is decomposed in the presence of the substrate and that the enzyme, now devoid of coenzyme, is susceptible to thermal inactivation. A case in point is rabbit muscle glyceraldehyde phosphate dehydrogenase, which Velick, Hayes, and Harting (*11*) have demonstrated to be very unstable at room temperature when deprived of the coenzyme, diphosphopyridine nucleotide, with which it is normally associated. Beevers (*3*) reported barley glutamic acid decarboxylase to be subject to thermal inactivation. The failure to observe the anomalous inactivation at pH 6.5 as opposed to lower pH in the absence of pyridoxal phosphate may also be explicable on the basis of the increased sensitivity of the enzyme to inactivation at lower pH values.

Citrus flavedo tissue is a relatively good source of the enzyme, being 10 times richer on a fresh-weight basis than the most active of the plant materials listed by Schales, Mims, and Schales (*7*). The activity of the 0.36 to 0.39 ammonium sulfate fraction from the purification study when expressed in terms of cubic millimeters of carbon dioxide per milligram protein nitrogen per hour is approximately 40,000 at 37° C. Assuming the reaction velocity to be halved at 30° C., this value becomes 20,000 as compared to 12,500 obtained by Weinberger and Clendenning (*12*) with a highly purified wheat leaf preparation. In view of the fact that the flavedo purification was of the most preliminary sort, it would seem that orange flavedo is the starting material of choice.

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Literature Cited

(1) Adamson, D. W., *J. Chem. Soc.*, **1939,** 1564.
(2) Axelrod, B., Symposium on Citrus Products, 9th Annual National Meeting, Institute of Food Technologists, San Francisco, Calif., July 11, 1949.
(3) Beevers, H., *Biochem. J. (London)*, **48,** 132 (1951).
(4) Metzler, D. E., Ikawa, M., and Snell, E. E., *J. Am. Chem. Soc.*, **76,** 648 (1954).
(5) Okunuki, K., *Botan. Mag. (Tokyo)*, **51,** 270 (1937).
(6) Rockland, L. B., personal communication.
(7) Schales, O., Mims, V., and Schales, S. S., *Arch. Biochem.*, **10,** 455 (1946).
(8) Schales, O., and Schales, S. S., *Ibid.*, **11,** 155 (1946).
(9) Tafel, J., and Stern, M., *Ber. deut. chem. Ges.*, **33,** 2224 (1900).
(10) Umbreit, W. W., Bellamy, W. D., and Gunsalus, I. C., *Arch. Biochem.*, **7,** 185 (1945).
(11) Velick, S. F., Hayes, J. E., Jr., and Harting, J., *J. Biol. Chem.*, **203,** 527 (1953).
(12) Weinberger, P., and Clendenning, K. A., *Can. J. Botany*, **30,** 755 (1952).
(13) Yanofsky, C., *J. Biol. Chem.*, **198,** 343 (1952).

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PECTIC PRODUCTS IN FRUIT

Determination of Galacturonic Acid in Tomatoes and Its Changes on Storage

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The degradation of pectic substances to D-galacturonic acid is a possible explanation of the fate of these compounds in fruits and vegetables. Using the tomato as the test fruit, the object of this investigation was to devise an analytical procedure for D-galacturonic acid in tomatoes and to determine the changes in galacturonic acid content of tomatoes during storage.

THE FATE OF PECTIC SUBSTANCES in fruits and vegetables was thought to be due to degradation of these compounds to D-galacturonic acid. Therefore an investigation was undertaken, using the tomato as the test fruit, with the objective of devising an analytical procedure for determining D-galacturonic acid in tomatoes and determining the changes in galacturonic content of tomatoes during storage. An extraction procedure and a method for separating interfering substances were required.

Preliminary Investigations

Two procedures using naphthoresorcinol—those of Kertesz (*7*) and of Winkler (*9*)—were studied. The Winkler procedure used 50 mg. of naphthoresorcinol per sample of galacturonic acid, so that a recrystallized lot of reagent sufficed to analyze only 7 to 15 samples, when adequate checks on the reliability of the reagent were made. The Kertesz method was investigated because only 4 mg. of naphthoresorcinol was used per analysis, and this permitted adequate checks and duplicate determinations; at least 100 samples could be analyzed with one sample of recrystallized naphthoresorcinol. Modifications in acid concentration and reaction temperature of the Kertesz procedure were developed which permitted the analysis of galacturonic acid solutions containing between 0.0050 and 0.0550 mg. per ml. with a precision within 0.0004 mg. per ml.

In the modified procedure the hydrogen ion concentration was reduced from 3.90*N* to 3.56*N* and the reaction temperature was lowered from 100° to 91° C. The reduction of the hydrogen ion concentration increased the range of galacturonic acid that could be determined.

Experiments indicated that neither color nor melting point was satisfactory as an index of purity of naphthoresorcinol. Therefore each lot of recrystallized naphthoresorcinol was checked with standard solutions of galacturonic acid before use.

In order to separate the tomato carbohydrates that react with naphthoresorcinol from the galacturonic acid, the ion exchange technique was used.

The excellent recovery of galacturonic acid from standard solutions and from ethanolic extracts of tomatoes indicated that this method made possible the determination of galacturonic acid in tomatoes.

Procedure

Extraction of Galacturonic Acid from Tomatoes The procedure was adapted from the standard procedure for precipitating alcohol-insoluble solids preparatory to determination of pectic substances (*2*). The tomatoes in each 1-kg. sample were cut into thin slices and dropped into 2750 ml. of boiling 95% ethyl alcohol. This gave a final concentration of 70% ethyl alcohol after addition of the sample. The mixture was boiled for a few minutes and allowed to cool overnight. The insoluble solids were crushed with a pestle while still in the alcohol, the mixture was filtered with suction, and the solution volume was measured. A sample of the solution was reserved for determination of galacturonic acid.

Purification of Naphthoresorcinol Flush a 250-ml. Erlenmeyer flask with nitrogen. Dissolve approximately 3.5 grams of C.P. naphthoresorcinol in 100 ml. of hot water in the flask and add a few granules of sodium hydrosulfite and then 0.3 gram of Norit activated carbon to the solution. Flush a funnel and Erlenmeyer flask with nitrogen. Place 7 grams of sodium chloride in the flask and continue to flush both the funnel and the flask with nitrogen while rapidly filtering the solution. Stopper the flask tightly and place in a refrigerator overnight. Filter the crystals, wash with cold 7% sodium chloride solution, and dry under vacuum. Grind the crystals in a glass mortar before use.

Naphthoresorcinol Reagent Add 0.1 gram of naphthoresorcinol to 50 ml. of water and hold the solution at 37° C. for 24 hours. Filter the solution and store under refrigeration in a dark bottle. Check the solution daily with standard galacturonic acid solutions.

Determination of Galacturonic Acid Add 2 ml. of the naphthoresorcinol reagent to 2 ml. of sample and 1.83 ml. of concentrated hydrochloric acid in a 25-ml. volumetric flask. Shake to mix the contents and place in a water bath thermostated at 91° C. for 45 minutes. Cool for 10 minutes in ice water, then add 2 ml. of 95% ethyl alcohol and 17 ml. of ether. Extract the violet color by shaking for 30 to 60 seconds. Determine the absorbance of the ether extract in cuvettes at 575 mμ with a water-cooled Beckman DU spectrophotometer. Use a 2-ml. water blank as a standard for 0.000 absorbance.

Standard Curve for Galacturonic Acid

A standard curve of absorbance *vs.* galacturonic acid concentration is shown in Figure 1. Each point on the curve is a mean of at least three absorbance determinations at the various galacturonic acid concentrations used. A straight line drawn between values for 0.0 and 0.02 mg. per ml. differs from the determined line by only 0.0005 mg. per ml. A straight line constructed between the values for 0.02 and 0.0550 mg. per ml. deviated only 0.0005 mg. per ml. from the determined curve. The mean deviation with the modified Kertesz method was 0.0004 mg. per ml. over the range from 0.01 to 0.0550 mg. per ml.

The naphthoresorcinol procedure is dependent upon color development in the reaction mixture. It is not specific for galacturonic acid, as other substances may react with naphthoresorcinol to produce interfering color complexes. Therefore, although the recommended procedure is precise for pure galacturonic acid solutions, compounds present in the tomato extract may interfere.

Separation of Galacturonic Acid from Interfering Substances

The procedure used for extracting galacturonic acid from tomatoes resulted in a 70% ethyl alcohol solution containing galacturonic acid, other organic acids, carbohydrates, nonpectic polygalacturonides, inorganic salts, and some protein. Only the organic acids, carbohydrates, and nonpectic polygalacturonides were suspected of interfering in the modified Kertesz naphthoresorcinol procedure.

When the modified procedure was used in testing prepared solutions of tomato acids, the naphthoresorcinol did not react with the acids to produce a color complex. However, carbohydrates, fructose, glucose, and sucrose did produce an interfering color complex.

In order to separate the tomato carbohydrates from the galacturonic acid, the ion exchange technique was used. Attempts to separate galacturonic acid from carbohydrates using the hydroxyl form of Amberlite IRA 400 were unsuccessful. Experiments indicated that IRA 400(CO_3) did not retain sugars and, therefore, could be used to achieve the desired separation. Selection of the proper elution conditions, such as pH, flow rate, and elution rate, enabled a recovery of galacturonic acid to within 0.15 mg. Standard solutions of galacturonic acid containing the tomato acids and carbohydrates were eluted to study the efficiency of recovery (Table I). The mean loss in three elutions was 0.08 mg.

Galacturonic acid added to three ethanolic extracts of tomatoes, originally containing 4.10, 3.86, and 3.50 mg. of galacturonic acid per 100 ml. of extract, was recovered with a mean loss of 0.08 mg., as shown in Table II.

After the ion exchange technique has been thoroughly tested by using solutions of galacturonic acid and acids and carbohydrates and also ethanolic extracts of tomatoes with added galacturonic acid, the following procedure was adopted.

Decant 30 ml. of Amberlite IRA 400 anion exchange resin several times with water to remove ultrafine particles. Place the washed resin in a glass column 25 cm. long and 22 mm. in diameter with a fritted-glass disk at one end. Place a separatory funnel fitted with a cork at the upper end of the column. Fit a short piece of rubber tubing with a 2-inch length of glass tubing and a Hoffman clamp at the lower end. Wash the column three times with 120 ml. of 1.2*N* hydrochloric acid, with 200 ml. of water, and with 60 ml. of 1.2*N* sodium hydroxide. Wash the chloride form of the resin with 500 ml. of 1.0*N* sodium carbonate at 3 ml. per minute, rinse with 200 ml. of water, and replace the water with 50 ml. of 35% ethyl alcohol.

Pipet 50 or 100 ml. of the ethanolic tomato extract into a beaker and dilute with an equal quantity of water. Adjust the pH of the solution to 8.0 with 1*N* sodium hydroxide, using the Beckman pH meter. Pass the sample through the column at a rate of 3 ml. per minute. Rinse the beaker with 50 ml. of 35% ethyl alcohol, rerinse with 50 ml. of water, and pass both washings through the column at 3 ml. per minute. Discard these eluates. Elute the galacturonic acid with 270 ml. of 1.0*N* sodium carbonate. Collect and mix the eluate in a 500-ml. graduate. Analyze 2-ml. portions of the eluate by the modified naphthoresorcinol procedure. Regenerate the column with 250 ml. of 1.0*N*

Figure 1. Standard curve of absorbance *vs.* concentration by modified Kertesz naphthoresorcinol method for determining galacturonic acid

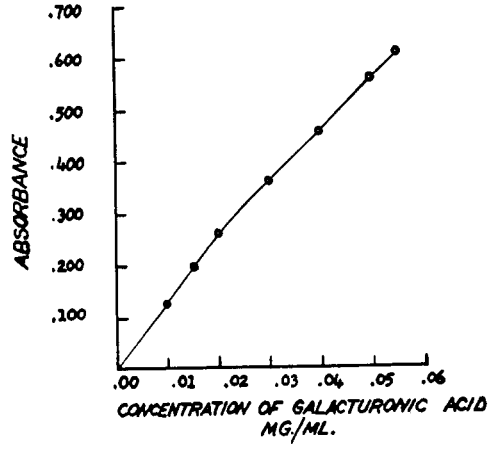

Table I. Recovery of Galacturonic Acid from Solutions Containing Carbohydrates and Acids

[Using IRA 400(CO_3)]

Sample Number	Galacturonic Acid in Sample, Mg.	Fructose in Sample, Mg.	Glucose in Sample, Mg.	Sucrose in Sample, Mg.	Galacturonic Acid Recovered, Mg.
1	6.00	200	200	200	6.07
2	6.00	400	400	400	5.86
3	6.00	640	200	...	5.99

Table II. Recovery of Galacturonic Acid Added to Ethanolic Extracts of Tomatoes

[Using IRA 400(CO_3)]

Sample	Vol. of Sample, Ml.	Galacturonic Acid, Mg.			
		Added	Found	Recovered	Loss
B-1	50.0	0.00	2.05	...	...
B-1	50.0	0.00	2.04	...	...
B-1	100.0	0.00	3.98	...	...
B-1	50.0	0.83	2.92	0.87	+0.04
B-1	50.0	10.21	12.17	10.12	−0.09
B-2	100.0	0.00	3.86	...	...
B-2	100.00	5.44	9.25	5.39	−0.05
B-2	100.00	2.61	6.32	2.46	−0.15
B-3	100.00	0.00	3.50	...	...
B-3	100.00	5.44	8.90	5.40	−0.04
B-3	100.00	8.72	12.11	8.61	−0.11

sodium carbonate at a flow rate of 3 ml. per minute before each use.

The galacturonic acid content of tomatoes was calculated as follows. The galacturonic acid concentration of the eluate was obtained from the experimental mean absorbance of two determinations by the modified naphthoresorcinol method and the standard curve of concentration *vs.* absorbance in Figure 1.

Sample Calculation.

Mean absorbance = 0.111

From Figure 1 the concentration of galacturonic acid = 0.0079 mg. per ml.

0.0079 mg. per ml. times 270 ml. of eluate = 2.13 mg.

Volume of aliquot passed through column = 50 ml.

Volume of total ethanolic extract = 2900 ml.

$$2.13 \text{ mg. times } \frac{2900 \text{ ml.}}{50 \text{ ml.}} = 123 \text{ mg. per kg. of tomatoes}$$

To separate galacturonic acid from carbohydrates in a nonalcoholic solution, the same procedure was followed, except that the sample was not diluted with water before the pH of the sample was adjusted. The sample eluted contained between 1 and 14 mg. of galacturonic acid and the total acid content did not exceed 270 mg.

This ion exchange procedure, used in conjunction with the procedures for extraction and determination of galacturonic acid in pure solution, was employed to find the galacturonic acid content of stored tomatoes.

Galacturonic Acid Content of Stored Tomatoes

Forty-five pounds of Queen's variety tomatoes were picked from a single plot of the New Jersey Agricultural Experiment Station Vegetable Research Farm on September 1, 1953. The seed had been sown on March 28, 1953, and the seedlings planted in the field on May 25, 1953. The ground was fertilized with equal quantities of nitrogen, phosphorus, and potassium. The plot was dusted twice with rotenone. The tomatoes were sorted after picking, so that fruit free from cracks, insect damage, and bruises were used. The fruit were red ripe with some yellow discoloration. Nine samples of from 6 to 9 tomatoes, each weighing 1 kg. ± 7 grams, were prepared. The tomatoes were stored in enamel trays in an open refrigerator at 42° F.

Three replicates were withdrawn after one day of storage and the galacturonic acid content was determined by the extraction, separation, and analysis methods. No visible changes had occurred during this storage period. After 20 days visible softening had occurred and the skin of three replicates had a slightly shriveled appearance. The last three replicates were removed from storage after 41 days. The fruit were soft to the touch, and had mold areas, although some firm areas remained. The results of the analyses are shown in Table III. Calculation of the mean galacturonic acid content at each storage date showed the expected difference in glacturonic acid content of the replicates. To determine whether the decrease in mean galacturonic acid with time was a chance occurrence due to variation between biological replicates or a significant change due to a correlation between galacturonic acid content and storage time, an analysis of variance was performed on the data. An F ratio of 3.03 was obtained. From the tables of distribution of F values, it was found that $F_{5\%} = 5.14$ (*7*).

The mean galacturonic acid content of Queen's variety tomatoes decreased slightly with time. An analysis of variance gave a variance factor of 3.03, which was not significant. If the F value were 5.14, it would mean that there were 19 chances in 20 that the galacturonic acid content changed significantly with time.

Both LeCrone and Haber (*3*) and Stier (*8*) found that the total pectic substances in tomatoes decreased during storage. If the pectic substances were degraded to monomer galacturonic acid, an increase in galacturonic acid would occur during storage, which would approximately equal the decrease in weight of the total pectic substances. In this study no such increase was found.

Neither digalacturonic acid nor trigalacturonic acid reacts with naphthoresorcinol (*5*, *6*). These compounds may be the reaction products of pectic degradation in tomatoes. Pectic acid depolymerase, which has been found in tomatoes, causes selective hydrolysis of polygalacturonic acids in vitro without producing monomer galacturonic acid (*4*). If the same reaction occurs in tomatoes, the products must by some unknown mechanism, form nonpectic substances.

Conclusions

A modification of the Kertesz naphthoresorcinol determination was devised which permitted the analysis of galacturonic acid solutions containing between 0.000 and 0.0550 mg. per ml. with a precision within 0.0004 mg. per ml. One lot of reagent sufficed to analyze 100 samples with duplicate determinations.

An ion exchange procedure using IRA 400 (CO_3) made possible the quantitative separation of galacturonic acid from standard solutions containing acids

Table III. Influence of Storage Time on Galacturonic Acid Content of Queen's Variety Tomatoes

	Storage Period		
	1 day	20 days	41 days
	Galacturonic Acid, Mg./Kg.		
	177	140	119
	153	156	101
	130	124	130
Means	153	140	117

and carbohydrates as well as ethanolic extracts of tomatoes.

From 1 to 14 mg. of galacturonic acid could be determined in an aliquot of standard solution or tomato extract containing up to 276 mg. of organic acids and 100 mg. of carbohydrate with an accuracy within 0.12 mg. using these procedures. Galacturonic acid content of Queen's tomatoes did not appreciably change with storage time. No increase in galacturonic acid, which would occur if the pectic substances were degraded to galacturonic acid, was found.

Literature Cited

(1) Kertesz, Z. I., "Pectic Substances," Interscience, New York, 1951.
(2) Kertesz, Z. I., and McColloch, R. J., N. Y. State Agr. Expt. Sta. Bull. **745** (1950).
(3) LeCrone, F., and Haber, E. S., *Iowa State Coll. J. Sci.*, **7,** 467 (1953).
(4) McColloch, R. J., and Kertesz, Z. I., *Arch. Biochem.*, **17,** 197 (1948).
(5) Phaff, H. J., and Luk, B. S., *Arch. Biochem. Biophys.*, **36,** 231 (1952).
(6) Rahman, M. B., and Joslyn, M. A., *Food Research*, **18,** 308 (1953).
(7) Snedecor, G. W., "Statistical Methods," Collegiate Press, New York, 1937.
(8) Stier, E. F., "Changes in Pectic Substances during Storage of Tomatoes," Ph.D. thesis, Rutgers University, 1951.
(9) Winkler, W. O., *J. Assoc. Offic. Agr. Chemists*, **34,** 511 (1951).

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IMPURITY MEASUREMENT IN FATS

Spectrophotometric Determination of Melamine and Formaldehyde in Lard

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Ultraviolet and visible spectrophotometric methods for the determination of micro amounts of melamine and formaldehyde in aqueous media have been modified in order to obtain the desired detectability in fats such as lard. These methods were used in the investigation of the degree to which melamine and formaldehyde might be extracted from wet-strength paper by lard.

PROPOSALS FOR THE USE OF MELAMINE-FORMALDEHYDE RESINS to impart wet strength to paper intended for food wrapping have prompted an investigation of the degree to which melamine and formaldehyde might be extracted from the paper by the food. Ultraviolet (*4*) and visible (*1, 5*) spectrophotometric methods were available for the determination of micro quantities of these compounds in aqueous media; however, modifications of these methods were required in order to achieve the necessary detectability for their application to fats such as lard. The present paper describes a procedure of extraction of the lard with dilute hydrochloric acid and a hydrocarbon, which yields as increase in detectability and eliminates certain interferences.

Apparatus and Materials

A Cary automatic recording spectrophotometer, Model 11, No. 67, and fused quartz cells of various light path lengths were used for the ultraviolet spectrophotometric work. A modified General Electric recording spectrophotometer, equipped with a photomultiplier tube, and 10-mm. glass cells were used for the visible spectrophotometric work. "Spectroscopically pure" isooctane and 12N and 0.1N hydrochloric acid were used. Solutions of phenylhydrazine hydrochloride (1%) and of potassium ferricyanide (5%) in distilled water were prepared fresh daily. Raw prime steam lard (Swift), containing no antioxidants or inhibitors, was used in the recovery and storage tests. (Commercial antioxidants have ultraviolet absorption which interferes with the detection of melamine.) Wet-strength wrapping paper, treated with PAREZ resin 607 (*2*) (a commercial trimethylolmelamine, American Cyanamid Co.) and a non-resin-treated wrapping paper were used in the storage tests.

Ultraviolet Spectrophotometric Method for Melamine

The strong absorption band of melamine at 235 mμ in dilute acid solution has been used for the determination of melamine in wet-strength paper, (*4*), and the theoretical detectability has been calculated to be 4 γ. The detectability of melamine in a material such as lard may be improved by an extraction procedure which serves to separate the melamine from interfering materials and to concentrate the melamine in a minimum volume corresponding to the volume of the absorption cell to be used. Although melamine and melamine resin (trimethylolmelamine) may be distinguished from one another by their spectra and a two-component analysis may be performed (*3, 4*) when these compounds are present in appreciable amounts, it is not possible to distinguish them in the extremely low concentrations encountered in the lard samples. As the resin and the melamine have very nearly the same absorptivity at the analytical wave length, 235 mμ, the data may be reported as "melamine and/or resin."

It was found that some absorbing material was present in the 0.1N hydrochloric acid extraction of an iso-octane solution of lard which had not been exposed to wet-strength paper. In order to compensate for this interfering absorption, a suitable correction term had to be devised. A simple subtractive term using absorbance at some longer wave length where melamine did not absorb (*3, 4*) was ineffective. Examination of 17 extracts of fresh lard solutions showed that the ratio of absorbances at 235 and 260 mμ was constant (Table I).

This allowed the use of a correction term based on the experimentally determined ratio of absorbances of "blank" lard samples stored under the same conditions as the test samples.

Procedural Details

Approximately 15 grams of lard were weighed by difference on an analytical balance, dissolved in 100 ml. of iso-