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Note

3,5,3',5'-Tetramethylbenzidine as a spray reagent in the thin-layer chromatographic detection of organochlorine pesticides

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Although efforts have been made to reduce the use of organochlorine pesticides, they are still widely used in certain parts of the world. In Central Africa, DDT and other organochlorine pesticides are not only used for agricultural purposes, but also to spray large forest areas for tsetse fly control. The tsetse fly bite causes sleeping sickness in humans, and cattle-breeding is virtually impossible in tsetse fly infested areas.

Thin-layer chromatography (TLC) using silver nitrate as a spray reagent has been widely used for the detection of organochlorine pesticides¹. However, it is not very sensitive: the spots are ill-defined for low concentrations of pesticides and they cannot be easily located because the background is dark, possibly because of impurities in the silica gel², the stationary phase commonly used. Improvements on the silver nitrate spray method have been obtained by adding 2-phenoxyethanol³ or ammonia^{4,5} to the silver nitrate spray, incorporating the silver nitrate into the silica gel slurry during the preparation of the chromatographic plates^{2,6-8}, the use of highly purified silica gel² and the use of aluminium oxide as adsorbent⁵, although DDT and its derivatives have been reported to decompose under UV light when using aluminium oxide plates⁹.

Other reagents besides silver nitrate have also been investigated. Adamovic¹⁰ tested various amines as locating agents for the TLC detection of organochlorine pesticides. The colour reaction for his test is based on Feigl's spot test for amine compounds¹¹. Amine bases react with 2,4-dinitrochlorobenzene to give yellow products, which for primary and secondary amines are thought to be N-substituted 2,4-dinitrobenzene analogues. For tertiary amines, the constitution of the coloured products is not known. In Adamovic's test, chlorinated pesticides take the place of 2,4-dinitrochlorobenzene. However, aromatic amines, and in particular benzidine, although sensitive, have not found favour as reagents because they are potential carcinogens. Although benzidine is a known carcinogen, its 3,5,3',5'-tetramethyl derivative (Fig. 1) has been found to be non-carcinogenic. Holland *et al.*¹² prepared and successfully tested this compound in place of benzidine for the detection of blood. They pointed out that carcinogenic action in aromatic amines is thought to involve *ortho*-hydroxylation of the amines; this is impossible in 3,5,3',5'-tetramethylbenzidine. In his paper on the use of aromatic amines as spray reagents in the detection of chlorinated organic pesticides, Adamovic¹⁰ pointed out that if the chro-

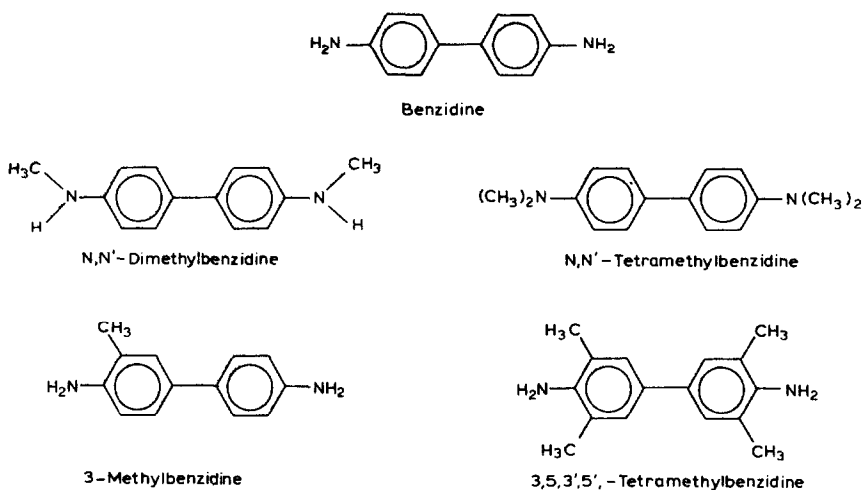


Fig. 1. Structures of benzidine and some substituted benzidines.

matograms sprayed with benzidine and dephenylamine are exposed to sunlight rather than irradiated with a UV lamp, spots still appear. In our work, a number of substituted benzidines have been investigated as possible spray reagents for organochlorine pesticides. Direct sunlight was used to induce colour development throughout. Emphasis was laid on 3,5,3',5'-tetramethylbenzidine because of its proven non-carcinogenicity.

EXPERIMENTAL

Apparatus

A chromatographic plate applicator (Quickfit Instruments), an MSE Ato Mix high-speed blender and a Buchi Rotavapor-R rotary evaporator were used.

Reagents

Basic aluminium oxide (Alumina Woelm B). A 35-g amount of alumina and 40 ml of distilled water were shaken thoroughly in a stoppered erlenmeyer flask. The slurry was spread on glass plates using an applicator set at 0.25 mm. The plates were then air-dried. Before use all plates were activated by heating in an electric oven at 110°C for 30 min and then cooled in a desiccator.

Solvents. Analytical reagent acetone and *n*-hexane were redistilled using a 1-m glass column insulated with cotton-wool.

Stock pesticide solutions. Standard solutions were prepared by dissolving 20 mg of pesticide in redistilled *n*-hexane and diluting to the mark with the same solvent in a 100-ml volumetric flask. Each standard solution contained 200 mg/l. The pesticide standards used were *p,p'*-DDT, *o,p'*-DDT, aldrin, *p,p'*-DDE, endrin, dieldrin, *o,p'*-DDD, *o,p'*-DDE, *m,p'*-DDD and *p,p'*-DDD. All solutions of pesticides were kept refrigerated at -15°C and were equilibrated at room temperature before use.

Spray reagents. 3-Methylbenzidine, N,N'-dimethylbenzidine and N,N'-tetramethylbenzidine were prepared according to the method described by Stephen¹³.

3,5,3',5'-Tetramethylbenzidine was prepared according to the method of Holland *et al.*^{1,2}. Solutions of spray reagents were freshly prepared before use by dissolving 100 mg of each reagent in 100 ml of *n*-hexane.

Developing solvent. The developing solvent was 20% (v/v) redistilled acetone in redistilled *n*-hexane in all instances.

Celite 545. This was obtained from Koch-Light.

Method

Testing of spray reagents. For testing of all spray reagents, 10 μ l of each organochlorine pesticide was spotted on to a plate using a micropipette; the plate was then developed in a saturated tank of the developing solvent until the solvent front reached a pre-marked line 15 cm from the spots. The plate was air-dried and then sprayed with the reagent in a fume hood. The plate was again air-dried and then immediately exposed to direct sunlight to develop the colours.

To find the approximate detection limits, the standard pesticide solutions were diluted by known factors and the experiment was repeated until spots could not be detected.

Application to samples. The method was tested on samples (tree bark, foliage) that had been collected from areas in Zambia where spraying for tsetse fly control had been carried out. Each sample was blended at high speed in a electrical blender. A known amount of sample (10 g) was then weighed, wrapped in a filter-paper, placed in a thimble and extracted in a Soxhlet extractor for 5 h using a acetone-*n*-hexane (1:1). The extract was then concentrated to about 5 ml by slow evaporation over a steam-bath.

For the clean-up, 30 g of Celite 545 were thoroughly mixed with 20 ml of concentrated sulphuric acid using a pestle and mortar. The mixture was packed into a column, the sample poured in at the top and then eluted with 150 ml of *n*-hexane. The eluate was collected and concentrated to about 5 ml using a rotary evaporator and then spotted on to TLC plates. For spiked samples, known amounts of standards were added to the 10 g sample before extraction. Five samples (three tree bark and two foliage) were tested for organochlorine pesticides. Two tree bark and the foliage samples had been collected from an area sprayed a fortnight prior to the date of collection; one tree bark sample was collected from an area that had not been sprayed. After collection all samples were stored in a freezer at -15°C until the date of analysis.

RESULTS AND DISCUSSION

All four spray reagents investigated, 3-methylbenzidine, N,N'-dimethylbenzidine, N,N'-tetramethylbenzidine and 3,5,3',5'-tetramethylbenzidine, produced colours with each of the ten organochlorine pesticides within 60 sec of exposure of the plate to direct sunlight. 3,5,3',5'-Tetramethylbenzidine, however, produced colours almost instantaneously on exposure of the plate to sunlight. The results are shown in Table I.

The spots obtained with 3-methylbenzidine, N,N'-dimethylbenzidine and N,N'-tetramethylbenzidine were stable for about 1 h, but those obtained with 3,5,3',5'-tetramethylbenzidine started to fade within 5-10 min. These spray reagents,

TABLE I
COLOURS OF SPOTS FORMED AND DETECTION LIMITS

Reagent	Colour	Detection limit (μg)
3-Methylbenzidine	Yellow	—
N,N'-Dimethylbenzidine	Yellow-brown	1.0 (2.0 for aldrin)
N,N'-Tetramethylbenzidine	Light khaki	—
3,5,3',5'-Tetramethylbenzidine	Green, changing to yellow for <i>p,p'</i> -DDD, <i>p,p'</i> -DDE, <i>o,p'</i> -DDE, <i>m,p'</i> -DDD Blue-green for: aldrin, <i>p,p'</i> -DDT, <i>o,p'</i> -DDT, endrin, dieldrin	0.2 (0.3 for aldrin)

particularly 3,5,3',5'-tetramethylbenzidine, therefore cannot be used in densitometry because of fading of the spots, unless under strictly controlled conditions of temperature and time. However, they can be used for identification; the background is clear and the spots are well defined and can be marked on the plate for later use.

The development times ranged from 27 to 30 min. The differences in time were presumably due to slight differences in temperature, humidity and thickness of the layers from plate to plate. The R_F values, for the organochloride pesticides investigated are given in Table II. The values quoted were obtained from at least ten replicate measurements for each pesticide. There were variations of R_F values from run to run, presumably because of differences in laboratory humidity¹⁴, temperature¹⁵ and adsorbent thickness. The R_F values relative to each other were, however, always the same from run to run.

3,5,3',5'-Tetramethylbenzidine and N,N'-dimethylbenzidine formed the most intense spots for a given concentration of organochlorine pesticide. Further tests were made with these two reagents to determine the limits of detection. For 3,5,3',5'-tetramethylbenzidine this was found to be 0.2 μg for all the organochlorines investigated except aldrin, which had a detection limit of 0.3 μg . N,N'-Dimethylbenzidine was less sensitive; the limit of detection was 1.0 μg for all the other pesticides and 2.0 μg for aldrin.

The four samples collected from areas that had been sprayed for tsetse fly control with DDT produced positive results for *p,p'*-DDT, using 3,5,3',5'-tetrame-

TABLE II

R_F VALUES FOR ORGANOCHLORINE PESTICIDES ON ALUMINA (0.25 mm THICK) AT ROOM TEMPERATURE

Pesticide	R_F value	Pesticide	R_F value
Aldrin	0.82 \pm 0.06	Endrin	0.68 \pm 0.05
<i>p,p'</i> -DDT	0.70 \pm 0.07	Dieldrin	0.65 \pm 0.05
<i>o,p'</i> -DDT	0.72 \pm 0.06	<i>o,p'</i> -DDD	0.59 \pm 0.06
<i>p,p'</i> -DDD	0.60 \pm 0.07	<i>o,p'</i> -DDE	0.73 \pm 0.05
<i>p,p'</i> -DDE	0.80 \pm 0.05	<i>m,p'</i> -DDD	0.59 \pm 0.06

thylbenzidine as spray reagent. Spiked samples also confirmed the presence of *p,p'*-DDT. No spots were detected with the control sample.

CONCLUSION

3,5,3',5'-Tetramethylbenzidine has been shown to be the most sensitive of the four spray reagents tested. Although the spots formed fade quickly, they last long enough to be marked on the plate. In the absence of sunlight the plates can be irradiated with UV light to induce colour formation.

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