Title: ANTHOCYANIN DEGRADATION IN FREEZE-DRIED STRAWBERRIES AND STRAWBERRY PUREE

Abstract approved: Dr. R. E. Wrolstad

Anthocyanin degradation in freeze-dried strawberry puree was studied under various relative humidity conditions at 37°C. Moisture was found to be the determining factor in the rate of pigment degradation. Storage at relative humidities below 11% provided good pigment retention in this low-moisture product. Enzymatic degradation of the anthocyanin pigment did not occur.

Pigment retention at 100% relative humidity was not improved when nitrogen was substituted for air as the storage atmosphere; however, the rate of browning was decreased with storage under nitrogen.

Gel filtration on acetylated Sephadex was applied to the study of anthocyanin degradation. Whereas three separate bands were observed on the gel column, only one major component, pelargonidin 3-glucoside, was positively identified.
Stannous, stannic and aluminum ions improved the color stability of strawberry puree stored at 50°C. However, the stability of the anthocyanin pigments was not improved. It was concluded, therefore, that some colored complex(es) was formed which was not extractable in the analytical procedure used. Quercetin could be the complexing agent.

A purplish-black discoloration appeared in puree samples containing both ferrous and ferric ions. Quercetin could also be the complexing agent in this reaction.
Anthocyanin Degradation in Freeze-dried Strawberries and Strawberry Puree

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of the requirements for the degree of

Master of Science

June 1972
APPROVED:

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Date thesis is presented __________ 9/2/71

Typed by Mary Jo Stratton for John Arthur Erlandson
ACKNOWLEDGEMENT

I would like to express my appreciation for the advice and encouragement given me by Dr. R. E. Wrolstad during this investigation and the rest of my Master's program.

I am grateful to the faculty and graduate students of the Food Science and Technology Department for their cooperation and helpful suggestions.

I would like to thank the Nutrition Foundation, Inc. whose grant provided me with a research assistantship.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>LITERATURE REVIEW</strong></td>
<td>2</td>
</tr>
<tr>
<td>Anthocyanin Degradation</td>
<td>2</td>
</tr>
<tr>
<td>Stability Problems in Low-moisture foods</td>
<td>4</td>
</tr>
<tr>
<td>Gel Filtration</td>
<td>6</td>
</tr>
<tr>
<td>Metal Ions and Strawberry Color</td>
<td>7</td>
</tr>
<tr>
<td><strong>EXPERIMENTAL</strong></td>
<td>8</td>
</tr>
<tr>
<td>Strawberries</td>
<td>8</td>
</tr>
<tr>
<td>Determination of Total Anthocyanin</td>
<td>8</td>
</tr>
<tr>
<td>Preparation of Anthocyanin Extracts for Thin-layer Chromatography</td>
<td>9</td>
</tr>
<tr>
<td>Cellulose Thin-layer Chromatography</td>
<td>9</td>
</tr>
<tr>
<td>Colorimetry</td>
<td>10</td>
</tr>
<tr>
<td>Gel Filtration</td>
<td>10</td>
</tr>
<tr>
<td>Moisture Determination</td>
<td>11</td>
</tr>
<tr>
<td>Anthocyanin Degradation Studies</td>
<td>13</td>
</tr>
<tr>
<td>Effect of Relative Humidity</td>
<td>13</td>
</tr>
<tr>
<td>Effect of Enzyme Inactivation</td>
<td>16</td>
</tr>
<tr>
<td>Effect of Headspace Air and Nitrogen</td>
<td>17</td>
</tr>
<tr>
<td>Effect of Metal Ions</td>
<td>17</td>
</tr>
<tr>
<td><strong>RESULTS AND DISCUSSION</strong></td>
<td>19</td>
</tr>
<tr>
<td>Anthocyanin Degradation in Freeze-dried Strawberry Puree</td>
<td>19</td>
</tr>
<tr>
<td>Effect of Relative Humidity</td>
<td>19</td>
</tr>
<tr>
<td>Effect of Air and Nitrogen</td>
<td>27</td>
</tr>
<tr>
<td>Effect of Browning</td>
<td>30</td>
</tr>
<tr>
<td>Gel Filtration Analyses</td>
<td>32</td>
</tr>
<tr>
<td>Effect of Metal Ions on Red Color and Anthocyanin Degradation in</td>
<td>38</td>
</tr>
<tr>
<td>Strawberry Puree</td>
<td></td>
</tr>
<tr>
<td><strong>BIBLIOGRAPHY</strong></td>
<td>47</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Comparison of pigment and water content in freeze-dried strawberry puree stored at various relative humidities at 37°C.</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gas-liquid chromatogram (30% THEED column) of a methanol extract of freeze-dried strawberry puree.</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>Acetone peak areas obtained for various concentrations of water.</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Pigment retention of freeze-dried strawberry puree at various RH's and 37°C (preliminary study).</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Pigment retention of freeze-dried strawberry puree at various RH's and 37°C.</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>Comparison of water sorption isotherm with pigment retention of strawberry puree after 180 hr at 37°C.</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>Effect of enzyme inactivation (heat treatment) on pigment retention of freeze-dried strawberry puree at 100% RH and 37°C.</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>Reaction rate curves for the degradation of anthocyanin pigments in freeze-dried strawberry puree at various RH's and 37°C.</td>
<td>31</td>
</tr>
<tr>
<td>8</td>
<td>Effect of air and nitrogen on the degradation of anthocyanin pigments in freeze-dried strawberry puree at 100% RH and 37°C.</td>
<td>29</td>
</tr>
<tr>
<td>9</td>
<td>Production of 5% NaOH soluble brown pigments in freeze-dried strawberry puree at 100% RH and 37°C.</td>
<td>31</td>
</tr>
<tr>
<td>10</td>
<td>Effect of air and nitrogen on the production of brown pigments in freeze-dried strawberry puree at 100% RH and 37°C.</td>
<td>33</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>11</td>
<td>Gel filtration curve of a methanol extract of freeze-dried strawberry puree on Sephadex LH-20 in 0.01% HCl in methanol.</td>
<td>35</td>
</tr>
<tr>
<td>12</td>
<td>Variation of maximum absorbance with pigment retention in gel filtration analysis of acidic methanol extract of freeze-dried strawberry puree at 100% RH and 37°C.</td>
<td>37</td>
</tr>
<tr>
<td>13</td>
<td>Effect of storage at 50°C on the Gardner α value and pigment concentration of strawberry puree.</td>
<td>39</td>
</tr>
<tr>
<td>14</td>
<td>Effect of stannous ion on the Gardner α value and pigment concentration of strawberry puree at 50°C.</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>Effect of stannic ion on the Gardner α value and pigment concentration of strawberry puree at 50°C.</td>
<td>41</td>
</tr>
<tr>
<td>16</td>
<td>Effect of aluminum ion on the Gardner α value and pigment concentration of strawberry puree at 50°C.</td>
<td>42</td>
</tr>
<tr>
<td>17</td>
<td>Effect of ferrous ion on the Gardner α value and pigment concentration of strawberry puree at 50°C.</td>
<td>43</td>
</tr>
<tr>
<td>18</td>
<td>Effect of ferric ion on the Gardner α value and pigment concentration of strawberry puree at 50°C.</td>
<td>44</td>
</tr>
</tbody>
</table>
ANTHOCYANIN DEGRADATION IN FREEZE-DRIED
STRAWBERRIES AND STRAWBERRY PUREE

INTRODUCTION

Color is a very important physical parameter affecting consumer acceptance of strawberry products. The pleasing red color of fresh strawberries is due to the anthocyanin (ACN) pigments.

ACN pigment degradation has been the subject of many investigations. Most studies have been concerned with isolating the various factors involved in the degradation process. These studies were carried out using extracted juices or purified pigments in model systems.

The introduction of freeze-dried strawberry products has brought about the need for better understanding of the stability of ACN pigments in such low-moisture systems. Part of this investigation was conducted on a freeze-dried strawberry puree to evaluate the effect of moisture, air and browning on the degradation of these pigments.

The purpose of the other part of this investigation was to assess the effect of stannic and other metal ions on color stability and their relation to ACN degradation in strawberry puree.
Anthocyanin Degradation

ACN degradation has been studied by a number of investigators. Among those factors found to affect degradation are: moisture, oxygen, sugar and sugar breakdown products (furfural and hydroxymethylfurfural) and enzymes.

Markakis, Livingston and Fellers (1957) observed that water was necessary for the decolorization of strawberry ACN's. They postulated two mechanisms for pigment degradation which involved water. The first was a hydrolytic cleavage of the glycosidic bond in the ACN which yields an unstable aglycone. The aglycone degrades much faster than does the ACN; however, no evidence of aglycone formation was observed.

A second mechanism involved a hydrolytic opening of the pyrylium ring at position 1, 2 forming an unstable ketone (substituted chalcone) which then polymerized to a brownish precipitate (insoluble polyphenolic compound). By radioactive tracer techniques, 85% of the original $^{14}$C labeled pelargonidin 3-glucoside activity was recovered in the brown precipitate. The precipitate was found to be soluble in 5% NaOH and partially soluble in methanol. This work was substantiated by Hamdy et al. (1961) who isolated a brown polyphenolic polymer from degraded pelargonidin 3-glucoside.
In their study of ACN pigment breakdown in model systems, Lukton, Chichester and Mackinney (1956) found that an insoluble red-brown precipitate and a soluble brown material were formed to a much greater extent in oxygen than in nitrogen. Their proposed pathway for the formation of this precipitate included hydrolysis of the pseudobase to the aglycone followed by polymerization. The polymeric material was thought to contain glucose.

Sugar and sugar breakdown products (furfural and hydroxymethylfurfural) are known to increase the rate of ACN degradation (Meschter, 1953; Decereau, Livingston and Fellers, 1956; Tinsley and Bockian, 1960; Daravingas and Cain, 1968).

Mackinney, Lukton and Chichester (1955) observed a linear relationship between pigment loss and browning in strawberry preserves. The authors also speculated that the brown product formed was either concomitant with or a direct result of pigment degradation.

Enzymes have been found which decolorize ACN's in red cabbage (Paech and Eberhardt, 1952) and in Coleus hybridus leaves (Bayer and Wegmann, 1957).

Huang (1955), using a fungal enzyme preparation (anthocyanase), studied the decolorization of chrysanthemin (cyanidin 3-β-glucoside). He proposed that the enzyme acted like a β-glucosidase. The removal of the sugar moiety from the ACN allowed the unstable aglycone to be transformed into various colorless entities, e.g., 2-carbinol or
chalcone. The major effect of this enzyme preparation on a concentrated ACN solution was the removal of pigment from solution as a precipitate of aglycone.

Scheiner (1961) described an enzymatic decolorization of ACN's in sweet and sour cherries. The mechanism included enzymatic oxidation of O-dihydroxyphenyls to quinone and subsequent reaction between the quinone and ACN forming a colorless compound. Pelargonidin 3-glucoside was also decolorized by the enzyme preparation.

**Stability Problems in Low-moisture Foods**

The effect of moisture content on some deteriorative reactions in low-moisture and intermediate-moisture foods has been comprehensively reviewed by Labuza, Tannenbaum and Karel (1970). Basic theories of water sorption were applied to the discussion of stability problems.

Water adsorbed in a food system exists in three different states:

1) monolayer coverage representing a range of relative humidities from 0 to 20% in which water is very tightly bound at hydrophilic sites; 2) multilayer (20-55% RH); and 3) capillary condensation (above 55% RH) in which water condenses in the porous structure of the food. A water sorption isotherm reflects these states of water.

Deteriorative reaction rates in foods depend on the state of
water which exists in the system. Lipid oxidation rates decrease with increasing water activity (equilibrium relative humidity). Microbial spoilage is enhanced at high water activities (capillary condensation region).

In a study of browning in a model system containing sucrose, Karel and Labuza (1968) found that even at low water activities, hydrolysis of sucrose can occur giving rise to reducing sugars which have a potential for browning. From this and other studies Labuza, Tannenbaum and Karel (1970) concluded that water has a dominant influence on the rate of browning in systems which contain carbonyls. Browning usually increases with water content to a maximum (intermediate-moisture range) in most food systems. Such a relationship was observed in dehydrated orange juice by Karel and Nickerson (1964).

The effect of water activity on the degradation of pigments both in low-moisture foods and in low-moisture model systems has been recently studied. As water activity increased above 0.32, chlorophyll degradation (conversion to pheophytin) also increased; below 0.32 very little degradation occurred (Lajolo, Tannenbaum and Labuza, 1971). β-carotene in a freeze-dried model system was more stable at high water activities (Chou and Breene, 1971).

The effect of water activity on enzyme activity has been reviewed by Acker (1969). He stated that enzymatic reactions in
low-moisture foods show a significant dependence on water activity and that a water sorption isotherm can be used to predict enzymatic reactions. Increasing substrate mobility was the cause of increasing enzyme activity at high water activities (capillary condensation region).

**Gel Filtration**

In studies conducted by Somers (1966, 1967), Sephadex gel filtration was used to separate condensed tannins from residual acylated and unacylated ACN's both in wine and in grape pigments. Similar techniques were applied to the study of simple and condensed leucoanthocyanins in tea plants by Forrest and Bendall (1969) and of polyphenols of apple peel by Durkee and Jones (1969).

Total exclusion of high molecular weight fractions from the gel suggested that separation was due to the true molecular sieve action of the gel. Estimates of the molecular weights were made. Separation of the lower molecular weight monomeric polyphenols (ACN's and leucoanthocyanins) was thought to be due to adsorptive partition.

Sephadex LH-20, a hydroxypropyl derivative of Sephadex G-25, is especially suited for filtration using organic solvents. It has been used in a number of studies of flavonoids and phenolic glucosides (Crispin, Payne and Swaine, 1968; Johnston, Stern and Waiss, 1968; Repas, Nikolin and Dursun, 1969). Adsorption to the gel is related to the structure of the phenolic glucosides and increases with the number
of hydroxyl groups on the molecule.

**Metal Ions and Strawberry Color**

Stannic chloride has been shown to improve the color stability of strawberry puree (Sistrunk and Cash, 1968, 1970). In the latter investigation stannous and stannic chloride salts also enhanced the stability of ascorbic acid. Their work indicated that the breakdown of ascorbic acid and pigment were not always directly related.
EXPERIMENTAL

Strawberries

Strawberries (Fragaria ananassa Duch. variety Northwest) were obtained from Oregon State University's North Willamette Experiment Station on June 23, 1970. Fruit was washed, individually quick frozen (IQF) at -35°C, packed in polyethylene bags and stored at -24°C. A representative sample had a pH of 3.6, contained 0.77% acid (determined as citric) and had a soluble solids content of 9.7%.

IQF whole berries were freeze-dried in a Hull freeze dryer (Model 651M-9WDF-20) at 49°C and at pressures from 300 to 900 μ. The final product (10% of original weight of berries) was stored under nitrogen at -24°C in sealed 603 x 700 tin cans containing silica gel desiccant.

Strawberry puree to be freeze-dried was prepared as follows: IQF berries were thawed at room temperature and blended in a large Waring blender. The puree was frozen in sheets of uniform thickness and freeze-dried as described above. The final product was broken up and stored under the same conditions as the IQF whole berries.

Determination of Total Anthocyanin

The total ACN assay of Swain and Hillis (1959) was used. Modifications included using 0.1% HCl in methanol as the extracting
solvent and measuring the absorbance at 510 nm. The ACN concentration was expressed as μM of pelargonidin 3-glucoside per g puree. Calculations were based on a molar absorbtivity of 36,600 (Wrolstad, Putnam and Varseveld, 1970).

**Preparation of Anthocyanin Extracts for Thin-layer Chromatography**

The pigments extracted in the total ACN assay were isolated using a modification of the procedure reported by Wrolstad, Putnam and Varseveld (1970). The acidic methanol extract (remaining portion from total ACN assay) was diluted ten-fold with water and poured on a damp polyvinylpyrrolidone (PVP) layer (2 in. thick). The ACN adsorbed to the PVP was eluted with 0.1% HCl in methanol and filtered through Whatman No. 1 filter paper in a Buchner funnel. The filtrate was concentrated in a Buchi rotary evaporator at 40°C and stored at -5°C.

**Cellulose Thin-layer Chromatography**

Cellulose TLC plates were prepared by blending 15 g MN300 cellulose powder and 90 ml distilled water at high speed in a Virtis homogenizer. The slurry was spread at a thickness of 0.25 mm on five 20 x 20 cm plates. The coated plates after setting at room temperature for 1-4 hr were dried at 100°C for 30 min and stored over silica gel desiccant.
Both one- and two-dimensional TLC were carried out. A solvent system of glacial acetic acid-water-HCl (15:82:3) was used for one-dimensional TLC. In two-dimensional work the solvent system used was: first direction, n-butanol-HCl-water (5:2:1); second direction, water-HCl-formic acid (8:4:1) (Nybom, 1968).

Colorimetry

The color of strawberry puree samples was measured with a Gardner CDM. The instrument was standardized against a red plaque having the following National Bureau of Standards values: \( L = 26.8; \) \( a = +45.4; \) and \( b = +15.1. \) The \( a \) value was used as a measure of redness (Sistrunk and Cash, 1970).

Gel Filtration

All Sephadex materials were purchased from Pharmacia Fine Chemicals, Inc.

Sephadex LH-20 (31 g) was swollen overnight at \( 2^\circ C \) in 0.01% HCl in methanol. A column (2.5 x 25 cm) was prepared by pouring the swollen gel into a solvent resistant Sephadex column (Model SR 25/45). Settling was allowed prior to placing a sample applicator on top of the gel. Flow rates of 2.1-2.5 ml/min of the elution solvent, 0.01% HCl in methanol, were maintained throughout the investigation.

The absorbance of the effluent was monitored at 510 nm by a
Beckman DB spectrophotometer equipped with a Flow Cell Assembly (Model 96160) and a strip chart recorder (chart speed - 4 in/min). After passing through the spectrophotometer the effluent was collected in 10 ml quantities in a fraction collector (ISCO - Model 272) coupled with an ISCO Model 400 Volumeter. This apparatus permitted the accurate measurement of the elution volume of separated pigment bands.

Extracts for the gel filtration analyses were prepared using two different procedures. In procedure 1, two-gram samples of freeze-dried strawberry puree powder were extracted with 10 ml methanol and filtered through two layers of Whatman No. 1 filter paper in a Buchner funnel. Three milliliters of filtrate was applied to the column.

Procedure 2 utilized the total ACN assay extract. A one-gram sample of freeze-dried strawberry puree powder was extracted with 200 ml of 0.1% HCl in methanol. Three milliliters of this extract was applied to the column.

**Moisture Determination**

A modified gas-liquid chromatographic method (Mary, 1969) was used to determine the moisture content of freeze-dried strawberry puree. The method is based upon the stoichiometric reaction of water with 2, 2-dimethoxypropane (DMP) in acid solution to yield acetone and methanol. The area of the acetone peak is calculated and used as a
measure of water content.

The major modification of Mary's procedure was the incorporation of an internal standard (2-pentanone) into each reaction mixture. This permitted correction of acetone peak areas for possible variations among samples in GLC sensitivity and sample injection.

The modified procedure follows: freeze-dried strawberry powder samples (1.000 g) were shaken with 50 ml absolute methanol (J. T. Baker Chemical Company). Ten milliliters of the clear extract was transferred quantitatively to a 25 ml volumetric flask to which 2 ml of DMP (Eastman Organic Chemicals) and 5 ml of 0.1 N methanesulfonic acid (Eastman Organic Chemicals) in methanol were subsequently added. These solutions were then shaken for 5 min on a wrist-action shaker to accelerate the reaction. After shaking, 5 ml of 2-pentanone standard solution (10.000 g per 250 ml methanol solution) were added. Methanol was added to volume and the solution was thoroughly mixed. Blank determinations were run in which absolute methanol replaced the methanol extract.

Approximately 2 μl of these solutions were injected onto an Aerograph 1520 gas chromatograph equipped with a hydrogen flame ionization detector. A 7-1/2' x 1/8" stainless steel column packed with 30% tetrahydroxyethylethlenediamine (THEED) on Chromosorb W was used. The operating conditions were: oven temperature, 75°C (isothermal); injection block temperature, 80°C; detector
temperature, 160°C; nitrogen (carrier gas) flow rate, 15 ml/min; hydrogen, 20 ml/min; air, 275 ml/min. A sample chromatogram is shown in Figure 1.

Peak areas were calculated by triangulation according to the procedure of Kingston (1964).

Determinations were run in triplicate. The acetone peak areas were corrected to zero by subtracting the peak area of the blank.

Standard solutions containing 4.6-23 mg water per 25 ml were prepared in the same manner as described above. The corrected acetone peak areas were plotted against the µg of water in the form of a standard curve as shown in Figure 2.

Anthocyanin Degradation Studies

Effect of Relative Humidity

Freeze-dried strawberry puree was ground using a mortar and pestle into a coarse powder. Powder samples (approximately 1 g) were transferred to weighed open-top vials (2.5 x 6.5 cm). These were stored in the dark in vacuo in a large desiccator containing silica gel desiccant. After 16 hr the sample vials were reweighed and the weights recorded. Six vials were placed in each of six relative humidity (RH) chambers which were kept in the dark at 37°C.

Vacuum desiccators served as the RH chambers. The RH's
Figure 1. Gas-liquid chromatogram (30% THEED column) of a methanol extract of freeze-dried strawberry puree. (Peak no. 1, 2, 2-dimethoxypropane; 2, acetone; 3, 2-pentanone; 4, methanol.)
Figure 2. Acetone peak areas obtained for various concentrations of water.
and the materials used to maintain them were: 0%, silica gel desiccant; 11%, saturated LiCl solution; 32%, saturated MgCl₂ solution; 57%, saturated NaBr solution; 75%, saturated NaCl solution; and 100%, distilled water. The RH's of the four saturated salt solutions are those reported for 35°C by Rockland (1960).

The chambers were then evacuated and the samples allowed to equilibrate with the constant humidity of the chambers. After the equilibration period (2 hr), 'original' moisture and total ACN content readings were recorded. Moisture uptake of the samples was measured gravimetrically.

Formation of acidic methanol-insoluble reddish-brown pigments was assayed by the method of Sistrunk and Cash (1970). The procedure was modified in that acidic methanol was used to extract the pigments. The residue which remained was extracted two times with 40 ml of 5% NaOH. The samples were stirred and then centrifuged. The supernatant was decanted into a 100 ml volumetric flask and made to volume. Absorbance was recorded at 410 nm.

**Effect of Enzyme Inactivation**

To determine if enzymes were responsible for ACN degradation, the following procedure was carried out:

Two hundred grams of thawed IQF berries were blended in a Waring blender. The puree was divided into two 100-g portions; one
sample was heated to $75^\circ C$ and held for 10 min in a water bath and the other was not heated.

The puree samples were freeze-dried in a laboratory model lyophilizer at a pressure of 100 μ. The freeze-dried material was ground up and six 1.000 g samples of each treatment group were weighed into open vials. The vials were stored at 100% RH and $37^\circ C$. ACN degradation was followed as previously described.

**Effect of Headspace Air and Nitrogen**

Samples of freeze-dried strawberry powder were divided into two groups, one stored in air and the other stored under nitrogen. ACN degradation and the formation of alkali-soluble brown pigments were followed as previously described.

One gram samples were weighed into open vials and placed in 500 ml filter flasks containing distilled water. The flasks were evacuated and the samples were allowed to equilibrate for two hours at 100% RH. After the equilibration period, one flask was filled with air by releasing the vacuum and the other was flushed with nitrogen and both stoppered. Samples were stored at $37^\circ C$.

**Effect of Metal Ions**

Twelve hundred grams of IQF strawberries were thawed at room temperature and blended in a Waring blender. The puree was divided
into six 200-g portions and stored under nitrogen at 2°C until needed.

Aqueous solutions of five metal salts——SnCl₂ · 2H₂O, SnCl₄ · 5H₂O, AlCl₃ · 6H₂O, FeCl₂ · 4H₂O and FeCl₃ · 6H₂O——were prepared in such a way that when 10 ml of these solutions were added to the 200 g portions of puree, the concentration of metal ion would be 0.2%. Ten milliliters of water were added to another puree sample as a control.

Test tubes containing 20 g portions of sample were covered with Parafilm and placed in a 50°C water bath. Gardner Color Difference Meter (CDM) readings and total ACN assays were taken periodically throughout the study.
RESULTS AND DISCUSSION

Anthocyanin Degradation in Freeze-dried Strawberry Puree

Effect of Relative Humidity

In a preliminary study ACN degradation at various RH's was followed. Results of this study are presented in Figure 3.

It was found that the rate of degradation increased with an increase in RH. Large differences in rate between the 11 and 32% RH storage conditions and between the 32 and 57% RH storage conditions can be observed. These differences suggested that the dependence of ACN degradation on water content was similar to that reported for other deteriorative reactions, e.g., non-enzymatic browning (Karel and Labuza, 1968), chlorophyll degradation (Lajolo, Tannenbaum and Labuza, 1971), and enzymatic degradation (Acker, 1969). It also seemed possible that a water sorption isotherm could be used to predict pigment stability in this low-moisture product.

These possibilities led to a second degradation study in which samples were stored for longer periods of time at the lower RH's. The initial moisture content of the freeze-dried powder as determined by a gas-liquid chromatographic method was 2.2%. Water uptake was gravimetrically determined on subsequent samples and reported as μM water per g dry powder.
Figure 3. Pigment retention of freeze-dried strawberry puree at various RH's and 37°C. (Preliminary study).
The degradation rate curves are shown in Figure 4. The relative rates are the same as those observed in the preliminary study. On the basis of these two studies, good pigment retention can be predicted in a freeze-dried strawberry product if stored at 11% RH or below. Storage at RH's above 32% would be undesirable.

The accompanying table presents a comparison of water and pigment content in samples analyzed in this study. Certain trends can be observed which relate water content with the rate of pigment degradation. The low rate of degradation below a value of 150, the intermediate rate between 150 and 700 and the rapid rate above 700 coincide with the theoretical division of the sorption isotherm into three regions (monolayer, multilayer and capillary condensation). It seemed reasonable to assume therefore that a sorption isotherm could be used to assess storage stability of ACN pigments in this low-moisture system.

A sorption isotherm was plotted and appears in Figure 5 along with the accompanying pigment retention data. An inverse relation between pigment retention and moisture content is observed. Sorption isotherms are generally sigmoidal; the curve obtained was similar in shape to that obtained by Karel and Nickerson (1964) for orange juice powder.

The dependence of pigment degradation on relative humidity can be compared to that of other deteriorative reactions occurring in foods.
Figure 4. Pigment retention of freeze-dried strawberry puree at various RH's and 37°C.
Table 1. Comparison of pigment and water content in freeze-dried strawberry puree stored at various relative humidities at 37°C.

<table>
<thead>
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<th>% Relative Humidity</th>
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Figure 5. Comparison of water sorption isotherm (-•-) with pigment retention (-△-) of strawberry puree after 180 hr at 37°C. (Moisture content = g H₂O per 10 g puree.)
Lipid oxidation decreases as water activity increases (Labuza, Tannenbaum and Karel, 1970). Oxidation of β-carotene in low-moisture model systems is affected in a similar manner (Chou and Breene, 1971).

Non-enzymatic browning increased and ascorbic acid retention decreased with increasing RH in dehydrated orange juice (Karel and Nickerson, 1964).

The rate of chlorophyll degradation was very slow at water activities below 0.32 for freeze-dried spinach and model systems (Lajolo, Tannenbaum and Labuza, 1971).

The activity of enzymes which require water either as a medium for reaction or as a reactant show the same dependence on water activity (Acker, 1970) as does pigment degradation in this study. Therefore, the possibility exists that enzymatic hydrolysis of the pigment might also be occurring in addition to the chemical hydrolysis mentioned by Lukton, Chichester and Mackinney (1956), Markakis, Livingston and Fellers (1957), and Hamdy et al. (1961).

The effect of a heat treatment, sufficient to inactivate all naturally occurring enzymes, on ACN degradation was studied. Figure 6 presents the results of this investigation.

The difference in the observed degradation rates is quite small. This would suggest that an enzyme was not responsible for the pigment degradation.
Figure 6. Effect of enzyme inactivation (heat treatment) on pigment retention of freeze-dried strawberry puree at 100% RH and 37°C. (—, unheated; •, held at 75°C for 10 min.)
The kinetics of the degradation reaction were examined by plotting the quantity, 100/\% pigment remaining at time "t" (a/x), on a logarithmic scale against time for the samples stored at the various RH's. Figure 7 is such a plot.

A straight line is expected if a reaction is first order in some reactant. Although no straight lines are observed for the entire storage time, reasonably straight lines can be drawn for the 75 and 100\% RH samples for the first 69 hr of storage. It can be assumed, therefore, that the degradation is first order in ACN pigment over this period of time. The deviation from first order reaction kinetics suggests that breakdown products have accumulated to such an extent that they interfere with the reaction.

No obvious pattern of first order kinetics is observed in samples stored at the other RH's.

This kinetic data can be used to explain chemical hydrolysis as the cause of ACN degradation. A direct relation would exist between amount of water and reaction rate if water was the determining factor in the reaction. This is plainly observed in all the data presented.

**Effect of Air and Nitrogen**

An investigation of the effect of air and nitrogen storage atmospheres on ACN degradation rate was carried out. The results are presented in Figure 8.
Figure 7. Reaction rate curves for the degradation of anthocyanin pigments in freeze-dried strawberry puree at various RH's and 37 °C. (a = 100%; x = % pigment retained.)
Figure 8. Effect of air and nitrogen on the degradation of anthocyanin pigments in freeze-dried strawberry puree at 100% RH and 37 °C. ( – , nitrogen; – , air.)
The degradation rate in air was always greater than the rate in nitrogen; however, the difference was quite small. Therefore, oxygen would not be a determining factor in ACN degradation in the presence of a substantial amount of water.

**Effect of Browning**

A reddish-brown compound was observed in increasing amounts with storage at the higher RH's (57, 75, 100). This compound appeared in the residue remaining after extraction of the pigment for the total ACN assay.

Various solvents were tried in attempts to extract the compound for further characterization. The compound was soluble in aqueous 5% NaOH and partially soluble in methanol. This finding agrees with that of Markakis, Livingston and Fellers (1957) in their investigation of strawberry pigment breakdown in model systems.

The procedure of Sistrunk and Cash (1970) as previously outlined was used to monitor the brownish pigment produced with storage at 100% RH and 37°C. Figure 9 represents the data compiled in this analysis.

The production of this brown pigment increases with storage and is therefore inversely related to the ACN content. It was thought that browning was involved in pigment degradation. Hydrolysis of the glucose from the anthocyanin would release this reducing sugar
Figure 9. Production of 5% NaOH soluble brown pigments in freeze-dried strawberry puree at 100% RH and 37°C.
for subsequent non-enzymatic browning reactions. This mechanism would be consistent with the conclusions drawn from the RH study.

Figure 10 shows how the production of brown pigments varied with storage time in air and nitrogen.

The rate of brown pigment production was greater in air than in nitrogen and differed by approximately the same amount throughout the study. Therefore the presence of oxygen in a storage atmosphere does affect the browning rate of this low-moisture product but does not affect the rate of pigment degradation. These findings agree with those of Lukton, Chichester and Mackinney (1956) and Tinsley and Bockian (1960) in their studies of pigment model systems.

Gel Filtration Analyses

Polymerization has been implicated as the cause of the insoluble reddish-brown precipitate formed during ACN degradation (Lukton, Chichester and Mackinney, 1956; Markakis, Livingston and Fellers, 1957; Hamdy et al., 1961). It was thought that the degree to which polymerization occurred could be measured using the gel filtration techniques previously outlined. Any pattern of molecular weights of polymers could then be used to better understand the ACN degradation process.

Since the adsorptive capacity of Sephadex for polymerized polyphenols is eliminated by preparation in acidic alcohol solutions
Figure 10. Effect of air and nitrogen on the production of brown pigments in freeze-dried strawberry puree at 100% RH and 37°C. (— nitrogen; — air.)
(Somers, 1966), 0.01% HCl in methanol was used as the elution solvent. The gel's normal molecular sieve capacity should have been restored. The acid in the solvent also aided in the visualization of the separate bands on the column.

The 5% NaOH extraction solution used to measure the production of reddish-brown pigments was not well suited for gel filtration. Neutralization with 10% HCl reduced the color intensity of the extract and caused the formation of a precipitate. As a result no visible band was observed on the column. Because the reddish-brown pigment was partially soluble in methanol, methanol was chosen as the extraction solvent.

Figure 11 is a sample elution pattern of the methanol extract. Despite the fact that there are four peaks, only three distinct bands were observed on the column. The effluent of each run was divided into three fractions (I, II, III) as shown in the figure, and concentrated.

Poor resolution of the concentrated fractions on cellulose TLC suggested that some interfering material was present in the concentrates. Adsorption of the pigment fraction on insoluble polyvinylpyrrolidone (PVP) and subsequent washing with water was used to remove the interfering material. Rechromatography of the PVP-treated samples resulted in all fractions having very nearly the same $R_f$. 

Figure 11. Gel filtration curve of a methanol extract of freeze-dried strawberry puree on Sephadex LH-20 in 0.01% HCl in methanol.
Two-dimensional cellulose TLC was carried out on selected samples along with a pelargonidin 3-glucoside standard. Each sample was spotted individually on TLC plates as was the standard. Also each sample along with the standard was applied as a single spot. Development in the two-dimensional solvent system (Nybom, 1968) resulted in no observable differences between samples and standards.

The results show that the three separate bands on the Sephadex column were composed of the same pigment, pelargonidin 3-glucoside. One explanation could be that each band was adsorbed to the gel to a different degree and that there was no actual difference in molecular weight. Some residual adsorptive capacity must have remained in the gel after treatment with acidic methanol.

Alternatively there could have been polymers of different molecular weights, but the PVP treatment somehow destroyed the polymeric structures leaving only the monomeric pelargonidin 3-glucoside. This explanation is consistent with the finding of Somers (1966) who speculated that the three-dimensional structure of tannins could physically trap monomeric ACN's. These monomers were found to be easily removed by mild acid hydrolysis.

Gel filtration was carried out using the acidic methanol extract of the total ACN assay. The elution patterns showed but one peak at 92 ml elution volume. The maximum absorbance of this peak varied directly with the ACN concentration as shown in Figure 12.
Figure 12. Variation of maximum absorbance (- - ) with pigment retention (- - ) in gel filtration analysis of acidic methanol extract of freeze-dried strawberry puree at 100% RH and 37°C.
Pelargonidin 3-glucoside was thought to be the main constituent of the band. The elution volume of a pelargonidin 3-glucoside sample was identical to that of the pigment extract. This confirmed the speculation.

**Effect of Metal Ions on Red Color and Anthocyanin Degradation in Strawberry Puree**

The effects of stannic and other metal ions on the stability of red color in strawberry puree in relation to the breakdown of ACN pigments were investigated. Figures 13-18 show the relationship between Gardner a value and total ACN concentration.

As shown in Figure 13, pigment degradation and the drop in Gardner a value are directly related. As degradation progressed the desirable red color became an undesirable reddish-brown. An increase in the amount of reddish-brown color in the residue remaining after acidic methanol extraction was also observed. This agrees with the findings of the other degradation study.

In the presence of stannous, stannic and aluminum ions, the red color of the puree was either enhanced or remained the same throughout the experiments (Figures 14-16). This is in agreement with the findings of Sistrunk and Cash (1970). The stabilization of the color, however, did not affect the total ACN pigment concentration which followed the same or nearly the same pattern as the control. The residue which remained after acidic methanol extraction appeared dull.
Figure 13. Effect of storage at 50°C on the Gardner a value and pigment concentration (µM pigment/g puree) of straw-berry puree. (•, pigment concentration; ●, Gardner a value.)
Figure 14. Effect of stannous ion on the Gardner a value and pigment concentration (µM pigment/g puree) of strawberry puree at 50°C. (Δ-, pigment concentration; ○-, Gardner a value.)
Figure 15. Effect of stannic ion on the Gardner a value and pigment concentration (µM pigment per g puree) of strawberry puree at 50°C. (-A-, pigment concentration; -○-, Gardner a value.)
Figure 16. Effect of aluminum ion on the Gardner a value and pigment concentration (µM pigment/g puree) of strawberry puree at 50°C. (-△-, pigment concentration; -○-, Gardner a value.)
Figure 17. Effect of ferrous ion on the Gardner a value and pigment concentration (µM pigment/g puree) of strawberry puree at 50°C. (-△-, pigment concentration; -○-, Gardner value.)
Figure 18. Effect of ferric ion on the Gardner a value and pigment concentration (μM pigment/g puree) of strawberry puree at 50°C. (-Δ-, pigment concentration; -○-, Gardner a value.)
red in color with these three treatments and its intensity appeared to increase with time.

This could mean that the degraded ACN was bound up in some form of insoluble complex. The rate of the actual complexing reaction, if it did occur, must then have increased with time. This finding agrees, in part, with that of Sistrunk and Cash (1970), who observed that the amount of reddish-brown pigments, not extractable with ethanol and extractable only with 5% NaOH, increased with extended storage time and was higher in those samples treated with stannous and stannic ions.

They discussed the possibility that colorless tannins, which react with metal ions to form red, brown, gray and other colored complexes, could be the cause of the reddish-brown pigments in the puree residue. Another possible explanation could be the colored complexes formed between quercetin and stannous and stannic ions (Kirk and Pockington, 1969; Raik and Timofeeva, 1969; Wieczorek, 1969). Quercetin has been identified as one of the flavonoid constituents of strawberries by Williams and Wender (1952). Heintze (1960) stated that stannous ion forms chelates in the acid range of canned fruits with polyphenols (anthocyanins, flavonals and catechols). The formation of such colored complexes could account both for the increase in the stability of the red color and the increase in the unextractable reddish-brown pigments.
Ferrous ion caused a rapid decline in the redness value of the puree (Figure 17). A purplish-black color, which darkened somewhat upon storage, was produced. However, the pigment degradation rate was the same as that of the control sample. The residue retained the purplish-black color.

Ferric ion caused a much more rapid drop in the redness value of the puree (Figure 18) than did the ferrous ion. The color of the puree turned purplish-black almost immediately upon addition of the metal ion solution. As in the case of ferrous ion, the pigment degradation rate was similar to that of the control. The residue which remained after pigment extraction was black in color.

Quercetin is known to form a dark colored complex when combined with ferric ion (Cruess, 1958). Oxidation of ferrous iron to ferric iron could account for the formation of the purplish-black color upon addition of either ferrous and ferric ions to the strawberry puree.


