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Note

A useful spray reagent to differentiate common phenolic compounds on thin-layer plates and paper chromatograms

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Various spray reagents have been described for the detection of phenolic compounds on thin-layer plates and paper chromatograms[1,2]. Some of the reagents most commonly used for phenolic acids are ammonia vapor, diazotized *p*-nitroaniline, diazotized sulphanilic acid and benzidine. For flavonoid compounds, 5% ethanolic aluminum chloride, 5% aqueous sodium carbonate, 1% aqueous ferric chloride + 1% aqueous potassium ferricyanide (1 : 1), 1% aqueous sodium hydroxide, *p*-toluenesulfonic acid and ammonia vapor are commonly used[3,4]. Although these spray reagents have proved useful for the detection of phenolic compounds, they do have certain limitations. For example, diazotized *p*-nitroaniline produces the same color reaction (blue) with syringic, *p*-coumaric, sinapic and gentisic acids[5]. Similarly, a 1% aqueous ferric chloride + 1% aqueous potassium ferricyanide produces a blue color reaction with flavones, flavonols, isoflavones, flavanones, aurones and chalcones; a 5% ethanolic aluminum chloride spray gives a yellow color reaction with flavonols, flavones, chalcones and aurones. A spray reagent which differentiates different classes of flavones, flavonols, etc. would be extremely useful for the detection of phenolic compounds.

In recent years, 2-aminoethyl diphenylboric acid ester sometimes referred to as "flavone reagent" or "Naturstoffreagens" has been used to detect the phenolic spots on thin-layer plates, since this compound, when used as a spray reagent, intensifies the fluorescence of phenolics[6–9]. It has been found that this compound when used in conjunction with certain spray reagents and ammonia vapor can differentiate some of the common phenolic compounds on thin-layer and paper chromatograms. This report describes the use of 2-aminoethyl diphenylboric acid ester (AEDBE) for the detection of phenolic compounds.

EXPERIMENTAL

Two-dimensional chromatography was carried out using Whatman No. 1 chromatography paper and thin-layer plates coated with cellulose. A blended mixture of 18 g of cellulose powder (Sigmacell, Type 19; Sigma, St. Louis, Mo., U.S.A.) and 90 ml of distilled water was spread at a thickness of 0.25 mm on 20 × 20 cm glass plates. The plates were dried for 1 h at room temperature prior to use. The plates

and chromatographic paper were spotted with a minimum aliquot of 10 μl (1.0 μg) methanolic solution of each phenolic compound. For the first direction, the paper chromatograms were developed in *n*-butanol–acetic acid–water (6:1:2) and the TLC plates developed in *tert.*-butanol–acetic acid–water (3:1:1). Acetic acid–water (15:85) was used for development in the second direction for both PC and TLC. The developed plates and chromatograms were dried and then sprayed with AEDBE (Sigma), diazotized *p*-nitroaniline (DNP), 5% ethanolic aluminum chloride ($AlCl_3$), and 5% aqueous sodium carbonate (Na_2CO_3). A number of plates and chromatograms were also exposed to ammonia vapor and observed under UV light.

The flavone reagent was prepared by dissolving 1 g of AEDBE in a mixture of 50 ml each of methanol and propanol; the DPN reagent consisted of 5 ml of 0.5% DPN in 2 *N* HCl plus 1 ml of 5% aqueous sodium nitrite and 15 ml of 20% sodium acetate.

The samples of flavonoids and hydroxy- and methoxycinnamic acids were purchased from K & K Labs., Plainview, N.Y., U.S.A. and J. T. Baker, Phillipsburg, N.J., U.S.A. The glycoflavones were isolated from the leaves of flax (*Linum usitatissimum* L.).

RESULTS AND DISCUSSION

The various color reactions of the phenolic compounds produced by the chromogenic reagents and by ammonia vapor are shown in Table I. It can be seen that both AEDBE and 5% $AlCl_3$ differentiate the anthocyanidins, pelargonidin, cyanidin and delphinidin by their color reactions. AEDBE reagent produced a greyish-red color with pelargonidin, deep blue with cyanidin and light blue with delphinidin, whereas $AlCl_3$ gave an orange-red color reaction with pelargonidin, pinkish-violet with cyanidin, and blue with delphinidin. Thus $AlCl_3$, which has largely been used to detect flavonols, flavones, isoflavones, flavanones, aurones and chalcones, can also be utilized to characterize certain classes of anthocyanidins. AEDBE and 5% $AlCl_3$ in conjunction with basic lead acetate and ammonia vapor, can be extremely useful to differentiate the anthocyanidins.

All the mono-, di-, and trihydroxyflavonols and the mono- and dihydroxyflavones and glycoflavones turned yellow with 5% $AlCl_3$ and 5% Na_2CO_3 except the flavonol myricetin, which turned green and later grey with Na_2CO_3. Most importantly, a color variation was noticed between the mono-, di-, and trihydroxyflavonols with the AEDBE reagent. Kaempferol with the monohydroxy B-ring turned pale yellow, whereas its dihydroxy derivative, quercetin, produced an orange color, and the trihydroxy derivative, myricetin, became orange-brown in color. With DPN, a spray which has been largely used to characterize the phenolic acids, an orange color was obtained with kaempferol, yellow with quercetin and yellowish brown with myricetin. The separation of kaempferol, quercetin and myricetin by AEDBE and DPN is extremely important as this helps tremendously in the identification of these three classes of flavonols. AEDBE when used in conjunction with DPN also differentiates the flavones apigenin and luteolin. With AEDBE apigenin turned light yellow, and luteolin pale yellow or yellow green, whereas with DPN apigenin became orange-red and luteolin yellow. In the glycoflavone group, the two monohydroxy compounds vitexin and isovitexin, and vicenin either showed no color

TABLE I

COLOR REACTIONS OF COMMON PHENOLIC COMPOUNDS (1.0-μg LEVEL) BY SPRAYING AEDBE AND OTHER REAGENTS ON TLC AND PC

Color key: bl = blue, br = brown, c = colorless, dp = deep, ft = faint, gy = grey, gr = green, lt = light, or = orange, pk = pink, pl = pale, r = red, vt = violet, y = yellow.

Compounds	*Visible light*				*UV light*
	AEDBE	*DPN*	*$AlCl_3$ (5%)*	*Na_2CO_3 (5%)*	*NH_3*
Anthocyanidins					
Pelargonidin	gy-r	or-y	or-r	gr	or-r
Cyanidin	dp-bl	or-y	pk-vt	gr	gy-bl
Delphinidin	lt-bl	or-y	bl	gr	bl
Flavonols					
Kaempferol	pl-y	or	y	y	y
Quercetin	or	y	y	y	y
Myricetin	or-br	y-br	y	gr-gy *	y
Flavones					
Apigenin	lt-y	or-r	y	y	y
Luteolin	pl-y, y-gr	y	y	y	y
Glycoflavones					
Vitexin and isovitexin	c	c	y	lt-y	y
Orientin and isoorientin	pl-y	y-br	y	y	or-y
Vicenin	ft-y	c	y	lt-y	y
Lucenin	y	y	y	y	y
Hydroxy- and methoxy-cinnamic acids					
p-Coumaric acid	c	or-br	c	c	vt
Caffeic acid	y	or-br	c	y	bl
Ferulic acid	c	or-r	c	c	bl
Sinapic acid	c	or-r	c	c	bl-gr
Chlorogenic acid	y	or-br	c	y	bl-gr

* Green turns grey.

reaction or changed into very faint yellow spots with AEDBE. Orientin, isoorientin and lucenin, on the other hand, exhibited yellow to pale-yellow colors with this spray reagent. With DPN, the orientin and isoorientin produced a yellow-brown color and lucenin became yellow.

In the case of hydroxy- and methoxycinnamic acids listed in Table I, the 5% $AlCl_3$ did not produce any color change in visible light. With the AEDBE reagent caffeic and chlorogenic acids turned yellow but *p*-coumaric, ferulic and sinapic acids gave no change in color reaction. Similar color reactions were produced with 5% Na_2CO_3. DPN reacted with *p*-coumaric, caffeic and chlorogenic acids to give an orange-brown color and with ferulic and sinapic acids an orange-red appearance. Thus AEDBE can differentiate *p*-coumaric and caffeic acids, both of which appear orange-brown with DPN, the commonly used spray reagent for determining phenolic acids.

CONCLUSIONS

This study has shown that (1) 5% $AlCl_3$ can also be used to differentiate certain anthocyanidins, (2) DPN, which has been largely used to characterize phenolic acids, is also a useful spray reagent to separate some of the common flavonols, and (3) AEDBE is extremely useful to differentiate some of the common phenolic compounds belonging to the anthocyanidin, flavonol, flavone and hydroxycinnamic acid groups. This latter compound (AEDBE), which previously has found widespread use as an agent to intensify phenolic spots, has gained added importance as an effective spray reagent for the differentiation of certain phenolics.

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