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A modified *p*-anisidine technique for the detection and permanent recording of free and combined fructose on paper chromatograms

Fructose, in addition to its occurrence as the free compound and combined with glucose as sucrose, is present throughout the green plant kingdom in many different non-reducing oligo- and polysaccharides. Various sucrosylfructans occur in algae[1], bryophytes[2,3], and higher plants, especially Compositae[4], Boraginaceae[5], Amaryllidaceae[6], Liliaceae[6] and Graminae[7], and various sucrosylgalactans in other higher plants[8]. The distribution of these compounds is undoubtedly considerably wider than known at present and a simple paper chromatographic technique for their detection and recording would facilitate their recognition.

Reagents incorporating *p*-anisidine have been widely used for the chromatographic detection of sugars since their introduction in 1950[9]. After spraying and heating at *ca.* 100°, different kinds of sugars give different colours (*e.g.* aldopentoses, red; aldohexoses and deoxyaldohexoses, green-brown; ketohexoses, lemon-yellow, with the intensity of spots enhanced by ultraviolet light). The original reagent (3% *p*-anisidine in moist *n*-butanol) has undergone several modifications[10,11] and, in conjunction with periodate, has been used to distinguish sugars from polyols[12,13]. *p*-Anisidine reagents are sensitive to less than 1 μg sugar, aldoses reacting more strongly than ketoses under the usual heating conditions employed. However, sprayed chromatograms cannot readily be stored since they stain other papers on contact and the spots become obscure on a darkening background.

This paper describes a modified reagent which can be used (a) specifically to reveal free and combined fructose (and some other ketoses) with an increased sensitivity over the original reagent and (b) subsequently to reveal free and combined aldoses. A simple method of preparing contact prints for the permanent recording of spots at each stage is also presented.

Materials and methods

Sources of sugars. In addition to commercially available sugars, including stachyose and inulin, hot water extracts of various plant tissues (see Tables I and II) served as sources of non-reducing fructans and sedoheptulose.

Chromatography. Solutions of authentic sugars and aqueous extracts of plant tissues, which, in addition to fructans, usually contained sucrose and free fructose and glucose, were applied to Whatman No. 1 paper and developed in an insulated box at room temperature for a suitable period (24–48 h) in *n*-propanol–ethyl acetate–water (6:1:3)[14]. (Other solvents, including those containing borate, *e.g.* methyl ethyl ketone–acetic acid–water saturated with boric acid (9:1:1)[15], have also been used without any effect on the reaction with the detection reagent.)

For tests of sensitivity 1, 10 and 100-μg quantities of fructose, sucrose, raffinose, stachyose, glucose, xylose, arabinose and ribose were applied in duplicate to Whatman No. 1 paper and developed as above. The duplicate spots were then compared using the two reagents described below.

Detection techniques. *p*-Anisidine·hydrochloride (3 g) was dissolved in 100 ml moist *n*-butanol and acidified with 2 ml of concentrated hydrochloric acid (Reagent I).

Chromatograms were either dipped or sprayed with this reagent and heated at 60° for 5–10 min. After recording of reactions (see below), the chromatogram was then either re-heated for 5–10 min at 100° or allowed to remain at room temperature for 24 h (preferably in the dark to minimize darkening of background) before re-recording of spots.

This modification of the original technique was compared with that of spraying with a reagent containing 2 ml of glacial acetic acid/100 ml instead of hydrochloric acid (Reagent II) and heating at 100° for 5–10 min. This second technique is comparable to the original[9].

Recording of spots. Under safe-light conditions, chromatograms were placed on top of sheets of "Ilfobrom" grade 5 paper, which is especially sensitive to blue light. Close contact between chromatogram and photographic paper was obtained by superimposing a 1/2-in. plate glass sheet. A blue "Cinemoid" filter was sandwiched on top of this by another glass sheet. After a suitable exposure (*e.g.* to normal room lighting from a 150 W tungsten lamp for 15 sec), the paper was processed in "Agfa" liquid developer 8-66, diluted as recommended by the manufacturer, for 1.5 min at 20°. After rinsing in 3% acetic acid and fixing in an acid fixing medium (sodium thiosulphate 300 g/l + sodium metabisulphite 15 g/l) for 10 min, the paper was washed for 30 min in running tap water and dried.

Results and discussion

Free fructose, sorbose, sedoheptulose, together with sucrose and all sucrosylgalactans and sucrosylfructans tested, reacted with Reagent I when heated at 60° to give lemon-yellow spots, intensely fluorescent under ultraviolet light. Tables I and II

TABLE I

$R_{fructose}$ VALUES OF AUTHENTIC AND NATURALLY OCCURRING SUGARS REACTING WITH REAGENT I AT 60°

Sugars with trivial names.

Sugars	$R_{fructose}$ *values*
Sedoheptulose[a]	105
Fructose	100
Sorbose	93
Sucrose	78
Isokestose[b]	60
Melezitose	58
Raffinose	44
Lychnose/isolychnose[c]	42
Planteose[d]	40
Stachyose	22
Verbascose[e]	14
Ajugose[e]	6
Inulin	0

[a] From leaves of *Sedum spectabile* (Crassulaceae).
[b] From rhizomes of *Tussilago farfara* (Compositae).
[c] From roots of *Dianthus barbatus* (Caryophyllaceae).
[d] From seeds of *Plantago major* (Plantaginaceae).
[e] From roots of *Verbascum thapsus* (Scrophulariaceae).

TABLE II

$R_{fructose}$ VALUES OF SOME NATURALLY OCCURRING SUGARS REACTING WITH REAGENT I AT 60°

Oligomers (without trivial names) present on chromatograms of aqueous extracts of plants listed as clearly defined spots. In all species listed in this table as well as *Dianthus* and *Plantago*, fructans of lower $R_{fructose}$ occurred between that of lowest listed and the base-line, but were not clearly defined under the conditions used.

Plant tissue	$R_{fructose}$ *values*					
Scapania undulata (Hepaticae) (apical segments)	63	54	45	37	30	24
Sphagnum palustre (Musci) (apical rosettes)	57	46	37	30		
Allium schoenoprasum (Liliaceae) (bulbs)	56	40	29	21		
Tussilago farfara (Compositae) (rhizomes)	48	37	29			
Agropyron repens (Gramineae) (rhizomes)	55	37	27			
Plagiochila asplenioides (Hepaticae) (apical segments)	60	49	39			
Symphytum officinale (Boraginaceae) (roots stocks)	56	43				

record their mobilities. Authentic compounds were readily detectable at <1 μg/spot. Since only the fructose residues react, this means that levels of combined fructose lower than the 0.25 μg present in 1 μg stachyose can be detected. It should be emphasized, however, that it is the weight/unit area on chromatograms that is important and compounds of low R_F spread out less than those of high R_F values. By contrast, free and combined aldoses (at levels of 100 μg/spot) only reacted faintly under these conditions (Reagent I at 60°). However, they could be readily revealed in their usual colours[9], either by leaving the chromatograms at room temperature for 24 h, or by re-heating at 100° for 5–10 min. The sensitivity of this technique to free and combined ketoses was greater, and to free and combined aldoses lower, than that achieved by spraying with Reagent II and heating at 100° immediately. Nevertheless, 1 μg of most aldoses could be detected.

Some idea of fructose:aldose ratios of fructans could be assessed by the degree of colour change when chromatograms, treated with Reagent I and heated to 60°, were re-heated at 100°. When fructose was a minor component of spots as in stachyose the original lemon-yellow colour turned green-brown, but spots with a high fructose component, *e.g.* inulin, remained lemon-yellow.

Since yellow spots absorb blue light, the intensity of the lemon-yellow spots after heating at 60° with Reagent I could readily be recorded as white spots on a black background by the contact print technique described. Less satisfactory results were possible after the re-heating step due to the differing colour of spots and enhanced background.

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