### Summary

The method of Neuman and Logan has been adapted to the determination of hydroxyproline as an index of connective tissue in muscle.

The procedure may be used on alkaline or acid hydrolyzates directly without preliminary separation of the fibrous proteins of the muscle tissue.

Acid hydrolysis of muscle tissue in presence of stannous chloride has been found superior to the alkaline hydrolysis for hydroxyproline determinations.

In determining hydroxyproline, corrections must be made for destruction during hydrolysis of tissue, the interference of tyrosine or tryptophan and the color of the hydrolyzate in color formation, and the fact of other amino acids present in the hydrolyzate may depress color development.

A method for determining relative amounts of collagen and elastin in a connective tissue is suggested.

The connective tissue (alkali insoluble proteins) of the *longissimus dorsi* muscle of cattle was found to be 12.39 ± 0.40% hydroxyproline and to consist of 84% collagen and 16% elastin.


During the course of this investigation it was observed that fat tends to destroy tryptophan during alkaline hydrolysis of tissue.

The tyrosine and tryptophan content of the *longissimus dorsi* muscle of cattle was found to be relatively constant 1.024 ± 0.047% and 0.330 ± 0.020%, respectively.

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*Received for review May 10, 1954. Accepted July 22, 1954.*


# FRUIT PECTIN

# Determination of a Soluble Pectin in Apples


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METHODS FOR THE DETERMINATION of the pectin content of natural materials have been, in most cases, very tedious and not applicable to small amounts of material. They have been critically discussed by McColloch (*11*).

It was found necessary to examine the methods as a basis for a study of the relation of maturity of apples to the soluble pectin content. Methods based on precipitation of calcium pectate have been used more frequently, perhaps, than others, but are tedious. The evolution of carbon dioxide in the Tollens-Lefèvre reaction (*10*) would seem to be most specific for polyuronic acid material, but is not conveniently applicable to large numbers of samples. After the work reported here was well advanced, the colorimetric carbazole method of Stark (*16*) appeared.

The authors have modified the calcium pectate method so as greatly to expedite its use, have developed a photometric method based on the results of Ikawa and Niemann (*6*), and have compared the results obtained by these methods with the uronic acid content as determined by applying the carbon dioxide method to apples.

### Extraction of Soluble Pectin from Apples

In order to remove all the soluble pectin from apples, Carré and Haynes (*3*) found it necessary to carry out 60 to 80 water extractions. This is obviously impractical when a considerable number of samples are under consideration, and more recent investigators have used four or five extractions (*1, 5, 12*). McColloch (*11*) described a "water-soluble" pectic fraction which he defined by the method of preparation. This involved two 2-hour extractions with water at 30° C. of the alcohol-insoluble portion of the sample. Carré and Haynes reported that as much as 20% of the pectin may remain behind after 10 extractions with cold water.

In view of such results, the whole procedure of repeated extractions seems of dubious validity, and it is not unlikely that the pectin appearing as "soluble" under these circumstances is different from that actually in solution in the apple. While the chemical nature of protopectin has not been established, it is known to be easily rendered soluble, and 80 extractions would clearly entail very long contact with water. Other unknown changes to pectic materials might also occur during such extensive treatment.

In the ripe apple, less than 4% of the edible material is insoluble in water (*9*). The solids in solution include pectin, so that the pectin content of a sample of the solution present in the apple may be considered to represent the dissolved pectin content of the whole edible portion of the apple within a small margin of error. Information on the composition of unripe apples is not extensive. Widdowson (*19*) found 3 to 7% alcohol-insoluble material in fresh apple tissue in June, with the level decreasing thereafter. This would include practically all the water-insoluble material, as well as soluble pectin, and perhaps other water-soluble polyoses. During ripening, starch is converted to sugar, but the maximum level of starch in McIntosh apples has been reported as only 1.5 to 2% in late July and early September (*9*).

**Methods available for the determination of pectin in apples are not particularly satisfactory for routine use. Modifications of the calcium pectate method are presented which greatly expedite its use. A new photometric method was developed based on the absorption at 295 mμ of a pectin sample heated in a boiling water bath with 9 volumes of 84% sulfuric acid. Both methods were used to estimate the pectin content of a fraction believed to represent the dissolved pectin from apples of various degrees of maturity. Results agreed fairly well with the polygalacturonic acid content as determined by the method of carbon dioxide evolution.**

The authors have made some determinations of the starch content of Delicious apples, which are considered to be particularly high in starch, and found about 2% starch in late July and early September apples, with the level decreasing thereafter. Analyses were made using the method of Neubert *et al.* (*13*). It seems unlikely that other insoluble constituents are present in any very significant amount. Cellulose, pentosans, and proteins have been reported (*15*) to increase or to show little change during ripening. Tannins are present at a very low level even in the unripe apple (*15*). Thus it appears safe to estimate the content of insoluble material in unripe apples (July) as no more than 5 or 6%. The authors have therefore taken the pectin content of apple juice to represent a soluble pectin level for the apple pulp. Correction factors for the insoluble content could be estimated if greater accuracy was desired. Analytical results based on volume of juice may be converted to the weight basis by dividing by an average density of 1.06 grams per ml. This figure holds for unripe, as well as ripe, apples.

Frozen apples are thawed, peeled, cored, put through a food grinder, and then briefly beaten in a Waring Blendor. The pulp is centrifuged in a basket centrifuge with a strip of filter cloth over the perforations. The early portion of the centrifuge is retained, recentrifuged in 250-ml. centrifuge bottles at about 1000 × G for 15 minutes and boiled gently for 15 minutes, and the evaporated water is replaced. It is then centrifuged for 10 minutes at 12,800 × G (10,000 r.p.m. in Servall angle centrifuge Model SS-1a), and the supernatant solution is carefully decanted. Samples from it may be analyzed for pectin by one of the methods outlined above.

Freezing, thawing, and homogenizing the pulp should break up the cell structure thoroughly. In earlier work, the juice was pressed out in a Carver hydraulic press. This procedure is satisfactory if preceded by homogenization. The authors found it best to divide the pulp into a number of cheesecloth bags placed between metal disks in the cylinder of the press. It is necessary to centrifuge the juice before boiling or starch will be solubilized, especially in unripe apples, and will interfere with the pectin determinations.

The later portions of the juice collected from either the press or the basket centrifuge were clearer and lower in pectin content as determined by any of the three methods. However, repeated experiments indicated that the pectin level by any of the methods showed no consistent decrease through at least the first 75% collected. The decrease is believed to be due to filtration of the pectin by the tight cake of residue in the press or on the wall of the basket centrifuge. There would seem to be no danger of error from this cause if the last half of the juice is discarded.

**Table I. Effect of High Speed Centrifugation on Determination of Soluble Pectin**

| Method | After 10 Min. at 10,000 R.P.M., Mg./Ml. | After 15 Min. at 2000 R.P.M., Mg./Ml. |
|---|---|---|
| Gravimetric | 1.03 | 1.48 |
| Photometric | 0.98 | 1.05 |
| $CO_2$ evolution | 0.79 | 0.80 |

Carré and Haynes (*3*) state that the extract must be boiled in order to destroy pectase. The present studies did not indicate significant amounts of pectase. With ripe apples, boiling the juice does not change the results, if the determination is carried out reasonably promptly. Boiling would serve to precipitate the small amount of protein present, and it does inactivate the enzyme system responsible for the development of the dark brown color in the juice. This darkening will result in high results by the photometric method, and because the browning is much more rapid with unripe apples, the boiling step is correspondingly more important.

Inclusion of the high speed centrifugation results in a decreased pectin value as measured by the calcium pectate method, but results by the carbon dioxide evolution method are unchanged. Typical results are given in Table I. It thus appears that the material removed contains no appreciable amount of galacturonic acid residues, and is not held to the pectin by very strong forces. Semiquantitative tests indicated that the material was, at least to a considerable extent, a polysaccharide yielding reducing sugars on hydrolysis. Results of pectin analyses by other investigators using the calcium pectate method usually include this material.

### *Calcium Pectate Method*

The principal steps in determining pectin as calcium pectate are the alkaline hydrolysis of the methyl ester groups from the polygalacturonic chain, followed by neutralization, addition of an excess of calcium chloride, and filtering, washing, and drying of the calcium pectate precipitate. Carré and Haynes (*3*) used a hydrolysis period of 1 hour or preferably overnight, and after adding calcium chloride, let the mixture stand for 1 hour and heated it to boiling before proceeding with the filtration. This procedure was necessary for reasonably rapid filtration. Such difficulties are avoided by the use of the centrifuge.

The sample of apple juice containing 20 to 30 mg. of pectin as calcium pectate is placed in a 250-ml. centrifuge bottle and diluted to 50 ml. The solution is mixed with 50 ml. of 0.25*N* sodium hydroxide and let stand 25 minutes. It is acidified with 50 ml. of 2*N* acetic acid and 50 ml. of 1*M* calcium chloride are added with stirring. After 15 minutes, the precipitate is centrifuged down at 2000 r.p.m. (ca. 1000 × G) for 15 minutes. The supernatant solution is carefully decanted off, leaving 10 to 20 ml. to aid in transferring the precipitate to the tared 15-ml. conical centrifuge tubes. The transfer is most conveniently done by way of a small beaker. The precipitate in the centrifuge tube is washed with hot water until free of chloride ion, by alternately centrifuging, decanting the supernatant liquid, and resuspending in water. The tube and contents are then dried at 100° C. for 48 hours and weighed.

Using a higher concentration of alkali

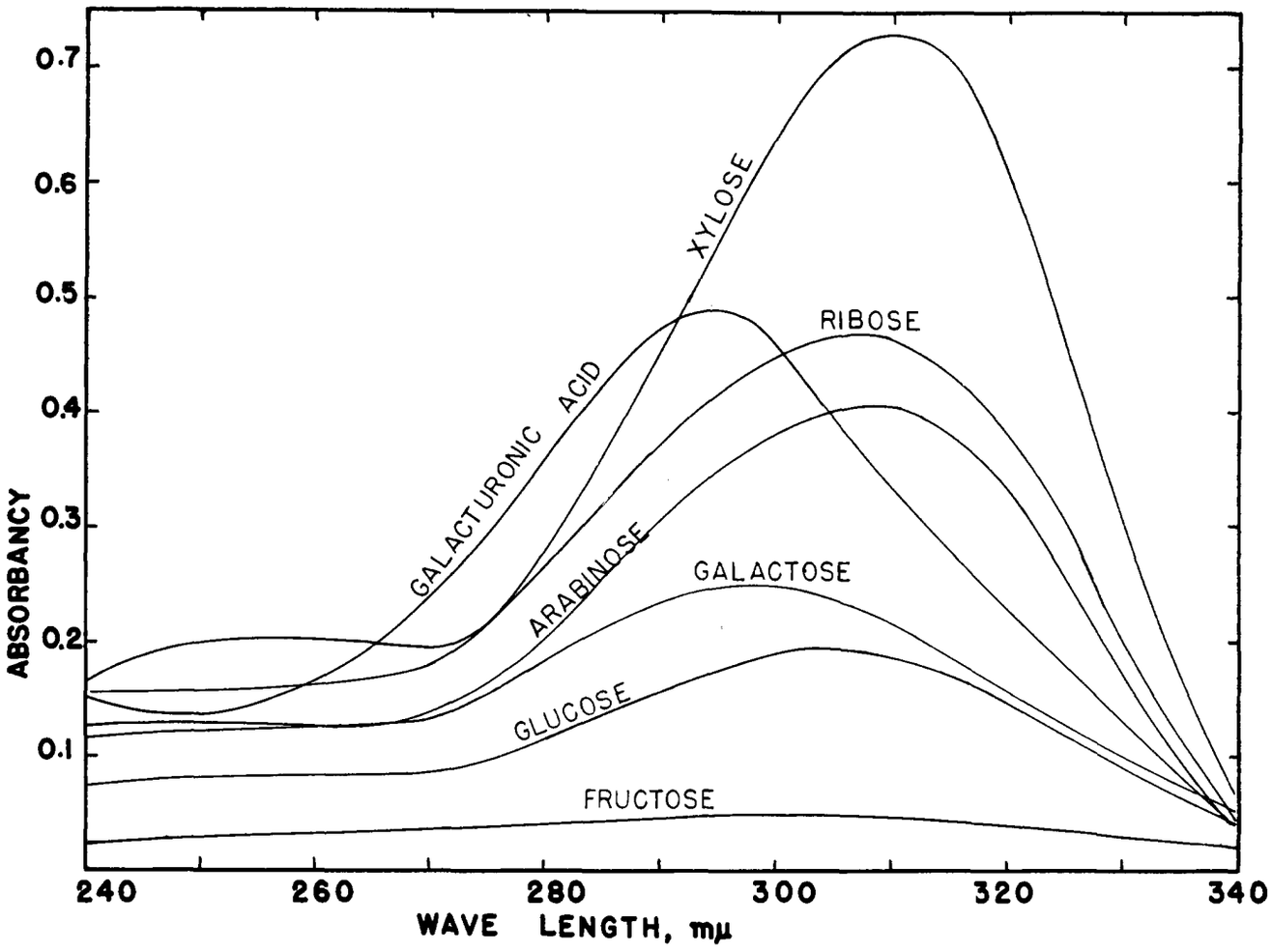


Figure 1. Ultraviolet absorption curves for 0.001*M* solutions of various sugars

than Carré and Haynes, the authors have found no significant differences in the results with hydrolysis times from 15 to 30 minutes, with a tendency for lower results with longer periods than this. The practicability of shorter hydrolysis has been reported (*17*). Results have not been significantly different whether the calcium precipitation was allowed to stand for 15 minutes or overnight. Calcium pectate precipitates are difficult to dry to constant weight, but decrease of weight is very slow after 24 hours, and the authors have standardized the procedure for 48 hours' drying.

The above method gives good checks with results obtained by the original Carré-Haynes method on samples of juice from ripe apples, "green" apples, and ripe pears. Calcium pectate obtained by this procedure from a sample of N.F. pectin represented 90 to 100% of the original weight, after correction for moisture, ash, and an assumed 8% calcium content of the precipitate (*8*). Results obtained using different levels of the same sample agree satisfactorily. The results of a large number of analyses showed an average sampling variation of about 6%. Occasional lack of homogeneity of the turbid apple juice samples is believed to cause the larger variations.

### *Photometric Method*

Ikawa and Niemann (*6*) have presented absorption curves for various carbohydrates after 15 minutes' treatment at 100° C. with 79% sulfuric acid. They found that galacturonic acid has an absorption maximum at 294 mμ after this treatment, and L-arabinose, D-galactose, D-glucose, and D-fructose absorb less strongly. Malic acid has no appreciable absorption under these conditions. Solutions of N.F. pectin and of apple pectin (calcium pectate redissolved with potassium oxalate) gave maxima at the same wave length and absorption curves of a generally similar shape. A group of absorption curves for sugars of interest in this connection is presented in Figure 1.

It was necessary to carry out a preliminary rough separation of the pectin before using this approach, as sugars are present in apple juice in such high concentration relative to the pectin. The findings of Emmett and Carré (*4*) were used for the preliminary separations.

To 10 ml. of juice in a 50-ml. centrifuge tube are added 1 ml. of 5*N* sulfuric acid and 40 ml. of 95% ethyl alcohol slowly and with constant stirring. After 30 minutes, the tube is centrifuged for 10 minutes at 2000 r.p.m., and the precipitate is washed twice by suspending it in acidified alcohol (1 part of 5*N* sulfuric acid, 10 parts of water, and 40 parts of 95% alcohol), recentrifuging, and decanting off the supernatant liquid. The precipitate is then redissolved in hot distilled water, cooled, and made up to a pectin concentration of 20 to 100 γ per ml.

Nine milliliters of 84% sulfuric acid are layered beneath 1 ml. of the redissolved pectin. The solutions are thoroughly mixed with a stirring rod, and placed in a boiling water bath for 15 minutes. The solution is cooled and read in the spectrophotometer at 295 mμ against a blank of 9 ml. of 84% sulfuric acid plus 1 ml. of water treated in the same way. The reading is compared with readings from a standard curve prepared by carrying standard galacturonic acid solutions of from 20 to 100 γ per ml. through the operations of this paragraph.

Emmett and Carré (*4*) found that acidified alcohol gave complete precipitation of apple pectin overnight. The authors have found no significant increase in results after half an hour with acidified alcohol. At least one washing is necessary to remove nonpectin material causing absorption at 295 mμ; readings remain unchanged with further washing. The acid treatment must be carried out in scrupulously clean glass-

ware, as the presence of dust affects the results. For delivery of the acid a shortened bent stopcock buret is used, with the stopcock lubricated by the acid itself. A fresh blank must be prepared with every group of unknowns. The heating time appears not to be critical; 5 minutes additional heating does not affect the results. When 0.5 ml. of water was substituted for 0.5 ml. of the 84% sulfuric acid, the results obtained were about 10% too low.

By this procedure, the sample of N.F. pectin was found to contain 95% galacturonic acid or about 92% pectin [considering pectin as 72% methylated polygalacturonic acid anhydride—i.e., 12% methoxyl—with unit molecular weight, 186 (*18*)] after correction for moisture and ash. A sample of purified demethylated polygalacturonic acid assayed 95% pectin. in agreement with results by the method of carbon dioxide liberation. Satisfactory agreement was obtained both when different dilutions of apple juice were precipitated by acidified alcohol and when the precipitate was redissolved to give various concentrations. The average sampling error of a large number of duplicate analyses was about 10%.

### Carbon Dioxide Evolution Method

Uronic acids evolve 1 mole of carbon dioxide when boiled with strong acids (*10*). Numerous reports have outline methods of applying this reaction to the determination of pectins and polyuronic acids, including gravimetric micromethods.

In this work a modification of Kemmerer and Hallett's (*7*) apparatus for the gravimetric microdetermination of carbonate carbon was used. The acid bulb was omitted. In its place a mercury trap was inserted to prevent carbon dioxide from backing up into the Ascarite guard tube. Ascarite was used as the absorbent for carbon dioxide and magnesium perchlorate as a drying agent. The sulfuric acid trap was filled with a solution of silver sulfate in sulfuric acid.

Because some carbohydrates yield as much as 0.5% carbon dioxide when hydrolyzed with acid (*2, 14*), a preliminary separation of pectin from the sugars in the apple juice was necessary. The pectin was precipitated from the juice as in the calcium pectate method, and the washed wet precipitate was transferred to the boiling flask of the carbon dioxide apparatus. The samples were hydrolyzed by boiling 5 hours with 12% hydrochloric acid. Practically quantitative yields of carbon dioxide were obtained from pure galacturonic acid.

### Comparison of Results by the Three Methods

After a soluble pectin preparation has been obtained from an apple by the above procedure, the three methods of determining pectin described here do not necessarily give the same result. Some typical figures are given in Table II. The calcium pectate and photometric methods frequently give approximately the same result until the apples become overmature. The former then yields a somewhat higher figure. The carbon dioxide evolution method would indicate only one third as much pectin as the other two in July apples, but as the apples mature the spread gradually becomes small.

Analyses of Golden Delicious apples showed that results by the gravimetric and photometric methods mostly agreed within 20%, with little consistency in which gave the higher figure. With Red Delicious apples the photometric result was, in most cases, decidedly higher than the result by the calcium pectate method. The empirical nature of the two methods gives no reason to expect that they are measuring the same materials in all cases.

As the method of analysis by carbon dioxide evolution gives a direct measure of the galacturonic acid polymer, it appears that in unripe apples the other two methods include nongalacturonide material intimately associated with the pectin molecule. Whether such material should be considered pectin is perhaps a matter of definition. At any rate it would appear that little such material remains in thoroughly ripened apples, since the various methods agree fairly well.

**Table II. Soluble Pectin Content of Various Apple Samples**

| Sample | Gravimetric, Mg./Ml. | Photometric, Mg./Ml. | $CO_2$ Evolution, Mg./Ml. |
|---|---|---|---|
| Golden Delicious, picked Aug. 9, 1952 | 0.32 | 0.29 | 0.11 |
| Red Delicious, picked Aug. 9, 1952 | 0.31 | 0.38 | 0.14 |
| Red Delicious, picked Sept. 18, 1950 | 0.30 | 0.30 | 0.13 |
| Golden Delicious, picked Nov. 3, 1952 | 0.64 | 0.59 | 0.42 |
| Red Delicious, picked Nov. 3, 1952 | 0.55 | 0.96 | 0.42 |
| Golden plus Red Delicious from cold storage (overripe) | 1.24 | 1.06 | 1.02 |
| Commercial pectin, N.F. | 95% | 92% | 84% |


### Acknowledgment

The authors wish to thank Archie Van Doren, Tree Fruit Experiment Station, Washington Agricultural Experiment Stations, for his assistance in the collection of apple samples, and Katherine Day of this laboratory for assistance in application of the methods.


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*Received for review January 4, 1954. Accepted July 16, 1954. Presented at the Northwest Regional Meeting,* AMERICAN CHEMICAL SOCIETY, *June 12 and 13, 1953. Scientific Paper 1281, Washington Agricultural Experiment Stations, Pullman, Project 999.*