CCC. THE ANALYSIS OF CARBOHYDRATES OF THE CELL WALL OF PLANTS.

II. THE DETERMINATION OF PENTOSE AS SINGLE SUBSTANCES AND IN MIXTURES CONTAINING URONIC ACIDS AND HEXOSES.

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In the first communication of this series [Norris & Resch, 1935] the importance of the accurate determination of the furfuraldehyde obtained from plant materials was demonstrated, and the relationship between uronic acids and the furfuraldehyde yielded by them was placed on a more satisfactory basis.

Whilst the above work was in progress and for some time following its publication, a critical survey of the methods available for the determination of furfuraldehyde was undertaken, and we now believe that the proximate analysis of plant materials is susceptible of greater accuracy than was formerly the case.

In this communication it is proposed to describe only investigations on the well-known method of Kröber [1901], which employs phloroglucinol as precipitating agent.

The inaccuracies which have become associated with the determination arise from a number of errors and inconsistencies in applying the original method. The duration and temperature of distillation and the apparatus used for it must be standardized, as also the conditions of precipitation and after-treatment of the precipitate. Strict adherence to a fixed set of conditions is essential.

In many cases the original method of Kröber has not been exactly followed, but the subsequent calculation has been made on the basis of Kröber's tables or formulae. This is particularly to be noted where the precipitated phloroglucide has been washed with alcohol; it is quite indefensible to use Kröber's tables in such cases, since treatment with alcohol was not included in his method.

Again, even supposing that the use of Kröber's results is permissible, there is inherent inaccuracy in the employment of formulae based on them. The formula, $F = (P + 0.0052) \times 0.5185$ (where F = furfuraldehyde and P = phloroglucide), is often used and is generally regarded as accurate within the limits $P = 0.03$ to 0.3 g. Analysis of Kröber's tables will show that this is not, in fact, the case: the relationship between furfuraldehyde and phloroglucide over the range indicated can be expressed graphically only by a slightly curved line, and the straight line relationship indicated in the formula is only an approximation and is only valid over a very small range.

We have therefore based our investigation on the premise that if the distillation and precipitation procedures are really standardized, then a given weight of pentose will correspond to a definite weight of phloroglucide, within the limits of experimental error, and that the relationship between product and phloroglucide may, over a small range of weights only, be represented by a

straight line. The equation best expressing the relationship has been calculated by the method of least squares and the standard error of experiment determined mathematically.

In this manner equations have been derived to indicate the relationship between phloroglucide and (i) furfuraldehyde; (ii) arabinose; (iii) xylose; (iv) galacturonic acid; (v) pectolic acid; (vi) glycuronic acid (as euxanthic acid).

The greatest source of error in modern usage of the Kröber method, and one which has apparently been largely overlooked, lies in the fact that in actual practice the investigator rarely, if ever, deals with single substances, but is concerned almost invariably with mixtures of greater or less complexity. The tacit assumption that the weight of phloroglucide produced by a number of substances in admixture exactly corresponds to the sum of the weights of phloroglucide produced by each separately is shown in this communication to be unjustified, nor does Kröber give any indication that his tables may be used for any but pure single substances.

To establish relationships for the large number of possible mixtures of two or more of the commonly occurring pentoses, uronic acids and hexoses, constitutes a laborious routine and requires a statistical investigation whose results would doubtfully justify the time expended. We have therefore confined ourselves to the examination of those mixtures commonly met with in cell wall chemistry and have outlined methods which may be employed for the more accurate analysis of such substances as pectin, the hemicelluloses etc.

EXPERIMENTAL

Distillation. In the first instance the apparatus described in the previous communication [Norris & Resch, 1935] was used, but all the joints involving rubber bungs were replaced by interchangeable ground glass connexions. This involved a change in the shape and diameter of the side tube leading to the condenser, and it was found necessary to "lag" this by means of asbestos paper. Also, in order to maintain the normal rate of distillation it was necessary to increase the temperature of the glycerol-bath to 175–180°. Comparative results using the old and the new forms of apparatus did not invariably show the superiority of the latter, but from every practical point of view the ground glass joints are preferable, and any remaining doubts as to the possible effect of decomposition of the rubber bungs in the older form are removed.

In order to maintain a more even distillation temperature and to avoid possible violent fluctuations in the concentration of the acid it was decided to revert to the original method of Kröber, in that the acid is now added in portions of 30 ml. every 10 min. instead of 60 ml. every 20 min. as hitherto.

Experiments with pure furfuraldehyde by direct precipitation and by precipitation after the usual distillation showed that there is a slight loss of furfuraldehyde—about 1%—during distillation. In order to remove furfuraldehyde from the influence of the acid as quickly as possible and to obviate possible oxidation, nitrogen was bubbled through the apparatus in several series of experiments. It was thought possible that the current of nitrogen might increase evaporation of furfuraldehyde at the delivery tube and to obviate this the tube was run directly into the solution of phloroglucinol in some experiments. The use of nitrogen brought no increase in yield, however, and was discontinued. There seems little doubt that, since in the case of furfuraldehyde itself the distillation loss is within the normal limits of experimental error, the low yields observed in the case of the pentoses are due to more deep-seated degradation of the

sugar molecule itself, depending upon differences in configuration [cf. Bott & Hirst, 1932].

Precipitation. The results of further experiments with phloroglucinol as precipitant have involved very little modification in the procedure previously described. It was established that, although the precipitate is collected in a thin layer in the sintered glass crucibles, 100 ml. of water for washing are insufficient, and we have now adopted 150 ml. as standard.

The practice of washing the precipitate with hot alcohol has been entirely abandoned. Statistical examination of the results after such washing has shown that the supposed beneficial effects on concordancy are illusory.

The hygroscopic nature of the precipitate has often been observed and means of obviating difficulties in weighing suggested. The practice of placing the crucibles in tared weighing bottles appeared on test to be of doubtful value, since large weighing bottles are necessary and the effect of moisture on the glass surface under varying conditions of humidity is appreciable. It is possible to form a close estimate of the weight of the crucible and contents and to place most of the weights on the balance pan before removing the crucible from the desiccator. Using the open crucible in this way it was found possible to complete the weighing within 60 sec., and no disturbing effects due to absorption of moisture have been observed.

Standard method. The final method adopted was essentially similar to that previously described, but with the following changes: (i) interchangeable ground glass connexions replace rubber bungs; (ii) the temperature of distillation is now 175–180° instead of 170°; (iii) additions of 12 % hydrochloric acid at the rate of 30 ml. every 10 min.; (iv) the precipitate is washed with 150 ml. of cold distilled water instead of 100 ml.

Relation between phloroglucide and furfuraldehyde-yielding substances.

1. *Furfuraldehyde.* For the purpose of these experiments specially purified furfuraldehyde was obtained from the manufacturers and then submitted to five distillations. The final product gave the correct boiling-point and refractive index and was directly distilled into the weighing bottle to avoid any possible oxidation. A known weight was made up to volume in 12 % hydrochloric acid and aliquot portions were precipitated direct as usual with phloroglucinol. By omitting the distillation procedure in this case we were able to ascertain the absolute relationship between furfuraldehyde and phloroglucide obtained by the usual precipitation method.

Table I. *Relation between furfuraldehyde and phloroglucide.*

Wt. of furfuraldehyde g.	Wt. of phloroglucide g.	Wt. of furfuraldehyde g.	Wt. of phloroglucide g.
0.0283	0.0525	0.0532	0.1015
0.0322	0.0606	0.0538	0.1029
0.0366	0.0686	0.0556	0.1064
0.0400	0.0755	0.0595	0.1145
0.0405	0.0765	0.0634	0.1229
0.0506	0.0981		

2. *Arabinose.* Purified specimens of arabinose were used in this series, aliquot portions of a standard solution being distilled and precipitated according to the standard procedure. The arabinose was calculated to anhydroarabinose,

since this is the more convenient form in practical applications. These remarks apply also to the other sugars dealt with.

Table II. *Relation between anhydroarabinose and phloroglucide.*

Wt. of anhydroarabinose g.	Wt. of phloroglucide g.	Wt. of anhydroarabinose g.	Wt. of phloroglucide g.
0.0624	0.0624	0.0946	0.0963
0.0719	0.0725	0.1135	0.1179
0.0757	0.0758	0.1270	0.1316
0.0930	0.0952		

3. *Xylose*. This was treated in a precisely analogous manner to arabinose.

Table III. *Relation between anhydroxylose and phloroglucide.*

Wt. of anhydroxylose g.	Wt. of phloroglucide g.	Wt. of anhydroxylose g.	Wt. of phloroglucide g.
0.0566	0.0649	0.0811	0.0953
0.0697	0.0812	0.0937	0.1100
0.0704	0.0821	0.0995	0.1183

4. *Galacturonic acid* and 5. *pectolic acid*. The yields of phloroglucide from these sources are given in the previous communication [Norris & Resch, 1935, p. 1593] and need not be repeated here. The results have been submitted to statistical analysis together with those obtained for the other products in the present communication (*vide infra*).

6. *Glycuronic acid* (as euxanthic acid). In this case the results quoted in the earlier communication cover too narrow a range for present purposes. A second series of experiments gave the results shown in Table IV, wherein the euxanthic acid is calculated to carbon dioxide. Uronic anhydride is readily obtained by multiplying by 4.

Table IV. *Relation between carbon dioxide from glycuronic anhydride and phloroglucide.*

CO ₂ equivalent of urone g.	Wt. of phloroglucide g.	CO ₂ equivalent of urone g.	Wt. of phloroglucide g.
0.0209	0.0275	0.0417	0.0594
0.0261	0.0353	0.0438	0.0636
0.0313	0.0432	0.0459	0.0640
0.0365	0.0517	0.0469	0.0666

Interpretation of results.

The foregoing results were subjected to mathematical analysis in two ways: (a) by the method of least squares the linear equation which best represented the relationships between product and phloroglucide was calculated; and (b) the standard error of the determinations was computed. In each case, then, it was possible to establish a linear relationship indicated by the following general equation:

$$S = a + bP \pm e,$$

where

S = substance under examination;

P = phloroglucide;

e = standard error of the determination;

a and b = constants.

The following equations were thus derived:

- (1) Furfuraldehyde: $F = 0.5030 P + 0.0018 \pm 0.0003$.
- (2) Anhydroarabinose: $A = 0.9253 P + 0.0050 \pm 0.0007$.
- (3) Anhydroxylose: $X = 0.7673 P + 0.0069 \pm 0.0009$.
- (4) Galacturonic anhydride, as carbon dioxide:
 $C_{Ga} = 0.5778 P + 0.0013 \pm 0.0009$.
- (5) Pectolic anhydride, as carbon dioxide:
 $C_p = 0.5744 P + 0.00076 \pm 0.0008$.
- (6) Glycuronic anhydride, as carbon dioxide:
 $C_{Gu} = 0.6613 P + 0.0026 \pm 0.0006$.

It will be observed that the smallest experimental error occurs in the first equation, a result to be anticipated, since the furfuraldehyde was determined directly, whereas all the other experiments involved distillation. It was shown by statistical methods that there is no significant difference in the yield of phloroglucide from galacturonic acid itself as compared with that from pectolic acid, a confirmation of the result obtained by inspection in the previous paper. It should be emphasized that the equations given are only valid for the range of weights indicated in the tables, which have been chosen to include the weights commonly dealt with by us in applications to cell wall substances. Furthermore, it has been repeatedly noticed by us that different workers in these laboratories invariably obtain slightly different equations based on their own tables of results, and the standard errors also show slight differences similarly. It is possible that the differences observed are not of great significance (in the mathematical sense of the term), but it seemed desirable to make this point as an indication that for completely satisfactory results each worker should prepare his own experimental data.

Application to the analysis of mixtures.

The statistical method was now applied to the case of mixtures of substances commonly met with in dealing with pectin and the hemicelluloses. In the former case mixtures containing arabinose, galacturonic acid and galactose occur, and the first set of experiments below was designed to enable us more accurately to analyse such mixtures.

Arabinose and galacturonic acid (as pectolic acid). Pectolic acid was used in these experiments since it is more readily prepared than galacturonic acid, and moreover approximates much more closely to the form in which galacturonic

Table V.

Anhydro- arabinose g.	CO ₂ equivalent to pectolic acid g.	Phloroglucide	
		Found g.	Calc. g.
0.0238	0.04943	0.1064	0.1050
0.0476	0.04943	0.1282	0.1307
0.0714	0.04943	0.1554	0.1565
0.0220	0.05830	0.1253	0.1186
0.0476	0.05698	0.1393	0.1439
0.0714	0.05698	0.1663	0.1697
0.0238	0.06575	0.1354	0.1334
0.0476	0.06575	0.1614	0.1591
0.0714	0.06575	0.1863	0.1849

acid occurs under normal conditions. Mixtures were prepared according to the requirements of a "Latin square" and were then submitted to the standard distillation and precipitation procedures. From the results obtained an equation correlating anhydroarabinose, the carbon dioxide equivalent of pectolic acid and the phloroglucide produced was calculated.

The equation deduced from the results (first three columns) was:

$$(7) \ A = 0.9942 P - 1.9081 C_p + 0.01313 \pm 0.00305.$$

It will be noted that the experimental error in the case of these mixtures is considerably greater than those for the substances treated separately (equations (2) and (5)). The figures in the final column were calculated from the individual equations quoted above, and these show quite clearly that the phloroglucide obtained from the mixtures is not the sum of the quantities obtained from the single substances. The differences observed are greater than the experimental error and therefore of definite significance.

In analysing such mixtures, then, it is possible to derive the weights of the two constituents present with greater accuracy than heretofore, since by the usual method of decarboxylation [Dickson *et al.* 1930] the amount of uronic anhydride may be obtained and, following a phloroglucide determination, the value of C_p may be substituted in the equation, whence the value of A may be calculated. The equation, however, is not necessarily valid outside the range of weights from which it was derived.

Pectolic acid and galactose. Apart from the alleged production of small amounts of ω -hydroxymethylfurfuraldehyde, galactose should make no contribution to the total phloroglucide produced from mixtures in which it occurs. At the same time it is possible that it may have some influence on the yield of phloroglucide normally obtained from the usual furfuraldehyde-yielding substances. This and the following set of experiments were accordingly designed to determine whether or not galactose had a significant effect.

Table VI.

Galactose g.	CO ₂ equivalent of pectolic acid g.	Phloroglucide g.
0.0221	0.06575	0.1121
0.0442	0.06575	0.1123
0.0663	0.06575	0.1114
0.0736	0.06555	0.1155
0.0819	0.07135	0.1251
0.1604	0.06983	0.1196
0.1839	0.07963	0.1351
0.2045	0.05935	0.1051
0.2157	0.06253	0.1106
0.2298	0.06643	0.1168

These results are discussed below.

Arabinose, pectolic acid and galactose. It remains to examine the effect of galactose when present in mixtures containing arabinose in addition to pectolic acid. To this end mixtures were prepared and analysed as shown in Table VII.

Analysis of these results, and those of Table VI, leads to the same conclusion, namely, that galactose exerts an effect on the yields of phloroglucide normally to be expected from the other constituent or constituents of the mixture, but that such effect is not significant until the proportion of galactose reaches more than

Table VII.

Anhydro-arabinose g.	CO ₂ equivalent of pectolic acid g.	Galactose g.	Phloroglucide g.
0.0220	0.05830	0.0205	0.1253
0.0220	0.06748	0.0205	0.1413
0.0238	0.06575	0.0221	0.1379
0.0476	0.06575	0.0221	0.1647
0.0238	0.06575	0.0442	0.1353

50 % of the total constituents. In general it may be said that where galactose is present in small proportions there is a slight relative increase in phloroglucide yield, but where the proportion is greater there is a slight relative decrease. Since it is not usual to work with mixtures containing more than 50 % of galactose, at least in pectin chemistry, its effect may usually be neglected.

Thus, in dealing with ternary mixtures of the above type, uronic acid may be determined by quantitative decarboxylation, arabinose may be calculated as already described (equation (7)) from the phloroglucide determination and galactose obtained by difference.

Xylose and glycuronic acid (as euxanthic acid). Mixtures in the xylose, glycuronic acid, glucose series were examined in a similar manner to that already described. The relation between xylose, glycuronic acid and phloroglucide was first determined in accordance with the results in Table VIII.

Table VIII.

Anhydroxylose g.	CO ₂ equivalent of glycuronic acid g.	Phloroglucide g.
0.0660	0.0021	0.0797
0.0660	0.0042	0.0838
0.0660	0.0078	0.0869
0.0660	0.0115	0.0926
0.0660	0.0156	0.0996
0.0704	0.0083	0.0943
0.0792	0.0021	0.0951
0.0792	0.0042	0.0990
0.0792	0.0078	0.1043
0.0968	0.0021	0.1189
0.0968	0.0042	0.1217
0.0968	0.0078	0.1271

The equation derived from these results was:

$$(8) \quad X = 0.7859 P - 1.146 C_{Gv} + 0.0060 \pm 0.0006.$$

In this instance, also, the yield of phloroglucide obtained from the substances in admixture was found not to coincide with the sum of the yields of the constituents taken separately. The yield of "mixed" phloroglucide is always higher than might be anticipated. The equation may be used for calculating xylose-glycuronic acid mixtures since C_{Gv} may be determined independently.

Xylose and glucose. The effect of addition of varying proportions of glucose to xylose on the phloroglucide yield was next examined as in Table IX.

The figures of the final column were derived from equation (3) and it was shown by statistical treatment that the differences between found and calculated values were not significant, i.e. the effect of glucose on the furfuraldehyde yield of xylose may be neglected for the range of weights taken.

Table IX.

Anhydroglucose g.	Anhydroxylose g.	Phloroglucide	
		Found g.	Calc. g.
0.0270	0.0748	0.0860	0.0885
0.0540	0.0748	0.0860	0.0885
0.0765	0.0748	0.0876	0.0885
0.1530	0.0748	0.0877	0.0885
0.3060	0.0748	0.0886	0.0885
0.0720	0.0704	0.0842	0.0828
0.0360	0.1056	0.1253	0.1286
0.0720	0.1056	0.1266	0.1286
0.1080	0.1056	0.1252	0.1286

It may be mentioned in this case that the precipitates obtained were submitted to alcohol washing in order to eliminate ω -hydroxymethylfurfuraldehyde phloroglucide if any were present. However, the results obtained confirmed our dissatisfaction with the procedure, since they showed a considerable and variable depression in the yield, with consequently a much larger standard error of experiment.

Xylose, glycuronic acid and glucose. The yield of phloroglucide from the mixtures of all the substances is shown in Table X, together with the weights of xylose calculated from equation (8).

Table X.

Anhydroxylose		CO ₂ equivalent of urone g.	Anhydro- glucose g.	Phloro- glucide g.
Taken g.	Calc. g.			
0.0704	0.0709	0.0083	0.0360	0.0947
0.0704	0.0722	0.0083	0.0720	0.0963
0.0704	0.0722	0.0083	0.1080	0.0963
0.0704	0.0728	0.0042	0.0720	0.0910
0.0704	0.0717	0.0125	0.0720	0.1019
0.0704	0.0732	0.0167	0.0720	0.1098

The differences between the weights of xylose taken and those calculated are in each case positive, and they are significant as shown by statistical analysis. The presence of glucose in the ternary mixture thus involves results which are not in accord with equation (8) obtained for xylose-euxanthic acid mixtures, and the case is by no means so clear-cut as the previous one involving arabinose, pectolic acid and galactose.

It would be possible to arrange a series of experiments so as to enable an equation relating phloroglucide with the three constituents to be calculated: $X = a + bP + cC_{Gy} + dG$; this would not be of much value, since although P and C_{Gy} could be determined we should still have an equation involving the two unknowns X and G . Since there is no satisfactory way of determining glucose in such a mixture, the values of X and G would remain indeterminate.

It may be possible, when experiments now proceeding with other furfuraldehyde precipitants are complete, to derive a second equation relating, for example, the thiobarbituric acid precipitate with the three constituents; in which case we might, by calculating two simultaneous equations in X and G , obtain values for these constituents. It is hoped to make an additional communication on this point.

For the present, however, in analysing such mixtures [Angell & Norris, 1936], the most satisfactory procedure appears to lie in calculating xylose and glycuronic acid from equation (8), obtaining glucose by difference. The approximate

composition being known in this manner, a correction is then applied to the weight of phloroglucide by comparison with results in Table X for a mixture of approximately similar constitution. Recalculation then follows as above. Whilst this gives a final result which is still only an approximation, there is little doubt that it represents a considerable improvement on results hitherto obtained.

In conclusion, it may be stated that the methods described in this communication have been applied to hemicelluloses [Angell & Norris, 1936] and pectin [Norris & Resch, in preparation], and that by their means much more satisfactory results have been obtained. Except in the former case mentioned, no assumptions are made, the results by standard methods being mathematically treated as general cases and later applied to particular ones met with in practice. In our view, individual standardization is essential in furfuraldehyde determination, and the method and apparatus require as close control as, for example, the well-known Reichert-Wollny determination.¹

SUMMARY.

1. Criticisms of the usual procedure in furfuraldehyde determination are made and remedies suggested.
2. A standardized procedure based on experiment is described.
3. The relation between phloroglucide and furfuraldehyde-yielding substances, singly and in admixture, is determined.
4. The effect of the presence of galactose and glucose in such mixtures is also determined.
5. The results are treated mathematically, equations indicating the relationships and standard errors of experiment being deduced.

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¹ It has not been possible in this contribution to include details of mathematical analysis, but these can be provided on request.

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