compound were obtained by x-ray diffractions. Nujol Mulls were then prepared and infrared scans made. The scans obtained are shown in Figure 4. A review of the scans shows that the material isolated from the liver homogenate corresponds closely to the 3-amino-5-nitro-*o*-toluamide.

Additional proof of the structure of the unknown compound was obtained with the Bratton-Marshall test. If the Bratton-Marshall procedure for the determination of aryl amines were carried out in butyl alcohol, distinct color curves were obtained for the 3-amino-5-nitro-*o*-toluamide and the 5-amino-3-nitro-*o*-toluamide. This reaction was carried out by adding 1 ml. of a 0.25% solution sodium nitrite to the compound in 5 ml. of 4*N* HCl. After 5 minutes, 1 ml. of 1.25% ammonium sulfamate was added. After allowing the solution to stand another 5 minutes, 85 ml. of butyl alcohol was added followed by 1 ml. of 0.25% solution of *N*-(1-naphthyl) ethylenediamine dihydrochloride. Typical spectral curves obtained are shown in Figure 5. The spectral curve for the unknown compound corresponds to the 3-amino-5-nitro-*o*-toluamide indicating that the unknown compound was probably the 3-amino-5-nitro-*o*-toluamide.

Sample II (Figure 1) was analyzed by the Bratton-Marshall, sodium methylate, tetramethylammonium hydroxide, diaminopropane, and potassium cyanide methods (*5*, *6*, *8*, *10*). The Bratton-Marshall test was negative while all the rest were positive (*8*). The colors obtained in these tests suggested that the compound was probably 3,5-dinitro-*o*-toluamide. Elemental analysis indicated an empirical formula of $C_8H_7N_3O_5$, and molecular weight determination gave 225. These values correspond to those obtained with 3,5-dinitro-*o*-toluamide.

Infrared scans of sample II were compared with the reference standard of 3,5-dinitro-*o*-toluamide and were found to be identical. From the results obtained, it was concluded that sample II was 3,5-dinitro-*o*-toluamide.

Apparently incubation of 3,5-dinitro-*o*-toluamide with liver homogenates results in the reduction of one of the nitro groups of 3,5-dinitro-*o*-toluamide with the formation of the 3-amino-5-nitro-*o*-toluamide. This latter compound apparently binds to the tissues when it is formed in the normal metabolism of the 3,5-dinitro-*o*-toluamide in the chicken. Other compounds are also produced by the metabolism of 3,5-dinitro-*o*-toluamide, but they do not appear to be bound to the tissue to any great extent.

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FRUIT PRESERVATIVES ANALYSIS

Determination of Calcium in Cherry Brines by Versenate Titration: Elimination of Anthocyanin Interference by Means of Carbonyl Reagents

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Carbonyl reagents were used to decolorize anthocyanin pigments in cherry brines which could then be titrated with disodium dihydrogen versenate (EDTA) solution. On brines ranging in calcium content from 500 to 9000 p.p.m., the EDTA method gave values that averaged 46 p.p.m. higher than the A.O.A.C. permanganate titration method. The precision of the EDTA method was significantly better. Recovery of calcium added to brines was 97.5 to 100%. Addition of 200 p.p.m. of ferric or phosphate ions, or 500 p.p.m. of aluminum, cupric, lead, or magnesium ions to brines did not interfere with the endpoint of murexide indicator.

The preservation of sweet cherries in calcium bisulfite brine is the initial step in processing maraschino and glacé cherries, as well as cherries for fruit cocktail and fruits for salad. Control of the amount of calcium in the brine is important in achieving the desired firm texture in the product. In the search for a simple, rapid method for determining calcium in the brine, attention was focused on complexometric titration techniques to circumvent precipitation and filtration steps that are employed in the conventional oxalate precipitation method (*1*).

Numerous reports on the rapid EDTA titration method for calcium are to be found, differing chiefly as to the indicator used. Schwarzenbach (7) described the complexometric titration of calcium using murexide as the indicator. Lewis and Melnick (*3*) reported studies on the accuracy of determinations of calcium and magnesium in which calcon and cal-red indicators were used. The accuracies they reported indicated that the EDTA titration method for calcium would be satisfactory for use in determining calcium in cherry brines. Moss (*4*) studied the limits of interference by various ions in the EDTA determination of calcium using cal-red indicator. He noted the use of hydroxylamine to reduce manganic ions and thus eliminate that source of interference. Schwarzenbach (*6*), likewise, mentions the use of hydroxylamine as a reducing agent for interfering ions, but its function as a carbonyl agent in removing interfering

colors due to anthocyanins has not been described previously.

The anthocyanins are decolorized by reaction with reagents generally used for carbonyl compounds (to be reported in detail in a future article). The mechanism likely involves the formation of ionized chalcones from the flavyllium salts of the anthocyanins at high pH as described by Pratt and Robinson (*5*). The chalcones react with hydroxylamine or hydrazine to form colorless oximation products such as demonstrated by Collins *et al.* (*2*) with 4,8-dimethoxyflavyllium chloride. Hydrazine can be used in place by hydroxylamine for decolorizing, although the latter is preferred because it is soluble in cold water, and, although irritating to the eyes and mucous membranes, it is less toxic than hydrazine.

Reagents

Standard Calcium Solution. Dissolve 1.000 gram of pure calcite or primary standard grade calcium carbonate in the minimum quantity of dilute hydrochloric acid (1:4). Boil, cool, and dilute to 1 liter with distilled water. 1 ml. = 0.4 mg. Ca.

Standard Versene (EDTA) Solution. Dissolve 4.0 grams of disodium dihydrogen ethylenediamine tetraacetate in 800 ml. of distilled water. Adjust pH to within the range 4.3 to 5.0. Dilute to 1 liter with distilled water. Standardize against standard calcium solution and adjust so that 1 ml. equals 0.4 mg. as calcium.

0.5*M* Hydroxylamine Hydrochloride. Dissolve 35 grams hydroxylamine hydrochloride in 1 liter of distilled water.

1*N* Sodium Hydroxide. Dissolve 42 grams of sodium hydroxide in 1 liter of distilled water. (This weight is taken to compensate for impurities in the sodium hydroxide.)

Calcium Indicator. Mix 0.2 gram of ammonium purpurate (murexide) with 100 grams of C.P. sodium chloride. Grind the mixture to 40–50 mesh. This dye is stable in the dry mixture.

Procedure

Preparation of Sample. If the brine is clear, no preparation is needed. Otherwise, filtration through a coarse filter is necessary to remove sediment which would interfere with the endpoint. The solution does not have to be free from cloudiness as subsequent dilution minimizes this.

Decolorizing Anthocyanin Pigments. Transfer 10 ml. of 0.5*M* hydroxylamine hydrochloride solution to a 400-ml. beaker containing 150 ml. of distilled water. Pipet 20 ml. of the cherry brine into the beaker and bring to pH 12.5 with 1*N* sodium hydroxide; this normally requires 30 ml. Transfer beaker contents to a 250-ml. volumetric flask and make to volume. (If the method is used for routine control to check the calcium content of standard brines, add a 20-ml. aliquot of the brine to 190 ml. of distilled water and 10 ml. of 0.5*M* hydroxylamine hydrochloride solution in a 250-ml. volumetric flask, and bring to volume with 1*N* sodium hydroxide.) The color will be blue on addition of sodium hydroxide if anthocyanins are present, but will change to green and then to greenish yellow or yellow in 15 minutes or less. Aliquots may be titrated immediately, or when convenient. However, on longer standing the color becomes a deeper yellow, and on standing overnight, a brownish flocculent precipitate may settle out. This does not affect the calcium determination. Substitution of 10 ml. of 0.2*M* hydrazine sulfate for hydroxylamine hydrochloride is a satisfactory alternative.

Titration of Calcium. Pipet 50 ml. of the treated brine into a 250-ml. Erlenmeyer flask. Add 50 ml. of distilled water and 0.4 gram of ammonium purpurate indicator (this can be measured with a calibrated scoop). Swirl to dissolve the indicator, and titrate with the 0.01*M* EDTA solution using a 25-ml. buret. The color, on addition of the indicator, will be salmon-orange if calcium is present. As the endpoint is approached, the color changes to rose. The endpoint is reached when the color changes to violet blue; no further change occurs on addition of more titrant. If the calcium content is very high, decrease the aliquot taken for titration, but maintain the 100-ml. total volume by addition of distilled water to the titrant. If the calcium content is very high, decrease the aliquot taken for titration, but maintain the 100-ml. total volume by addition of distilled water to titration flask. For the blank, follow the procedure outlined, but omit the brine.

Calculations.

$$\frac{\text{ml. of titrant} \times 400}{\text{ml. of sample}} = \text{p.p.m. calcium}$$

Results

Samples of brine from several lots of brined cherries were analyzed by the official A.O.A.C. permanganate titration method (*1*) and by the method described here. The considerable number of manipulations and the time involved in the A.O.A.C. method are in marked contrast with the simplicity of the EDTA method.

The EDTA titrimetric method gave slightly higher values than the A.O.A.C. method, the average difference being 46 p.p.m. (Table I). The precision of the EDTA method was significantly better than that of the A.O.A.C. method. The ratio of their error variances was 6.2 (significant at $P < 0.02$).

Accuracy of the EDTA method was tested by pipetting exactly 10 ml. of a standard solution containing 4 mg. per ml. of calcium into a 200-ml. volumetric flask and adding cherry brine to volume. A second flask was prepared by replacing the calcium solution with exactly 10 ml. of distilled water. This diluted the original brine 5% in both flasks, with the first flask containing 200 p.p.m. of added calcium. Table II shows the results of analyses after dilution and after the addition of calcium. Samples were brines from Bing and Royal Anne cherries. Recoveries determined in this fashion were 97.5 to 100%.

Table I. Comparison of Methods for Determining Calcium in Cherry Brines

(Two determinations on each sample were made by each method)

	Calcium, P.P.M.	
Brine Sample	EDTA method with NH_2OH	A.O.A.C. permanganate method
1	475	495
2	1325	1295
3	1335	1295
4	1360	1305
5	1370	1310
6	1370	1310
7	1390	1330
8	1445	1430
9	9120	9000
Av.	2132	2086
Pooled std. dev.	15	37

Table II. Recovery of Calcium Added to Cherry Brines

Sample	Brine, P.P.M. Ca	Brine with 200 P.P.M. Added, Ca	Recovery, %
A 1	1300	1460	
2	1290	1500	
3	1290	1490	
4	1280	1490	
Av.	(1290)	(1485)	97.5
B 1	570	770	
2	570	765	
Av.	(570)	(768)	99.0
C 1	530	730	
2	530	730	
Av.	(530)	(730)	100.0

Discussion

In the absence of a carbonyl reagent, the naturally occurring anthocyanin pigments render the test solution deep blue. This obscures the color of indicators and renders the method useless. The carbonyl reagents did not interfere with the EDTA complexometric titration of calcium.

As noted by Strachan (*8*), dilution minimizes interference by other cations. This is confirmed in the work with cherry brines; at the $^1/_{25}$ dilution, the endpoint is sharp and readily seen. Interference

Table III. Calcium Assay on Cherry Brine Samples with Added Ions

(Original brine contained 1665 p.p.m Ca^{+2}; murexide indicator used)

Ion Added	P.P.M.	Ca^{+2} Meosured[a]
Fe^{+3}	50	1665
	100	1675
	200	1665
	500	No endpoint
Al^{+3}	50	1660
	200	1690
	500	1675
Mg^{+2}	50	1670
	200	1695
	500	1695
Pb^{+2}	50	1660
	200	1690
	500	1675
PO_4^{-3}	50	1673
	200	1660
	500	No endpoint
Cu^{+2}	500	1665

[a] Averages of triplicate samples.

Table IV. Comparison of Calcium Determination with Four Different Indicators in Complexometric Titrations

	P.P.M. Calcium			
Brine Sample	Calcein	Calcon	Superchrome Blue B Extra	Murexide
Brine I	1800	1760		1715
+ 50 p.p.m. Fe^{+3}		1770		
+100 p.p.m. Fe^{+3}	1770	No endpoint		
+500 p.p.m. Fe^{+3}	1800	No endpoint		1705
+50 p.p.m. PO_4^{-3}	1770	1780		
+100 p.p.m. PO_4^{-3}	1770	1780		
+200 p.p.m. PO_4^{-3}	1790	1780		
+500 p.p.m. PO_4^{-3}	1790	No endpoint		1745
+50 p.p.m. Mg^{+2}	1845			
+200 p.p.m. Mg^{+2}	1845	No endpoint		1760
+500 p.p.m. Mg^{+2}	No endpoint	No endpoint		1745
Brine II			1000	1000
+50 p.p.m. Fe^{+3}			No endpoint	
+500 p.p.m. Fe^{+3}			No endpoint	995
Brine III			1450	1440
Brine IV			1650	1645
Brine V			1650	1630
Brine VI	1570		1700	1680
Brine VII	3760		4040	3740
Brine VIII	1605		1860	1590

of several ions was tested by adding known amounts to cherry brine and proceeding with the analysis using murexide indicator. The results are in Table III. The ions are those that might be found in brined cherry samples, but that normally occur in considerably smaller amounts than tested here. Ferric and phosphate ions interfered at 500 p.p.m. but did not interfere at 200 p.p.m. No interference was attributed to the other ions at levels of 500 p.p.m.

Several other indicators were tested, also, to compare their endpoints with that of murexide and to determine their sensitivity to interfering ions. Calcein, calcon, and Superchrome Blue B Extra showed more easily distinguishable endpoints in some cherry brine samples, but were somewhat more sensitive to interfering ions (Table IV). Calcon gave no endpoint in the presence of 100 p.p.m. ferric ion, 500 p.p.m. phosphate ion, or 200 p.p.m. magnesium ion. Calcein gave higher values than murexide, especially in the presence of added magnesium, and gave no endpoint in the presence of 500 p.p.m. added Mg. Superchrome Blue B Extra gave no endpoint in the presence of 50 p.p.m. added ferric ion. Ferric ion and phosphate ion at 500 p.p.m. interfered with the murexide endpoint in the complexometric titration of the brine sample used for the experiment summarized in Table III. However, in other brine samples (Table IV), a satisfactory endpoint was observed in the presence of these ions at the 500 p.p.m. level. Of the indicators tested, murexide was the most reliable in the presence of interfering ions.

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