hydrogens. These changes were attributed to increase in basic strength and hydrogen-bonding ability on methylation.

The pH of the amide solution did have some effect upon the particular amide solution's tendency to damage human blood cells. The low pH of the DEF solutions appears to be the reason for the total destruction of cells at very low amide concentrations. When buffered to a near-neutral pH, the critical concentration was raised from 0.4 to 10% (Table II). With the other amide solvents, there was little change in the critical concentration by the addition of an isotonic phosphate buffer since none of these amide solutions had inherently high or low pH values.

REFERENCES

(1) D. E. Cadwallader, *J. Pharm. Sci.*, **52**, 1175(1963).

(2) D. E. Cadwallader, B. W. Wickliffe, and B. L. Smith, *ibid.*, **53**, 927(1964).

(3) B. L. Smith and D. E. Cadwallader, *ibid.*, **56**, 351(1967).

(4) D. E. Cadwallader and J. P. Drinkard, *ibid.*, **56**, 5(1967).

(5) L. Knazko, *Farm. Obzor*, **35**, 200(1966).

(6) P. H. Elsworthy and H. E. C. Worthington, *J. Pharm. Pharmacol.*, **20**, 830(1968).

(7) A. J. Spiegel and M. M. Noseworthy, *J. Pharm. Sci.*, **52**, 917(1963).

(8) T. Waaler, *Medd. Norsk Farm. Selskap*, **9**, 121(1965).

(9) K. Davis and P. M. Jenner, *Toxicol. Appl. Pharmacol.*, **1**, 576(1959).

(10) T. S. Grosicki and W. J. Husa, *J. Am. Pharm. Assoc., Sci Ed.*, **43**, 632(1954).

(11) A. N. Martin, "Physical Pharmacy," Lea & Febiger, Philadelphia, Pa., 1960, p. 191.

(12) T. Nash, *J. Gen. Physiol.*, **46**, 167(1962).

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Spectrophotometric Analysis of Glucose and Mixtures of Glucose, Fructose, and Sucrose

EDWARD R. GARRETT and JOHN F. YOUNG

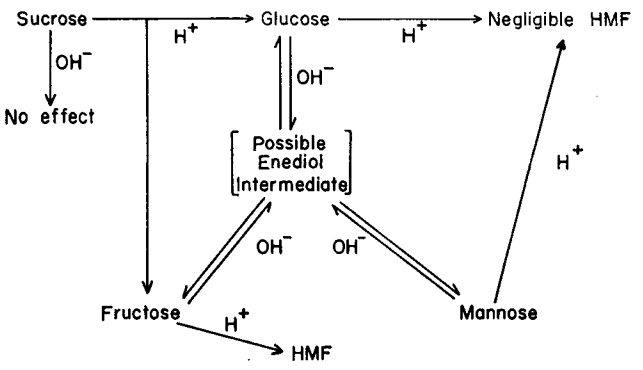
Abstract □ The aldose-ketose equilibrium in dilute alkali is utilized to transform a reproducible fraction of glucose to fructose; *e.g.*, 0.20 *N* NaOH, 40°, 3.5 hr. Acid treatment, *e.g.*, 1.0 *N* HCl, 80°, 10 hr., of this alkaline equilibrium solution results in the production of spectrophotometrically assayable hydroxymethylfurfural (HMF) ($\lambda_{max.}$ = 283 mμ) from the fructose produced. Glucose yields negligible HMF under these acid conditions prior to alkaline treatment. These two techniques permit the assay of fructose and glucose in mixtures. After 30 hr. of alkaline treatment under the stated conditions, fructose and glucose do not yield any HMF on acidification. However sucrose is stable under these conditions and on acidification hydrolyzes to fructose which yields a proportional amount of HMF. These facts permit the assay of fructose, glucose, and sucrose in mixtures. This assay is sensitive to concentrations for all three sugars as low as 10^{-4} *M*.

Keyphrases □ Glucose—analysis □ Fructose, glucose, sucrose mixture—analysis □ Aldose-ketose equilibrium, dilute alkali—glucose transformation to fructose □ Hydroxymethylfurfural formation—acid treatment aldose-ketose equilibrium □ UV spectrophotometry—analysis

The primary chemical methods for the analysis of glucose have been based on general methods for reducing sugars, such as oxidation by alkaline copper or ferricyanide solutions (1–5). Glucose has been analyzed in effluents after paper or column chromatographic separations of mixtures of sugars (6). Glucose has also been analyzed by GC after derivatization of sugar mixtures (7). Carbazole in sulfuric acid reactions (8), differential reaction-rate techniques (9), and dialysis based on differential kinetics (10) have also been applied to the determination of sugar mixtures.

Haworth and Jones (11) showed that acidification of an alkaline glucose solution could result in the production of hydroxymethylfurfural (HMF). Thus it is anticipated that with selective conditions of sequential alkali and acid treatment, a quantitative chemical analysis could be established for glucose based on the spectrophotometric assay of the HMF produced.

The Lobry de Bruyn-Alberda van Ekenstein transformation (12, 13) is the alkaline catalyzed equilibration of aldoses and ketoses through a possible enediol intermediate. The hexoses, mannose, glucose, and fructose, undergo such an alkaline equilibration (Scheme I).



Scheme I—Acid and base transformations of sugars

Table I—Results of Assays of Mixtures of Glucose and Fructose[a]

Mixture	10^4[Glucose] Concn.	10^4[Glucose] Found	10^4[Fructose] Concn.	10^4[Fructose] Found
1	3.20	3.24(0.11)	0.20	0.25(0.02)
2	3.20	3.33(0.17)	0.40	0.42(0.03)
3	3.20	3.14(0.14)	0.80	0.82(0.03)
4	3.20	3.31(0.21)	1.60	1.57(0.06)
5	3.20	3.72(0.28)	3.20	3.12(0.08)
6	1.60	1.73(0.14)	3.20	3.24(0.05)
7	0.80	0.74(0.14)	3.20	3.22(0.10)
8	0.40	0.50(0.10)	3.20	3.21(0.01)
9	0.20	0.21(0.07)	3.20	3.25(0.09)
10	0.40	0.27(0.02)	1.60	1.62(0.04)
11	0.80	0.57(0.08)	0.80	0.86(0.06)
12	1.60	1.37(0.07)	0.40	0.45(0.04)
13	10.00	9.64(0.84)	1.00	0.92(0.04)
14	10.00	9.84(0.91)	2.01	1.85(0.07)
15	10.00	10.61(0.66)	4.01	3.81(0.09)
16	10.00	10.52(0.54)	8.02	7.93(0.30)
17	10.00	10.40(0.16)	10.03	9.76(0.32)
18	8.00	8.59(0.41)	10.03	10.20(0.14)
19	4.00	4.21(0.19)	10.03	10.19(0.12)
20	2.00	1.96(0.39)	10.03	10.20(0.26)
21	1.00	0.95(0.39)	10.03	10.16(0.18)
22	2.00	1.81(0.28)	8.02	7.94(0.20)
23	4.00	3.86(0.16)	4.01	3.97(0.10)
24	8.00	8.15(0.21)	2.01	1.96(0.06)

[a] The parenthetical standard deviations are based on four values for Mixtures 1–12 and on eight values for Mixtures 13–24.

Fructose has been shown to yield HMF under selective acidic conditions (14). This reaction occurs only to a minor extent with aldohexoses such as glucose and mannose.

Since the rates of fructose appearance and disappearance as monitored by HMF formation varied with fructose, glucose, mannose, or sucrose as the starting material, it was feasible to establish standard controlled conditions to assay for each of these components in mixtures. Judicious use of sequential alkaline and acidic treatment resulted in the analysis of each component.

This paper presents the statistically evaluated methods and procedures based on these principles for the analysis of glucose alone and in mixtures with sucrose and fructose.

EXPERIMENTAL

Materials—A spectrophotometer (Beckman model DU), slit width 0.1 mm., was used to measure the absorbance of HMF. Glucose and fructose (Distillation Products Industries); and sucrose (Everglades Sugar Refinery) were used. All other chemicals were of analytical reagent grade.

Glucose Analysis—A 5-ml. aliquot of glucose solution was added to 20 ml. of preheated 0.25 *N* NaOH at 40° to prepare a solution 0.20 *N* in NaOH. After 3.5 hr. of alkaline treatment, a 5-ml. aliquot was acidified with 20 ml. of HCl to result in a 1.0 *N* HCl solution. This acidic solution was placed in an 80° constant-temperature bath for 10 hr. An aliquot was taken, cooled, appropriately diluted, and read at 283 mμ against a water blank. The absorbance of this HMF chromophore was linear with respect to the original glucose concentration over a range of 1 to 30 × 10^{-4} *M*.

Sugar Mixture Analysis—Sugar mixtures of glucose, fructose, and sucrose were analyzed by determining the HMF content on the acidification of three aliquots; two of which were first reacted with alkali for specific times. Analogous to the glucose assay, a 5-ml. sample of the sugar mixture was placed in 20 ml. of preheated (40°) 0.25 *N* NaOH to prepare a solution 0.20 *N* in NaOH. A 5-ml. sample was taken from this alkaline solution after 3.5 hr. and another after 30 hr. of alkaline treatment. The alkaline aliquots were acidified with 20 ml. of HCl to result in a 1.0 *N* HCl solution. One milliliter of the original sugar mixture solution was directly acidified with 24 ml. of HCl to result in a 1.0 *N* HCl solution. When sucrose was not present, the 30-hr. sample was omitted.

These acidic solutions may be stored in the refrigerator before heating in the 80° bath without affecting the final absorbance.

After placing the acidic solution in a 80° constant-temperature bath for 10 hr., the absorbances at 283 mμ of the appropriately diluted three samples were read on cooling against a water blank.

Standards must be run daily for each individual sugar in a similar manner. Satisfactory calibration curves were obtained by making two replicate assays on one high and one low sugar concentration.

Statistical Design—The analysis of mixtures of glucose and fructose was replicated twice for each of 2 days. Calibration curves (five points) were obtained for fructose at 0 hr. and for fructose and glucose after 3.5 hr. of alkaline treatment. Hexose mixtures ranging in concentrations from 0.2 to 10 × 10^{-4} *M* for each sugar (Table I) were analyzed.

Analysis of mixtures of glucose, fructose, and sucrose were replicated twice on each of 3 days to estimate the errors associated with these procedures. Two concentrations (2 and 4 × 10^{-4} *M*) of each sugar were used in all combinations to make eight mixtures (Table II, Mixtures 1–8). Each individual sugar at the stated concentrations was analyzed in duplicate each day to determine the calibration curves.

In addition three other ternary mixtures were replicated five times within 1 day (Table II, Mixtures 9–11). Appropriate standards were run concurrently.

RESULTS AND DISCUSSION

The alkaline transformation between ketoses and aldoses was first characterized by Lobry de Bruyn and Alberda van Ekenstein in the late 1800's (12, 13). Since an accurate and simple procedure has previously been reported from the authors' laboratories (14) for the assay of fructose, it was conceived that if glucose would give some proportionate amount of fructose *via* this alkaline transformation, assaying for fructose would permit an indirect determination of glucose. This hypothesis has proven correct.

Fructose when placed in 1.0 *N* HCl at 80° resulted in a maximum yield of HMF after 10 hr. (14); glucose and mannose when treated similarly yielded a negligible amount of HMF. When the alkaline

Table II—Results of the Assay of Mixtures of Glucose, Fructose, and Sucrose[a]

Mixture	10^4 Glucose Concn.	10^4 Glucose Found	10^4 Fructose Concn.	10^4 Fructose Found	10^4 Sucrose Concn.	10^4 Sucrose Found
1	2.00	1.84(0.64)	2.00	2.21(0.25)	2.00	1.97(0.14)
2	4.00	3.82(1.21)	2.00	2.68(0.09)	4.00	3.79(0.17)
3	2.00	2.37(0.54)	4.00	4.28(0.34)	4.00	3.91(0.22)
4	4.00	3.37(0.96)	4.00	4.30(0.49)	2.00	2.16(0.16)
5	4.00	4.32(0.84)	4.00	4.21(0.33)	4.00	4.04(0.12)
6	2.00	1.82(0.21)	4.00	4.37(0.21)	2.00	2.01(0.08)
7	4.00	3.93(0.91)	2.00	2.04(0.15)	2.00	2.09(0.13)
8	2.00	2.80(0.91)	2.00	2.10(0.46)	4.00	3.93(0.24)
9	4.02	3.71(0.22)	2.01	1.93(0.28)	3.00	3.33(0.09)
10	2.01	1.81(0.36)	3.01	2.80(0.07)	4.00	4.17(0.05)
11	3.02	2.81(0.27)	4.02	3.53(0.07)	2.00	2.21(0.04)

[a] The parenthetical standard deviations are based on six values for Mixtures 1–8 and on five values for Mixtures 9–11.

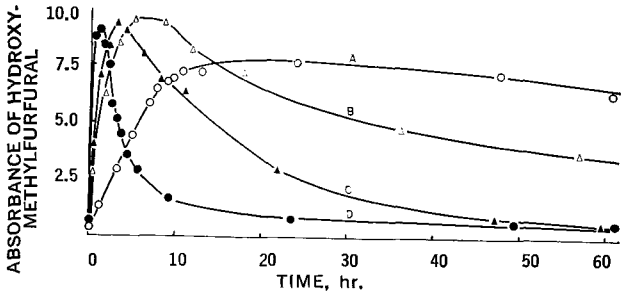


Figure 1—*Temperature effects on the absorbance of hydroxymethylfurfural derived from glucose (0.006 M) in 0.20 N NaOH as a function of time. Aliquots of the alkaline equilibrium mixture are acidified to 1.0 N HCl, reacted at 80° for 10 hr., appropriately diluted, and the hydroxymethylfurfural absorbance measured (283 mμ). The plotted absorbances of the solutions were corrected for the dilutions. Key:* A, *25°;* B, *35°;* C, *40°;* D, *50°.*

glucose transformation mixture was acidified and the amount of fructose formed was measured by obtaining the absorbance of the derived HMF, a means of monitoring the rate of achievement of the equilibrium and subsequent degradation of the transformed sugar was obtained. The degradation product of sugars in alkali are most probably due to the aldol condensations, oxidation-reduction disproportionations, and polymerizations which readily occur with aldehydes and alcohols in alkaline solution. The facts that fully alkaline-degraded glucose and fructose (40°, 0.20 *N* NaOH, 30 hr.) do not give rise to an HMF chromophore on acidification, and that their degradation products do not interfere with the HMF yield from the fructose derived from the alkaline-undegraded sucrose on acidification are proof that these degradation products do not interfere with the HMF absorbance derived from fructose.

Both the temperature (Fig. 1) and alkaline concentration (Fig. 2) affect the rate and yield of this transformation. The conditions chosen for the conversion of glucose to fructose were 40° in 0.20 *N* NaOH.

The glucose assay was applicable to a concentration of 1×10^{-4} *M* with an accuracy of about 4%. The linear correlation coefficient was 0.999 for a calibration curve over the range of 1 to 30×10^{-4} *M* in glucose (Fig. 3).

Mixtures of glucose and fructose were analyzed at concentrations from 0.2 to 10.0×10^{-4} *M* in hexose. The error for fructose was about 4% and for glucose about 9%. There was no significant variation among days when daily calibration curves were prepared. Representative data are presented in Table I.

The analysis of mixtures of glucose, fructose, and sucrose were based upon the following experimental facts: (*a*) glucose, without prior alkaline treatment, did not form significant amounts of HMF under these conditions (14); (*b*) a maximum conversion of glucos

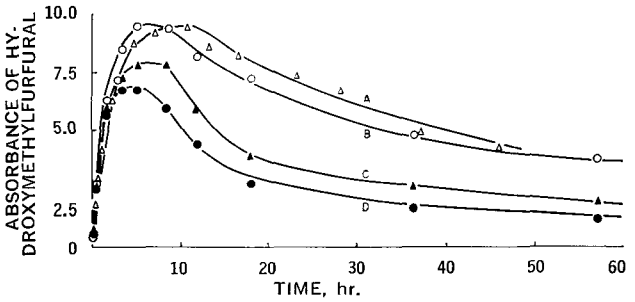


Figure 2—*Alkaline effects on the absorbance of hydroxymethylfurfural derived from glucose (0.006 M) at 35° as a function of time. Aliquots of the alkaline equilibrium mixture are acidified to 1.0 N HCl, reacted at 80° for 10 hr., appropriately diluted, and the hydroxymethylfurfural absorbance measured (283 mμ). The plotted absorbances of the solutions were corrected for the dilutions. Key:* A, *0.10 N NaOH;* B, *0.20 N NaOH;* C, *0.40 N NaOH;* D, *0.60 NaOH.*

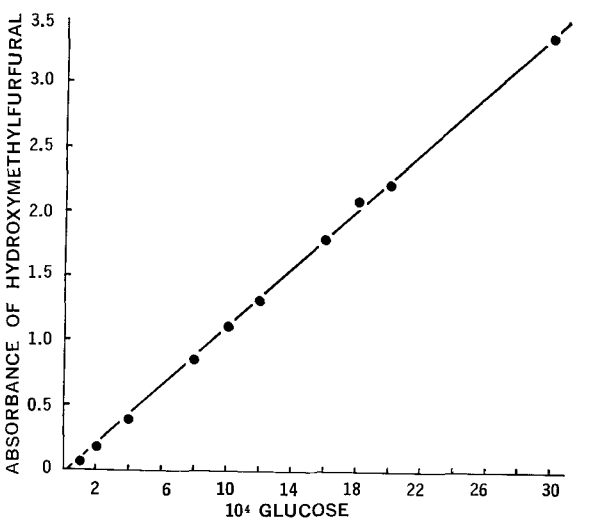


Figure 3—*Calibration curve for the spectrophotometric analysis of glucose. Glucose is reacted at 40° for 3.5 hr. in 0.20 N NaOH; an aliquot is obtained, acidified to 1.0 N HCl, reacted for 10 hr. at 80°, appropriately diluted, and the hydroxymethylfurfural absorbance measured (283 mμ). The plotted absorbances of the solutions were corrected for the dilutions.*

to fructose occurred after 3.5 hr. at 40° in 0.20 *N* NaOH (Figs. 1 and 2); (*c*) fructose, after the initial equilibration, degraded in alkali by an apparent first-order process; (*d*) sucrose was stable in 0.20 *N* NaOH at 40° (Fig. 4); and (*e*) neither glucose nor fructose at 10^{-4} *M* concentrations yielded significant HMF upon acidification after 30 hr. at 40° in 0.20 *N* NaOH. Analyses of HMF after 0.20 *N* NaOH treatment of 0, 3.5, and 30 hr. at 40° were picked as the basis for the assay of the sugar mixtures.

Since glucose is not significantly contributing to the chromophore at zero time relative to the alkaline treatment, the total absorbance (*A*) due to the HMF formed by acidification of molar concentrations of sucrose (*S*), glucose (*G*), and fructose (*F*), was represented by:

$$A_0 = \alpha_0^S S + \alpha_0^F F \qquad \text{(Eq. 1)}$$

where α represents the molar absorptivity of HMF times the fraction of that sugar available to form HMF on acidification. After 3.5 hr. of alkaline treatment, the total absorbance of the acid-generated chromophore was due to the contribution of all three species:

$$A_{3.5} = \alpha_{3.5}^S S + \alpha_{3.5}^F F + \alpha_{3.5}^G G \qquad \text{(Eq. 2)}$$

The total absorbance of the acid generated chromophore after 30 hr. of alkaline treatment was due only to the sucrose:

$$A_{30} = \alpha_{30}^S S \qquad \text{(Eq. 3)}$$

The α values were obtained from the slopes of the calibration curves for the individual sugars degraded in alkali for the specific times of 0, 3.5, and 30 hr. at 40° in 0.20 *N* NaOH. Calibration curves should be prepared concomitantly with the analysis of unknowns.

Slight negative but reproducible intercepts were observed in all standard calibration curves against the water blanks. Therefore more accurate versions of Eqs. 1–3 were the following.

$$A_0 = \alpha_0^S S + \alpha_0^F F - I_0 \qquad \text{(Eq. 4)}$$

$$A_{3.5} = \alpha_{3.5}^S S + \alpha_{3.5}^F \mathrm{F} + \alpha_{3.5}^G \mathrm{G} - I_{3.5} \qquad \text{(Eq. 5)}$$

$$A_{30} = \alpha_{30}^S \mathrm{S} - I_{30} \qquad \text{(Eq. 6)}$$

where the I_{HR} represents the positive values of the negative intercepts and

$$I_0 = I_0^S + I_0^F \qquad \text{(Eq. 7)}$$

$$I_{3.5} = I_{3.5}^S + I_{3.5}^F + I_{3.5}^G \qquad \text{(Eq. 8)}$$

$$I_{30} = I_{30}^S \qquad \text{(Eq. 9)}$$

Table III—Intercept (I) and Apparent Molar Absorptivity Value (α) for the Pure Sugars[a]

Sugar Involved	F_0	$F_{3.5}$	$G_{3.5}$	S_0	$S_{3.5}$	S_{30}
I	−0.020	−0.035	−0.060	−0.037	−0.015	−0.065
α	3240 ± 209	1307 ± 77	1334 ± 138	3443 ± 225	3501 ± 148	3559 ± 269

[a] Based on the measured absorbance (A) at 283 mμ [after the necessary alkaline treatment of fructose (F_{HR}), glucose (G_{HR}), and sucrose (S_{HR}) for the specified hours in 0.20 N NaOH at 40°, given by the lettered subscripts, and subsequent acidification in 1.0 N HCl for 10 hr. at 80°] plotted against the individual sugar concentration in accordance with the equation: $A = \alpha$ [sugar] + I.

Rearranging of Eqs. 4–6 for the calculation of the sugar concentration resulted in the following forms:

$$S = (A_{30} + I_{30})/\alpha_{30}{}^{S} \quad \text{(Eq. 10)}$$

$$F = (A_0 + I_0 - \alpha_0{}^{S}S)/\alpha_0{}^{F} \quad \text{(Eq. 11)}$$

$$G = (A_{3.5} + I_{3.5} - \alpha_{3.5}^{S}S - \alpha_{3.5}^{F}F)/\alpha_{3.5}{}^{G} \quad \text{(Eq. 12)}$$

Due to the small errors of the multistep process, the $\alpha_{HR}{}^{S}$ values show a small amount of variance (Table III).

This assay procedure is only as accurate as the calibration curve that obtains the α and I values. Repeated experiments have indicated that the intercepts are fairly constant for each particular time and sugar. The slopes however varied somewhat from day to day (Table III).

The error of the sucrose assay in such ternary mixtures is the smallest (4%). The errors of fructose (9%) and glucose (18%) analyses are larger due to the inherent nature of the assay. The analysis of glucose has the largest error since the difference calculations add both the sucrose and fructose experimental error to the glucose error.

Mixtures of glucose, fructose, and sucrose were run at concentrations ranging from 1.6 to 10.0 × 10^{-4} M in sugar. There was no significant variation among days when daily calibration curves were prepared. Representative data are presented in Table II.

Similar procedures can also be applied for the analysis of mixtures of mannose, fructose, and sucrose. If glucose and mannose are in the same mixture, an additional sample would be needed (7 hr. in 0.20 N NaOH at 40°) to be able to differentiate between the glucose and mannose.

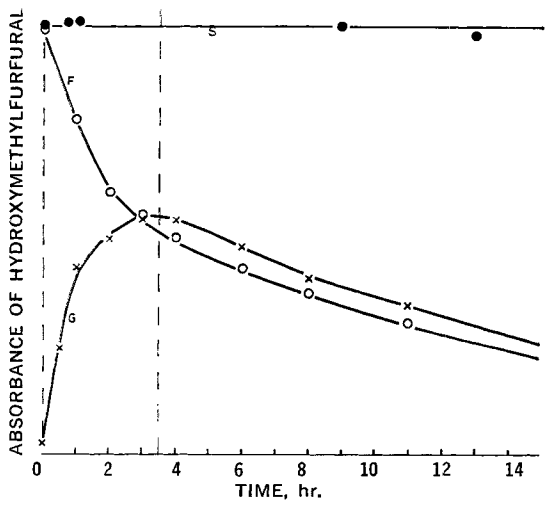


Figure 4—*The conversion of 0.006* M *glucose* (G) *to fructose, the loss of 0.006* M *fructose* (F), *and the invariant availability of fructose from 0.006* M *sucrose* (S) *in alkali with time. The reactions were followed by acidifying aliquots of the 0.20* N *NaOH solution at 40° to 1.0* N *HCl, reacting at 80° for 10 hr., and measuring the absorbance of the hydroxymethylfurfural derived from fructose at 283 mμ.*

SUMMARY

An accurate method has been devised for the indirect analysis of glucose (±4% in the range of 1.0 to 10.0 × 10^{-4} M glucose) utilizing the Lobry de Bruyn-Alberda van Ekenstein transformation of glucose to proportional amounts of fructose under specific conditions of alkali (0.20 N NaOH) and temperature (40°). The fructose formed was converted in acid to the spectrophotometrically assayable hydroxymethylfurfural at 283 mμ.

Glucose and fructose in mixtures can be analyzed by determining the HMF content on the acidification of two aliquots; one of which was first reacted in alkali for 3.5 hr.

A simple procedure has been devised for the simultaneous analysis of mixtures of glucose, fructose, and sucrose by determining the HMF content on the acidification of three aliquots; two of which were first reacted with alkali for specific times.

The assay of a solution of an individual sugar was accurate to ±4%. The error with additional sugars in a mixture was higher due to the inherent calculations of the assay.

REFERENCES

(1) N. Nelson, *J. Biol. Chem.*, **153**, 375(1944).
(2) M. Somogyi, *ibid.*, **160**, 61(1945).
(3) *Ibid.*, **195**, 19(1952).
(4) G. Guinn, *J. Chromatog.*, **30**, 178(1967).
(5) J. P. Marais, J. L. de Wit, and G. V. Quicke, *Anal. Biochem.*, **15**, 373(1966).
(6) L. Hough, in "Methods of Biochemical Analysis," vol. 1, D. Glick, Ed., Interscience, New York, N. Y., 1954, p. 205.
(7) H. W. Kircher, *Anal. Chem.*, **32**, 1103(1960).
(8) Z. Dische and E. Borenfreund, *J. Biol. Chem.*, **192**, 583 (1951).
(9) S. Siggia, J. G. Hanna, and N. M. Serencha, *Anal. Chem.*, **36**, 638(1965).
(10) H. B. Mark, Jr., L. M. Backes, D. Pinkel, L. J. Papa, *Talanta*, **12**, 27(1965).
(11) W. H. Haworth and W. G. M. Jones, *J. Chem. Soc.*, **1944**, 667.
(12) C. A. Lobry de Bruyn and W. Alberda van Ekenstein, *Rec. Trav. Chim.*, **14**, 203(1895).
(13) *Ibid.*, **16**, 262 (1897).
(14) E. R. Garrett and J. Blanch, *Anal. Chem.*, **39**, 1109 (1967).

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