Colorimetric Method for Starch Determination

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Starch was determined in the wood, bark, and leaves of various tree species by two methods. The first one was based on the precipitation of starch with iodine, its subsequent hydrolysis, and determination of the reducing sugar set free. The other method made use of the colorimetric determination of starch with iodine. Based on results obtained with the first method, colorimetric calibration curves were constructed for the tested starches of various origins. The curves for different genera varied significantly, whereas no such differences were established for different citrus species or different varieties belonging to the same species. Factors were calculated for the colorimetric determination of the tested starches based on values for potato starch. A method was developed for the calculation of the amylose-amylopectin ratio, based on colorimetric calibration curves for various starches.

The determination of starch in tree wood and bark by hydrolytic or polarimetric methods is difficult owing to the small amount of starch present in these tissues and to the interference of nonstarchy compounds. These methods are of little value, particularly when only small amounts of plant material are available. In such cases, starch determinations may be performed by the highly specific method of Pucher *et al.* (1948), which is based on the extraction of the starch with perchloric acid, its precipitation as an iodine complex, acid hydrolysis after removal of the iodine, and finally the determination of sugar with Somogyi's reagent. This procedure is very tedious and unsuitable for routine determinations. Another choice may be the colorimetric determination of starch according to Nielsen (1943), or a similar colorimetric procedure.

The main disadvantage of these methods is due to variation in the amylose and amylopectin content of starches from various sources. The color intensity obtained per weight unit of starch varies for different plant species. To overcome this difficulty, calculation of factors for the determination of starches from various plant species has been proposed (Nielsen and Cleason, 1945; Humphreys and Kelly, 1961), based on a standard colorimetric extinction curve for potato starch.

Some doubt as to the value of such factors is cast by the fact that considerable varietal differences in the amylose–amylopectin ratio were found in seeds (Deatherage *et al.*, 1955; Goering *et al.*, 1957; Hilbert and MacMasters, 1946; Whistler and Weatherwax, 1948) and bulbs (Lansky *et al.*, 1949) of several plant species. A slight influence of environmental conditions on the amylose–amylopectin ratio has been claimed for barley and maize seeds (Fergason and Zuber, 1962; Goering *et al.*, 1957). Other work has shown, however, that with oat and rice seeds, potato tubers, and apple fruits no (or only negligible) varietal differences occurred (Carter and Neubert, 1954; Deatherage *et al.*, 1955; Halsall *et al.*, 1948).

Variation in the amylose–amylopectin ratio is not the only source of differences in the relative color intensity obtained with various starches when iodine is added. The chain length of the amylose molecule influences color intensity as well (Baldwin *et al.*, 1943), but colorimetric methods for the determination of the amylose–amylopectin ratio in a given starch (Humphreys and Kelly, 1961; MacCready and Hassid, 1943) are necessarily based on disregard of this factor. However, considering the large variation in the figures for the amylose–amylopectin ratio as obtained for a given species by various investigators using other methods, the colorimetric method may be regarded at least as a rough estimate.

The purpose of the present investigation was to test the adaptation of colorimetric starch determinations for wood and bark of several species of fruit trees, including the use of specific factors based on potato starch for each species. In addition, estimates of the amylose–amylopectin ratios of the species under investigation were established.

MATERIALS AND METHODS

Plant Material. The plant material was obtained from various experimental and commercial orchards in Israel.

Samples from citrus trees were obtained from the trunk of trees 1½, 23, and 30 years old. The trees belonged to the following species: *Citrus sinensis* (Linn.) Osbeck vars. Shamouti and Baladi (sweet orange), *Citrus aurantium* Linn. (sour orange), *Citrus limettioides* Tanaka (Palestinian sweet lime), *Citrus medica* Linn. var. Ethrog, *Citrus jambhiri* Lush. (rough lemon), and *Citrus grandis* (Linn.) Osbeck var. Goliath (Pummelo). Samples were obtained from November to January, and analyses were made on both bark and wood samples.

The following varieties of pear trees (*Pyrus communis*, L.) were sampled: Bartlett, Beurré Diel, Bergamotte Esperén, Duchesse d'Angoulême, and Beurré Superfin. Bark samples were taken from the trunks of 2- and 6-year-old trees. Bark samples were taken from the stems of 2-year-old quince (*Cydonia vulgaris*, Pers.) trees of the type E.M.A. and from the trunks of 5-year-old apple (*Malus sylvestris*, Mill.) trees of the Delicious variety. Leaves and whole canes of grapes (*Vitis vinifera*, L.), variety Dabouki, were sampled.

Reagents. PUCHER *et al.* (1948) I-KI REAGENT. Dissolve 7.5 grams of iodine and 7.5 grams of potassium

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iodide in 150 ml. of water and make up to 250 ml. The solution is filtered with suction.

ALCOHOLIC NaCl SOLUTION. Mix 350 ml. of ethanol (97%) with 80 ml. of water and 50 ml. of a 20% aqueous NaCl solution and make up to 500 ml. with water.

ALCOHOLIC NaOH SOLUTION. Mix 350 ml. of ethanol (97%) with 100 ml. of water and 25 ml. of 5*N* NaOH solution.

MORELL'S (1941) STOCK SOLUTION A. Forty grams of potassium ferricyanide and 40 grams of sodium carbonate monohydrate are dissolved and diluted to 2 liters with water.

MORELL'S STOCK SOLUTION B. A 40% sodium carbonate monohydrate solution.

MORELL'S REAGENT. Fifty milliliters from stock solution A are mixed with 50 ml. from stock solution B and made up to 2 liters with water. The reagent is prepared fresh daily.

Analytical Procedures. To stop enzymatic activity, the samples were autoclaved for 5 minutes at 5 p.s.i., dried at 60° C., and ground to pass through a 40-mesh sieve. Extraction was done twice on 200 mg. of the material with 10 ml. of 31% perchloric acid, each time for 20 minutes. The combined extracts were made up to 50 ml. with water. Analysis for starch was made on 8 ml. of the extract according to the method of Pucher *et al.* (1948). From the same extract, 1, 2, or 3 ml., depending on the starch content, were analyzed colorimetrically for starch according to Nielsen's (1943) method.

The procedure of Pucher *et al.*, slightly modified, was as follows. To 8 ml. of plant extract, 2 ml. of water, 5 ml. of 20% aqueous NaCl solution, and 2 ml. of the I-KI reagent were added. After 20 minutes, the precipitate was centrifuged, cautiously decanted, and subsequently suspended in 5 ml. of alcoholic NaCl solution. After centrifugation and decantation, 2 ml. of alcoholic NaOH solution were added to the precipitate, and the tubes were gently shaken until all the blue color was discharged. The liberated starch was centrifuged and washed with 5 ml. of alcoholic NaOH solution. Two milliliters of 0.7*N* HCl were added and the tubes were heated in a water bath for 2½ hours under reflux. The solution, containing the glucose obtained by hydrolization of the starch, was neutralized with 0.5*N* NaOH, with the aid of a pH meter.

Contrary to the method of Pucher *et al.*, the glucose in the solution was determined with Morell's (1941) method. According to this method, 2 ml. of the neutral solution were added to 25 ml. of Morell's reagent and kept in a boiling water bath for 10 minutes. After cooling, absorbance was measured with a Klett-Summerson photoelectric colorimeter (Filter 42). The glucose content was determined according to a previously constructed calibration curve.

Starch determinations according to Nielsen's method were performed as follows. The aliquot taken from the plant extract was made up to 7 ml. with water and neutralized with 2*N* NaOH, using phenolphthalein as indicator. Acetic acid, 2*N*, was added till the pink color of the indicator disappeared and then an excess of 2.5 ml. of acetic acid was added. One milliliter of a 5% solution of KI and 5 ml. of a 0.01*N* KIO_3 solution were added, and the solution was made up to 25 ml. with water. After 10 minutes, absorbance was measured with a Klett-Summerson photoelectric colorimeter (Filter 64). The units of this instrument were translated into absorbance ($A = 2R/1000$ if R is a Klett-Summerson unit).

Readings for absorbance obtained with Nielsen's method were plotted against the amount of starch present in the 1- to 3-ml. sample used for this method, the amount being calculated according to the results of the parallel determinations carried out according to the method of Pucher *et al.* (1948).

Such double determinations of starch were performed with eight to 19 samples per species, obtained from different trees and varying greatly in their starch content. In addition, 37 samples of dried, analytical potato starch were weighed and analyzed according to Nielsen's method, thus establishing a standard curve for potato starch.

Calculations. Since with Nielsen's method the regression curves of the amount of starch against absorbance were more or less linear, within a certain range, straight lines were fitted for the curves of different species, each one fitting the formula:

$$\text{Amount of starch } (S) = a \times \text{absorbance } (A) + b \quad (1)$$

All regressions obtained for the various species were subjected to an analysis of covariance. For each species, the amount of starch giving a reading of absorbance of 0.100, A_{100}, and that amount giving the maximal absorbance still within the range of linearity, A_{max}, were calculated. Then the absorbances obtained by the same amount of potato starch, A_{p100} and $A_{p\ max}$, were calculated from the regression curve of potato starch. For both readings and all tested species, a "potato factor," f, was calculated as follows:

$$f = \frac{f_{100} + f_{max}}{2} \quad (2)$$

when

$$f_{100} = \frac{A_{100}}{A_{p100}}$$

and

$$f_{max} = \frac{A_{max}}{A_{p\ max}}$$

The existence of the factor b in the regression curves was the reason for the differences found for the values f_{100} and f_{max}.

The estimation of the percentage of amylose, z, in the tested starch was based on the finding that with light of approximately 640 mμ, the absorbance obtained by adding iodine to pure amylose is, according to graphs presented by Wolff *et al.* (1955), 7.74 times greater than that obtained with amylopectin. Therefore, if k represents the absorbance obtained with 1 μg. of amylopectin, c the amount of amylose, and d the amount of amylopectin present in e μg. of a given starch, the absorbance obtained with this starch is:

$$A = 7.74(ck + dk) = k[7.74(c + d)]$$

The amylose content of potato starch has been estimated by different workers as being between 17 and

25.5% (Carter and Neubert, 1954; Kerr and Trubell, 1943). Accepting the medium value of 22%, as found by Bates *et al.* (1943), the absorbance obtained with e μg. of potato starch is:

$$A_p = \frac{7.74 \times 22e_pk}{100} + \frac{78e_pk}{100} = 2.483e_pk$$

Dividing both absorbances gives:

$$\frac{A}{A_p} = \frac{7.74(c + d)}{2.483e_p} = \frac{6.74(c + e)}{2.483e_p} \text{ (as } e = c + d)$$

hence

$$c = \frac{2.483e_pD}{6.74D_p} - \frac{eD_p}{6.74D_p} = 0.368\,\frac{e_pD}{D_p} - 0.148e$$

The amylose content of the given starch (in per cent) is therefore

$$z = \frac{100c}{e} = 36.8\,\frac{e_pD}{eD_p} - 14.8$$

Comparing equal amounts of the given starch and potato starch

$$z = 36.8\,\frac{D}{D_p} - 14.8 = 36.8f - 14.8 \qquad (3)$$

We have seen that f is not the same for different amounts of various starches. The average values for z were calculated in the same manner as for f:

$$z = \frac{z_{100} + z_{\max}}{2} \qquad (4)$$

RESULTS

Figure 1 shows the regression curves of citrus starch against absorbance, as obtained with Nielsen's (1943) colorimetric method. The curve is practically linear between 100 and 360 μg. of starch. Above this value the ascent of the curve is much less steep, but it may still be used for starch determinations. Owing perhaps to interfering substances which cause considerable deviations at low starch concentrations, the regression line does not pass the zero point. Bark and wood samples fit well into the same curve. Samples from six citrus species and two varieties of one species (sweet orange) show no significant deviations from the common curve. Similarly, samples from five pear varieties, all of the species *Pyrus communis*, fit well in one common regression line.

The significant constants for the regression curves of the tested starches are given in Table I. The differences between the lines, as tested by an analysis of covariance are, with one exception, significant at the 5% level or higher, many of them being highly significant at the 1% level. The common F is also highly significant. The only nonsignificant difference was that between the curves for apple and quince starch, which may be regarded as practically identical. The curves for the leaves and canes of grapevine were practically identical.

The b values are negligible for the pure, analytical potato starch, but were particularly high with citrus starch, and somewhat less with quince starch (here b was negative). As a result, determinations of the potato factor and the percentage of amylose at absorbances below 0.100 were unreliable, particularly with citrus and quince starch. Between the absorbance 0.100 and the upper limit of lin-

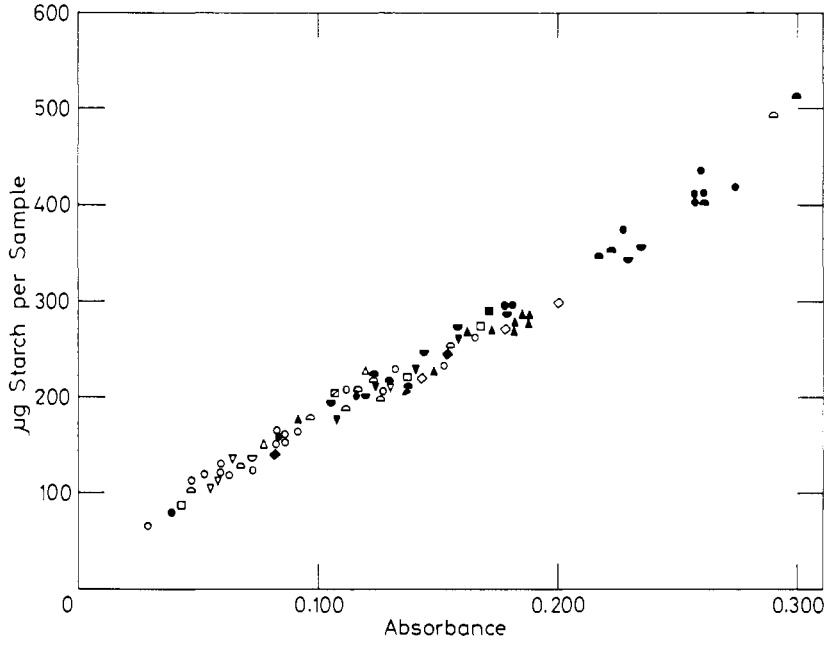

Figure 1. The regression of the amounts of starch in the sample on the absorbance obtained with iodine for various citrus species

○ — Sweet orange, var. Shamouti; △ — Sweet orange, var. Baladi; ▽ — Sour orange; ⌓ — Palestinian sweet line; ⌔ — Rough lemon; □ — Ethrog; ◇ — Pummelo. Open symbols, bark. Filled-in symbols, wood

Table I. Parameters of Colorimetric Calibration Curves for Starch of Various Tree Species and Their Amylose Content[a]

Species	a[b]	b	Upper Limit of Linearity, μg. Starch in Sample	Potato Factor, f	Amylose Content of Starch, %
Citrus	1382	+38	360	0.955 ± 6%	20.3 ± 2.2
Grapevine	1529	+13	480	0.972 ± 3%	21.0 ± 0.9
Pear	1588	−7	450	1.017 ± 1%	22.6 ± 0.5
Apple	1537	−8	450	1.059 ± 2%	24.2 ± 0.6
Quince	1537	−19	450	1.110 ± 5%	26.0 ± 2.0
Potato tubers	1578	−1	500	...	22[c]

[a] The formula for the linear part of the calibration curves is: μg. starch in final colorimetric solution = $s \times$ absorbance + b
[b] $F = 4.4$, being significant at the 1% level. All differences between the lines, with the exception of apple *vs.* quince, are significant at the 5% level or higher.
[c] According to Bates *et al.* (1943).

earity of the calibration curves, the errors for f and z are relatively small, as may be seen in Table I. This upper limit was about the same for most of the starches tested. It was relatively low only with citrus starch.

This method for the determination of starch has been applied in several investigations (Gur and Samish, 1965; Mendel and Cohen, 1967). Figure 2 shows, as an example, the seasonal changes in the starch content of the bark from scion and rootstock stem of pear trees grafted on quince rootstock.

DISCUSSION

The establishment of potato factors for assaying various kinds of starch colorimetrically, as advocated by Nielsen and Cleason (1945) and by Humphreys and Kelly (1961), may be applied to the wood and bark of various tree species, provided that only readings above the absorbance 0.100 (corresponding to about 150 to 180 μg. of starch per sample) are considered. Reliable results may be obtained with readings down to 0.050 absorbance (70 to 100 μg. of starch) only if, instead of using potato factors, calibration curves similar to the authors' are constructed. If the potato factor is used for values within this low range, errors of 4% for apples and pears and up to 16% for citrus may arise. The starch determination in the wood and bark of the species tested may also be undertaken by using these calibration curves. In this case the absorbance obtained with several samples of potato starch has to be compared with the present values, as not all photoelectric instruments are calibrated precisely. The results of such a comparison may be used for calculating a correction factor for the instrument used.

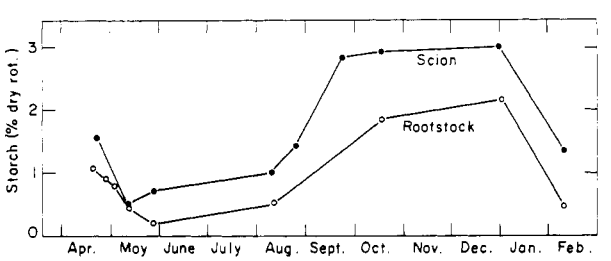

Figure 2. Seasonal changes in the starch content of stem bark, from the rootstock and scion of Bartlett pear trees grafted on quince *A* rootstock

The objection against the use of colorimetric starch determinations, that the amylose–amylopectin ratio depends not only on species, but also on variety (Deatherage *et al.*, 1955; Goering *et al.*, 1957; Hilbert and MacMasters, 1946; Whistler and Weatherwax, 1948), could be disproved in the case of citrus bark and wood, as well as pear bark. Similarly, no differences between varieties were reported for apple fruits (Fergason and Zuber, 1962) and other species (Carter and Neubert, 1954; Deatherage *et al.*, 1955). With citrus, different species did not differ in the composition of their wood and bark starch, but significant differences were found between starches belonging to different genera. An environmental influence on the amylose–amylopectin ratio has been claimed in certain cases (Goering *et al.*, 1957; Halsall *et al.*, 1948). However, the reported differences are small and may not, according to present knowledge, be regarded as a serious obstacle for the colorimetric method of starch determination. Large differences in the composition of starch between leaves, shoots, and tubers of potato have been reported (Meyer and Heinrich, 1942). In the authors' determinations, citrus wood did not differ in this respect from citrus bark, or grapevine leaves from canes, although citrus leaves seem to differ from citrus bark and wood.

The establishment of calibration curves for starches from wood and bark of woody species is worthwhile, though tedious, if a large number of routine determinations is intended. This is due to the fact that the determinations with Nielsen's (1943) method, as modified for dried material, are very rapid and convenient after such curves are established. For the sake of accuracy, it seems advisable to use only the linear part of the calibration curves. A modification of Nielsen's method, which was very important when analyzing dried material, is the double starch extraction, instead of the single one proposed by Nielsen (1943).

With regard to the estimation of the amylose–amylopectin ratio, this method resembles that used by Humphreys and Kelly (1961) for wood starch, with two differences: Whereas the latter authors disregarded the fact that amylopectin also yields some color with iodine, this factor was included in the present calculations; Humphreys and Kelly (1961) accepted the exceptionally high value (25.5%) obtained by Kerr and Trubell (1943) for the amylose content of potato starch, whereas the authors used the somewhat lower value (22%) obtained by Bates *et al.* (1943). For establishing this ratio, only readings above 0.100 absorbance should be considered, as lower values introduce large errors.

The per cent of amylose found in the starch of apple fruits by Carter and Neubert (1954) was 25 to 26%, which is almost identical with the amount found by the authors in apple bark (24 ± 0.5%).

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