DETECTION OF *o*-DIHYDROXY PHENOLIC COMPOUNDS

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In studies involving polyphenolic substances, a rapid and accurate method for the detection of compounds containing *o*-dihydroxy groups would be desirable. Among naturally occurring phenolic compounds, the *o*-dihydroxy grouping is a common phenomenon. Caffeic acid and combined forms thereof, as glycosides and acid derivatives, such as for example, chlorogenic acid, are among the most prevalent phenolics in nature[1,2]. Protocatechuic acid and aldehyde also are widely distributed in nature[2,3]. Of the more complex natural phenolics, flavonoids commonly have an *o*-dihydroxy arrangement in the B-ring[4–6]. Gallic acid, its derivatives and condensation products, may also be considered as *o*-dihydroxy compounds.

There is an interest in developing a useful spray reagent for the detection of *o*-dihydroxy phenolic groups in low-molecular-weight lignin sulfonates. Recently, *o*-dihydroxy groups have been determined in lignin sulfonates[7]. In chlorine bleaching of pulp, demethylation of the residual lignin occurs, with subsequent formation of *o*-dihydroxyl groups[8]. A reliable method to determine the presence of *o*-dihydroxy groups would be important in the chemistry of naturally occurring phenolic substances and their derivatives.

The most common procedures presently employed for the detection of *o*-dihydroxy phenolic compounds involve the formation of a complex utilizing the *o*-dihydroxy groups. Ferric chloride, a reagent with which most phenols give an intense color, will give a green color with *o*-dihydroxy phenolics[9]. However, this is an empirical generalization having no absolute validity, since substitutions on the aromatic ring can result in non-*o*-hydroxylated compounds giving a green color and *o*-dihydroxy phenolics giving colors other than green when treated with a solution of ferric chloride.

Acidified molybdate solutions have been used in spot tests[10] or as spray reagents in chromatography[11] for the detection of *o*-dihydroxy compounds. An intense orange-brown color is a positive indication of complex formation. A buffered solution of molybdate was used by PRIDHAM[12] in paper electrophoresis and paper chromatography of phenolic compounds. Through the use of molybdate-treated paper, the R_F values for *o*-dihydroxy phenolics were reduced, often considerably, when compared to untreated-paper chromatography. The molybdate reagent is specific for *o*-dihydroxy and *ene-diol* groups. Carbohydrates gave a negative test, but L-ascorbic, an *ene-diol* compound, gave a strong positive reaction. This reactivity towards *ene-diol* compounds appears to be the only exception to the molybdate ion being a selective reagent for *o*-dihydroxy phenolics; however, since this test is dependent on the formation of a complex conceivably other dihydroxy compounds having the necessary spatial arrangement would also give a positive test.

The borate ion is often used as a complexing agent, and it has been employed to detect the presence of *o*-dihydroxy compounds[13–16]. Unlike the molybdate complex, the borate complex is colorless and the presence of the complex is determined either by a bathochromic shift in the ultraviolet spectrum[15] or by a lower R_F value in paper chromatography when compared to the non-complexed substance[16]. The use of borate complexes for the detection of *o*-dihydroxy phenolics is more common than the use of molybdate complexes, despite serious shortcomings. The borate ion will complex not only *o*-dihydroxy groups but also *o*-hydroxy-carboxy groups and glycol groups. Carbohydrates will complex with borate ions[17], consequently phenolic glycosides will form complexes although an *o*-dihydroxy phenolic group may be absent. The possibility of the phenolic group being ionized by alkaline borate solutions is always present. JURD[15] advocates the use of a sodium acetate–boric acid solution in ultraviolet spectroscopy and paper treated with this solution in paper chromatography as a means of eliminating this ionization of the phenolic hydroxy[16]. The chromatographic procedure cannot be applied to compounds containing a free acidic function, since the carboxyl group is ionized by the reagent.

NEALES[18] utilized this ability of *o*-dihydroxy compounds to form complexes for the detection of substances containing boron, tungsten, molybdenum, and germanium. He used chlorogenic and caffeic acids as the *o*-dihydroxy reagents.

Another approach to the detection of *o*-dihydroxy phenolic compounds would be through utilizing the ease with which they form *o*-quinones and then testing for the quinone formed. FEIGL *et al.*[19] detected both *o*-diketone and *o*-quinone compounds through treatment with phenylhydrazine in dilute mineral acid. The formation of a yellow to red coloration was a positive test. A similar reaction in which *o*-quinones condense with diamines containing primary amino groups on adjacent carbon atoms is of importance in recognizing *o*-diamino compounds[20]. These tests are not exclusive and other substances, including aromatics and α,β-unsaturated aldehydes, such as cinnamyl aldehydes, also give a positive test.

Quinones can be detected by their colored reaction products when treated with sodium thiosulfate. ROSENTHALER[21] employed this procedure as a spot test, using, however, only *p*-quinones. In FEIGL's *Spot Tests in Organic Analysis*[22], several methods are given for the detection of quinones or related compounds. The only procedure which appears adaptable to the detection of *o*-dihydroxy phenolic compounds is that employing phloroglucinol. Both the sodium thiosulfate and the phloroglucinol methods were modified and tested in their ability to detect *o*-dihydroxy phenolic compounds.

EXPERIMENTAL

Test solutions containing 1 mg of known compounds per ml were prepared as standards and are listed in Table I. Spots of various concentration of these compounds were prepared by repeated addition of drops of standard solutions on filter paper. One drop of standard solution was approximately one lambda. Paper chromatograms of selected standards at various concentrations were also prepared. The phloroglucinol spray reagent was prepared by dissolving 5 g of phloroglucinol in 300 ml of methanol. The sodium thiosulfate spray was a 0.1*M* aqueous solution and the alkaline spray was a 0.1*N* sodium hydroxide solution. The paper was first sprayed with either the

phloroglucinol reagent or the sodium thiosulfate solution and allowed to dry, after which it was sprayed with the alkaline solution.

RESULTS AND DISCUSSION

In using both the phloroglucinol and the sodium thiosulfate spray reagent, no color development occurs until after treatment with the alkaline spray. Many of the compounds tested, especially among the flavonoids, immediately become yellow when treated with alkali. This is not a positive test. The dark color indicating a positive test requires approximately 5 minutes to develop to a maximum intensity and then slowly becomes dull and fades, occasionally with a change in coloration. In general, both the phloroglucinol and the sodium thiosulfate spray reagents give the same color with specific positive substances; however, the phloroglucinol reagent is easily the more preferred. The phloroglucinol reagent is much more sensitive and selective than the other spray reagent. The phloroglucinol spray reagent is essentially unreactive toward *p*-dihydroxy compounds, whereas the other reagent does react in producing a coloration with this grouping. A color reaction with the sodium thiosulfate reagent appears to be inhibited when the *o*-dihydroxy compound also contains a carbonyl group adjacent to the aromatic ring. Neither reagent gave a positive reaction with L-ascorbic acid, an *ene-diol* compound.

Because of these advantages of the phloroglucinol reagent, this one was further investigated and Table I gives the results obtained. The sensitivity of the reagent is such that for most compounds one drop of 1 mg per ml of standard solution is sufficient for detection. A slightly higher concentration is often desirable to better develop the color produced, since the various positive substances produce different colors and this would further aid in identification. In paper chromatography, a two- to three-fold increase in the concentration of the substance to be tested is usually required, and this concentration is acceptable in the paper chromatography of hydroxycinnamyl compounds. As a result, the phloroglucinol spray reagent would be useful as a detecting agent in the paper chromatography of low-molecular-weight lignin substances. In the paper chromatography of flavonoids, a substantially lower concentration is required because otherwise streaking will result; consequently, the phloroglucinol spray cannot be used in detecting these substances in paper chromatography.

No exceptions have been noticed in the compounds tested, nor have interfering substances been detected. Under the test conditions, acetylated phenolic hydroxyls are slowly hydrolyzed, and consequently acetylated *o*-dihydroxy compounds give a positive test. *o*-Dihydroxyls in which one of the hydroxy groups is linked to a carbohydrate by a glycosidic bond do not give a positive test. This fact may be helpful in determining the position of carbohydrate attachment in polyphenolic glycosides.

A fresh preparation of sinapyl alcohol gave an extremely weak positive test, whereas an older preparation gave a more positive reaction. This difference is probably attributable to a loss of methoxy content of sinapyl alcohol during storage, which evidently resulted in the formation of an *o*-dihydroxy group. This loss of methoxy content from sinapyl alcohol had been previously noted by FREUDENBERG and DILLENBURG[23]. There is also a loss of methoxy content during polymerization to form artificial lignin[24].

Myricetin, a flavonol containing a pyrogallol configuration on the B-ring, gives

TABLE I

RESULTS OF THE TESTING FOR *o*-DIHYDROXY COMPOUNDS WITH PHLOROGLUCINOL AND ALKALINE SPRAY REAGENTS

Compound	*Result of test (color)*
Simple phenolic compounds	
Phenol	Negative
Resorcinol	Negative
Orcinol	Negative
Pyrocatechin	Positive (blue-green)
Pyrogallol	Positive (red-brown)
p-Hydroxybenzaldehyde	Negative
Vanillin	Negative
Vanillyl alcohol	Negative
Syringaldehyde	Negative (yellow in base)
Protocatechuic aldehyde	Positive (brown-black)
5-Methoxyprotocatechuic aldehyde	Positive (brown-black)
Anisyl alcohol	Negative
Veratric aldehyde	Negative
Piperonal	Negative
2,5-Dihydroxyacetophenone	Negative (yellow in base)
n-Propyl gallate	Positive (brown)
Cinnamyl-type phenolic compounds	
p-Coumaraldehyde	Negative (yellow in base)
Ethyl *p*-coumarate	Negative
p-Coumaryl alcohol	Negative
Ferulic acid	Negative
Coniferaldehyde	Negative (yellow in base)
Ethyl ferulate	Negative
Isosafrole	Negative
Isoeugenol	Negative
Ethyl 3,4-methylenedioxycinnamate	Negative
Sinapic acid	Negative (sl. yellow in base)
Ethyl sinapate	Negative (yellow in base)
Sinapyl alcohol	Negative to slight positive*
Caffeic acid	Positive (black)
Ethyl caffeate	Positive (black)
Caffeyl alcohol	Positive (black)
α-Conidendrin	Negative
β-Conidendrol	Positive (blue-gray)
Flavonoid compounds	
Kaempferol	Negative (pale yellow in base)
Aromadendrin	Negative
Naringenin	Negative
Phloretin	Negative
Quercetin	Positive (brown)
Quercetin-3′-glucoside	Negative (bright yellow in base)
3,5-Dimethoxyquercetin	Positive (brown)

(*continued on p. 541*)

TABLE I (*continued*)

Compound	*Result of test (color)*
Tamaraxetin	Negative (yellow in base)
Dihydroquercetin	Positive (brown)
Dihydroquercetin-3′-glucoside	Negative
Luteolin	Positive (tan)
Diosmetin	Negative
Chrysoeriol	Negative (pale yellow in base)
Astilbin	Positive (dull red)
Quercitrin	Positive (olive green)
Isoquercitrin	Positive (green)
Fustin	Positive (brown)
Rhamnetin	Positive (gray-brown)
d-Catechin	Positive (tan)
l-Epicatechin	Positive (tan)
Morin	Negative (yellow in base)
Dihydromorin	Negative
Eriodictyol oxime	Positive (red-tan)
Homoeriodictyol	Negative
Hesperetin	Negative
Myricetin	Positive (intense deep blue)*

* Specific results discussed in text.

an immediate intense deep blue coloration with the alkaline spray alone, and no difference in coloration is noted if this compound has previously been sprayed with either the phloroglucinol or the sodium thiosulfate reagent. This is a known reaction of a 5,6,7- or 3′,4′,5′-trihydroxyflavone to base[25]. Neither pyrogallol nor propyl gallate reacted similarly.

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SUMMARY

A procedure has been developed for the detection of *o*-dihydroxy compounds. Compounds possessing an *o*-dihydroxy group are spotted on filter paper and will develop a dark color when sprayed first with methanolic phloroglucinol and then sprayed with dilute alkali. The test appears to be very selective, and no exceptions have been noticed in the compounds tested, nor have interfering substances been detected. Model compounds of interest in lignin chemistry and numerous flavonoid compounds have been tested.

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