# MODERNE METHODEN DER PFLANZENANALYSE

HERAUSGEGEBEN VON

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MIT 48 ABBILDUNGEN

# MODERN METHODS OF PLANT ANALYSIS

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# Inhaltsverzeichnis. — Contents.

# Mono- and Oligosaccharides and Acidic Monosaccharide Derivatives.

By

**D. J. Bell.**

With 5 Figures.

## Introduction.

The analyst of plant tissue is confronted by a complex system containing saccharides of varying chemical natures, different stabilities and a variety of physical properties. Cellulose, so chemically resistant and so physically inert, occupies one end of the system; the other is occupied by chemically active and readily soluble monosaccharides. A host of intermediaries may also be present according to the biological origin of the tissue. In addition, inorganic ions, characteristic of vegetable organisms are certain to accompany the organic components which usually include non-carbohydrate compounds of types which interfere with carbohydrate analysis.

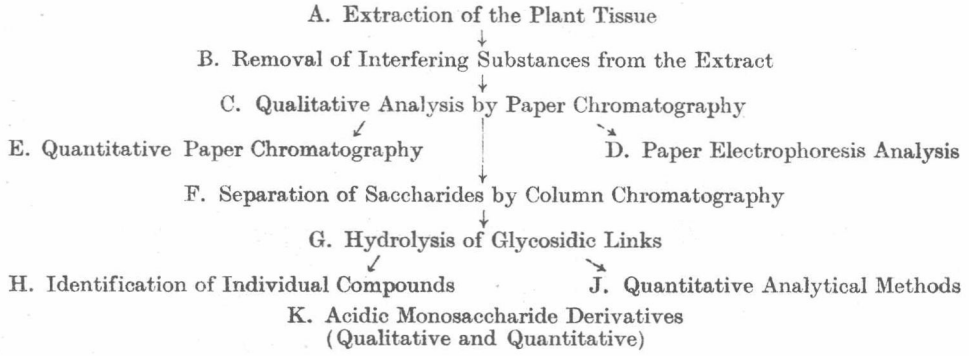
The first problem of the analyst is to select a procedure which will quantitatively eliminate non-carbohydrate interfering substances. His second problem is to discover what carbohydrates are present in the material he is attempting to analyse. His third problem is to find the most specific methods appropriate to the required analyses.

In carbohydrate analysis there is always danger of destruction or alteration of labile compounds. There is moreover the possibility of loss due to unexpected adsorption on solid particles.

The advent of modern chromatographic methods has revolutionised carbohydrate analysis. It is now clear that the detection and determination of water soluble saccharides is reliable only when aided by chromatographic procedures (cf. TÄUFEL and IWAINSKY, 1952, and WANNER, 1952).

This article is not directly concerned with the detection and assay of polysaccharides. Nevertheless it is difficult to define a boundary between oligo- and polysaccharides. The writer has therefore decided to confine his attention to analysis of the materials which can be extracted from plant tissues by means of hot 80% ethanol, an extractive in frequent use by the plant analyst. In this way it is considered that the conventional polysaccharides will remain undissolved. Investigation of glycosidic compounds frequently necessitates hydrolysis of the inter-radical linkages. Therefore some attention will be paid to this operation, one which is of course equally applicable to the polysaccharide field.

The following scheme illustrates the general lay-out of this chapter in relation to a progressive analytical procedure: Capital letters refer to appropriate sections.




## A. Extraction of Tissues.

The supposition is not infrequently encountered that plant enzymes act much more slowly than their animal homologues; this is incorrect. Analysis carried out on tissues which have spent some time in transit between the field and the laboratory may give an erroneous picture of the composition of the living plant. (Cf. Davies, Daish and Sawyer, 1916; Greenhill, 1933; Ferguson, 1948.) Rapid drying in air, ovens, etc., requires such a high temperature that not only destruction of carbohydrate may take place, but in the earlier stages of "warming up", the enzymes may be transiently stimulated. Laidlaw and Wylam (1952) have shown that freeze-drying inhibits enzymic change in the water soluble carbohydrate fraction of grass samples, but the enzymes themselves remain active. These authors emphasise the necessity for using a method of enzymic inhibition, such as dropping the tissue into hot alcohol, which can readily be carried out at the actual time of collection.

Wylam (1953) has made a study of the changes which take place in grass samples after cutting, with respect to changes in several free sugars and fructosan. One batch was immediately plunged into the alcohol, a large sample was allowed to wilt in the air and a third sample packed into stoppered bottles to prevent escape of moisture and imitate grass lying at the bottom of a pile in an open field. Wylam's results may be summarised as follows:—

*Table 1.*

| | Dry matter % | Carbohydrates as % dry weight | | | |
|---|---|---|---|---|---|
| | | Glucose | Fructose | Sucrose | Fructosan |
| Fresh Grass . . . | 18.4 | 1.3 | 1.7 | 6.2 | 9.6 |
| Wilted 2 hrs . . | 20.6 | 1.3 | 1.7 | 5.1 | 9.2 |
| Wilted 24 hrs . . | 44.6 | 1.9 | 2.9 | 5.4 | 5.2 |
| Wilted 8 days . . | 78.1 | 1.6 | 1.4 | 5.2 | 3.5 |
| Bottled 4 hrs . . | 18.4 | 1.7 | 2.1 | 4.8 | 7.1 |
| Bottled 96 hrs . . | 18.4 | 3.2 | 2.4 | 3.4 | 2.8 |

Seasonal variations in carbohydrates have long been noted (e. g. Waite and Boyd, 1953). It is therefore essential that note be taken of the actual condition of the material to be investigated.

The introduction of the "blendor" apparatus for tissue disintegration has greatly facilitated the rapid extraction of plant material. Several procedures are detailed below. Previous treatment with organic solvents is sometimes used, e. g. dropping the freshly gathered material into boiling 95% ethanol; while this

procedure seems to be generally satisfactory the possibility of a chemical transglycosidation, yielding non-reducing ethyl glycosides, should not be disregarded. For example the ethyl α-D-galactoside isolated by NOTTBOHM and MAYER (1938) from sweet yellow lupin may well have arisen during the preliminary benzene-ethanol extraction to which the plant was subjected.

Having obtained quantitative extraction of the soluble saccharides the "clarification" or purification by removal of non-carbohydrate interfering substances must next be carried out. In recent years, due largely to the possibility of chromatographic sugar analysis, much work has been done on this operation which is a necessary preliminary to the further examination of most extracts. In general, the use of ion-exchange resins has been found to provide a procedure superior to others, both with respect to accuracy and convenience. (See, however, B II, 3.)

The following selected procedure are described to illustrate successful techniques which have been applied to various types of tissue.

**1. Aqueous Extraction of Freshly Gathered Material.** (REIFER and MELVILLE, 1947.)

This method is claimed to reduce enzyme action to a minimum. 10—20 g. of rapidly cut tissue (~ 1 cm. lengths) is placed in a 500 ml. screw-top blendor along with 50 g. distilled water — crushed ice mixture. 10 ml. of peroxide free diethyl ether is added along with a further 70—100 ml. of ice-water. After "blending" for 2—5 minutes a further 50 g. of crushed ice is added, the lid of the apparatus washed down with water and blending resumed for 2—5 mins. After again washing down the lid, a few drops of anti-foaming agent is added, the slurry transferred to a graduated vessel and after shaking to remove air-bubbles, made up to volume. The final temperature of the macerate is about 8° C.

**2. 70% Ethanolic Extraction of Forage Plants.** (THOMAS, MELIN and MOORE, 1949.)

This procedure was used for simple sugar determinations. The authors state that their results compared well with parallels by the A. O. A. C. (1945) extraction method; indeed the prolonged reflux treatment recommended by the A. O. A. C. results in loss of up to 2% of "sugar content measured after inversion".

**(a) Dried Material** (finely ground). Approximately 2 g. (the amount should vary inversely with the sugar content) are blended with 200 ml. of 75% ethanol for 5—7 mins. The macerate is then filtered, on a Büchner funnel, through a layer of filter aid (e. g. Hyflo Super-cel). The solid residue is washed five or six times with aqueous ethanol (50—70%).

**(b) Fresh Green Material.** 3—10 g. of tissue is cut as finely as possible without expressing large quantities of juice, and then blended (5—7 minutes) with ethanol (~ 200—250 ml.) sufficient to give a final concentration of over 70%. Filtration and washing of the solid residue is conducted as in (a) above.

**3. 80% Ethanolic Extraction of White Potato.** (WILLIAMS, POTTER, BEVENUE and SCURZI, 1949.)

This procedure was used for sugar determinations; the authors specify the use of freshly distilled ethanol and found that clarification neither by neutral lead acetate nor by decolorising charcoal satisfactorily removed non-sugar reducing substances from solutions after evaporation of the alcohol. Ion exchange resins, however, proved satisfactory for this purpose. (See, however, B II 3.)

Raw potatoes, quickly peeled, sliced and finely chopped are added to boiling 95% ethanol, sufficient to give with the water in the tissue a final concentration of 80%. $CaCO_3$ (1—2 g.) is added and the mixture stirred for one hour at the boiling point. The supernatant liquid is then decanted and filtered through a pleated paper. The residual solid is covered with 80% ethanol and the whole heated for half an hour, the resulting supernatant liquid being filtered through the original paper. The residue is then blended with 80% ethanol and the resulting macerate transferred to a Soxhlet apparatus and extracted for half an hour. The residual solid is exhausted for 4 to 5 hours with a fresh batch of 80% ethanol. All the ethanolic extracts are combined and stored in the dark until required.

## B. Elimination of Interfering Substances from Plant Tissue Extracts.

### I. Examination of Lead and Charcoal Methods.

Bevenue and his collaborators have made an exhaustive study of this problem; their important results are summarised here. Bevenue (1949) working, not with plant extracts but with aqueous solutions of glucose, fructose and sucrose has assessed the absorptive power of various kinds of charcoal on these sugars (cf. Forsee, 1938; Hiscox, 1942; Lott, 1945; Morris and Wesp, 1932). *Animal* charcoals did not absorb any detectable amount of the three sugars, except sucrose when in very low concentration (0.05%). With *non-animal* charcoals, according to the source, sucrose recovery varied from 43% to 87%. Pre-treatment of the sugar solutions by lead acetate followed by dibasic sodium phosphate showed similar adsorption by the charcoals studied.

Bevenue and Washauer (1950) have made a critical comparative study on the determination of reducing sugars in 29 different plant tissues primarily subjected to extraction by 80% ethanol (cf. A I, 3). Determinations were made by two methods ($Cu^{++} \rightarrow Cu^{+}$, Somogyi, 1926; and $Fe(CN)_6^{3-} \rightarrow Fe(CN)_6^{4-}$, Hassid, 1936—1937). Five types of extract were compared:

(a) The untreated 80% ethanolic extract.

(b) (a) treated with decolorising charcoal, "Baker and Adamson, Code 1551"; cf. Bevenue (1949), Williams, Potter, Bevenue and Scurzi (1949).

(c) The fluid from (b) freed from ethanol by evaporation in a steam bath[1], filtered through a mat of filter-aid (Celite) (Waldron, Ball, Miller and Benne, 1948) and analysed for sugars without further clarification.

(d) The aqueous fluid from (c) was treated for 15 minutes with an excess of neutral lead acetate, the excess $Pb^{++}$ removed by addition of disodium hydrogen phosphate and the precipitate filtered off.

(e) The aqueous filtrate from (d) was treated for 10 minutes with the decolorising charcoal used in (b) and then filtered.

In about 50% of the tissues examined it was found that total reducing sugars could accurately be determined directly on the 80% alcoholic extract, without removal of the alcohol and without further clarification. The remainder of the tissues required only treatment of the extract with a suitable decolorising charcoal. The original paper should be consulted for details.

From such results it is obvious that an appropriate series of preliminary experiments must be carried out before accurate determinations be attempted on a plant tissue extract.

[1] The writer suggests that, in general, all evaporations of solutions containing reducing sugars should be effected below 40° C.

## II. Purification by Ion-Exchange Resins.

WILLIAMS, BEVENUE and their collaborators, by exhaustive studies summarised below, have demonstrated the superiority of ion-exchange resins as means of eliminating interfering ionic substances from plant extracts. (See, however, B II 3.)

### 1. 80% Ethanolic Extracts of White Potato. (WILLIAMS, POTTER, BEVENUE and SCURZI, 1945.)

The following comparative procedure was used on 80% ethanolic extracts of white potato (A I, 3) after removal of the ethanol by evaporation on the steam-bath. Reducing sugars were determined by several methods; for the HASSID (ferricyanide) method clarification by resins alone gave satisfactory results, in comparison with copper reduction.

Three clarification procedures were compared, (a) clarification with lead acetate-disodium hydrogen phosphate (B I), (b) treatment of filtrate from (a) with charcoal (B I) and (c) treatment, successively, with basic- and acid-binding resins.

The procedure is as follows. A 24 mm. diameter column of 15 g. Amberlite IR-100 H-AG (base binding) is arranged so that its effluent drips onto the surface of a 24 mm. diameter column of 20 g. Amberlite IR-4 B-AG. Both columns are back-washed with distilled water for 1—2 hours before use.

Two 30 ml. aliquots of the aqueous extract (A I, 3) previously treated with 2 g. of Celite analytical filter-aid and filtered, are passed through the two columns, to eliminate dilution errors; the eluates are discarded. Then, 100—125 ml. of the solution to be analysed are passed through the columns at a rate of 3 ml./min. This amount of solution represents about 70 g. of raw, or 30 g. of dehydrated potatoes. Control experiments using pure glucose, fructose and sucrose showed no change in concentrations of these sugars.

### 2. 80% Ethanolic Extracts of Fresh and Dried Vegetable Tissues. (WILLIAMS, BEVENUE and WASHAUER, 1950.)

The general procedure was similar to that used above. A large variety of resins were examined; in these experiments the resins were not used in columns but in the following manner. After washing thoroughly in distilled water, 2.0 g. (dry weight) of each of the acid-binding and base-binding resins were mixed in

Table 2. *Pairs of resins*[1] *found suitable for clarifying plant extracts.* (WILLIAMS et al., 1950.)

| Base Binding | Acid Binding | Base Binding | Acid Binding |
|---|---|---|---|
| Duolite C3 | Duolite A4 | Ionac C 200 | Duolite A4 |
| Zeo Rex | Duolite A4 | Amberlite IR-100 H | Permutit S |
| Zeo Carb | Duolite A4 | Amberlite IR-100 H | Duolite A4 |
| Amberlite IR-100 H | Ionac A 293 M | Amberlite IR-100 H | Duolite A4 |
| Amberlite IR-120 | Duolite A4 | Amberlite IR-100 H | Ionac A 300 |
| Dowex 50 | Duolite A4 | Amberlite IR-100 H | Amberlite IR-A400 |
| Ionac C 200 | Permutit S | Amberlite IR-100 H | Amberlite IR-4 B |
| | | Ionac C 200 | Ionac A 293 M |

a 250 ml. conical flask. 50 ml. of the alcohol-free, celite-filtered, extract was added and the mixture agitated every 10 minutes during a period of two hours. The writer suggests the use of motor driven rollers to provide regular and continuous mixing. The solution was then filtered by gravity through a fluted paper, the

[1] See B II 3.