such as the use of uniform and extremely thin layers or the automated application of derivatives, appear to raise the accuracy considerably. A successful increase in the accuracy of the method eventually may extend its application to the analysis of estrogens in peripheral human plasma or non-pregnancy urine.

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Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances

A simple bioautographic technique according to WELTZIEN[7], and modified by DEKHUIJZEN[1] for detection of fungitoxic substances has been in use for many years in this laboratory. Chromatograms on Whatman No. 3MM paper are developed with propanol–water (85:15) and after drying are sprayed with a conidial suspension of *Glomerella cingulata*. After incubation, clearly visible inhibition zones indicate the presence of fungitoxic compounds. Chromatography thus permits not only the detection of fungitoxic substances *per se*, but also makes the study of the conversion reactions and of decomposition of such compounds possible.

Although this method is elegant for many reasons (high sensitivity, possibility of keeping records), it has the disadvantage of paper chromatography in general, the development being rather time consuming (16 h). This proved to be especially inconvenient in the study of fungitoxic compounds which are gradually converted non-enzymatically into other compounds either by oxidation (*e.g.* phenylthiosemicarbazide and derivatives[2]) or hydrolysis (*e.g.* benomyl[3]). The rather slow development did not give a satisfactory separation of the various components, confluent spots being obtained instead. Therefore, silica gel thin-layer chromatography on DC-Alufolie Kieselgel F_{254} plates (Merck) was considered as an alternative to the paper chromatographic technique.

With the widespread usage of TLC, many bioautographic methods have been introduced which make use of this technique for the more rapid separation of antimicrobial substances. On perusing the literature we did not really come across a very simple bioautographic technique based on TLC. Moreover several additional manip-

ulations had to be performed for the bioassay to be carried out. General routine normally includes either pressing the thin-layer plate on agar seeded with a suitable sensitive microorganism[4] or pouring a molten nutrient agar on the thin-layer chromatogram, which after solidification is then seeded with the test organism[5]. Neither method is very appropriate if one wishes to save the plates; moreover, with the first technique at least some skill is required to handle the thin-layer plates properly. To overcome some of the difficulties WAGMAN AND BAILEY[6] introduced the use of Chrom-AR® silicic acid/glass fiber sheets for bioautography of antimicrobial compounds, although according to this method the material to be investigated again has to be transferred from the sheet to agar.

We found that direct spraying of the thin-layer chromatograms with a spore suspension of the test fungus in a glucose–mineral salts medium was by far the easiest technique, and also gave the most reliable results. The thin-layer plates are developed for 20–60 min depending on room temperature and the solvent system employed. Ether and ethyl acetate proved the most suitable solvents because no trace of these remains in the silica gel after drying, as opposed to other systems, as for instance butanol–acetic acid–water (4:1:1) where even prolonged drying does not completely remove the acetic acid present. After locating the UV-absorbing spots, the chromatograms are usually sprayed with a conidial suspension of *Cladosporium cucumerinum* in a medium prepared as follows. A stock solution contains: 7 g KH_2PO_4, 3 g $Na_2HPO_4 \cdot 2H_2O$, 4 g KNO_3, 1 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl per l of tap water. The solution is autoclaved at 120° for 20 min. Just before making the conidial suspension 10 ml of a 30%

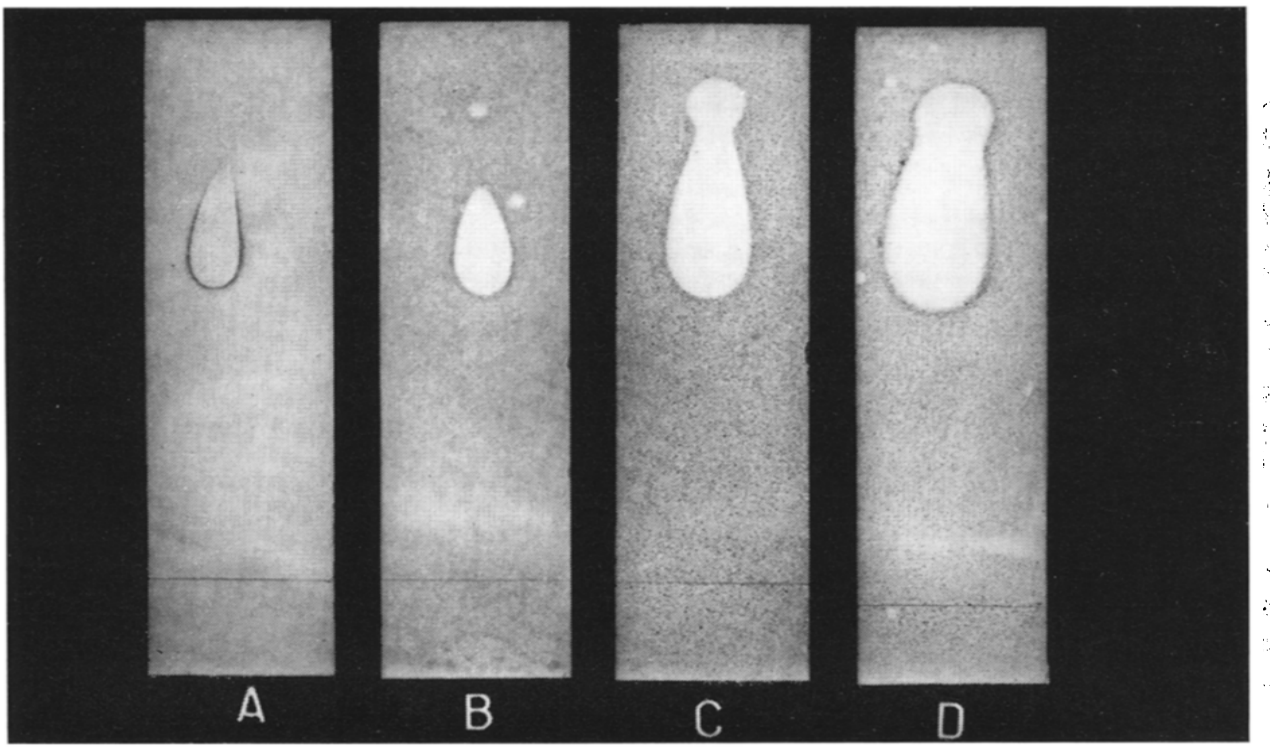

Fig. 1. Differential sensitivity for benomyl (= 1-(butylcarbamoyl)-2-benzimidazole carbamic acid, methyl ester) of: (A) *Ascochyta pisi*; (B) *Fusarium culmorum*; (C) *Penicillium expansum*; and (D) *Colletotrichum lindemuthianum*. The quantity spotted was in each case 1.25 μg active ingredient.

aqueous solution of glucose is added per 60 ml of this solution. During spraying, care should be taken to avoid the plates becoming too wet. After spraying the thin-layer plates are incubated in a moist atmosphere[1] for 2–3 days at 25°. Inhibition zones indicate the presence of the original fungitoxic product plus, if present, conversion or decomposition products, which are fungitoxic. It should be realized, that not all UV-absorbing spots will be fungitoxic as well, nor will all fungitoxic spots absorb at 254 nm.

This technique has been successfully used in the study of phenylthiosemicarbazide and various derivatives[2] and benomyl and its conversion products[3], among others fungicides, and is presently being used to investigate the non-enzymatic and metabolic conversion of a fungitoxic piperazine derivative, W 524 (= N,N'-bis-(1-formamido-2,2,2-trichloroethyl)piperazine) (Boehringer Sohn, Ingelheim am Rhein, G.F.R.). In addition to *Cladosporium cucumerinum*, many other fungi proved to be excellent test organisms for the detection of fungitoxic compounds. For instance, benomyl and its conversion products could be bioassayed by using, among others, *Aspergillus niger*, *Ascochyta pisi*, *Botrytis cinerea*, *Colletotrichum lindemuthianum*, *Fusarium culmorum* and *Penicillium expansum*, as is shown for four of these in Fig. 1; *Glomerella cingulata*, however, proved not to be sensitive to benomyl.

All results obtained so far show, that direct spraying of thin-layer plates with conidial suspensions of fungi is a most useful, easy and rapid technique for the detection of fungitoxic substances. The pliable aluminum thin-layer plates have the additional advantage of being easily cut with scissors, and they also can be preserved without the risk of loosening the silica gel layer.

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