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Note

Interference by flavonoids in the phenol–sulfuric acid analysis of carbohydrates

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The phenol–sulfuric acid method[1], originally devised by Smith and coworkers[2], is one of the most used methods for the analysis of carbohydrates. It has the advantage that, like other procedures using strongly acidic conditions, it responds to reducing sugars, glycosides, and polysaccharides. The method is therefore often used in ecological and nutritional studies in order to determine total carbohydrates, *e.g.*, in plant extracts. Such extracts, however, often contain other classes of compounds that yield chromophores with strong acid and the flavonoids constitute one such common type of material. We have recently commenced studies of flavonoid–carbohydrate complexes in plants[3], and needed a simple procedure, such as the phenol–sulfuric acid method, for determination of total carbohydrates of extracts and fractions. We now describe the interferences and uncertainties in such systems.

The flavonols are present in most plant materials either as simple glycosides or as end groups in polymeric proanthocyanidins. Catechin [**1**, (+)=form; all of the following observations are found to apply equally to epicatechin] is one of the commonest examples and we have found that, with the phenol–sulfuric acid reagent, this yields a visible spectrum that includes a peak at 485 nm, identical to the absorption maximum given by glucose, but with only 6% of the intensity (see Fig. 1). The same reaction between catechin and sulfuric acid alone (*i.e.*, a phenol-free "blank") gives a different spectrum with increased absorbance at 485 nm (see Fig. 1). When these procedures are extended to systems containing catechin with added D-glucose, using the conventional blank containing phenol, the increase in absorbance at 490 nm (the conventional wavelength for measurement of glucose[1]) due to the added D-glucose is found to be 94% of that expected for the added D-glucose (see Table I). However, in the analytical situation where flavonoids of unknown nature and amount are present together with reducing sugars or glyco-

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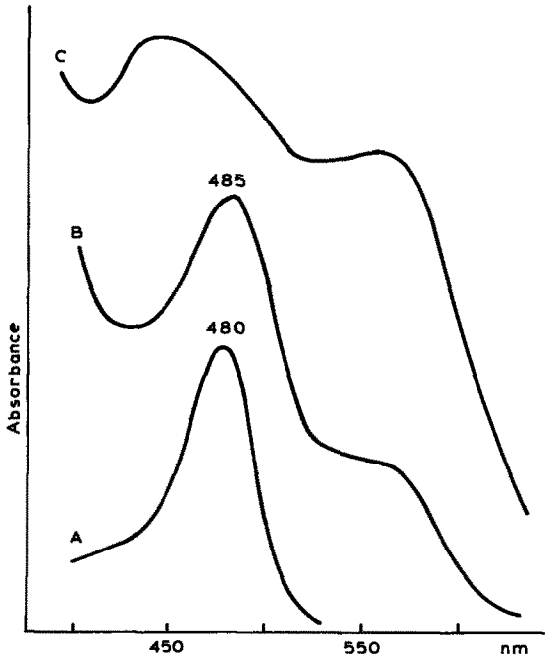


Fig. 1. Spectra from phenol–sulfuric acid reaction of (A) D-glucose (13 μg) and (B) (+)-catechin (317 μg); also, spectrum (C) of (+)-catechin with sulfuric acid alone (phenol-free).

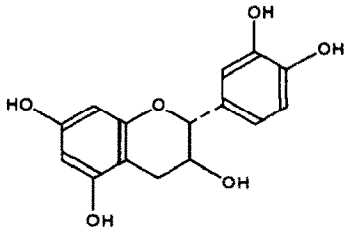
sides, or both, it would not be possible to correct for the absorbance due to the flavonoid reaction.

The foregoing problems do not occur with all classes of flavonoids. The glycoside rutin (2) gives a phenol–sulfuric acid response at 490 nm that, after deduction of the small phenol-free blank, is "correct" for its carbohydrate content (see Table I), using a calibration determined with pure rutinose (the calibration for

TABLE I

EFFECTS OF FLAVANOIDS ON PHENOL–SULFURIC ACID ANALYSES

Flavonoid, carbohydrate content	*Absorbance at 490 nm*
0.317 mg of (+)-catechin	0.19
phenol-free blank	0.285
0.030 mg of D-glucose	0.31
0.317 mg of catechin + 0.030 mg of D-glucose	0.48
0.048 mg of rutin (0.0256 mg of rutinose)	0.27
phenol-free blank	0.01
0.0256 mg of rutinose	0.26
0.082 mg of PPC	0.51
phenol-free blank	0.205



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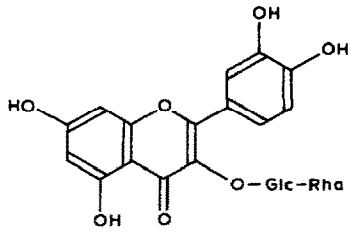

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rutinose is virtually identical with that for glucose). The spectra (see Fig. 2) indicate the formation of two distinct chromophores, at 477 and 430 nm, from carbohydrate and flavonoid components, respectively. The addition of known amounts of glucose to rutin increased the phenol–sulfuric acid response after deduction of the phenol-free blank by 94% of the theoretical amount. Unlike the case of catechin therefore, solutions containing rutin can be analyzed for total carbohydrate, within at most 6% error, simply by correction *via* a phenol-free blank.

We have encountered such problems in the case of polymeric proanthocyanidin glucosides (PPC) from softwood barks[4,5]. Analysis of these compounds, as mixtures before fractionation, regularly indicates >30% of carbohydrate by the phenol–sulfuric acid method after deduction of the relatively large phenol-free

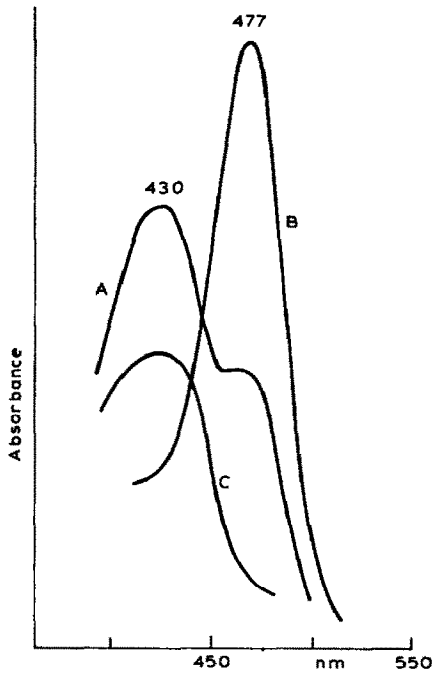

Fig. 2. Spectra from phenol–sulfuric acid reaction of (A) rutin and (B) rutinose and spectrum (C) of rutin with sulfuric acid alone (phenol-free).

blank (*e.g.*, in Table I). However, the determination of glucose by acid hydrolysis, reduction, acetylation, and gas–liquid chromatography using an internal standard, gave typical values of glucose content of ~7%. Enzymic hydrolysis and n.m.r. spectroscopy confirmed the latter value.

These observations suggest that the determination of carbohydrate by the phenol–sulfuric acid method, *e.g.*, in plant extracts, although quite likely to have good precision, may be subject to major errors when non-carbohydrate components are found to generate significant visible color on treatment with acid under the conditions of the analysis. Similar problems are likely to occur with other analytical procedures using strong acid. Under such circumstances, the validity of the colorimetric analysis for carbohydrate must be checked by some unequivocal method such as enzyme hydrolysis and gas–liquid chromatography, or n.m.r. spectroscopy.

The low (94%) response of the phenol–sulfuric acid analysis to added glucose, described for catechin and rutin, was also observed in the presence of similar amounts of the proanthocyanidin glucosides. This small, but significant effect is apparently due to interference by the flavonoids in the complex reactions leading to the chromophore having λ_{max} 485 nm.

EXPERIMENTAL

Materials. — Catechins, rutin, and rutinose were used as supplied by K and K Laboratories (New York) after drying at 40°/133 Pa. The polymeric procyanidin D-glucosides were isolated from aqueous ethanolic extracts of the bark of *Picea englemannii*.

Phenol–sulfuric acid analyses. — Procedures were as described earlier[1]. In a typical analysis, an aqueous solution (1 mL) containing a known amount of flavonoid and carbohydrate was mixed with 5% phenol solution (1 mL), 96% sulfuric acid (5 mL) was added, and the spectra were observed at 350–650 nm. For D-glucose, we found λ_{max} 485 nm, but, in all single-wavelength measurements, used absorbances at 490 nm as described[1]. Phenol-free blanks utilized water (1 mL) instead of the 5% phenol solution.

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