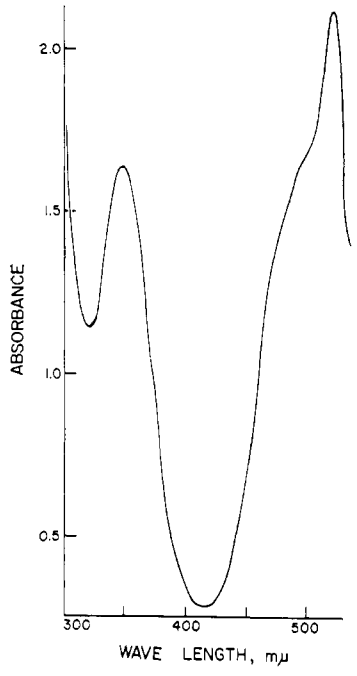

Figure 6. Absorption spectrum of pink solution obtained from *Allium giganteum* juice

Leaf and stem samples of *Allium giganteum* and bulb, stem, and flower samples of *Allium christophii* (both central Asian species) were crushed with and without acetic acid and with and without formaldehyde. The *christophii* samples remained white and no water-soluble pigment formed in the leaf tissue of *giganteum*. A mercaptan odor was given off immediately after cutting the *christophii* bulbs. The flowers are similar but larger than typical onion flowers and contain a small amount of preformed red pigment. This pigment was not identified, and no additional pigment formed after crushing. *Giganteum* leaves yielded a strong manure-like odor when cut. The stems became red-orange in a very few seconds after cutting. The juice came rapidly to the cut surface and reddened almost immediately. This juice was filtered and passed over Magnesol, giving one pink and one orange band. These were eluted readily by acidified 95% ethyl alcohol and the absorption spectrum of the pink solution is shown in Figure 6. A similarity to the onion pigment is noticeable in the visible region, but the ultraviolet absorption is very intense and is probably due largely to impurities. The pink solution turned brown on storage in air at room temperature for a few hours.

Literature Cited

(1) Acher, R., Crocker, C., *Biochem. Biophys. Acta* **9,** 904 (1952).
(2) Cruess, W. V., *Fruit Prods. J.* **23,** 305 (1944).
(3) Fraenkel-Conrat, H., Singer, B., *Arch. Biochem. Biophys.* **65,** 296 (1956).
(4) Hulme, A. C., *Food Science Abstr.* **28,** 345 (1956).
(5) Joslyn, M. A., Peterson, R. G., J. AGR. FOOD CHEM. **6,** 754 (1958).
(6) Joslyn, M. A., Sano, T., *Food Research* **21,** 170 (1956).
(7) Kohman, E. F., *Science* **106,** 625 (1947).
(8) Lukes, T. M., *Food Technol.* **13,** 391 (1959).
(9) Ottaway, J. H., *Biochem. J.* **68,** 239 (1958).
(10) Peterson, R. G., M.S. thesis, University of California, Berkeley, 1956.
(11) Peterson, R. G., Ph.D. dissertation, University of California, Berkeley, 1958.
(12) Peterson, R. G., Joslyn, M. A., *J. Org. Chem.*, in press.
(13) Sugihara, J., Cruess, W. V., *Fruit Prods. J.* **24,** 297 (1945).
(14) Yamaguchi, M., Shannon, S., Dept. Vegetable Crops, University of California, Davis, Calif., private communication, 1959.
(15) Yemm, E. W., Cocking, E. C., *Analyst* **80,** 209 (1955).

Received for review June 12, 1959. Accepted September 21, 1959.

STARCH ANALYSIS

Determination of Amylose in Starch

B. CARROLL and H. C. CHEUNG
Department of Chemistry, Rutgers, the State University, Newark, N. J.

Congo red is used colorimetrically for the determination of amylose in starch. Adsorption of the dye is independent of the chain length of the polysaccharide over a wide range. The extinction coefficient of the adsorbed dye is the same for both amylose and dextrin. The results are discussed and compared with those obtained from the usual iodine sorption and colorimetric methods.

THERE ARE SEVERAL METHODS for the determination of amylose in starch. Some of these methods (*4*, *8*) are based on analysis of the hydrolysis products, which procedure is often time-consuming. Iodine has been used colorimetrically (*1*, *6*, *7*). The latter method is based on the fact that amylose binds iodine, whereas amylopectin does not. Optical measurements of the iodine-starch mixtures are usually made at a fixed wave length, in the vicinity of 610 mμ, which is approximately the absorption maximum of the mixture. Iodine interaction depends not only on the degree of branching, but also on chain length, thereby leading to conflicting analytical results (*5*). It seems desirable to obtain another indicator which does not depend on chain length, although responding to changes in the degree of branching in the starch. Congo red appears to be a suitable adsorption indicator for this purpose.

Experimental

Materials. A histological grade of Congo red (National Aniline Division, Allied Chemical and Dye Corp.) was used, without further purification. Buffers were prepared from reagent grade potassium hydrogen phthalate and so-

dium hydroxide for pH ranges 4 to 6, and from monopotassium phosphate and sodium hydroxide for pH ranges 6 to 8.

Materials used as reference substance were corn amylose 14SSP and corn amylopectin 14SSP (Northern Utilization Research and Development Division, Peoria, Ill.). The potato amylose 13SSP and potato amylopectin 13SSP were obtained from the same source. The Superlose (potato amylose), Dutch R-A (native potato), and Ramalin G (potato amylopectin) (Stein Hall & Co.) had been prepared by a salt fractionation process. The defatted corn and potato starches (National Starch & Chemical Corp.) were defatted during the course of this work. The high linear cornstarch and wrinkled pea starch (Corn Products Refining Co.) as well as the other starches were used directly without further purification.

All starch solutions were prepared by dissolving 0.1 gram (dry basis) of the starch in 5 ml. of standardized 1*N* sodium hydroxide. A uniform solution was obtained within 1 hour. The alkaline mixture was then neutralized with an equivalent amount of standardized 1*N* hydrochloric acid, and diluted to 100 ml. In all cases, neutralization was reached within 1.5 hours. The neutralized solution was used within 2 to 3 hours.

Apparatus. A Beckman Model G pH meter was used for all pH measurements and for the potentiometric titrations; calomel-platinum electrodes were used in the latter procedure. All optical measurements were made in a Beckman DU spectrophotometer, equipped with cells of 1-cm. path length.

Procedure. Amylose was determined as follows: Five milliliters of pH 4.2 buffer and 5 ml. of a 0.1% starch solution were introduced into a 50-ml. volumetric flask. Approximately 29 ml. of water were then added, followed by 10 ml. of 0.0004*M* Congo red. The resulting mixture was diluted to volume and the absorbance at 500 mμ was immediately measured. An appropriate starch solution was used as a blank. The final mixture contained $2 \times 10^{-5}M$ Congo red and 0.01% starch. For the iodine mixtures, no buffer was used. The final solution contained 0.003% starch, 0.003% iodine, and 0.03% potassium iodide. The absorbance was measured at 615 mμ. The iodine sorption values were obtained potentiometrically (*2*).

Results and Discussions

Aqueous solutions of Congo red follow Beer's law to within ± 3%, up to a concentration of about 2×10^{-5} *M* (*3*). The addition of starch to the dye causes the extinction at the spectral peak to increase. The absorption spectrum of Congo red in the presence of an excess of starch is shown in Figure 1. This curve shows the spectral property of the bound dye to be independent of the type and size of the substrate. Unlike iodine, which causes a blue color with amylose or native starch, a plum color with amylopectin, and a red color with dextrin, Congo red causes no shift in color with these materials. Such changes in iodine behavior have been attributed to changes in the degree of branching and chain length of the substrate.

Figure 2 shows the effect of variation of the type of substrate upon the affinity of the adsorption process, as carried out at low concentration (0.01%). The starches used were amylose, a native starch, and dextrin. The only difference among these curves is the intensity. The difference in absorbance for these substances is assumed to be, to a large extent, a function of the degree of branching. For mechanical mixtures of solutions of corn amylose and corn amylopectin such a relation has been found to be linear. The relationship can be represented by the equation

$$R_c = (D_s - D_{ap})/(D_{am} - D_{ap}) \times 100$$

where R_c is per cent of amylose in the sample, D_s is the absorbance of the sample, D_{ap} is the absorbance of corn amylopectin, and D_{am} is the absorbance of corn amylose. A series of starches was used to test this equation. The results are shown in Table I. These are compared with results obtained with the usual iodine colorimetric and sorption methods. A fair agreement is seen for most of the samples.

The colorimetric iodine method yields more precise values, because the spread in optical scale between amylose and amylopectin is considerably larger than that for Congo red. However, precision in these determinations may be misleading, as indicated subsequently by the effects of variation in chain length in the starch sample. In this connection, the addition of Congo red to an acid-converted corn dextrin yields an R_c value of 20%, a figure slightly lower than that for native cornstarch.

There is a large discrepancy between the Congo red results and those for iodine for the second and the third samples in Table I. Both were corn amylose and had been heated, one at 150° C. for 1 hour, and the other at 150° C. for 48 hours. Heat treatment usually has two major effects on the starch molecules. One is degradation (the chain length is reduced) and the other is branching. If it is assumed that the binding of Congo red is strongly dependent upon the degree of branching, one could readily explain the considerably lower results with Congo red (see Table I). The heat treatment for the second sample in Table I was relatively mild, so that the reduction of the chain length was probably slight; nevertheless, the degree of branching was sufficient to reduce the Congo red binding.

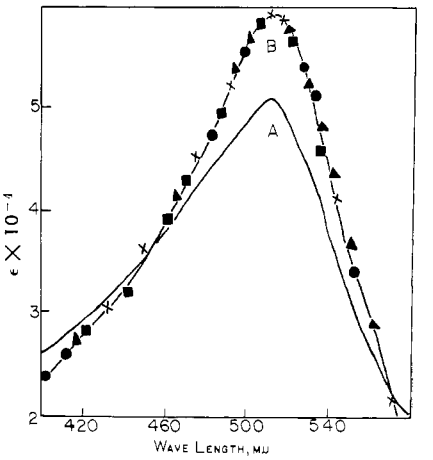

Figure 1. Congo red solution in presence of excess starch

Solutions buffered at pH 5, dye concentration = 2×10^{-5}M, temperature = 25° C.

- A Dye alone
- B. Dye with starch
- ● Corn amylose, 14SSP
- × Potato amylose, 13SSP
- ■ Potato starch
- ▲ Corn dextrin

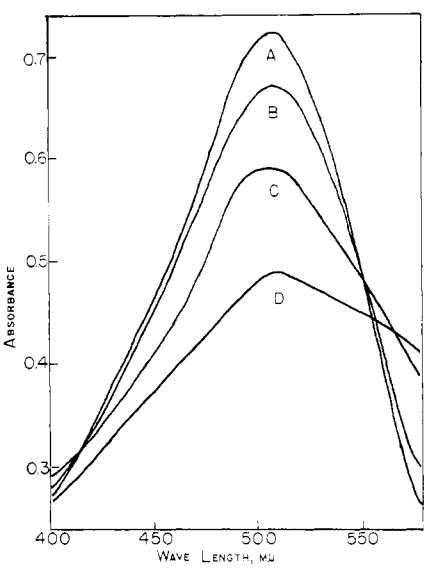

Figure 2. Spectral curves of aqueous Congo red in presence of starch and dextrin

All solutions buffered at pH 4.2, total substrate concentration = 0.01%, dye concentration = 2×10^{-5} M, temperature = 22° C.

- A. Superlose (potato amylose)
- B. Wrinkled pea, containing about 70% amylose Table I
- C. Acid-converted dextrin
- D. Congo red alone

The sample that had been heat-treated for 48 hours at 150° C. was hydrolyzed subsequently in the presence of β-amylase and found to yield only 48% of the theoretical content of maltose. This indicated that linkages other than those in the 1,4 positions were present, and branching was indeed obtained.

Throughout all measurements the concentration of sodium hydroxide used in dissolving the starch was kept constant,

Table I. Amylose Content in Starch

Sample	Iodine Sorption	Amylose, % R_i (iodine)	Amylose, % R_c (Congo red)
Corn amylose, 14SSP	19.4	100.0[a]	100.0[a]
Washed 6 times with methanol, dried at 150° C. for 1 hour	18.7	96.0	73.0
Washed 6 times with methanol, dried at 150° C. for 48 hours	14.6	73.5	54.5
Corn amylopectin, 14SSP	0.8	0.0[a]	0.0[a]
Potato amylose, 13SSP	20.6	104.5	103.9
Superlose	19.0	96.0	105.0
Defatted potato starch, vacuum-dried at 100° C. for 1 hour	3.8	28.0	23.0
Defatted cornstarch, vacuum-dried at 100° C. for 1 hour	5.2	24.5	20.0
Dutch R-A	3.7	29.5	27.0
High linear corn	12.0	46.5	42.0
Wrinkled pea	15.0	76.5	70.0
Ramalin G	1.1	10.0	0.3
Potato amylopectin, 13SSP	1.5	12.0	11.2
Washed 6 times with methanol, dried at 105° C. for 1 hour	0.8	10.1	9.9

[a] Basis of calibration. Congo red results obtained at starch concentration = 0.01%, pH = 4.2, temperature = 32° C., and dye concentration = $2 \times 10^{-5} M$.

Table II. Change in Extinction of Congo Red in Presence of Amylose

pH	$\%\Delta\epsilon_{500m\mu}$	$\%\Delta\epsilon_{600m\mu}$
3.2	32	25
4.2	80	84
4.6	22	66
5.1	9	50

Dye concentration = $2 \times 10^{-5} M$, temperature = 32° C., starch concentration = 0.01% of corn amylose, 14SSP.

and hence the concentration of sodium chloride was fixed at about $0.005M$. To ascertain whether variation of the amount of sodium chloride would affect the optical behavior of the dye itself, measurements were taken over a range of 0.0 to $0.02M$ sodium chloride. The extinction coefficient of Congo red was found to be independent of the sodium chloride concentration.

Table II shows the effect of pH on the spectral change of Congo red with starch. A pH of 4.2 is seen to be a suitable choice, because at this pH, the change in extinction is optimal. This pH should be expected, because the pK of the dye is about 4.1.

Acknowledgment

The authors are indebted to C. E. Weill for his suggestions and for the work involving β-amylase. They also acknowledge a grant from the Corn Industries Research Foundation.

Literature Cited

(1) Baldwin, R. R., Bear, R. S., Rundle, R. E., *J. Am. Chem. Soc.* **66,** 111 (1944).
(2) Bates, F. L., French, D., Rundle, R. E., *Ibid.*, **65,** 142 (1943).
(3) Carroll, B., Van Dyk, J. W., *Ibid.*, **76,** 2506 (1954).
(4) Hestrin, S., Mager, J., *Anal. Chem.* **19,** 1032 (1947).
(5) Kerr, R. W., Cleveland, F. C., Katzbeck, W. J., *J. Am. Chem. Soc.* **73,** 3916 (1951).
(6) Kerr, R. W., Trubell, O. R., *Paper Trade J.* **117,** No. 15, 25 (1943).
(7) McCready, R. M., Hassid, W. Z., *J. Am. Chem. Soc.* **65,** 1154 (1943).
(8) Shaffer, P. A., Somoggi, M., *J. Biol. Chem.* **100,** 695 (1933).

Received for review April 14, 1959. Accepted August 4, 1959.

SILAGE ANALYSIS

Determination of Sugar in Silages and Forages

H. G. WISEMAN, J. C. MALLACK, and W. C. JACOBSON

Dairy Cattle Research Branch, Animal Husbandry Research Division, ARS, Agricultural Research Center, Beltsville, Md.

A copper reduction method is described for the determination of sugar in silages and forages, whereby the usual sequence of dealcoholization and clarification is reversed. Clarification and deionization are performed on 80% alcoholic extracts, prior to dealcoholization to avoid handling of gummy residues. Somogyi's phosphate sugar reagent has been modified to give equal response to glucose and fructose.

QUANTITATIVE ANALYTICAL methods for sugars in silages and forages have been considerably shortened by the use of Waring Blendors for extraction (*8, 9*), ion exchange resins for clarification (*5, 10*), and Somogyi's copper microreduction reagents for ease of manipulation (*6, 7*).

Although extracts of many types of fresh green plants may be safely analyzed for sugar without clarification (*1, 8*), Williams and coworkers (*10*) have shown that extracts of dehydrated plant material must be clarified to remove the nonsugar reducing substances present. These may be removed by ion exchange resins according to the technique of Williams *et al.* (*10*).

The latter workers have also demonstrated the reliability of the Somogyi carbonate reagent for sugar analyses in various types of plant materials and the method is listed in the 6th, 7th, and 8th editions of "Methods of Analysis of the