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Note

Dicyclohexylcarbodiimide as a simple and specific fluorogenic spray reagent for some di- and tricarboxylic acids

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On thin-layer chromatographic (TLC) plates or chromatographic papers, di- and tricarboxylic acids can be detected colorimetrically with pH indicators¹⁻³, 2,7-dichlorofluorescein-aluminium chloride-iron (III) chloride⁴, copper acetate-potassium hexacyanoferrate⁵ or dicyclohexylcarbodiimide-hydroxylammonium chloride-iron(III) chloride⁶. These methods detect the acidity or the carboxyl group of the acids, and therefore they will not be specific for the detection of these acids. Lang and Lang⁷ recommended a fluorogenic spray reagent composed of pyridine and acetic anhydride, or of acetic anhydride, acetic acid and a small amount of potassium carbonate, for the specific detection of citric, itaconic and aconitic acids. In this paper a sensitive, specific and simpler method for the detection of citric, isocitric, *cis*- and *trans*-aconitic acids and some other biogenic compounds by using dicyclohexylcarbodiimide (DCC) as a spray reagent is described.

EXPERIMENTAL

Reagents and materials

The sources of the test compounds used were Fluka (Buchs, Switzerland; citraconic and itaconic acids), Sigma (St. Louis, MO, U.S.A.; sodium pyruvate, α -ketoglutaric, uric and amino acids), Riedel de Haën (Hannover, G.F.R.; fumaric and glyoxylic acids), Kanto Chemical Co. (Tokyo, Japan; hippuric acid) and E. Merck (Darmstadt, G.F.R.; glycolic and palmitic acids). All of these compounds were used as received.

cis-Aconitic, isocitric and anhydrous citric acids were prepared by common procedures from *cis*-aconitic anhydride, sodium isocitrate (Fluka) and citric acid (Kanto Chemical Co.). *trans*-Aconitic acid (m.p. 206.5–207.0°C) was prepared by the method described by Bruce⁸. Tricarballic acid (m.p. 163–164°C) and trimethyl citrate (m.p. 79.0–79.6°C) were obtained by hydrogenation of *trans*-aconitic acid with 2.5% sodium amalgam and methylation of citric acid with methanol and sulphuric acid, respectively.

The other test compounds and solvents were of analytical-reagent grade.

The sole reagent for the fluorogenic reaction was DCC (E. Merck). A 0.8 M solution of DCC was prepared from *n*-butanol and used as the spray reagent.

Thin-layer and paper chromatography

Pre-coated aluminium TLC sheets (silica gel 60, 5 × 10 cm, 0.2 mm layer) from

E. Merck and Whatman No. 1 papers were used without any treatment. The sample solutions (0.1–10 mM in methanol) were spotted with micro-pipettes (Camag, Muttenz, Switzerland). Ascending chromatography was performed in a glass tank at room temperature (*ca.* 22°C). After development, the air-dried plates or papers were sprayed (twice, if necessary) with the DCC solution in a fume-hood. The spots of aconitic acids could be observed immediately under longwave ultraviolet light, whereas those of the others were observed under the same light after heating in an oven at 70°C for 5 min.

The solvent systems used in TLC and paper chromatographies were freshly prepared and were *n*-butanol–acetic acid–water (3:1:1) and diethylether–formic acid (90%)–water (7:2:1), respectively.

RESULTS AND DISCUSSION

The limits of detection, R_F values and the colours of the fluorescence are listed in Table I. It can be seen that DCC is a sensitive reagent for the fluorometric detection of citric and *trans*- and *cis*-aconitic acids on both TLC plates and papers and can also be used for the semimicroanalysis of leucine, glyoxylic, α -ketoglutaric, isocitric, itaconic, malic and hippuric acids. In addition, DCC behaved as a highly specific reagent for these acids, as demonstrated by a specificity investigation on other carboxylic acids. The following esters and carboxylic acids were negative to DCC: phthalic anhydride, potassium phthalate, trimethyl citrate and benzoic, *p*-hydroxybenzoic, acetic, propionic, palmitic, acetoacetic, succinic, lactic, tartaric, tricarballic, ascor-

TABLE I

LIMITS OF DETECTION OF THE ACIDS ON TLC PLATES OR PAPERS

Solvent systems: *n*-butanol–acetic acid–water (3:1:1) for TLC and diethyl ether–formic acid (90%)–water (7:2:1) for paper chromatography.

Compound	Limit of detection (nmole)*	R_F	Colour of fluorescence
<i>cis</i> - or <i>trans</i> -aconitic acid	0.2 (1.0)	0.52 (0.89)	Yellow-green (blue)
Citric acid	0.5 (1.25)	0.12 (0.54)	Blue-green (blue)
Isocitric acid	25 ((50)	0.34 (0.54)	Blue (blue)
Itaconic acid	50	0.74	Red-orange
Malic acid	100	0.45	Violet
Maleic acid	> 150**	0.64	Yellow
Glyoxylic acid	150	0.58	Blue
α -Ketoglutaric acid	150	0.53	Blue-violet
Sodium pyruvate***	150	0.40	Violet
Hippuric acid	40	0.73	Violet
Leucine†	50	0.36	Blue

* The data in parentheses were obtained by paper chromatography.

** Maleic acid was detected as a dark but not fluorescent spot when a small amount of the acid was applied.

*** Before dilution to volume, the sample solution was acidified with 1 *N* hydrochloric acid.

† The sample solution was prepared with 0.1 *N* hydrochloric acid.

bic, sorbic, orotic, nicotinic, pantothenic, uric, glycolic, malonic and amino acids (glycine, alanine, valine, proline, threonine, serine, methionine, cystine, tyrosine, tryptophan, lysine, arginine, histidine and aspartic and glutamic acids).

The reactions of *trans*- and *cis*-aconitic acids with DCC were rapid and might occur without heating. However, heating was necessary for the other acids. Although the fluorescence intensity of the spots diminished slowly, they remained detectable for several hours when the plates or papers were kept in the dark.

The reaction was investigated in various solvents for citric and aconitic acids. The results, which will be reported elsewhere, reveal that both citric and aconitic acids reacted readily and gave the strongest fluorescence in *n*-butanol. For this reason, *n*-butanol was chosen as a solvent for the spray reagent. In this solvent DCC retained its activity for at least 1 week.

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