grinding and extraction, the picture would be simple—all nitrogen other than this would be extracted. However, the work of Bawden & Pirie (1944) and Crook (1946) suggests that the unextractable residue represents a cross-section of the nitrogenous material present in the cells fixed there by the extraction process itself. The plant cell contains a complex mixture of proteins of varying solubility and in various states of dispersion. These might be expected to be extracted at different rates and to different degrees of completeness. It is surprising therefore that extraction from all species of plants should proceed in such a manner that the residue is representative of all proteins and yet at the same time, the degree of extraction is so accurately expressed by a linear regression.

SUMMARY

1. The extraction of nitrogenous materials from the leaves of various plant species has been investigated by a procedure previously worked out.
2. The extraction varied from $41\frac{1}{2}$ to $93\frac{1}{2}$ % of the total leaf nitrogen, the average for all species being 75%.
3. The extraction was mainly influenced by the nitrogen content of the leaves, increasing with increasing nitrogen content.
4. The extraction was also influenced by the dry-matter content, as the wetter leaves have a higher nitrogen content.

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A Manometric Method for the Estimation of Milligram Quantities of Uronic Acids

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The most widely applicable method for the estimation of uronic acids is that based on the demonstration by Mann (1894) and Mann & Tollens (1896), that uronic acids are decomposed by heating with 12% (w/w) HCl giving a 25% yield of CO₂ (calculated to the anhydride). The method was further explored by Lefèvre (1907) and Lefèvre & Tollens (1907).

In the latter method, the sample was boiled with 12% HCl under reflux for 3.5 hr., and a stream of CO₂-free air passed through the apparatus. After washing and drying, the CO₂ carried from the flask was absorbed in a tube containing KOH. The CO₂ evolved was estimated by the increase in weight of the absorption tube. In subsequent years, many modifications of the original method were introduced, mainly concerned with alterations in the time of decomposition sometimes extended to 5 hr. and even to 8–10 hr. (Ehrlich & Schubert, 1929), and in the methods employed for washing, trapping and estimation of the CO₂. Barium hydroxide became a favoured absorption agent, and titrimetric methods were substituted for gravimetric (cf., for example, Dickson, Otterson & Link, 1930). More recently, attention has been directed to the use of stronger acid to

shorten the time needed for decomposition (Colin & Lemoyne, 1938b, 18% HCl; McCready, Swenson & Maclay, 1946, 19% HCl), and to measurement of rates of CO₂ evolution in order to estimate traces of uronic acid derivatives in the presence of polysaccharides (Norman, 1939; Whistler, Martin & Harris, 1940).

The most radical departure in the determination of uronic acids by means of the CO₂ evolved on decomposition is that of Voss & Pfirschke (1937), who used an approximately 20M-solution of ZnCl₂ at its b.p. of 146–147° instead of HCl. All these methods have in common the disadvantage of a complex apparatus and a long decomposition time, usually 5–8 hr.

The method to be described is based on the decomposition of not more than 50 mg. of the sample with 0.25 ml. of 12% HCl in a sealed tube at 111°. The CO₂ evolved is then measured by a modification of the method of Van Slyke & Folch (1940) for manometric carbon determination. The present method has the advantage that samples do not need attention during decomposition, and that, as a consequence, determinations may be made at the rate of 4/hr. for long periods.

METHOD

Preparation of tubes. Soda-glass tubing, 7 mm. external diameter, 5 mm. internal diameter, is cut into lengths of 8–10 cm. and cleaned by immersion in cleaning mixture for 3 hr. The tubes are washed, rinsed in distilled water, dried, and a bulb of diameter *c.* 15 mm. blown on the end of each. If carbonate- CO_2 measurements are to be made, the side wall of the tube above the bulb is softened in the flame and a pocket of capacity about 0.5 ml. is blown. The tubes are numbered with a commercial black glass-marking ink, dried in the oven, and stored in a desiccator. Bulbs prepared in this way have a volume of about 1 ml., and a total volume of about 2 ml. after sealing; 60–80 bulbs can be blown/hr.

Weighing of sample. The sample should not exceed 50 mg. in weight. It must be deposited in the bulb with no contamination of the walls of the tube, otherwise charring will occur on sealing the tube, and extraneous CO_2 will be formed. A convenient spatula for the introduction of solids into the bulb may be made by sealing a V-section piece of 'sealing-in metal' (obtained from an old radio valve) into a piece of glass tubing that is a sliding fit in the sample tube. If the tubing from which the spatula handle is made fits well, it is almost impossible to deposit material on the walls of the bulb tube if the metal portion is not overloaded. The spatula is introduced holding the tube and spatula horizontal and, when the metal scoop is inside the bulb, it is rotated through 180° , thus depositing the sample, turned to its original position and withdrawn. Liquids are introduced by a capillary pipette. Samples for analysis must be dry. Up to 1 ml. of solutions may be dried off in a weighed bulb and then reweighed.

Addition of acid. HCl (12% w/w) is used. A pipette is made from the tubing used for the spatula handle. The drawn-out tip of the pipette is bent at an angle of 45° from the axis of the pipette, and ground so that it just clears the wall of the tube when the pipette is inserted to the neck of the bulb. This ensures complete delivery and draining of the pipette into the bulb with no loss of acid in the upper parts of the tube. The pipette is fitted with a rubber teat and may conveniently be graduated to deliver four portions of 0.25 ml. If carbonate determinations are being made, the acid is delivered into the side pocket instead of into the bulb.

Decomposition of sample. After the addition of the acid, the tube is sealed off by drawing out in the flame, taking care that a narrow prolongation of at least 2 cm. is secured. In sealing, 'carbonate' tubes must be held horizontal in order to avoid contact between acid and sample. The sealed tubes are secured vertically in a rack, which is immersed in boiling toluene for 5 hr. The toluene may be boiled in the bottom of a tall beaker, into the upper half of which a condenser made from a loose-fitting tin cannister is inserted. Such an arrangement permits the toluene to be boiled vigorously for long periods without loss. After 5 hr., the rack and tubes are removed from the beaker and cooled in water. Twenty-five samples can be weighed into tubes, the acid added, and the tubes sealed ready for decomposition in 1 hr.

Estimation of liberated CO_2 . A sealed tube is inserted as far as the bulb into one end of a piece of pressure tubing *c.* 8 cm. long. The other end is attached to the bent tube leading from the gas chamber of the Van Slyke-Neill manometric apparatus. The pressure tubing is exhausted twice by lowering the mercury and expelling the air in the usual way.

The second exhaustion serves to verify that there is no leak in the pressure tubing connexion. Two ml. of 0.5 N-NaOH are introduced into the chamber, the mercury lowered to about half-way down the large bulb, and the tap turned to connect the bulb to the chamber. The sealed tip of the bulb is then broken by bending the pressure tubing. The subsequent course of the analysis is substantially that described by Van Slyke & Folch (1940). Ten excursions of the mercury are sufficient for transfer of the CO_2 , the process being assisted by tapping the bulb during exhaustion, causing the liquid to coat the walls of the bulb, and warming the bulb in the hand during the mercury upstroke.

Estimation of carbonate CO_2 . Samples that contain carbonate are analyzed in the carbonate tubes described above. Two tubes are used for each analysis. In each the HCl is kept from the sample till after sealing, when it is brought into contact with it by bringing to the vertical. One tube is placed in an incubator at 40° for 5 hr. and the other in the toluene bath. The percentage carbonate CO_2 obtained by the analysis of the first tube is subtracted from that found for the second to obtain the uronic- CO_2 value.

RESULTS

Seven samples of galacturonic acid varying in weight from 4 to 12 mg. gave a CO_2 value of 22.33%, S.D. 1.00% (theoretical yield, 22.79%). Since the samples were weighed on a balance reading only to 0.1 mg., these figures show that results are reproducible. Some fifty compounds have been examined for possible interference. The results obtained are listed in Table 1.

Fig. 1. Variation of CO_2 yield with time of heating; citrus pectin.

Time of decomposition. From Fig. 1, which shows the course of CO_2 evolution in the decomposition of a sample of citrus pectin, it is seen that a period of 5 hr. appears ample for decomposition, and that the continued CO_2 evolution after 5 hr., due possibly to the decomposition of sugar impurities in the pectin and to the further decomposition of the uronic material, is steady, and in very accurate work can be allowed for (Norman, 1939; Whistler *et al.* 1940).

Table 1. *The CO₂ yield of substances tested for interference*

Substance tested	CO ₂ yield (mg. CO ₂ /100 mg. dry substance)	
	Present paper	Other values
Yields >2%:		
Allantoin	cf. Fig. 2	—
Alloxan	8.9, 9.8	—
<i>p</i> -Aminobenzoic acid	cf. Fig. 2	—
Ascorbic acid	17.1, 18.7	—
Hypoxanthine	10.3	—
Pyruvic acid	4.7	5.4(2)
Urea	27.4, 25.6	—
Yields >1%:		
Adenylic acid	1.8, 1.9	—
Agar	1.2	1.04(1)
Tannic acid	1.1	—
Tomato bushy-stunt virus*	1.1	—
Yields <1%:		
Acetic acid	—	Trace (<0.08)(2)
Amylose (potato)	0.65	—
Arabinose	0.6	0.47(8), c. 0.7(11)
Asparagine	<0.1	0(6, 7)
<i>iso</i> Barbituric acid	0.15	—
Caffeine	<0.1	—
Cellulose	0.3	0.45(7), 0.17(8), 0.16(9)
Cholesterol	<0.1	—
Citric acid	0.2	0(6, 7)
Creatinine	0.3	—
Diethylbarbituric acid	0.2	—
Egg albumin	<0.1	0(6, 7)
Ellagic acid	—	0.12(12)
Erythritol	<0.1	—
Formic acid	—	Trace (<0.08)(2)
Fructose	—	0.55(8), 0.80(7), 0.59(9)
Fucose	0.75	—
Fumaric acid	<0.1	—
Galactose	0.5	—
Gallic acid	—	0(7)
Gelatin	—	c. 0.25(11)
Glucono- γ -lactone	—	c. 0.25(11)
Glucosamine	<0.1	—
Glucose	0.7	0.18(4), 0.73(6, 7), 0.40(8), 0.19(9), 0.26(10), c. 0.25(11)
Glutaric acid	<0.1	—
Gluten (wheat)	0.25	—
Glyceric acid	—	0(2)
Glycogen	0.7	—
Glycollic acid	—	0(2)
Glyoxylic acid	—	0(2)
<i>p</i> -Hydroxybenzoic acid	—	0.53(12)
Inositol	<0.1	—
Inulin	0.85	0.60(8), c. 0.25(11)
Lactic acid	—	0.08(2)
Laevulinic acid	0.2	—
Maleic acid	<0.1	—
Malic acid	0.3	c. 0.25(11)
Maltose	—	0.40(8)
Mannitol	—	0(8)
Mannose	—	0.55(8)
Melibiose	0.7	—
Melzitose	0.7	—
2:3:6-Trimethylglucose	—	0.20(8)
Mucic acid	0.3	—
Nicotine	<0.1	—
Nicotinic acid	<0.1	—
Nucleic acid (yeast)	0.75, 0.95	—
Oxalic acid	—	0.42(2), 0.8(3), 2(6, 7), c. 0.25(11)
Peptone	—	0.4(7), 0.5(6)
Phenolic acids from redwood bark	—	0.58(12)
Phenylethylbarbituric acid	<0.1	—

Table 1 (cont.).

Substance tested	CO ₂ yield (mg. CO ₂ /100 mg. dry substance)	
	Present paper	Other values
Yields <1% (cont.):		
Rhamnose	—	0.90(8)
Ribose	0.6	—
Salicylic acid	—	0.60(12)
Serine	<0.1	—
Starch:		
Potato	—	0.26(5)
Tobacco (pptd. by ethanol)	—	0.57(5)
Tobacco (pptd. by freezing)	—	0.38(5)
Potato	—	0.30(8)
Rice	—	0.45(8)
Wheat	—	0.40(8)
Horse chestnut	—	0.50(8)
Potato	—	0.20(9)
Potato	—	c. 0.25(11)
Commercial soluble	0.65	—
Sucrose	—	0.75(7), 0.78(6), 0.52(6), 0.24(9), c. 0.25(11)
Tannin (Mimosa)	0.5	—
Tannin (Quebracho)	0.3	—
Tartaric acid	—	0 (6, 7)
Tauroglycollic acid	<0.1	—
Theobromine	<0.1	—
Theophylline	<0.1	—
Thymine	<0.1	—
Tobacco mosaic virus	0.35	—
Trehalose	0.5	—
Uracil	<0.1	—
Uric acid	<0.1	—
Xylose	0.6	0.40(6)

(1) Nanji, Paton & Ling (1925).

(2) Norman & Martin (1930).

(3) Link (1931).

(4) Anderson (1931).

(5) Spoehr & Milner (1935).

(6) Colin & Lemoyne (1938a).

(7) Colin & Lemoyne (1938b).

(8) Campbell, Hirst & Young (1938).

(9) Norman (1939).

(10) Whistler, Martin & Harris (1940).

(11) McCready, Swenson & MacLay (1946).

(12) Fuller, Bartholomew & Norman (1947).

* Higher value than for tobacco-mosaic virus, probably due to carbohydrate contaminant (Bawden & Pirie, 1938).

Interfering substances. It has been known for some time that substances other than uronic acids or their derivatives evolve small amounts of CO₂ under the conditions used in the determination of uronic acids. One of the earliest statements is that of Dickson *et al.* (1930), who found that the CO₂ yield of various plant acids, sugars, sugar acids, and polysaccharides 'is very small'. Since then, many substances have been tested and the majority have been found to give CO₂ yields negligible for practical purposes (Table 1). A number of substances not previously tested has been found to interfere seriously, however. Some of the previous work has been repeated in order to estimate the magnitude of the effects using the procedure described above, and it will be seen from Table 1 that these effects are of a similar order of magnitude to those found in earlier methods. In addition, other substances have been tested that may be encountered in the analysis of plant material. Some were found to give considerable CO₂ yields under the conditions used. Substances found to give a CO₂ yield over 2% of their weight were *p*-aminobenzoic acid (Weith, 1879),

ascorbic acid, allantoin, alloxan, hypoxanthine, pyruvic acid (Norman & Martin, 1930), and urea.

Fig. 2. Variation of CO₂ yield with weight of sample, A, *p*-aminobenzoic acid; B, allantoin.

When present in material analyzed by the method described, their CO₂ yield may vary with the amount present. Two examples are given in Fig. 2. If it is

suspected that any of these substances are present it is well to include controls with the amount of interfering substance equivalent to that in the sample. Figures for the percentage of CO₂ given by these substances are included in Table 1.

Since the CO₂ yield of *p*-aminobenzoic acid and allantoin varied with the amount used in analysis, it was of interest to determine whether a similar phenomenon was likely to occur with uronic groupings. A series of nine determinations was made on varying weights of the same pectin sample. The average CO₂ yield for five samples (wt. 1.3–9.3 mg.) was 12.4 %, s.d. 1.0 %, while that for four samples (wt. 13.1–18.2 mg.) was 12.0 %, s.d. 0.1 %. Variation in weight of sample used for analysis does not therefore seem to affect the value obtained materially.

DISCUSSION

The accuracy of the method is perhaps not as high as some of the previous methods, but it is well adapted to the routine analysis of small quantities of material. It has been successfully used in this Station for the routine analysis of samples of plant fibre. The method is not well suited to the study of rates of CO₂ evolution carried out by Norman and

others (Fuller, Bartholomew & Norman, 1947), in order to distinguish between different polyuronides; but these methods have recently been shown not to yield as clear-cut results as had been hoped (Fuller, 1947). That a number of substances present in plant, animal, or soil material may interfere to a marked extent is of importance, since it has often been assumed that such interference is unlikely to occur. The interfering substances may assume greater quantitative importance in material that has been fractionated; they might, in fact, be concentrated or introduced during the preparation of fractions.

SUMMARY

1. A rapid method for the analysis of small quantities of materials containing uronic acids or uronides is described.

2. The applicability of the method has been gauged by studying the interference by a number of substances, and results compared with those of previous workers.

3. It has been shown that a number of these substances may interfere seriously with the estimation by the present modification of the Lefèvre & Tollens method.

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