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EXTRACTION OF TOTAL PHENOLICS IN THE PRESENCE OF REDUCING AGENTS

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Abstract—The incorporation of reducing agent during the extraction of plant tissues with ethanol and acetone yielded extracts with higher content of total phenolics reacting with Folin–Denis reagent.

INTRODUCTION

THE MOST convenient method for the determination of total phenolics in plant tissues is by oxidation with the phosphomolybdate–tungstate reagent of Folin and Denis.[1] The quantitative extraction of the phenolics, prior to estimation, is effected with ethanol, methanol, or acetone. Whereas ethanol and methanol are used in the hot, acetone may be employed in the cold so that the tissue residue may be used for enzyme studies.

In view of the sensitive nature of the Folin–Denis reagent, it is essential to prevent the oxidation of phenolic compounds during extraction. According to Guadagni *et al.*[2] and Craft,[3] as much as 70–75 per cent of the total phenolics of peaches lost their chromogenicity towards Folin–Denis reagent under optimal conditions of oxidation, that is, when the tissue was ground in water or buffer and aerated. It was presumed that the brown enzymatically oxidized compounds were either unable to reduce Folin–Denis reagent or they retained only a small fraction of the phenolic groups originally present. Joslyn and Goldstein[4] found that color equivalents of different leucoanthocyanin preparations with Folin–Denis reagent were different and assumed that at a higher degree of polymerization less molybdate–tungstate was reduced under the test conditions. Another source of error resulting from oxidative reactions is the formation of quinones which polymerize to products entering into covalent linkages with proteins, from which the phenolics are not extractable by polar solvents.

The following is a report of the findings on the extraction of phenolics with ethanol and acetone in the presence of reducing agent.

RESULTS

The results obtained for the "apparent" and "true" total phenolic content of five different plant tissues are recorded in Table 1.

[1] O. FOLIN and W. DENIS, *J. Biol. Chem.* **22**, 305 (1915).
[2] D. G. GUADAGNI, D. G. SORBER and J. S. WILBUR, *Food Technol.* **3**, 359 (1949).
[3] C. C. CRAFT, *Proc. Am. Soc. Hort. Science* **78**, 119 (1961).
[4] M. A. JOSLYN and J. L. GOLDSTEIN, *Agric. Food Chem.* **12**, 511 (1964).

TABLE 1. EFFECT OF INCORPORATION OF CYSTEINE AND DITHIONITE DURING THE EXTRACTION OF PHENOLICS WITH ETHANOL AND ACETONE

Plant species and extraction procedure	Alone (−) or with added cysteine (+) or dithionite (++)	Cysteine* content	Phenolics† equivalent of cysteine or dithionite	"Apparent" total phenolics†	"True" total phenolics†
Acacia arabica leaves					
Direct extn. with	−	7·42	1·90	1126	1124
EtOH‡	+	121·8	39·40	1443	1404
EtOH extn. of	−	9·97	3·22	1116	1113
water homogenate	+	161·1	52·15	1364	1312
Cuscuta reflexa filaments					
Direct extn. with	−	2·33	0·75	44·97	44·22
EtOH	+	276·2	89·35	162·3	73·0
EtOH extn. of	−	4·51	1·50	21·70	20·20
water homogenate	+	240·4	77·75	140·1	62·35
Dendropthoe falcata leaves					
Direct extn. with	−	11·81	3·69	262·5	258·8
EtOH	+	198·7	62·1	427·5	365·4
EtOH extn. of	−	11·18	3·50	223·7	220·2
water homogenate	+	186·8	58·4	315·8	257·4
Nicotiana tabacum leaves					
Direct extn. with	−	2·28	0·73	22·55	21·82
EtOH	+	164·1	53·1	106·7	53·60
EtOH extn. of	−	1·88	0·61	10·91	10·30
water homogenate	+	216·2	69·9	83·7	13·80
Orobanche whole plant					
Direct extn. with	−	1·08	0·39	154·3	153·9
EtOH	+	156·3	58·3	338·4	280·1
	++		10·62	301·3	290·7
EtOH extn. of	−	0·93	0·34	37·2	36·86
water homogenate	+	109·2	39·85	214·8	174·9
	++		10·62	217·1	206·5
Acetone extn.	−	1·74	0·52	104·0	103·5
	+	140·0	42·16	360·0	317·8

* Measured by ninhydrin reaction and expressed as mg/10 g tissue equivalent.
† Measured by Folin–Denis reagent and expressed as mg tannic acid equivalent/10 g tissue.
‡ Results are the averages obtained in two separate determinations except in the case of the acetone extractions.

The incorporation of cysteine into ethanol during extraction of tissue resulted in an increase in the total phenolics content of 25, 41, 146, 65 and 82 per cent, respectively in the leaves of *Acacia arabica*, *Dendronthoe falcata* and *Nicotiana tabacum* and the tissues of *Cuscuta* and *Orobanche*. The increase was especially pronounced in the tissues of tobacco,[5] *Orobanche* and *Cuscuta*[6] known to be rich in phenolase. The unsupplemented ethanolic

[5] R. A. CLAYTON, *Arch. Biochem. Biophys.* **81**, 404 (1959).
[6] L. N. BEREZNEGOVASKAYA, *Dokl. Akad. Nauk. U.S.S.R.* **76**, 859 (1951).

extract of *Orobanche* was heavily pigmented, but in the presence of reducing agent the pigmentation was decreased markedly.

The total phenolic content of unsupplemented homogenate in water was the same, or nearly the same, as of ethanolic extract of the fresh tissue in the case of the leaves of *A. arabica* and *D. falcata*, but was much lower in *C. reflexa*, *N. tabacum* and *Orobanche*. Cysteine incorporated during homogenization in water resulted in 17 per cent increase of ethanol-extractable phenolics in the leaves of *A. arabica* and *D. falcata*, 34 per cent in the leaves of *N. tabacum*, while the increase in *Cuscuta* and *Orobanche* was 2- and 4-fold. A homogenate of *Orobanche* in water was brown, but the homogenate in reducing agent was not pigmented. Except in the case of *N. tabacum*, the values for ethanol-extractable phenolics in the supplemented aqueous homogenates were close to, but not identical with, those of ethanolic extracts of fresh tissue prepared in the presence of reducing agent.

Dithionite, tested with *Orobanche*, was as effective as cysteine in yielding extracts with enhanced chromogenicity towards Folin–Denis reagent and gave a 90 per cent increase in phenolics in ethanolic extract of tissue and a 5-fold increase in the ethanolic extract of the homogenate in water. The use of cysteine during extraction of *Orobanche* with cold acetone resulted in an almost trebling of the phenolics in the extract. Since any cystine in the various extracts would have reacted with ninhydrin leading to calculation as cysteine and since cystine does not react with Folin–Denis reagent, the correction factor of phenolics-equivalent of cysteine in the extracts should actually have been a lower value and hence the true phenolics content a higher value. However, this error was not appreciable since the color intensity of cystine with the ninhydrin reagent was only a fraction of that due to cysteine.

DISCUSSION

Joslyn and Goldstein,[4] who employed methanol for the extraction of phenolics from dehydrated persimmons, obtained considerably higher values when the samples had been sulfited. The authors also stated that when the extracts of persimmons were prepared by blending with aqueous glycerol, the addition of sulfite increased the content of total phenolics in one of three samples tested when the blending was at high speed.

Since the oxidation of tissue ascorbic acid may be retarded during extraction in the presence of reducing agent, an increased ascorbic acid content in the extracts will be indicated as an increase in total phenolics in extracts prepared in the presence of reducing agents. The contribution by ascorbic acid to the difference between the "apparent" and "true" phenolics will be proportionately greater in tissues which have a comparatively low content of phenolics and high content of ascorbic acid, as in the tips of the filaments of *Cuscuta*.[7] Separate determination of forms of ascorbic acid in the tissues above studied revealed that the content of dehydroascorbic acid and diketogulonic acid was negligible and that the concentration of ascorbic acid was of an order which could not introduce any appreciable error in the determination of phenolics.

Tannins are known to form complexes with plant constituents in addition to protein.[8] If any of these are chromogenic towards Folin–Denis reagent, an increase in their concentration, by suppressing the formation of tannins, would lead to an increased color value when reducing agents are employed during the extraction of phenolics.

[7] G. WALZEL, *Protoplasma* **41**, 260 (1952).
[8] T. ROBINSON, in *The Organic Constituents of Higher Plants*, p. 242, Burgess, Minneapolis (1963).

The inactivation of phenolases of peaches by the addition of metaphosphoric acid during extraction with aqueous ethanol, presumably at room temperature,[9] or the addition of acetic acid to methanol used as extractant of rhubarb petioles, with initial application of heat,[10] may be attended with hydrolysis of labile linkages and cannot be recommended for general use. The increased extraction of phenolics in the presence of reducing agents, observed in the present investigation, has to be attributed, in large measure, to a suppression of oxidation. Thiols, in general with other reducing agents, are known to function by rapidly reducing any quinones formed during the homogenization of plant tissues.[11] Thiols are known also to inhibit *o*-diphenol oxidase[12] and to react with quinones to form thioethers;[13] if this latter reaction was to take place to any significant extent in the extracts, there will be a diminution of color reaction with Folin–Denis reagent. In the present investigation, cysteine and dithionite had practically the same effect, but a role of metal chelation by the sulfhydryl compound and consequent retardation of oxidative reactions cannot be discounted.

The possibility that the reducing agent may bring about also a reduction of preformed phenolic oxidation products endogenous to the tissue cannot be excluded. When the ethanolic and acetone extracts of tissues prepared in the absence of reducing agent were stored for about 6 hr in contact with 0·013 M cysteine at room temperature, it was found that the values for total phenolics were increased almost to the same levels as those of extracts of tissues prepared in the presence of reducing agent.

"Acetone powders" constitute a convenient starting material for the sudy of some plant enzymes. The incorporation of a suitable reducing agent during the treatment with acetone, by extracting additional phenolics, may be expected to yield preparations better suited for enzyme study.

EXPERIMENTAL

Tissue and Sampling

The tissues used were the leaves of *Acacia arabica* Willd., *Dendrophthoe falcata* (L.f.) Ettingsh and *Nicotiana tabacum* L.; the filaments of *Cuscuta reflexa* Roxb. growing on *Pithecellobium dulce* (Roxb.) Benth. and the whole tissues of *Orobanche* species growing on *Solanum melongena* L. (for experiments with ethanol) and on *Brassica campestris* L. (for experiments with acetone). About 1 kg each of the leaves was collected fresh, the midribs removed and the blades torn to shreds and randomized. The filaments of *Cuscuta* were cut into small pieces and randomized. *Orobanche* was sliced into four longitudinal segments, each cut into pieces and pooled separately for use in ethanol extraction. For acetone extraction, the parasite was halved longitudinally.

Preparation of Extracts

(*a*) *Ethanol extraction of tissue.* Two 20 g samples were ground in a Waring blendor for 3 min with 95 per cent ethanol (final concentration 80 per cent), one without any supplement and the other with 0·04 M freshly neutralized cysteine. The two ethanolic suspensions were quickly taken to boiling and refluxed on a steam bath for 4 hr, the extracts filtered at the pump and the respective residues re-extracted twice with 80 per cent ethanol for a total period of 3 hr. The extracts from each set were pooled and made up to a known volume.

(*b*) *Ethanol extraction of homogenates.* Simultaneously, a third 20 g sample was ground with water and a fourth with 0·04 M cysteine (freshly neutralized) to give 20 per cent (w/v) homogenates, without straining through cloth. A 1·90 ml aliquot of each was extracted five times in succession initially with 95 per cent and subsequently 80 per cent ethanol by heating in a bath of boiling water for 2 min each. The centrifuged supernatants were made up to a known volume. In the case of *Orobanche*, 0·04 M sodium dithionite, adjusted

[9] S. LEONARD, B. LUH and E. HINREINER, *Food Technol.* 7, 480 (1953).
[10] H. A. W. BLUNDSTONE, *Phytochem.* **6**, 1449 (1967).
[11] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).
[12] J. W. ANDERSON and K. S. ROWAN, *Phytochem.* **6**, 1047 (1967).
[13] S. ROSTON, *J. Biol. Chem.* **235**, 1002 (1960).

to pH 6·0, was tested in place of cysteine, in the extraction both of tissue and of homogenate. All extractions were repeated with duplicate samples of the tissues.

(c) *Acetone extraction in the cold.* 50 g tissue was extracted in the cold in a Waring blendor with acetone, one lot without any supplement and another with added cysteine. Anhydrous acetone was employed for the first three extractions (one batch contained 0·04 M cysteine, incorporated in the form of a freshly neutralized M solution), to be followed by 80 per cent (v/v) acetone for six extractions (the cysteine concentration was reduced to 0·01 M). The residual solid in either case was extracted twice finally with anhydrous acetone (cysteine was not added).

Determination of Phenolics

Total phenolic content in every extract was determined, in suitably diluted samples, according to Goldstein and Swain,[14] with the reagent of Folin and Denis,[1] using tannic acid (B.P. grade from Messrs. W. J. Bush and Co. Ltd., England) as standard. The values represented the "apparent" total phenolics since cysteine and dithionite also reacted with the reagent. The cysteine content in each extract was determined, in suitably diluted aliquots, by the specific HCl-containing ninhydrin reagent of Gaitonde.[15] The color intensity of standard cysteine with the phenol reagent was separately determined and a value was calculated for the phenolics equivalent of the cysteine in the extracts, in terms of tannic acid. For arriving at the correction factor for use with dithionite, an aliquot of the reducing agent was refluxed with 80 per cent ethanol and suitable aliquots of the final solution allowed to react with Folin–Denis reagent. The "true" phenolics content in the extracts was arrived at by substracting the phenolics equivalent to cysteine or dithionite from the "apparent" total phenolics.

Tannic acid did not react with the ninhydrin reagent. Cystine yielded about 8 per cent of the color intensity of equimolar cysteine with ninhydrin, although this is considerably higher than the value reported by Gaitonde.[15] Cystine did not react with Folin–Denis reagent. The color intensity of cysteine with Folin–Denis reagent was about a third of that due to an equal weight of tannic acid; that of ascorbic acid was 80 per cent. The content of ascorbic acid and of dehydroascorbic acid and diketogulonic acid in the various tissues was determined with the 2,4-dinitrophenylhydrazine method of Roe,[16] as modified by Nair *et al.*[17]

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[14] J. L. Goldstein and T. Swain, *Phytochem.* **2**, 371 (1963).
[15] M. K. Gaitonde, *Biochem. J.* **104**, 627 (1967).
[16] J. H. Roe, in *Methods of Biochemical Analysis* (edited by D. Glick), Vol. 1, p. 115, Interscience, New York (1954).
[17] K. K. G. Nair, C. P. Tewari and P. S. Krishnan, *Ind. J. Chem.* **1**, 131 (1963).