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Note

Separation of dihydroxybenzoic acids and trihydroxybenzoic acids by two-dimensional thin-layer chromatography

HAZEL PECK*
Department of Biological Sciences, Sheffield City Polytechnic, Sheffield S1 1WB (U.K.)
A. W. STOTT
Department of Biochemistry, Scarborough District Hospital, Scarborough (U.K.)
and
J. B. TURNER
Department of Chemistry, Sheffield City Polytechnic, Sheffield S1 1WB (U.K.)
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Chromatographic methods for the separation of phenolic acids in general using paper[1–5] and thin-layer chromatography (TLC)[6,7] are well established, but little attention has been directed to the polyhydroxybenzoic acids in particular[8,9] and R_F data are incomplete. Resolution of an isomeric mixture using high-performance liquid chromatography[10] and packed column gas–liquid chromatography (GLC)[11–15] has proved unsatisfactory as have attempts using capillary column GLC with a SE-30 stationary phase in our laboratories.

These, and related compounds, are dietary components, being present in both naturally occurring foods, especially fruit and vegetables[16] and matured distilled alcoholic beverages[17]. The presence in urine of some of the dihydroxybenzoic acid isomers has been reported[15] and originates from both dietary intake and metabolic processes[18], including ingestion of aspirin (acetylsalicylic acid)[19]. Benzoic acid derivatives are increased in ureamic patients on dialysis and may be toxic[20]. Ingestion of benzoic acid, a widely used food preservative has been shown to provoke migraine attacks[21], and measurement of chemically related compounds may prove useful in such patients.

Owing to scant information regarding the chromatographic properties of the polyhydroxybenzoic acids conclusive interpretation of chromatograms for their presence has previously been difficult. We, therefore, describe a simple two-dimensional TLC method for the separation of dihydroxybenzoic acids and trihydroxybenzoic acids. It is rapid, does not require the use of potentially carcinogenic solvent systems and may be applied to a wide range of samples.

EXPERIMENTAL

Materials

Cellulose-coated (1-mm layer) foil-backed TLC plates were purchased from Merck (Darmstadt, F.R.G.). Commercially available (highest purity) phenolic acids

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TABLE I

R_F VALUES IN BOTH DIMENSIONS OF COMPOUNDS STUDIED AND THEIR COLOUR REACTION WITH DIAZOTISED *p*-NITROANILINE REAGENT

The common name is given in parenthesis when this differs from the accepted IUPAC name.

Compound No.	*Phenolic compound*	*Trivial name*	*R_F value*		*Colour*
			First dimension	*Second dimension*	
1	2,3-Dihydroxybenzoic acid	–	53	60	Brown/purple
2	2,4-Dihydroxybenzoic acid	β-Resorcylic acid	38	57	Orange/brown
3	2,5-Dihydroxybenzoic acid	Gentisic acid	60	51	Yellow-bleached
4	2,6-Dihydroxybenzoic acid	γ-Resorcylic acid	81	28	Orange
5	3,4-Dihydroxybenzoic acid	Protocatechuic acid	19	43	Pale grey/brown
6	3,5-Dihydroxybenzoic acid	α-Resorcylic acid	29	31	Bright orange
7	2,3,4-Trihydroxybenzoic acid	Pyrogallolcarboxylic acid	22	17	Pale yellow
8	2,4,6-Trihydroxybenzoic acid	Phloroglucinolcarboxylic acid	44	18	Orange
9	3,4,5-Trihydroxybenzoic acid	Gallic acid	19	23	Pale grey/brown
10	4-Hydroxy-3-methoxybenzoic acid	Vanillic acid	28	74	Purple
11	2-Hydroxy-2-(4′-hydroxy-3′-methoxy-phenyl)ethanoic acid (4-Hydroxy-3-methoxymandelic acid)	– Vanillylmandelic acid	41 35	48 41	Blue Purple/blue
12	2-Hydroxy-3-(4′-hydroxy-3′-methoxy-phenyl)propanoic acid (4-Hydroxy-3-methoxyphenyllactic acid)	–	41	48	Blue
13	4-Hydroxy-3-methoxyphenylacetic acid	Homovanillic acid	40	72	Blue/green
14	4-Hydroxy-3,5-dimethoxybenzoic acid	Syringic acid	24	73	Blue
15	2-Hydroxyphenylacetic acid	–	72	68	Purple
16	3-Hydroxyphenylacetic acid	–	48	64	Pink/purple
17	4-Hydroxyphenylacetic acid	–	43	63	Purple
18	2-(2′-Hydroxybenzoylamino)ethanoic acid (2-Hydroxyhippuric Acid)	Salicyluric acid, salicylglycine	32	60	Pink/red
19	2-Hydroxy-2-(4′-hydroxyphenyl)ethanoic acid (4-hydroxymandelic acid)	–	39	29	Pink

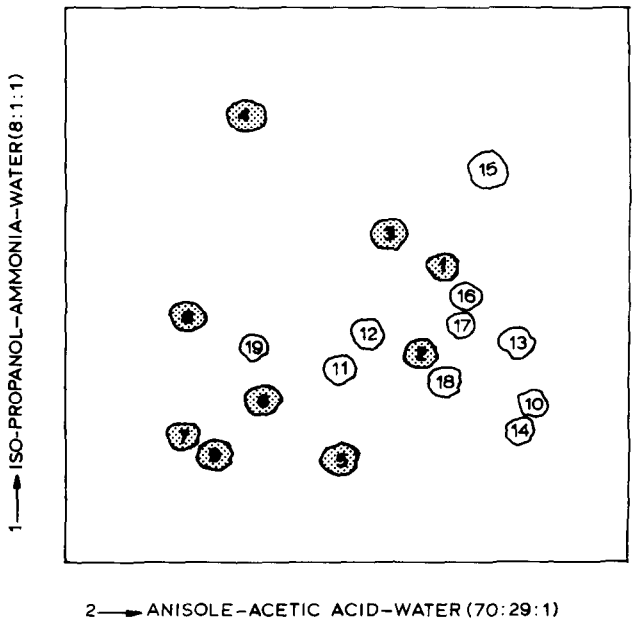

Fig. 1. Relative positions of compounds studied after two-dimensional TLC. The numbering corresponds to that shown in Table I. The hatched spots show the positions of the dihydroxybenzoic acids and the trihydroxybenzoic acids.

were used as reference compounds and solutions were prepared in ethanol. All solvents and chemicals were Analar grade with the exception of anisole, which was technical grade, and were purchased from BDH (Poole, U.K.).

Chromatography

Two-dimensional chromatography was carried out on 10 cm × 10 cm TLC plates; the eluent in the first dimension was isopropanol–0.88 ammonia–water (8:1:1), that in the second dimension anisole–glacial acetic acid–water (70:29:1). Approximately 10 μg of each compound were applied in a 10-μl volume. The compounds were detected by spraying with saturated potassium carbonate solution followed by diazotised *p*-nitroaniline[22].

RESULTS AND DISCUSSION

All the dihydroxybenzoic acids and trihydroxybenzoic acids produced various shades of yellow, and these are given in Table I with their R_F values in both dimensions. Fig. 1 shows their relative positions on the plate together with those of related phenolic acids. Run times in solvents 1 and 2 were approximately 90 min and 20 min, respectively.

This method can be applied to a wide range of samples, with prior extraction of the hydrobenzoic acids when concentrations are less than 1 g · l^{-1} (ref. 23). We have examined fresh random urine samples from 15 subjects on unrestricted diets in this way and have verified the previously reported presence of the 3,4-, 3,5- and

2,5-dihydroxybenzoic acid isomers. We have also observed spots which have been tentatively identified as 2,6- and 2,4-dihydroxybenzoic acids and 3,4,5-, 2,4,6-, and 2,3,4-trihydroxybenzoic acids. The metabolic processes involved in the production of some of the dihydroxybenzoic acids are known but information relating to the origin and, in particular, the significance of the trihydroxybenzoic acids in human fluids requires investigation.

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