The Carbohydrates of *Agave vera cruz* Mill.

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In a preliminary communication from this Laboratory, Srinivasan, Bhalerao & Subramanian (1952) reported the presence of polyfructosans in the stem of *Agave vera cruz* (hereinafter referred to as the agave; for description of this species, see Firminger, 1905; Duthie, 1915; Bailey, 1919). This material has since been shown by chromatographic methods to contain a number of other water-soluble carbohydrates (Bhatia, Satyanarayana & Srinivasan, 1953). The carbohydrate make-up in the stem of the agave appears, in general, to be similar to that reported by Dedonder (1950) and by Bacon & Edelman (1951) in Jerusalem artichoke. However, neither of the two polyfructosans isolated from the agave seems to be identical with inulin.

EXPERIMENTAL

Material. The plants used were all from wild growth in the fields adjoining Mysore South. The leaves and the root-end were discarded and the stem, after scraping off the fibrous skin, cut into small pieces and shredded in a brass grater. The grated material was squeezed through muslin by hand. The juice was heated on a water bath till it coagulated completely and then allowed to settle. The supernatant solution was centrifuged and filtered. The filtrate, an opaque, pale-yellow fluid, had a pH varying from 5 to 6. It contained malic acid (detected by chromatography). The following tests were positive: test for phenolic substances (with diazotized aniline), Molisch's test, Fehling's test (weak reduction) and Seliwanoff's test (strongly positive). Starch was absent.

Methods for the determination of sugars. Hydrolysis was carried out with $N-HCl$ for 9 min. at 67° . Total reducing sugar (T.R.S.) was determined by the method of Lane & Eynon (1923) and expressed as 'fructose'. Aldose was determined by the hypiodite method of Klein & Acree (1930) and the results expressed as 'glucose'. Optical activity was measured at 25° in a 1 dm. tube and with a sodium lamp as the light source. Soluble solids in the juice (referred to as solids) were determined with the Abbé's refractometer (sugar scale); the juice was diluted to 10% solids before determining optical rotation.

Chromatographic methods. For reference spots, 1% solutions of known sugars were used. The juice was applied directly, i.e. after heat coagulation and filtration. The solvent was n -butanol:acetic acid:water (4:1:5, v/v) mixture (Partridge, 1948). The period of development was 4–10 days for the ascending chromatogram and about 16 hr. for the descending one. The benzidine trichloroacetic acid reagent, as recommended by Bacon & Edelman (1951), was used for

spraying. Aldoses were located with the aid of aniline hydrogen phthalate (Partridge, 1949) and ketoses with the phloroglucinol reagent (Horrocks & Manning, 1949).

RESULTS

Variation in carbohydrate content of the plant

A survey of plant juices for their solids content and optical rotation was made with a view to choosing plants with maximum solids as starting material for the isolation of polyfructosans. Typical results of such a survey of about fifty plants selected at random over a period of about 10 months are presented in Table 1. Although there is no strict correlation between solids and rotation, plants having the maximum solids seem to possess also maximum laevorotation.

Table 1. *Variation in optical rotation and in solids from plant to plant of expressed juice from the stem*

Date of collection	α_D^{25} ($^\circ$)	Solids range (%)
21–31. iii. 52	+1.85 to -3.5	16.8 to 31.0
8–14. iv. 52	+1.67 to -5.25	12.0 to 31.5
7–20. v. 52	-0.8 to -2.8	13.3 to 23.0
2–25. vi. 52	-0.45 to -5.95	7.5 to 21.7
14. vii. 52	-6.55, -6.85	30.0, 31.5
12–24. viii. 52	-6.1 to -10.0	29.5 to 34.0
12. ix. 52	+0.65, -4.5	8.3, 15.1
13. xii. 52	-9.22	31.5
i. 53	-9.1	28.7

Distribution of carbohydrates in the stem

This study was undertaken in order to select portions with most solids. Transverse sections were taken at different levels of the stem. Juice expressed from these sections was analysed and the results are given in Table 2. The optical rotations calculated on the basis of glucose and fructose of the hydrolysed juices were found to be in close agreement with the observed rotations (Table 2A, plant no. XII). The assumption that the sugars obtained on hydrolysis are glucose and fructose, needs, however, to be confirmed by further evidence based on specific estimations like that of glucose by glucose oxidase.

Carbohydrate gradient across the stem. In a particular cross-section, two portions were taken at widely separated distances thus: portion (1), from periphery down to 1.5 in. (3.8 cm.) all round;

Table 2. Carbohydrate gradient along the stem: results obtained on three different plants

(Experimental material: expressed juice from the different sections; α_D = optical rotation; T.R.S. = total reducing sugar; I-IV denote descending serial sections of the stem.)

After hydrolysis								
	Initial		T.R.S. (%)	Glucose (G) (%)	Fructose (T.R.S.- G/1.06) (%)	Fructose:glucose	α_D	
	α_D (°)	Solids (%)					Observed (°)	Calculated (°)*
(A) Plant no. XII/28. iv. 1952								
I	-1.85	15.1	14.0	4.35	9.9	69.5:30.5	-6.6	-6.66
II	-0.20	15.0	14.64	5.25	9.69	63.4:36.6	-5.8	-6.0
III	+0.49	14.2	12.2	4.91	7.57	60.7:39.3	-4.06	-4.26
IV	+1.5	12.5	10.4	5.05	5.64	52.8:47.2	-2.46	-2.44
(B) Plant no. XLI/8. xi. 1952								
I	-5.03	27.2	27.3	4.66	22.9	83.1:16.9	—	—
II	-5.03	27.2	28.6	4.74	24.13	83.6:16.4	—	—
III	-2.49	25.0	25.5	5.8	20.03	77.5:22.5	—	—
IV	-1.95	23.4	23.7	6.2	17.85	74.2:25.8	—	—
(C) Plant no. LV/27. i. 1953								
I	-9.64	-29.3	28.4	4.79	23.88	83.3:17.7	—	—
II	-8.74	-28.5	28.7	5.0	23.98	82.8:17.2	—	—
III	-8.7	27.75	26.4	4.05	22.58	84.8:16.2	—	—
IV	-9.36	29.1	28.4	4.55	24.11	84.1:15.9	—	—

* Based on $[\alpha]_D^{20}$ glucose + 52.76° and for fructose - 90.46°.

portion (2), central section 1.5 in. (3.8 cm.) in radius. The optical rotation and initial solids of the expressed juice from the two sections were as follows: (1) periphery: +1.67°, 12.0%; (2) centre: -2.0°, 24.0%.

Studies by chromatographic methods

The juice from the stem of the plant was analysed by ascending chromatography with the use of the following sugars as reference compounds: rhamnose, xylose, arabinose, fructose, mannose, sorbose, glucose, galactose, sucrose, maltose and raffinose. The spots were developed for 10 days by ascending flow of the solvent, dried and sprayed. The following sugars were detected in the juice: fructose, glucose, sucrose, four carbohydrates yet unidentified (possibly oligosaccharides) and polyfructosan (R_f , zero). Of the four unidentified carbohydrates (one more than previously reported by Bhatia *et al.* 1953), one had an R_f value higher than raffinose, while the other three had lower R_f values. Hydrolysis of the carbohydrates present in the juice yielded only two sugars: glucose and fructose. This has been shown in the following experiment. The juice was applied at two spots on the opposite bottom corners of the paper (40 × 40 cm.) and developed (ascending technique) for 4 days. After locating the chromatogram on a guide strip, the corresponding area over the other spot was hydrolysed with trichloroacetic acid by spraying with 10% acid and heating at 110° for 10 min., and developed at right angles by the descending flow of the solvent for 16 hr., dried and

sprayed with benzidine trichloroacetic acid reagent. It was then found that all the component spots of the juice had given rise, as a result of hydrolysis, to glucose and fructose.

Isolation of polyfructosans

It was previously reported (Srinivasan *et al.* 1952) that hot extraction of the dried agave powder with methanol results in two fractions, one soluble and the other insoluble in this solvent, and that the two fractions have different optical rotations. In subsequent experiments to purify these two fractions, the aqueous solutions were first treated with ethanol up to 60% concentration. Thereby a precipitate, with little organic matter in it, was obtained. On filtering off this precipitate and adding ethanol to the filtrate to 88% concentration, a further precipitate was obtained. This was freed from ethanol and dried; $[\alpha]_D^{20}$ of the material thus obtained from the methanol-insoluble fraction: initial, -38.0°; after hydrolysis, -82.0°; from the methanol-soluble fraction: initial, -37.2°; after hydrolysis, -83.5°.

Next, the isolation of polyfructosans directly from the expressed juice, without the use of methanol, was undertaken. The steps were as shown in scheme on p. 288.

Polyfructosan I. This is a snow-white powder, light and hygroscopic; completely water-soluble. After a few reprecipitations from 90% ethanol, acetylation (Haworth, Hirst & Lyne, 1937) and deacetylation (Zemplan, 1926), it gave $[\alpha]_D^{20}$ initial,

—40.0°; after hydrolysis, —88.5°. The deacetylated material was homogeneous chromatographically and gave rise only to fructose on hydrolysis.

Polyfructosan II. This is a snow-white powder and hygroscopic: it reduces Fehling's solution, but does not contain free reducing sugars. It does not move on the chromatogram and gives rise to both fructose and glucose after hydrolysis. Glucose released after hydrolysis amounts to 15.0%; $[\alpha]_D^{25}$ initial, —20°; after hydrolysis, —69°. These characteristics remained unchanged after acetylation and deacetylation. Tentatively, this material also is referred to as a polyfructosan.

presented in Table 2. These results suggest that the content of the laevorotatory (fructose-containing) carbohydrates increases with maturity. This conclusion, however, needs to be confirmed, by a study of plants reared under known conditions. Plants used in our experiments were all collected from their native surroundings and were of unknown age, but far from their full maturity, since not one of them had 'poled'. Although the results reported here were all obtained with the stem of *Agave vera cruz* as the experimental material, possibly other species of the Agave family might give similar results. A trial chromatogram developed with the juice from the

DISCUSSION

Of the water-soluble carbohydrates in the stem of the agave, polyfructosans are the major constituents. The minor fractions contain sucrose and other carbohydrates composed of both glucose and fructose residues, besides free fructose and glucose. Though the carbohydrate content varies from plant to plant, the qualitative make-up of the carbohydrates appears to be the same; chromatograms obtained with different stem juices or with water extract of the dried material are similar (cf. Bhatia *et al.* 1953). The variations in the carbohydrate content (Table 1), have been recorded throughout a 10-month period. Also, when different plants were collected and examined on the same day, the stem juice of some showed a positive rotation (this only rarely), while in the majority the rotation was negative. It is, therefore, difficult to correlate these variations with a seasonal factor, contrary to what has been shown to be the case with Jerusalem artichoke (Thaysen, Bakes & Green, 1929; Bacon & Edelman, 1951; Bacon & Loxley, 1952). That this variation in the agave is possibly related to the age and maturity of the plant is indicated by results

stem of *Fourcroya gigantea* appeared similar to one obtained with *Agave vera cruz*.

SUMMARY

1. Among the water-soluble carbohydrates in the stem of *Agave vera cruz* the following have been detected by chromatographic methods: fructose, glucose, sucrose, four other substances, possibly oligosaccharides and two polyfructosans.

2. The optical activity and the content of total soluble solids obtained from various plants vary a great deal. While generally the juice is laevorotatory, instances of dextrorotatory juices have been noticed.

3. In some plants, there is a gradient in the distribution of carbohydrates along the length of the stem, as also radially in a cross-section.

4. Preliminary details regarding the isolation and characteristics of two polyfructosans are described.

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The Determination of Lactic Acid in Microgram Quantities

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The conversion of lactic acid to acetaldehyde by hot concentrated sulphuric acid was first reported by Denigès (1909) and its use as a basis for the determination of lactic acid was described by Mendel & Goldscheider (1925). Eegriwe (1933) described a reaction between *p*-hydroxydiphenyl and acetaldehyde to yield a violet colour and this colour reaction was first adopted as a sensitive means of estimating lactic acid by Miller & Muntz (1938).

The method most widely employed for determining lactic acid on a microscale is that of Barker & Summerson (1941). In the course of work in this laboratory requiring the estimation of very small amounts of lactic acid, usually in the presence of pyruvate, reproducible results could not be achieved with this technique; further, the final optical densities were not directly proportional to the amounts of lactic acid present.

This paper contains the results of our investigations whereby several modifications of Barker & Summerson's procedure were made. With these changes, 1–8 µg. of lactic acid could be determined with an accuracy of $\pm 2\%$. When interfering pyruvic acid is present, the procedure involves a dilution so that the method in this case is applicable to 2–10 µg. lactic acid.

METHODS

Reagents

p-Hydroxydiphenyl (1.5 g.) is dissolved in 10 ml. of 5% (w/v) NaOH and diluted to 100 ml. with water.

Standard lactic acid solution: 0.2133 g. of pure, dry lithium lactate (Hillig, 1937), was dissolved in about 100 ml. of water, 1 ml. of concentrated H_2SO_4 (A.R.) added and the solution made up to 1 l. with water.

Recommended method

The protein-free solution (2 ml.) containing 10–80 µg. of lactic acid, is pipetted into a 150 × 25 mm. test tube which contains 1 ml. of 20% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and the final volume is made up to 10 ml. with water. Approximately 1 g. of solid $\text{Ca}(\text{OH})_2$ is added and, after thoroughly mixing and allowing to stand for 30 min. or more, the solution is centrifuged.

If the original 2 ml. of protein-free solution contains 100–600 µg. of pyruvic acid, 6 ml. of the supernatant solution from the first treatment with copper-lime is pipetted into a second tube containing 0.6 ml. of 20% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.6 g. of solid $\text{Ca}(\text{OH})_2$ added. This procedure is then repeated a third time with 3 ml. of supernatant solution, 0.3 ml. of 20% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.3 g. of $\text{Ca}(\text{OH})_2$, whereby the amount of pyruvic acid present is reduced to a minimum and the colour interference due to it becomes a small constant value which may be deducted from the final optical density.

1 ml. of the supernatant solution from the third copper-lime treatment is transferred to another 150 × 25 mm. test tube, held in the arm of a mechanical shaker with its lower end immersed in an ice-water mixture. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.05 ml. of 12% (w/v) solution) is added followed by 6 ml. of conc. H_2SO_4 (A.R.) dropwise from a burette with vigorous shaking, the tap of the burette being lubricated with the concentrated acid. After complete addition, the contents are poured and allowed to drain into a small ground-glass stoppered Pyrex tube which is then heated for 30 min. in a water bath maintained at $60 \pm 1^\circ$. The tube is allowed to