XII. THE ESTIMATION OF PECTIN AS CALCIUM PECTATE AND THE APPLICATION OF THIS METHOD TO THE DETERMINATION OF THE SOLUBLE PECTIN IN APPLES.

By MARJORY HARRIOTTE CARRÉ AND DOROTHY HAYNES.

*Department of Plant Physiology and Pathology, Imperial College
of Science and Technology.*

(Received January 18th, 1922.)

IN order to follow the changes which take place in the pectic constituents of stored apples, a number of preliminary investigations have been found to be necessary. In the first place a method was required by which pectin could be estimated accurately in dilute solution. This having been established, it has become possible to proceed to an investigation of the means by which the various pectic constituents of the fruit can be separately extracted. The present communication, in so far as the extractions are concerned, is confined to an examination of methods for extracting the soluble pectin of fruits. The difficulties which are encountered in the course of this extraction are almost entirely mechanical—difficulties inseparable from the process of washing out this soluble colloidal material from the insoluble colloids of the cell wall with which it is intimately associated. The clearing up of these mechanical difficulties is a necessary preliminary to any further investigation of methods of extraction, for unless suitable methods of washing out are adopted much soluble pectin may remain behind to form a mixture with the pectin dissolved out by subsequent treatment.

It has been the general practice hitherto to estimate the pectin content of solutions by precipitating the pectin with alcohol. This method at its best is inconvenient and lacking in accuracy. The pectin obtained is necessarily of variable composition, since pectin probably exists in a number of forms intermediate between neutral pectin and pectic acid. Moreover, as dilution increases precipitation becomes increasingly difficult, until at very low concentrations no precipitate is obtained, even after prolonged standing with a large excess of alcohol. By the method now adopted the difficulty of precipitation is avoided, for calcium pectate can be made to flocculate from solutions of very low concentration, and a product of definite chemical composition is obtained. A comparison of the results of precipitating pectin with alcohol and as calcium pectate is given in the sequel.

THE PRECIPITATION OF CALCIUM PECTATE.

Since calcium pectate is a colloid, its state of aggregation varies greatly with the conditions under which it is precipitated, and in order to obtain a product which can be easily manipulated it is requisite to adjust these conditions carefully. Great difficulties were experienced in the early stages of this investigation by the occurrence of a sticky condition in which the precipitate could only be washed and filtered with extreme difficulty. This has been found to be the result of precipitating in alkaline solution, in which case calcium hydroxide is absorbed by the gel [Haynes, 1914]; if the period of hydrolysis with sodium hydroxide is too prolonged, or if the alkali is too concentrated, a similar result tends to be produced. On the other hand, too small an excess of alkali above the theoretical amount produces unsatisfactory coagulation, and as the concentration of pectin is increased the excess of alkali required increases also. In the opinion of the writers, both effects are probably a consequence of the tendency which alkalies exhibit to replace the loosely combined water molecules of the pectin sol.

To avoid these difficulties the precipitation of calcium pectate is carried out in the following stages:

- (1) Hydrolysis with sodium hydroxide.
- (2) Acidification with acetic acid.
- (3) Addition of calcium chloride.

Since no independent method exists for checking the results of pectin estimations, it has been necessary to carry out series of experiments and to ascertain how the weight of the calcium pectate varies when variations are made from a standard procedure arbitrarily adopted. In most cases determinations of the ash content of the precipitate calculated as calcium were also carried out. Since the number of interdependent factors is considerable, this has involved a large amount of work. No useful purpose would be served by giving a complete account of the details of this preliminary investigation, but some remarks on the successive stages of the process are required, and the results of a few experiments will be quoted in support of the statements made. The results of ash determinations require more detailed discussion, and will be treated separately below. The pectin solutions used for the investigation were in almost every case obtained from apple. Many determinations were made directly on the liquid obtained by expressing and washing out apple pulp, previously killed by freezing; but confirmatory series were also carried out on pectin from a similar source which had been precipitated by alcohol, re-dissolved and allowed to stand under toluene until suspended impurities were deposited. The juice in each case was heated immediately after pressing, to destroy pectase. It was found convenient to work with a quantity of solution giving from 0.02–0.03 g. of calcium pectate, and to dilute to a concentration of approximately 0.01 % before precipitation. Larger quantities

give so great a bulk of precipitate that it is difficult either to wash thoroughly or to dry satisfactorily.

Hydrolysis. Fellenberg [1918] has stated that pectin hydrolyses very rapidly in the presence of a small excess of alkali. The following series show the general results obtained in the course of the present investigation.

Time	Weight of calcium pectate obtained			
	SERIES I		SERIES II	
	(Concentration of NaOH)		(Concentration of NaOH)	
	N/100		N/50	
10 mins.	Very little		0.029	0.026
30 "	0.031	0.0285	0.0315	0.032
60 "	0.028	0.0285	0.0305	0.0305
4 hrs.	0.028	0.028	—	—
24 "	0.028	0.028	0.0295	0.030
48 "	0.028	0.0275	—	—

It will be observed that the rate of hydrolysis increases very rapidly with the concentration of alkali. As it is advisable to keep this concentration low, it is necessary to allow the mixture to stand at least an hour. The best results have been obtained by leaving it overnight, in which case the precipitate flocculates readily and is easy to wash and filter. It is noticeable that rather high results were obtained in both series for the half-hour period. This is a very frequent result of incomplete coagulation which renders effective washing extremely difficult.

Acidification and addition of calcium salt. Calcium pectate can be boiled with water without change, but with dilute acids it tends to pass into colloidal solution. The conditions of this change have not been fully investigated, but it appears that prolonged boiling reverses the peptising effect of acid. This is shown by the results of the following series of experiments carried out on equal quantities of pectin precipitated as calcium pectate.

Treatment				Weight of calcium pectate	
Boiled with water				0.0275	0.028
Boiled with N/2 acetic acid				0.0205	0.0225
"	"	"	5 mins.	0.0215	0.024
"	"	"	30 "	0.026	0.0265
"	"	"	60 "	0.0245	0.027
Boiled with N/10 acetic acid				0.0285	0.028
"	"	"	5 "	0.026	0.023
"	"	"	30 "	—	—
"	"	"	60 "	—	—

The precipitates which had been boiled with acid were found to contain a low percentage of ash. The action of acid must therefore be primarily attributed to the decomposition of calcium pectate; this being so, it is not surprising to find that an excess of calcium salts tends to reverse the effect and that calcium pectate may be boiled with dilute solutions of weak acids in the presence of a sufficient excess of calcium chloride without change of weight. The amount of calcium salt required increases very rapidly with the amount of pectin present. This is shown by a comparison of the two following series of precipitations:

Precipitation carried out with: 100 cc. $N/10$ NaOH; 100 cc. $N/1$ acetic acid; x cc. $M/1$ CaCl_2 :

Cc. $M/1$ CaCl_2 added	SERIES I 200 cc. apple pectin		SERIES II 400 cc. apple pectin as used in Series I	
	(1)	(2)	(1)	(2)
2	0.0300	0.0300	0.0565	0.0510
5	0.0300	0.0290	0.0560	0.0575
10	0.0295	0.0300	0.0600	0.0555
25	0.0305	0.0310	0.0605	0.0600
50	0.0300	0.0300	0.0605	0.0600
100	—	—	0.0605	0.0615

Effect of acid concentration.

Precipitation carried out with: 100 cc. $N/10$ NaOH; x cc. acetic acid; 50 cc. $M/1$ CaCl_2 :

Cc. acetic $N/10$	SERIES I 200 cc. dilute apple pectin		SERIES II 400 cc. dilute apple pectin as in I	
	(1)	(2)	(1)	(2)
2	0.1042	0.1270	—	—
10	0.0375	0.0365	—	—
20	0.0300	0.0305	—	—
50	0.0305	0.0290	—	—
100	0.0300	0.0295	—	—
$N/1$				
15	0.0310	0.0290	0.0645	0.0660
30	0.0300	0.0295	0.0615	0.0620
50	0.0300	0.0300	0.0610	0.0615
100	0.0300	0.0300	0.0605	0.0610

It will be seen that in the more concentrated series II the lowest limit of acid is only reached at 50 cc. of $N/1$ acetic acid, whereas in series I, 20 cc. $N/10$ acetic acid are seen to be sufficient. The higher values in both series are due to the carrying down of calcium carbonate and occluded impurities which are only removed by excess of acid.

It appears from these results that for ordinary concentrations of pectin the optimum concentration of calcium chloride is approximately $M/1$, and of acid $N/5$. Where the quantity of pectin is very small it has been found advisable to reduce the acid concentration to $N/10$, since the larger amount tends to produce stickiness. In this case the quantity of calcium chloride should be correspondingly reduced.

METHOD OF ESTIMATING PECTIN AS CALCIUM PECTATE.

As a result of these investigations the following method of estimating pectin has been adopted:

A quantity of pectin is taken which will yield from 0.02 to 0.03 g. of calcium pectate; this is neutralised and then diluted to a volume such that after addition of all reagents the total volume measures about 500 cc. 100 cc. of $N/10$ NaOH are then added and the mixture is allowed to stand at least an hour, but preferably overnight. 50 cc. of $N/1$ acetic acid are then added, and after five minutes 50 cc. of $M/1$ calcium chloride. The mixture is then allowed to stand for an hour, after which it is boiled for a few minutes and filtered through a large fluted filter. If the precipitation has been properly carried out, filtration should take place very rapidly and subsequent washing

should be easy. The washing is continued with boiling water until the filtrate is free from chloride, after which the precipitate is washed back into the beaker, boiled, and filtered again. It is then again tested for chloride, and these processes are repeated until the filtrate from the boiled precipitate gives no indication of chloride with silver nitrate. It is then filtered into a small fluted filter, from which it can be transferred to a dish and finally to a Gooch crucible which has been previously dried at 100°. The precipitate is dried to constant weight at 100° which has been found to require about 12 hours.

If the quantity of pectin is increased, the quantities of soda and calcium chloride should be correspondingly increased. If very small quantities of pectin are dealt with, the acid and calcium chloride should be reduced.

Example of estimations using dilute apple pectin.

Precipitations carried out with: 50 cc. pectin solution; 50 cc. *N*/10 soda; 100 cc. *N*/10 acetic acid; 100 cc. *M*/1 CaCl_2 :

SERIES I		SERIES II	
Purified pectin solution		Unpurified pectin solution	
0.0236	0.0237	0.0280	0.0285
0.0237	0.0236	0.0287	0.0300
0.0240	0.0231	0.0265	0.0275
0.0240	0.0230	0.0280	0.0280
0.0229	0.0233	0.0280	0.0285
Mean = 0.0235		Mean = 0.0282	

The quantity of acid used for these estimations is less, and the quantity of CaCl_2 greater, than prescribed in the text. It has been found that the alteration of these amounts facilitates the washing of the precipitate.

If precipitated according to the method given above, calcium pectate is so insoluble that quantitative precipitation can be carried out in solutions of extreme dilution, the limiting factors being rather those governing the drying and weighing of very small quantities of colloidal material. It is thus possible to carry out estimations by the calcium pectate method over a large range of concentrations at which precipitation by alcohol is impossible. The lower limit of concentration at which pectin can be even partially precipitated by alcohol has been found to be 0.06 %.

Example of method applied to very dilute solutions.

Precipitations carried out with: 50 cc. dilute pectin solution (apple extract); 50 cc. *N*/10 NaOH ; 50 cc. *M*/1 CaCl_2 ; 50 cc. *N*/1 acetic acid:

Weight of calcium pectate	0.0053	0.0055
	0.0050	0.0055
	0.0050	0.0053
	0.0055	0.0050
Mean = 0.00525		

A few examples of estimation by precipitation with alcohol are given below for comparison. They are all carried out on similar quantities (10 cc.) of apple juice to which a small quantity of water extract has been added.

		Weight of precipitate	
		(1)	(2)
25 cc. of alcohol		0.019	0.021
50 " "	(1)	0.018	0.015
	(2)	0.012	0.015
100 " "		0.018	0.020

The weights of calcium pectate obtained from 10 cc. of this extract were: (1) 0.023; (2) 0.023. It is probable that all the alcohol precipitations are too low. The solubility of pectin in alcohol is shown clearly by the following series of results, carried out on the same material as above:

(1) The weight of precipitate obtained by adding 50 cc. of alcohol to 10 cc. of extract diluted with 25 cc. of water was found to be (1) 0.019; (2) 0.020.

(2) With 50 cc. of water no precipitate was obtained in two days in either sample. After this a small precipitate was obtained from one, but not from the other.

(3) With 100 cc. of water no precipitate was obtained after standing for a week or even after doubling the amount of alcohol.

These experiments have not been repeated on purified pectin owing to lack of material. Such experiments would probably give more regular results, since in ordinary juice sugar acids and other substances are present which may themselves tend to favour coagulation.

In the foregoing account it has been assumed that the method of pectin estimation there described is dependent upon the production of a definite chemical compound—calcium pectate. This assumption obviously needs justification, more especially since pectin is a colloidal substance which is likely to form adsorption compounds and is known to form solid solutions with alkalies and alkaline earths [Haynes, 1914].

Pectic acid was among the first of the pectic compounds to be described; indeed the formation of pectic acid by hydrolysis still serves to define the class of substances known as pectins. The chemical nature of pectic acid is still obscure, but it is universally recognised as possessing definite acidic properties, and it has been found to possess the same chemical composition expressed by the empirical formula $C_{17}H_{24}O_{16}$ [Schryver and Haynes, 1916]¹ when derived from very different sources (strawberry, turnip, and rhubarb stalks).

The pectic acid from apples alone showed a slight variation, and the present work indicates that this may very possibly have been due to the presence of impurities.

Fellenberg [1918] assigns the formula $C_{62}H_{96}O_{52}(CO_2CH_3)_8$ to pectic acid on somewhat arbitrary grounds, and his own analyses of barium salts agree more nearly with the empirical formula given above.

The number of decomposition products obtained by Ehrlich and others from pectin indicates, however, that the molecule of pectin is large, and it is therefore hardly likely that pectic acid can be represented by a simple multiple of $C_{17}H_{24}O_{16}$. It probably differs from a simple multiple by some small

¹ In this paper the name pectin was used as the equivalent of pectic acid. There is however an increasing tendency to reserve the term pectin for soluble products, especially those occurring naturally in fruits, and to denote by pectic acid the last term in a series of pectic compounds obtained by hydrolysis of these soluble substances. This usage is advocated by Fellenberg, and is followed in the present paper.

quantity but the true formula cannot be ascertained with any certainty until the constitution of pectic acid is established; in the meantime it seems advisable to adopt with reservation the formula given above, and it is to this formula, assumed to be that of a dibasic acid that the results of the analyses given below are referred.

THE COMPOSITION OF CALCIUM PECTATE OBTAINED FROM APPLES.

Two methods of analysis have been employed:

- (1) Ignition to CaO.
- (2) Conversion of this to sulphate.

The following results were obtained by the ignition of calcium pectate obtained from a purified solution of apple pectin which contained at most 0.01–0.03 % of ash when precipitated by alcohol. When filtered carefully before precipitation the amount of ash was negligible.

Weight of Ca pectate	Weight of CaO	Percentage Ca
0.0160	0.0017	7.59
0.0226	0.0025	7.90
0.0270	0.0029	7.66
0.0266	0.0028	7.51
0.0746	0.0080	7.65
0.0308	0.0033	7.64

Mean = 7.658

In order to afford a comparison of the results of the two methods of ash determination, the following series are given. These were carried out on material similar to that used for the previous series. The calcium pectate was obtained by exactly similar methods of estimation in each case.

As CaO			As CaSO ₄		
Weight of Ca pectate	CaO	Percentage Ca	Ca pectate	CaSO ₄	Percentage Ca
0.0746	0.0080	7.65	0.0240	0.0062	7.60
0.0266	0.0028	7.52	0.0250	0.0065	7.65
0.0270	0.0029	7.60	0.0236	0.0062	7.70
0.0260	0.0017	7.59	0.0234	0.0062	7.65
0.0226	0.0025	7.70	0.0240	0.0064	7.67

Mean = 7.612

Mean = 7.654

Mean of the two series = 7.63.

It is evident that these results agree very closely with the theoretical percentage, 7.66, and the same percentage has been obtained repeatedly from calcium pectate precipitated under widely different conditions, it may therefore be regarded as established that calcium pectate obtained by this method is a definite chemical compound, the empirical formula of which approximates closely to $C_{17}H_{22}O_{16}Ca$.

Since estimations of pectin have usually to be made in a water extract containing sugars, salts and other compounds, it has been necessary to ascertain how nearly the composition of the precipitate from this unpurified material corresponds with theory. It has been found that the ash is usually

rather high, but that if sufficient precautions are taken the error can be reduced to an amount which probably seldom exceeds 1 % of the total ash.

The following are representative examples, each set being estimated on different material:

	Ca pectate	CaSO ₄	Percentage Ca
I	0.0243	0.0062	7.50
	0.0293	0.0076	7.63
II	0.0553	0.0144	7.66
	0.0577	0.0173	8.30
	0.0294	0.0077	7.70
III	0.0514	0.0138	7.90
	0.0151	0.0040	7.79
	0.0503	0.0129	7.54
	0.0425	0.0111	7.68

In the second set, which gives the highest ash, much iron was found to be present. It must be borne in mind that an increase in the weight of ash in the calcium pectate precipitate may be due either to the carrying down of suspended mineral matter or to the occlusion of organic substances such as the salts of organic acids. These latter if present may introduce considerable error, but if the juice is carefully filtered and if the precipitation is carried out in the presence of sufficient acid, it has been found that the difference can be reduced to a very small amount, which as is shown below may almost certainly be regarded as mainly of mineral origin.

It is significant to find that the impurities associated with pectin vary with different stages of pressing. This is illustrated by the following estimations:

Ash content of calcium pectate obtained from successive extractions of apple.

Number of pressings	Proportion of total Ca pectate present	Ash (calculated as percentage Ca)
1-10	79.5	7.62
10-20	14.0	9.20
20-30	6.5	9.70

In other cases final pressings were found to contain as much as 10-12 % of ash calculated as calcium. There seems much reason to think that this excess of ash is due to colloidal mineral matter, possibly associated with the protoplasm, which can only be pressed out when the cell is completely disintegrated. This, together with a variable amount of colloidal iron, appears to constitute the principal source of the impurities carried down with the calcium pectate precipitated from apple juice. The mineral nature of this impurity cannot be completely demonstrated without elementary analysis, but there seems to be very little doubt that it is largely, if not entirely, composed of inorganic material, in which case the error introduced into the weight of the calcium pectate precipitate will be very small.

It may accordingly be stated as a general rule that pectin should be estimated on material obtained by exhaustive extraction, but that if ash

determinations are made in order to ascertain whether or not the material affords calcium pectate in a state of comparative purity the later pressings should be rejected.

A few words may also be added here as to the precautions which it is advisable to take if satisfactory ash determinations are to be carried out. It must be remembered that pectin absorbs and carries down other substances very readily, and that these—once occluded—are removed only with very great difficulty.

Thus if calcium carbonate is contained in the precipitated gel, it is practically impossible to remove it by subsequent treatment with acid. Pectin should therefore be precipitated in dilute solution and in the presence of considerable excess of acid. The excess of CaCl_2 should not be greater than is sufficient for satisfactory coagulation, and at each stage of the process sufficient time should be allowed for the reagents to penetrate the large particles of the pectin sol.

THE EXTRACTION OF SOLUBLE PECTIN FROM APPLES.

A considerable amount of investigation has been necessary in order to work out a practicable method for the complete extraction of the soluble pectin. After the first few extractions have been carried out, further extraction gives a very dilute solution, and it is easy to imagine that the whole of the pectin has been extracted at this stage. It has been found, however, that a large proportion—sometimes more than 50%—comes out at so great a dilution that no precipitate is obtained from the solution even after prolonged standing with a large excess of alcohol.

The process is of necessity laborious. The following has been found the best method of procedure: 50 g. of finely minced apple are frozen in an efficient freezing mixture, and maintained at a low temperature for a considerable number of hours to ensure that the pulp is completely killed. The pulp is then warmed to the temperature of the air, and pressed through a cloth in a small hand press. After this the residue, which should be as dry as possible, is ground with fine purified sand in a mortar, and repeatedly washed with cold water and pressed. The residue should be well mixed after each addition of water. Sixty to eighty extractions are usually required, and the bulk of extract obtained is about two litres. Warm water should not be used, as this has been found to increase the amount of pectin obtained—presumably by promoting hydrolysis.

After extraction the liquid is boiled to destroy the action of pectase, and before estimations are carried out it is filtered, first through muslin and finally through a fluted filter paper. An aliquot part, containing a suitable quantity of pectin, is then taken for estimation. The amount varies from about one-half in the case of rather immature apples to one-tenth in the case of apples juicy or over-ripe.

The following estimations have been carried out on equal quantities of

apple previously finely minced and mixed to as uniform a condition as possible. They show the order of accuracy with which the process of extraction can be carried out.

Weight of calcium pectate	
In 1/10 of extract of 50 g. of apples	From 100 g. of apples
0.0257	0.514
0.0246	0.492
0.0271	0.542
0.0252	0.504
0.0245	0.490
Mean = 0.0256	Mean = 0.508

[Note added Feb. 17, 1922.—Other series of results have been obtained which show similar differences. These differences appear to be due rather to the difficulty of mixing the material uniformly than to incomplete extraction. Thus in the following series, in which pressings were collected in successive fractions, there is no indication that later fractions tend to contain more pectin where the first fraction is relatively low.

Weight of Ca pectate from 50 g. of apples			
Main portion	2nd fraction	3rd fraction	Total
0.339	0.0050	—	0.344
.345	.0100	0.0060	.361
.305	.0085	.0030	.3165
.328	.0070	—	.3345
.325	.0040	—	.329]

SUMMARY.

The precipitation of pectin as calcium pectate is described. It is shown that by a careful adjustment of conditions this can be used as an accurate method of analysis, and that the precipitate corresponds in composition closely to the empirical formula $C_{17}H_{22}O_{16}Ca$. A method is given for the extraction of soluble pectin from apples, and the results of a number of similar estimations are compared.

REFERENCES.

- Fellenberg (1918). *Biochem. Zeitsch.* **85**, 118.
 Haynes (1914). *Biochem. J.* **8**, 553.
 Schryver and Haynes (1916). *Biochem. J.* **10**, 539.