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.

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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).

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SILAGE ANALYSIS

Determination of Sugar in Silages and Forages

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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

Association of Official Agricultural Chemists." Somogyi's improved phosphate reagent (*7*) is used for the determination of starch (*3*, *4*). An extraction of sugar by 80% alcohol or 80% acetone is common to most methods. It serves to minimize the solubility of protein, pectin, starch, etc. However, the extracts still contain pigments, ionized substances, and gummy materials. The subsequent removal of alcohol before clarification results in a gummy residue which requires scrubbing to suspend it in water. This suspension often filters with extreme slowness and invites occlusion. Furthermore, the sugar has been exposed to interaction with these substances at steam bath temperatures during concentration.

An additional criticism is that the copper-reduction values of glucose and fructose are usually not equal, the yields of the cuprous precipitate in each case varying differently with pH. Thus, with more alkaline copper reagents, glucose has a greater reducing power than fructose, and vice versa (*2*).

The method of analysis of sugar in silages and forages reported here in detail departs from the usual procedures, in the sequence of dealcoholization and clarification. The latter step is completed directly on the 80% ethyl alcohol extract to avoid gummy residues in the ensuing dealcoholization step.

In addition, the Somogyi reagent has been modified in alkalinity to effect equal response by glucose and fructose.

Reagents

Sugar reagent, Somogyi 1945 (*7*) modification. Potassium iodate, 0.3567 gram per liter; disodium phosphate, 56 grams per liter; sodium hydroxide, 82.5 meq. per liter.

Sodium thiosulfate, ACS grade.

Glucose, National Bureau of Standards, sample 41.

Sucrose, National Bureau of Standards, sample 17.

Fructose, National Bureau of Standards, purified sample.

Starch solution, 1%, freshly prepared.

Potassium iodide solution, 2.5% from ACS grade compound.

Sulfuric acid, 1.8*N* and 1.5*N*.

Dowex 50 (Dow Chemical Co., Midland, Mich.) (for activation see company specifications), or Dowex 50-W-X8, 200 to 400-mesh, sold activated.

Duolite A-4 (Chemical Process Co., San Francisco, Calif.) (for activation see company specifications), or Dowex 3, 20 to 50-mesh, sold activated.

Ion exchange resin mixtures. 0.5 gram of Dowex 50 plus 1.75 grams of Duolite A-4 (activated resins) are allowed to soak overnight in 75 to 100 ml. of distilled water. Alternatively, 0.4 gram of Dowex 50-W-X8 plus 1.4 grams of Dowex 3 may be used.

Method

Extraction. Silage or forage materials are chopped by means of a paper cutter into approximately 0.25-inch lengths and are loosened and distributed over a flat surface for sampling. Twenty-gram samples are comminuted for 10 minutes in a Waring Blendor with approximately 400 ml. of 80% ethyl alcohol.

Filtration. The contents of the blender are poured onto a sintered-glass filter containing a filter bed of 60 grams of Darco G-60 and Celite 535 (1 + 3) sandwiched between two layers of Celite 535, each 1 cm. in depth. Three hundred milliliters of 80% ethyl alcohol are used for successive washings. The combined filtrate and washings are made up to 750 ml. in volume.

Deionization. An aliquot, 30 ml., is stirred for 15 minutes (magnetic stirrer) with the ion exchange resin mixture. The deionized solution is decanted and filtered through a glass-wool plug inserted in the neck of a powder funnel placed over a 150-ml. beaker. The resins are rinsed with 80% ethyl alcohol, which is also decanted onto the filter, and the resins are then stirred for 10 minutes with 25 ml. of hot water. The water extract and subsequent rinsing of the resin with approximately 25 ml. of hot water are filtered through the glass wool. The beaker with the filtrate is set on the steam bath. The sugar solution is concentrated to 1 to 2 ml. to remove the alcohol.

Inversion. Hydrochloric acid, 1*N* (25 ml.), is added to the cooled beaker, and the sugar solution is allowed to stand overnight at room temperature to invert.

Neutralization. Sufficient sodium hydroxide (20%) is added to bring the solution nearly to neutrality. Diluted, 2% sodium hydroxide is used to complete the neutralization to pH 7, the adjustment being controlled by use of a pH meter with glass and calomel electrodes. The solution is made to a volume, 50 ml. Aliquot volumes are usually 5 ml. for analysis of the extracts from fresh green material and 10 ml. in the case of silage.

Copper Reduction. Aliquots are pipetted into 25-mm. diameter test tubes as specified by Somogyi. An equal volume of sugar reagent is added to the aliquot. Five milliliters of sugar solution and 5 ml. of reagent are used in the analysis of fresh plant material while 10 ml. of sugar solution and 10 ml. of reagent are taken for silages. The tubes are placed in a rack and immersed in a boiling water bath for 15 minutes. The rack is removed and placed in a pan of cold tap water to cool the solutions.

Table I. Recovery of Glucose from Darco and Resin Treatments

	Recovery, 1.500 Mg. Glucose Treated	
	Darco treatment	Resin treatment
	1.497	1.495
	1.505	1.502
	1.507	1.499
	1.502	1.500
	1.503	1.494
	1.500	1.492
Av.	1.502+	1.497

Titration. Potassium iodide solution (2.5%) is added slowly to form a layer over the solutions in the test tubes; 1 ml. is used for each 5 ml. of sugar solution placed in each tube. Immediately before titration, sulfuric acid is added rapidly to the tubes, 5 ml. of 1.8*N* sulfuric acid for 10-ml. aliquots and 3 ml. of 1.5*N* sulfuric acid for 5-ml. aliquots. A Teflon-covered stirring bar is dropped into the solution and thorough oxidation of the precipitated cuprous oxide by the liberated iodine is obtained by vigorous magnetic stirring for 1 minute. The stirring is continued at a reduced rate, while the excess of iodine is titrated with 0.005*N* sodium thiosulfate to a faint yellow. Four drops of a 1% starch solution are added and the titration is continued until the blue starch-iodine color disappears.

Standardization. Five- or 10-ml. samples of sugar reagent are mixed with equal volumes of distilled water and with equal volumes of freshly prepared standard glucose solutions. Analyses are carried out as described under copper reduction and titration. The difference in titer for the tubes containing water and sugar is divided into the milligrams of sugar present in the aliquot, thereby giving the glucose equivalent for 1 ml. of sodium thiosulfate, *G*.

Calculation. A blank is run to include any contribution to the analysis from reagents or resins. From this value, *B*, is subtracted the titer, *T*, of a silage or forage aliquot. The difference, $B - T$, is multiplied by *G* to obtain the amount of glucose present in the aliquot taken for analysis. Thus, when a 10-ml. aliquot has been taken from 50 ml. of a sugar solution representing 0.8 gram of wet silage material, the total sugar expressed as glucose may be calculated on the dry matter (D.M.) basis as follows:

$$\text{Sugar, \% of dry matter} = \frac{(B - T)G \times 100}{10/50 \times 0.8 \times 10^3 \times \text{D.M.}(\%)/100}$$

$$= \frac{(B - T)G \times 62.5}{\text{D.M. (\%)}}$$

Dry matter determinations on silage

Table II. Sugar Analyses on Silage Extracts

Sample	Sugar, % Oct. 9	Oct. 28
246	1.85	1.85
248	1.36	1.32
249	1.64	1.69
251	1.47	1.44
267	1.74	1.83
268	2.57	2.54
270	2.11	2.03
272	1.38	1.26
273	1.60	1.60
275	2.12	2.08
278	1.79	1.71
288	2.58	2.55
292	2.20	2.16
295	1.67	1.64
300	1.53	1.54
303	2.71	2.66
304	1.78	1.93
305	2.51	2.45
326	1.97	1.94

Table III. Recovery of Clarified and Inverted Sucrose

(Theoretical recovery, 1.500 mg. inverted = 1.579 mg. "glucose found")

Sample	Glucose Found, Mg.	Theory, %
1	1.572	99.56
2	1.567	99.24
3	1.573	99.62
4	1.563	98.99
5	1.566	99.18
6	1.562	98.92
7	1.573	99.62
Av.	1.568	99.30

may be conveniently made by toluene distillation in a Bidwell-Sterling apparatus.

Results

Recovery experiments with standard glucose from the National Bureau of Standards were made to test the effect of the Darco G-60 and Celite 535 and resin treatments. In Table I, satisfactory recoveries are evident.

Satisfactory reproducibility of analyses is shown in Table II. Comparisons of analyses made 19 days apart on silage extracts are listed. Somogyi's reagent of 1945 was used to obtain the data shown in Tables I and II. Dowex 50 and Duolite A-4 were used in the clarification work.

In Table III, recoveries are listed for sucrose samples subjected to clarification, dealcoholization, and inversion. Seven 250-ml. aliquots of sucrose taken from a stock solution containing 3 grams of sucrose in 2 liters of 80% ethyl alcohol were filtered through Darco-Celite. The combined filtrate and washings were made up to 750 ml. in each case. From this volume, 30-ml. aliquots—0.0150 gram of sucrose—were resin-treated, dealcoholized, inverted, neutralized, and made up to 100-ml. volumes. The modified Somogyi reagent giving equal response to glucose and fructose was used to obtain the data in Table III. A Dowex 50-W-X8 and Dowex 3 mixture was used for the resin treatment. The inversion was carried out for 24 hours in the following manner. The acidified sugar solutions were placed in a desiccator vessel which was rapidly evacuated and refilled with nitrogen three times in succession. The temperature of inversion was maintained at 25° C. by placing the vessel in a temperature-controlled water bath.

Discussion

In this laboratory, reducing substances have been encountered in preliminary water extracts of ion exchange resins. The use of resins therefore necessitates adequate water washing to minimize the blanks, as well as the inclusion of resins in blank determinations.

The filtration of the Waring Blendor contents through the Darco-Celite bed yields an essentially colorless filtrate. The deionization by resins is also conducted on the 80% alcoholic filtrate, in contrast to the usual resin treatment of aqueous solutions. These two clarification steps remove ions and organic matter before the sugar solutions are concentrated on the steam bath to remove the alcohol. The solutions, when nearly evaporated to dryness, are free of gummy residues and require no filtration. Because a further increase in volume from the clarification steps is eliminated, a wider choice of final volume is permitted.

Attention is invited to the advantages offered by magnetic stirrings during titrations of the solutions in the 25-mm. diameter test tubes as used in the Somogyi sugar method. Although magnetic stirring bars, 1.25 inches long, stand on one end at an angle in test tubes of this diameter, the bars will rotate to give efficient mixing when held over a magnetic stirring apparatus. Under these conditions, titrations are performed rapidly without repeated removal of the tube to mix the contents by shaking. In addition, larger aliquots can be smoothly handled without danger of spillage and poor mixing. Of greater importance, however, is the need to ensure that the cuprous oxide precipitate formed in the reduction is stirred sufficiently well to react completely with the iodine formed upon addition of potassium iodide and sulfuric acid. Experience in this laboratory has shown that 1 minute of rapid stirring by the magnetic apparatus leads to consistent titration values; without this precaution results often gave considerable variation.

Preparations of the copper reagent at different alkalinities were tested against these sugars to obtain equal response to glucose and fructose. In each of these trials the disodium phosphate concentration prescribed by Somogyi was doubled to increase its buffer action. The intersection of the copper reduction curves of glucose and fructose came at a pH of approximately 9.6. The sodium hydroxide addition, possibly more precisely expressed than the pH, was 82.5 meq. per liter. The two sugars were further tested for equivalence at this alkalinity of the copper reagent. The relative reducing power of 1.5-mg. quantities of glucose and fructose was compared from triplicate determinations on each sugar in each of six trials. The fructose-glucose reducing ratios were 1.005, 1.006, 1.002, 1.005, 1.005, and 0.994.

Because the modified reagent reacts equally to glucose and fructose, the reducing action of inverted sucrose is conveniently twice that of glucose on a molecular basis. In Table III, inverted sucrose would be calculated to yield $(2 \times 180)/342$ times the reduction of glucose. Thus, 1.5 mg. of sucrose when inverted would be equivalent to 1.579 mg. of glucose (Table III). With glucose test solutions containing 0.5, 1.0, and 1.5 mg. per ml., the response of the modified reagent, within experimental error, was found to be linear.

When solutions of sucrose—National Bureau of Standards, sample 17—were inverted in air, consistent losses of approximately 3 to 4% were obtained, which could be tolerated in routine determinations. However, if the extent of aerial oxidation is a function of the concentration of fructose, the exclusion of air should be of more concern in the inversion of solutions relatively rich in fructose.

Literature Cited

(1) Bevenue, A., Washauer, B., *J. Assoc. Offic. Agr. Chemists* **33,** 122 (1950).
(2) Jackson, R. F., McDonald, E. J., *J. Research Natl. Bur. Standards* **34,** 213–26 (1945).
(3) *J. Assoc. Offic. Agr. Chemists* **39,** 84 (1956).
(4) Pucher, G. W., Leavenworth, C. S., Vickery, H. B., *Anal. Chem.* **20,** 850–3 (1948).
(5) Serbia, G. R., *Sugar* **42,** No. 6, 26 (1947).
(6) Somogyi, M., *J. Biol. Chem.* **70,** 599 (1926).
(7) *Ibid.*, **160,** 61 (1945).
(8) Thomas, J. W., Melin, C. G., Moore, L. A., *Anal. Chem.* **21,** 1363 (1949).
(9) Waldron, D. R., Ball, C. D., Miller, E. J., Benne, E. J., *J. Assoc. Offic. Agr. Chemists* **31,** 708 (1948).
(10) Williams, K. T., Bevenue, Arthur, Washauer, Barbara, *Ibid.*, **33,** 986 (1950).

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