by application of the Waller-Duncan K-ratio T test (Waller and Duncan, 1969).

RESULTS & DISCUSSION

Anthocyanin leakage from thawed samples

The blueberry samples employed in this study varied in acidity and soluble solids, indicative of possible ripeness differences (Woodruff et al., 1960), as well as in total anthocyanin content and berry surface area, both of which might be expected to influence leakage (Table 1). Differences in composition and surface area between samples harvested in 1982 and 1983 generally were small.

Anthocyanin leakage from thawed blueberries usually could be represented by linear absorbance vs time curves (Fig. 1, see Burlington). Occasionally, samples yielded two-

phase linear leakage curves, the second slope being greater than the first (Fig. 1, see Earliblue), indicating the occurrence of an event causing the leakage rate to increase during the trial. On the other hand, many dewaxed samples (Fig. 1, see Bluetta) yielded two-phase linear leakage curves with the second slope smaller than the first, suggesting depletion of the leakage source or an increase in the resistance of the berries to leakage.

Leakage rates (slopes) varied over a 20-fold range (Table 2), depending on cultivar, ripeness, condition, and dewaxing. The highest leakage rates were obtained with samples that underwent extensive skin rupturing during the leakage determination, an indication of poor condition. Comparisons of Berkeley, Bluetta, and Earliblue samples, harvested on two dates during the 1982 season, indicated an effect of berry ripeness on anthocyanin leakage, higher leakage rates being obtained with samples of each cultivar having the higher SS/A ratios (effect significant at 0.01 level by F-test).

Mean anthocyanin leakage rates for 1982 and 1983 samples of nine highbush blueberry cultivars are given in Table 3. The relatively large differences in leakage rates between seasons cannot be explained in terms of differences in sample ripeness (see Table 1). With the exception of the 1982 Earliblue and Weymouth berries discussed previously, all samples appeared to be in good condition, based on the turbidity of the water in which they were stirred. However, the 1982 samples may have been subjected to transient thawing during frozen storage due to unrecorded power outages and/or equipment malfunctions. Such thawing is indicated by visual observation of frozen berry cross sections, showing the occurrence of a narrow zone of pigment diffusion from the skin into the mesocarp. The diffusion zones were wider with 1982 samples than with 1983 samples. One would expect leakage rates to be greater in berries damaged by freeze-thaw cycling. Cultivar differences in leakage rates for samples of similar ripeness and condition, obtained in the same season, were similar in magnitude to ripeness effects on leakage, the rates falling within a three-fold range. Bluecrop and Bluetta berries tended to leak less while Weymouth tended to have a higher leakage rate.

Table 1—Composition and surface area of highbush blueberry samples

Cultivar	Harvest date	Titrateable acidity (% citric)	Soluble solids (% at 20°C)	SS/A ^a	Total anthocyanin ^b	Total surface area (cm ²) ^c
Berkeley	7-22-82	0.53	13.8	26.0	154	206
	8-2-82	0.40	13.6	33.6	148	185
	7-19-83	0.36	12.4	35.1	102	173
Bluecrop	7-16-82	0.56	11.9	21.4	86	177
	7-23-82	0.64	12.5	19.6	84	175
	7-19-83	0.65	13.6	21.0	66	185
Bluetta	6-28-82	0.72	10.9	15.3	168	201
	7-9-82	0.48	12.2	25.8	154	211
	7-5-83	0.50	11.1	22.4	135	198
Burlington	8-2-82	0.70	14.8	21.5	270	223
	8-2-83	0.98	13.2	13.5	152	235
Coville	8-2-83	0.65	14.1	22.0	143	192
Earliblue	6-28-82	0.50	12.5	25.0	140	196
	7-2-82	0.34	12.2	36.3	135	193
	7-5-83	0.31	14.0	44.8	153	194
Elliott	8-3-82	1.26	11.3	9.0	224	204
	8-9-82	1.36	11.5	8.4	204	207
	8-2-83	1.37	16.2	11.9	233	227
Jersey	7-19-83	0.71	14.8	20.9	164	224
Weymouth	6-28-82	0.54	11.4	21.0	152	204
	7-6-82	0.45	11.0	24.4	132	207
	7-5-83	0.84	11.2	13.2	129	217

^a Soluble solids ÷ titrateable acidity

^b Absorbance of ethanolic extract at 543 nm × dilution factor

^c For 50g sample

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The highest leakage rates obtained with thawed berries were substantially smaller than rates measured previously with cooked samples (Sapers et al., 1985).

Dewaxing increased the leakage rate with Bluetta, Burlington, Coville, Elliott, and Jersey blueberries, presumably by removing a barrier to the diffusion of polar anthocyanins from the underlying epidermal cells. It is not clear why the other cultivars did not respond similarly. However, their behavior was consistent over two seasons. Norris (1974) observed an increase in the penetration of 2,4-D through isolated plant cuticles after dewaxing. The removal of wax from blueberry skin also might affect its mechanical properties, making it more vulnerable to cracks and tears that would represent potential leakage sites.

The linear relationship found between absorbance and stirring time suggests that the leakage process may be described by Fick's law of diffusion, according to which the anthocyanin diffusion rate across the blueberry cuticular membrane should be directly proportional to the berry surface area and anthocyanin concentration gradient and inversely proportional to the membrane thickness. Our data were not consistent with this hypothesis, however; correlations between leakage rates and the sample anthocyanin content (assumed to be proportional to the anthocyanin concentration in epidermal cells), the sample surface area, or the product of the anthocyanin content and surface area lacking significance except with the dewaxed 1983 berries (Table 4). Although not significant at the 5% level, correlation coefficients for the 1982 samples also were higher for dewaxed berries than for corresponding untreated samples. The lack of significance in 1982 may be a reflection of the smaller number of cultivars compared, data for Earliblue and Weymouth samples being excluded because of atypical berry condition.

Leakage rates for 1983 Elliott and Bluetta samples, untreated or dewaxed (see Table 3), were compared with

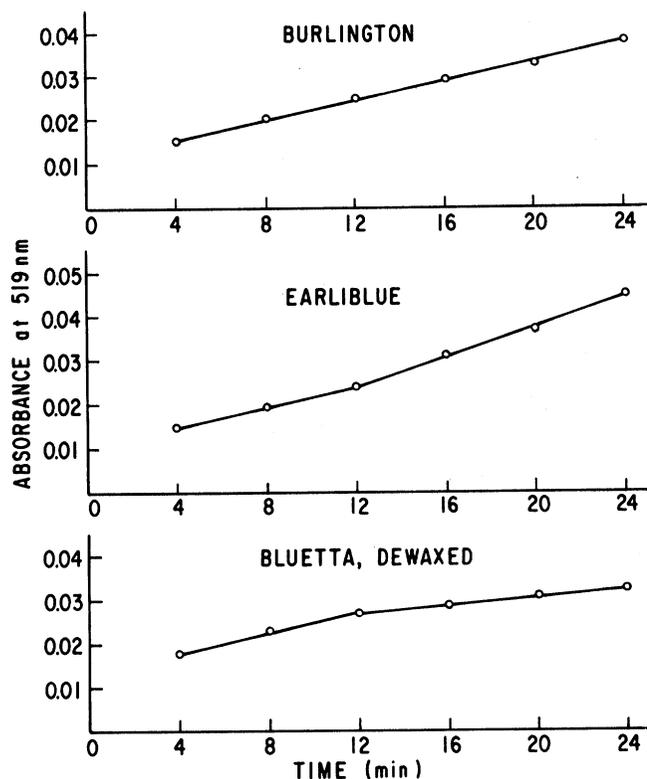


Fig. 1—Anthocyanin leakage curves for thawed highbush blueberries (1983 season).

measured cuticle and epidermal cell wall thicknesses (1.8 and 3.6 μm for Elliott, and 1.2 and 3.0 μm for Bluetta, respectively) to determine conformity to Fick's law. Leakage rates were not inversely proportional to the thickness of either or both structures, as would be expected if Fick's law were operative, even when rates were corrected for differences in berry anthocyanin content and surface area. Norris (1974) found no correlation between the leaf or fruit cuticle thickness of various plants and the penetration of 2,4-D. Likewise, Schönher (1976) reported that permeability coefficients for the diffusion of tritiated water across cuticles isolated from the leaves of several plants were not inversely proportional to cuticle thickness.

These results indicate that anthocyanin leakage is a more complex phenomenon than simple diffusion through the cuticle. Apparently, the cuticle per se is not an effective barrier to diffusion. The epicuticular wax is an effective barrier, and its removal by dewaxing in chloroform makes the leakage process appear more like diffusion through a membrane, perhaps the semipermeable plasma membrane of epidermal cells. With berries that are not dewaxed, leakage rates may be limited by diffusion through more permeable sites or discontinuities in the epicuticular wax.

Correlation between leakage rate and drip

Although the thawed berry samples evaluated in this study were visibly wet, they did not leak sufficient exudate during thawing to produce a measureable drip volume. Therefore, the extent of drip loss was estimated by rinsing the thawed berries with water and measuring the total quantity of anthocyanin in the rinsings (Table 3). In prin-

Table 2—Effect of ripeness and condition on anthocyanin leakage from thawed blueberries—1982 season

Cultivar	Harvest date	SS/A ^a	Condition	Leakage rate ($\times 10^{-3}$) ^b	
				Not dewaxed	Dewaxed
Berkeley	7-22-82	26.0	Good	2.2	3.3
	8-2-82	33.6	Good	4.9	3.7
Bluetta	6-28-82	15.3	Good	1.8	4.0
	7-9-82	25.8	Good	4.0	—
Earliblue	6-28-82	25.0	Fair	5.3	8.1
	7-2-82	36.3	Poor	16.7	13.1
Weymouth	6-28-82	21.0	Fair	8.9	8.5
	7-6-82	24.4	Poor	42.6	22.1

^a Soluble solids (%) \div titratable acidity (% citric)

^b Absorbance units per min per 100g berries

Table 3—Cultivar differences in anthocyanin leakage from thawed blueberries

Cultivar	Leakage rate ($\times 10^{-3}$) ^a					Drip (1983) ^b
	1982		1983			
	Not dewaxed	Dewaxed	Not dewaxed	Dewaxed		
Berkeley	3.6 ^{ef}	3.5 ^g	2.1 ^{cd}	2.5 ^{ef}	42.2 ^d	
Bluecrop	3.1 ^{ef}	3.2 ^g	0.7 ^e	0.7 ^g	3.0 ^g	
Bluetta	2.8 ^f	4.0 ^{fg}	0.8 ^e	2.1 ^f	14.0 ^f	
Burlington	4.0 ^{ef}	4.9 ^f	2.1 ^{cd}	3.4 ^d	50.2 ^c	
Coville	—	—	1.9 ^d	2.9 ^{de}	18.2 ^f	
Earliblue	11.0 ^d	10.6 ^d	1.9 ^d	2.4 ^{ef}	18.3 ^f	
Elliott	4.6 ^e	8.7 ^e	1.7 ^d	4.1 ^c	37.8 ^{de}	
Jersey	—	—	2.1 ^{cd}	3.3 ^d	15.6 ^f	
Weymouth	20.6 ^c	15.3 ^c	2.6 ^c	2.3 ^{ef}	37.4 ^e	

^a Absorbance units per min per 100g berries

^b Absorbance units per 100g berries

^{c-g} Means with different superscripts in same column are significantly different ($p < 0.05$)

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that this leakage represents anthocyanin that diffused from epidermal cells within the skin into the mesocarp during thawing and was released along with accumulating cellular fluids when the barrier represented by the skin was removed. Examinations of cross sections of fresh, frozen, and thawed blueberries clearly show the formation of a pigment gradient from the skin to the interior during thawing.

To determine the site of leakage in berries not having visible skin defects, the surfaces of individual berries were examined by the blotting technique and by microscopic observation. Leakage appeared to come from scattered point sources on the berry surface rather than from the entire surface as would be the case if anthocyanin diffusion from epidermal cells through the cuticle were uniform. Under the microscope, the point sources of leakage could be seen as small droplets of juice flowing from cracks or punctures in the skin. Leakage also appeared at the blossom end of the berries, apparently coming from the calyx. No leakage was seen at the stem scar.

These results suggest that differences in leakage between samples may be due at least in part to differences in the mechanical strength, thickness, and/or condition of the skin which affect the development of point source leaks. Additional differences between samples in the accumulation of cellular fluids under the skin, due to tissue damage during freezing and storage, may affect the pressure exerted by such fluids. Leakage will occur when the fluid pressure is sufficient to break through weak points in the skin. According to our hypothesis then, sample leakage rates reflect the number of point source leaks (a characteristic of the berry skin), the volume leaked (a manifestation of tissue damage and ice formation), and the anthocyanin concentration of the leaking fluid (determined by cultivar and ripeness). Linearity implies a constant flow of exudate from multiple point source leaks. Two-phase linear leakage curves where the second slope is greater than the first slope may represent the occurrence of a major new point source leak (i.e., an extensive skin rupture) during the period of measurement. Leakage curves where the second slope is less than the first may result from the cessation of one or several major point source leak due to decreasing internal pres-

sure. Research to confirm this hypothesis and examine its implications is continuing.

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Ms received 6/15/84; revised 9/14/84; accepted 10/4/84.

The authors thank Gavin R. Graff, Sandra P. Graham, Michael J. Kelley, and Beverly E. Maleeff, employees of the Eastern Regional Research Center, for their technical assistance; and Eric G. Stone of the USDA, Rutgers University Blueberry and Cranberry Research Center, who provided the blueberry samples.

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ciple, this estimate of drip should be related to the leakage rate, as determined in the dynamic system, since drip represents leakage over an extended period of time. However, the correlation between these two measurements, while significant, was too low ($r = 0.57$) to be of any predictive value.

A number of factors can explain the poor correlation between drip and leakage rates. First, drip represents a slow process occurring over a wide temperature range while leakage rates are measured over a brief time interval at a constant and higher temperature. Second, the drip process differs qualitatively from leakage (as we measured it), resulting from the slow melting of ice crystals in the intercellular spaces, diffusion of pigments and other solutes from damaged epidermal cells, and the dilution of these cellular fluids by the melted ice. The quantity of intercellular ice and extent of cell damage depend on freezing rate and storage conditions (Joslyn, 1966). During thawing, the pigmented cellular fluids accumulate within the berries and also penetrate the skin as exudate. Only this last step is measured as leakage in our system. In spite of the poor correlation between drip and leakage. We believe that both measurements are useful in understanding the basis of processibility and the effects of cultivar and sample condition on processability.

Anthocyanin leakage from individual berries

Observations made with individual thawed berries in the static model system shed considerable light on the leakage process. Of primary importance is the fact that leakage

Table 4—Correlations between anthocyanin leakage rates and total anthocyanin content and surface area for untreated and dewaxed blueberry samples

	Correlation Coefficient	
	1982 ^a	1983 ^b
Untreated		
Leakage rate vs total anthocyanin	0.41	0.30
Leakage rate vs surface area	0.16	0.29
Leakage rate vs total anthocyanin X surface area	0.37	0.30
Dewaxed		
Leakage rate vs total anthocyanin	0.63	0.90 ^c
Leakage rate vs surface area	0.46	0.71 ^d
Leakage rate vs total anthocyanin X surface area	0.59	0.90 ^c

^a Five cultivars compared

^b Nine cultivars compared

^c Significant at $p = 0.01$

^d Significant at $p = 0.05$

within a berry sample is not uniform but varies greatly from berry-to-berry, some berries even exhibiting no leakage (Table 5). The percentages of nonleaking and leaking berries were not highly correlated with leakage rates for 50g samples, probably because of the large contribution to anthocyanin leakage made by a relatively small number of berries. Nevertheless, clear differences in the distribution of leaking berries can be seen with widely differing samples such as Burlington and Weymouth. With the latter cultivar, the effects of berry condition on the leakage rate and distribution are evident. Similarly, dewaxing increases the proportion of leaking berries as well as the leakage rate.

Close examination of leaking berries revealed an association between the severity of leakage and the occurrence of skin rupturing that resulted in the release of seeds and other particulate matter as well as anthocyanin. However, not all anthocyanin leakage was accompanied by turbidity or visible skin rupturing. To determine the effects of skin abrasion, rupturing, and fragmentation on anthocyanin leakage rates, these defects were simulated in sets of berries matched according to the extent of leakage in the static system by scraping, slitting, or removing a section of skin on each berry. Leakage rates determined for these sets with the scaled down dynamic model system are given in Table 6. As might be expected, leakage rates for berries classified in the "slight leakage" category were higher than rates for berries classified as nonleaker. Leakage rates for the latter were not increased by abrading the skin with a razor blade. However, slitting the skin did increase the leakage rate by permitting the escape of pigmented cellular fluids released from cells that had collapsed during thawing. Skin fragments trimmed from the berries did not release much anthocyanin in spite of their high pigment content. However, the berries from which skin fragments were taken gave relatively high leakage rates. It is likely

Table 6—Anthocyanin leakage from simulated defects in skin of Berkeley blueberries^a

Trial	Appearance in static system		Treatment	Leakage rate ($\times 10^{-3}$) ^b
1	No leakage	None		2.4
	No leakage	Skin abraded		1.5
	Slight leakage	None		8.1
2	No leakage	None		1.2
	No leakage	Skin slit		3.5
	No leakage	Skin fragments		1.6
3	No leakage	None		2.8
	No leakage	Skin fragments		4.1
	No leakage	Berry after skin fragments removed		7.6

^a Harvested 8/2/82

^b Slope of absorbance vs time curve (absorbance units per min per 100g berries)

Table 5—Anthocyanin leakage from individual berries

Sample ^a	Treatment	Condition	Leakage rate ($\times 10^{-3}$) ^b	Percentage of berries		
				No leakage	Slight leakage ^c	Moderate leakage ^c
Burlington 8/2	None	Good	4.0	87	13	0
Bluetta 6/28	None	Good	1.8	67	25	8
	Dewaxed	Good	4.0	26	44	30
Elliott 8/3	None	Good	5.0	38	47	15
	Dewaxed	Good	12.6/8.4 ^d	9	59	31
Weymouth 6/28	None	Fair	8.9	64	21	14
	7/6	None	42.6/29.8 ^d	22	44	34

^a 1982 season

^b Slope of absorbance vs time curve (absorbance units per min per 100g berries)

^c Slight = trace of pink color; moderate = light pink.

^d Two-phase linear curve with rates corresponding to slope 1/slope 2