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A SCREENING METHOD FOR ORGANOCHLORINE AND -PHOSPHORUS PESTICIDE RESIDUES IN VEGETABLES USING THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A screening method is described for the semi-quantitative determination of the contamination level of organochlorine and -phosphorus pesticides in fruit and vegetables using thin-layer chromatography. The separations and R_F values are reproducible using this method that is sensitive and useful in confirmation of identity of the most widely used and typical pesticides; common organochlorine and organophosphorus pesticides are selectively eluted from extracts without a preliminary purification procedure and detected. The method allows one to determine whether a vegetable is contaminated below the tolerance limits, detecting residues as low as 0.05 p.p.m. of organochlorine and p.p.b. of organophosphorus pesticides.

INTRODUCTION

In the framework of our collaboration with the "European Economic Community (EEC) Committee for Standardisation of Analytical Methods for Pesticide Residues", we developed a screening method in fruit and vegetables using thin-layer chromatography (TLC) in parallel to gas chromatographic (GC) procedures. In our opinion, the aims of such a screening method are (a) to show whether a vegetable is contaminated above a certain level, (b) to determine by which class of pesticides it is contaminated, (c) to identify, if possible, the contaminating pesticides, and (d) to determine if the contamination level is lower than the tolerance limits allowed by law (Table I).

Several methods have been proposed in literature for separation and identification of different pesticides. Each method, generally, separates a chosen mixture of one single class by developing a series of chromatograms on different adsorbents, by different eluents (also binary and tertiary mixtures of different ratios) and, what is worse, with non-reproducible results, always needing spots of single components for the identification.

By the method described here a contaminated vegetable is examined by separating 16 out of 17 more representative and used pesticides on two plates under re-

TABLE I

TOLERANCE LIMITS (in p.p.m.) PROPOSED BY EEC FOR SOME OF THE MOST WIDELY USED PESTICIDES[1]

Pesticide	*Tolerance (p.p.m.)*
Azinphos-ethyl	0.4
Malathion	3.0
Parathion	0.5
Methylparathion	0.5
Paraoxon	0.5
pp'-DDT	1.0
Lindane	2.0
Aldrin	0.2
Dieldrin	0.2
Carbaryl	3.0

producible conditions. The method that we propose is perhaps more time-consuming than classical TLC procedures, but, in our opinion, this drawback is greatly compensated for by the additional information obtained and the excellent sensitivity.

In practice, we studied a chromatographic method that was able (a) to elute organochlorine and organophosphorus pesticides; (b) to have the lowest detection limits; (c) to have the best possible resolution for some of the most widely used and typical pesticides used for vegetables; (d) to be easily reproducible.

For (a) we observed that hexane on alumina eluted only organochlorine while methylene chloride on silica gel brought these substances to the solvent front, separating the organophosphorus compounds. The differentiation between the two classes is also favoured by using the detection methods on alumina, incorporating $AgNO_3$, as described by ABBOTT *et al.*[2] for organochlorine, and of the cholinesterase inhibition of organophosphorus pesticides as described by ACKERMANN[3]. Condition (b) the sensitivity of the two methods was 50 ng and approx. 0.5 ng respectively. To satisfy condition (c), only activity gradient techniques aid in separation of complex sample mixtures with components varying widely in polarity; for such a purpose we found the Vario-KS-Chamber* (ref. 4) useful because it permits several possibilities of gradients and a continuous development. Regarding condition (d), reproducible results are guaranteed by using the same adsorbent, controlling its activity[5] via relative humidity in a specially adapted chromatographic chamber. If R_F values are reproducible, the R_{st} and R_k values and the separation are reproducible as well[6]. In Figs. 2 and 4 the influence of a change in relative humidity (which controls the layer activity) is shown.

To characterise a contamination, we have chosen 17 pesticides (8 chlorinated, 8 phosphorated and 1 carbamate), and we looked for the best selective separation. Using a test solution (Table II) containing the above-mentioned pesticides, we spike pesticide-free extracts to the tolerance limits. The spiked extracts are used as reference for a simultaneous analysis of several samples under investigation. A comparison of the corresponding spot dimensions permits one to estimate the contamination level in a semi-quantitative way.

* Manufactured by Camag (Muttenz, Schweiz).

TABLE II

TEST SOLUTION (IN DISTILLED CHLOROFORM) USED FOR SPIKING THE EXTRACTS

Solution	*mg/l*	*Solution*	*mg/l*
Aldrin	4.0	Parathion	10.0
pp'-DDE	20.0	Methylparathion	10.0
op'-DDT	20.0	Malathion	60.0
pp'-DDT	20.0	Azinphos-ethyl	8.0
pp'-DDD	20.0	Carbaryl	60.0
Lindane	40.0	DDVP	10.0
Endrin	4.0	Paraoxon	10.0
Dieldrin	4.0	Malaoxon	60.0
Ethion	10.0		

EXPERIMENTAL

Extraction

The extracts are prepared according to the EEC method actually under investigation. Basically this method is a twofold acetonitrile /water–chloroform/ chloroform extraction, but any other extraction method can be used as well. Starting with a 100-g sample, we obtain a final 5-ml volume of extract in chloroform.

Spiked samples

Based on the proposed[1] tolerance limits (adding some arbitrary ones when not given in ref. 1) we prepare the test solution of the composition given in Table II. The contamination is such that when mixing the test solution and extract (1:1), the vegetable has a resulting contamination of the tolerance limits.

First plate: organochlorine pesticides

A 250-μ thick layer of Alumina DS-5 (Camag) is prepared from a slurry formed by shaking 55 g of adsorbent with 60 ml of 0.4% (w/v) aqueous silver nitrate solution (dose for five 20 × 20 cm plates) for 2 min. The prepared plates are dried in an oven at 110° for 1 h. On the cooled plate, 10 μl of the sample solution are spotted as a single application and an equal amount of extract, spiked to the tolerance limits, is spotted nearby. The plate is afterwards placed on a Vario-KS-Chamber for conditioning for 60 min at 18% rel. humidity, over conditioning trays filled uniformly with 60.6% sulphuric acid solution. Then the plate is eluted continuously (no front line was made) for 1.5 h. For the detection, the plate is exposed to moisture for some minutes before irradiating with a germicidal UV light source (Philips TUV, 15 W); within 20 min pesticides will appear as black spots on a white background.

Second plate: organophosphorus pesticides

At first, it is necessary to prepare (a) a rat liver homogenate (To 1 part of rat liver 3.5 parts (w/v) of iced distilled water are added and the mixture is homogenised at 3000 r.p.m. for 10 min. The solution is filtered on paper and the filtrate is centrifuged at 3000 r.p.m. for 15 min before decantation. To maintain the initial activity, the homogenate is stored in 1-ml tubes in a freezer.); (b) a bromine-saturated solution

in distilled water, freshly prepared; (c) a freshly prepared solution containing 4 ml of 2-naphthyl acetate solution (125 mg/100 ml of ethanol) and 16 ml of Fast Blue B solution (20 mg in 16 ml of distilled water).

1 μl of the sample solution is spotted on a 250-μ thick Silica Gel G (Merck) plate with an equal amount of extract spiked to the tolerance limits as reference. The plate is placed on a Vario-KS-Chamber arranged with the conditioning trays for an "anti-parallel" humidity gradient as shown in Fig. 3. 3, 3, 3, 3, 3, 13, 47, 58, 64 and 72% rel. humidities correspond to 77, 77, 77, 77, 77, 64.7, 45, 40, 36 and 33% sulphuric acid solutions. After conditioning for 60 min, chromatography is carried out using methylene chloride, the solvent front travelling 15 cm from the start. After evaporation of the solvent, the detection is obtained in four steps[3]. (1) The pesticides are activated by spraying lightly with solution (b). (2) When the odour of bromine is no longer present on the plate, it is sprayed with about 10 ml of liver homogenate (a) previously diluted with distilled water (1:3). (3) The plate is stored in a climatized atmosphere at 37°, having a high humidity (80–90%), for 30 min. (4) Then it is vapourized with about 5 ml of solution (c). Pesticides will appear as white spots on a violet background.

RESULTS AND DISCUSSION

Up to ten extracts can be simultaneously analysed on the two plates having as reference the corresponding extract spiked to the tolerance limits. The first plate gives information about the contents of organochlorine pesticides. In Fig. 1 extracts of lettuce, apple, cabbage and carrot are compared with the corresponding ones spiked

TABLE III

MIGRATION DISTANCES (IN CM) OF THE REFERENCE COMPOUNDS AS IN FIG. 1

References compounds	*cm*
pp'-DDE	11.5
op'-DDT	10.4
pp'-DDT	9.1
Aldrin	7.5
Lindane	3.5
pp'-DDD	3.0
Endrin	1.9
Dieldrin	1.4

to the tolerance limits; the migration distances (in cm) of the reference pesticides are listed in Table III with a standard deviation of 0.5 cm.

In Fig. 1 the relative spot dimensions are in relation to the tolerance limits (*e.g.* 2 p.p.m. for Lindane, 0.2 p.p.m. for Aldrin).

The detection limit for each reference chlorinated pesticide is 50 ng. For our extracts (20 g of sample per ml chloroform), spotting 50 μl, a contamination as low as 0.05 p.p.m. is easily detectable.

For organochlorine pesticides the best adsorbent is Alumina DS-5 (Camag).

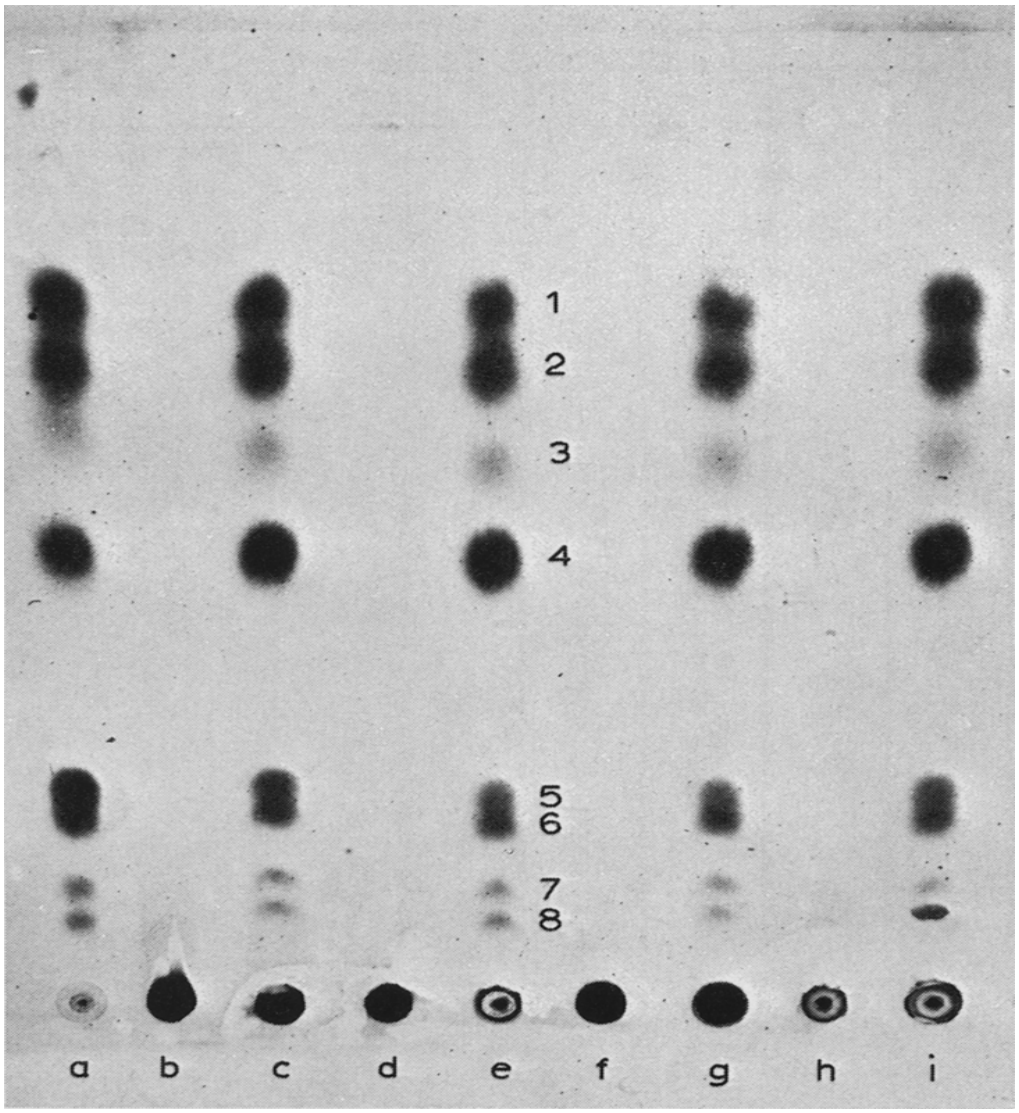

Fig. 1. Chromatogram of 4 extracts "pesticide free" and spiked to the tolerance limits with organochlorine pesticides: (a) test solution; (b) lettuce; (c) spiked lettuce; (d) apple; (e) spiked apple; (f) cabbage; (g) spiked cabbage; (h) carrot; (i) spiked carrot. All spots, 10 μl: (1) DDE, (2) *op'*-DDT, (3) *p,p'*-DDT, (4) Aldrin, (5) Lindane, (6) *pp'*-DDD, (7) Endrin and (8) Dieldrin. Alumina DS-5 (Camag) incorporating $AgNO_3$ conditioned 60 min at 18% relative humidity, Vario-KS-Chamber, 90 min continuous elution with cyclohexane.

We preferred this adsorbent because of its particular selectivity* towards the separation of DDE and Lindane and for the slower tendency to darken also during the irradiation process. In Fig. 2 the importance of controlling the humidity is shown; going from 65 to 18% rel. humidity the order is modified twice, passing from Aldrin–*pp'*-DDT–*pp'*-DDD–Lindane to *pp'*-DDT–Aldrin–Lindane–*pp'*-DDD. An elution time twice the normal one (15 cm elution) is required for ameliorating the separation.

The second plate gives information about the content of organophosphorus pesticides. In Fig. 3 extracts of lettuce, apple, cabbage and carrot are chromato-

* This selectivity was observed to be fading from one charge to another, perhaps due to different degrees of hydration of the gypsum binder.

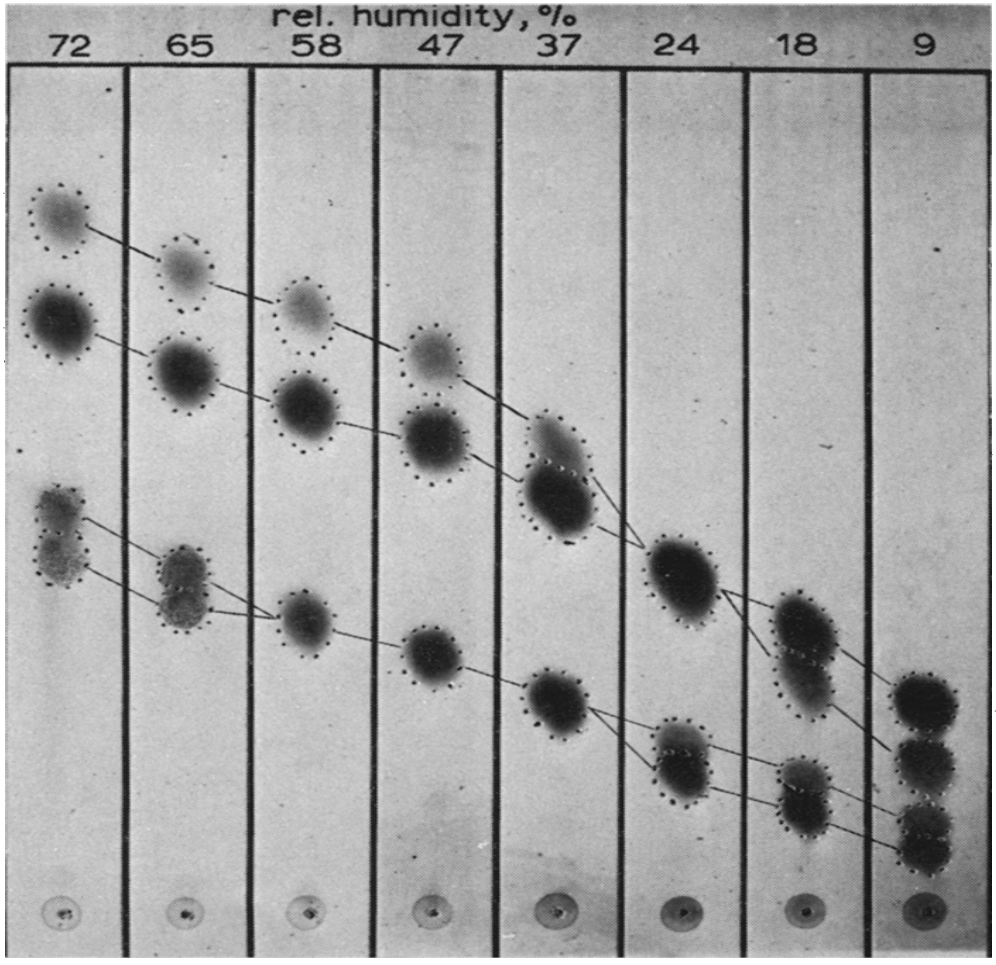

Fig. 2. Influence of humidity on the separation of chlorinated pesticides. Order of separations at 9% relative humidity: *pp'*-DDT, Aldrin, Lindane, *pp'*-DDD; at 72% relative humidity: Aldrin, *pp'*-DDT, *pp'*-DDD, Lindane. Alumina DS-5 (Camag) incorporating $AgNO_3$, Vario-KS-Chamber, orthogonal humidity gradient, cyclohexane.

graphed with the corresponding extracts spiked to the tolerance limits. The nine phosphorus pesticides* are listed in Table IV with the corresponding R_F values (standard deviation 5 hR_F) and sensitivities.

Pigments and other vegetable substances do not generally interfere seriously since they have different R_F values and bright colours, but difficult cases cannot be excluded.

In order to justify the experimental technique used for separating the organophosphorus pesticides, some considerations have to be made. A standard TLC plate (15 cm distance) has just sufficient space for the separation of, let us say, 10–15 spots, if these are equidistant from each other, which, of course, never happens. In the literature[3,7–11] R_F values of more than 80 organophosphorus pesticides are listed. Hence, it is impossible to separate all of them on a single TLC plate. Thus, for example, ACKERMANN[3] chose different mixtures as benzene–acetone or *n*-hexane–benzene–

* Carbaryl (Sevin), a carbamate pesticide, is here considered as an organophosphorus pesticide because of its similar chromatographic properties and its ability to inhibit cholinesterase[3].

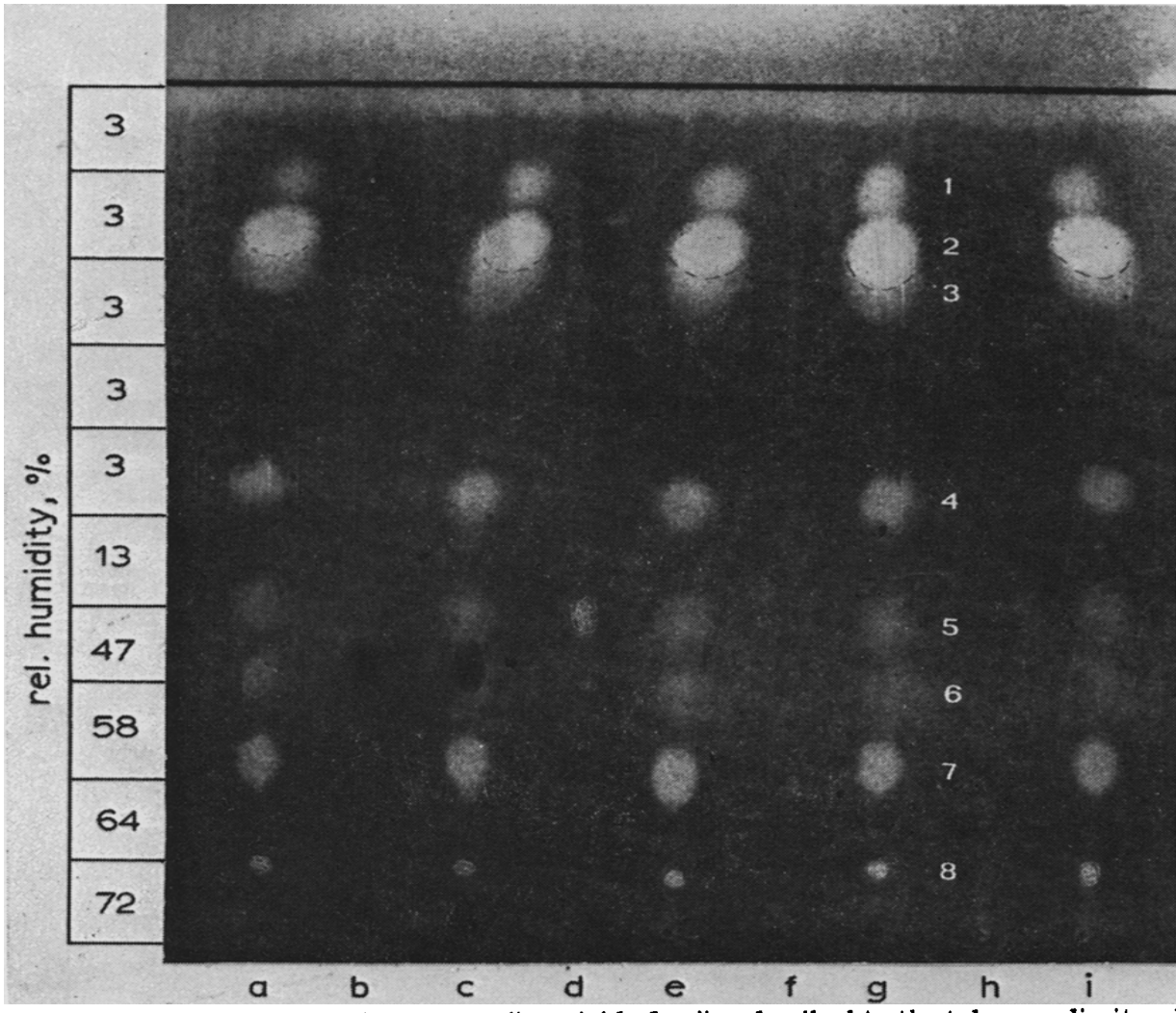

Fig. 3. Chromatograms of 4 extracts "pesticide free" and spiked to the tolerance limits with organophosphorus pesticides: (a–i) as in Fig. 1. Spot 0.5 μl: (1) Ethion, (2) Parathion, (3) Methylparathion, (4) Malathion + Azinphos-ethyl, (5) Carbaryl, (6) DDVP, (7) Paraoxon and (8) Malaoxon. Silica Gel G (Merck), Vario-KS-Chamber, antiparallel humidity gradient, methylene chloride.

TABLE IV

hR_F VALUES AND SENSITIVITIES OF REFERENCE ORGANOPHOSPHORUS PESTICIDES, AS IN FIG. 3

Pesticide	hR_F	*Sensitivity (ng)*
Ethion	89	1.0
Parathion	81	0.2
Methylparathion	77	0.2
Malathion	48	0.2
Azinphos-ethyl	48	0.2
Carbaryl	37	0.5
DDVP	28	0.2
Paraoxon	19	0.2
Malaoxon	7	10.0

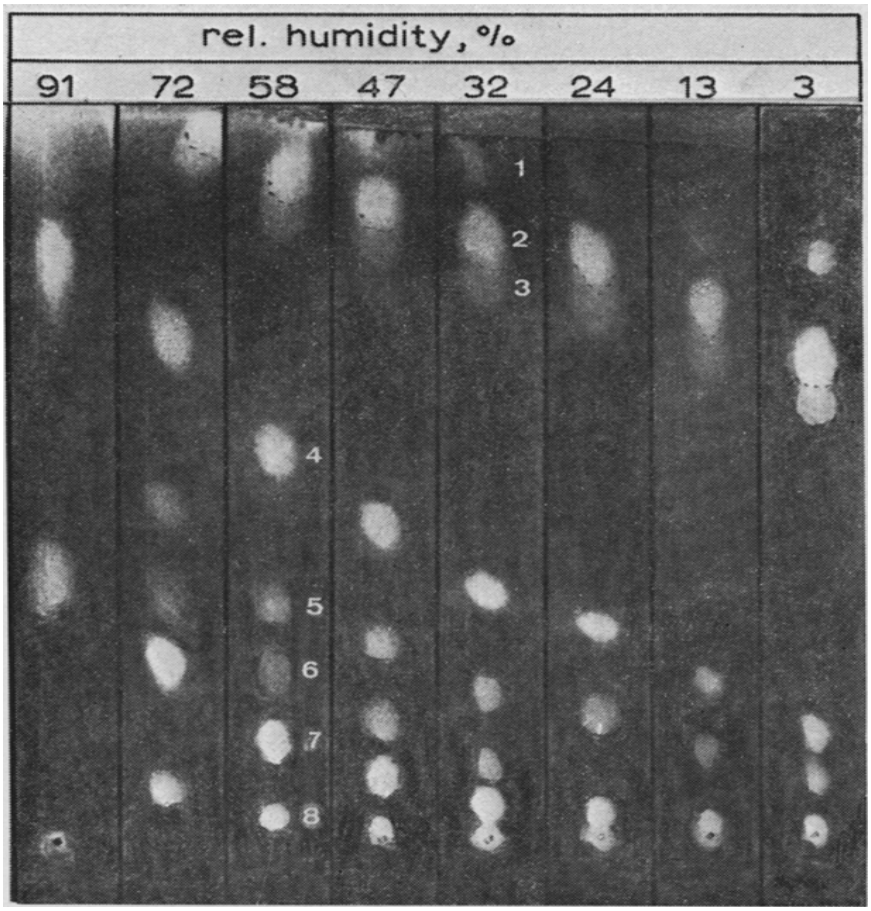

Fig. 4. Influence of humidity on the separation of organophosphorus pesticides. The order of separation is as in Fig. 3. Silica gel G (Merck), Vario-KS-Chamber, orthogonal humidity gradient, methylene chloride.

acetone of different composition, each of them separating* some (4–5) of the 38 compounds investigated, but never all of them. Other authors made other selections of compounds and hence had to use other solvents[7–11]. Therefore it is not astonishing that the separation of the 9 organophosphorus compounds chosen in the EEC list is not obtained by any of the forementioned methods. For example, by using an N-chamber and acetone–benzene mixture[3] or hexane–acetone mixture[7,10] as eluents, dependent on the solvent composition, the compounds are poorly resolved either in the upper or in the lower part of the plate, or, at the best, divided in two narrow regions, one in the upper and the other in the lower part of the plate. Thus we had to look for another technique. In Fig. 4 the influence of relative humidity on the separation is shown; only at a humidity as low as 3%, are Ethion, Parathion and Methyl parathion separated, while only at a higher humidity (62%) are the other five compounds satisfactorily separated. So, if we arrange a chromatographic system having in the lower part of the plate a high humidity, there the low-lying 5 compounds will separate; if the upper part of the plate is conditioned at 3% rel. humidity, the remaining 3 fast moving compounds will be resolved. Both regions are linked by steps of inter-

* The criterion for separation is a mean distance of 5 hR_F.

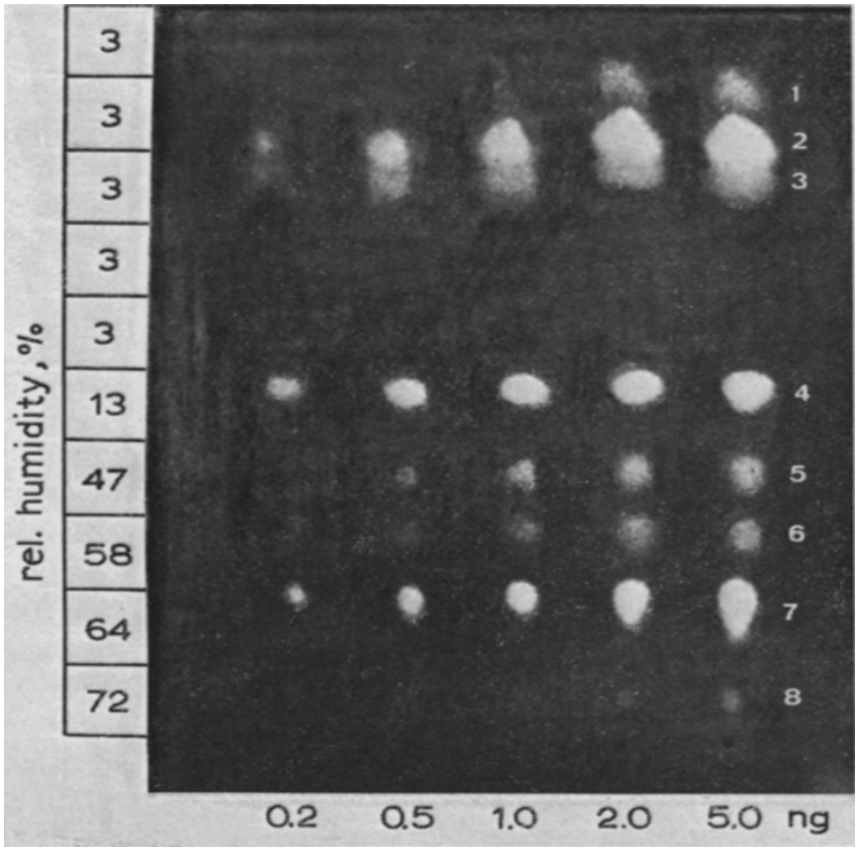

Fig. 5. Sensitivity test for organophosphorus pesticides: concentrations in ng/compound (Malaoxon is 10 times more concentrated). Silica Gel G (Merck), Vario-KS-Chamber, antiparallel humidity gradient as in Fig. 3, methylene chloride.

mediate humidities. Thus on the same plate in a single development we have well separated 8 or 9 spots (and there is space for others, if necessary!). Such an arrangement (humidity decreasing, *i.e.* increasing activity in the direction of the solvent flow) is a so-called "anti-parallel" activity gradient*, which has proved to be one of the more promising ways for separating a complex mixture of compounds varying widely in polarity[6,13].

In Fig. 5 a sensitivity test for the reference pesticides is shown at equal concentrations under identical chromatographic conditions. From these data, it is demonstrated that for a pesticide with 0.5 ng sensitivity, a contamination as low as the p.p.b. level can be easily detected.

Occasionally, as in Fig. 3, we observe that our unspiked apple extract contains a pesticide with a hR_F value equal to that of Carbaryl.

CONCLUSIONS

Detection methods

We have tested, as far as possible, all methods described in the literature balancing sensitivity and simplicity. For organochlorine pesticides, in our opinion,

* "Antiparallel" related to the solvent flow[12].

the method of ABBOTT *et al.*[2] is the most sensitive and simple. For organophosphorus pesticides, several papers have been published on the cholinesterase inhibition by MENDOZA *et al.*[7,14], ACKERMANN[3], WINTERLIN *et al.*[15] and quite recently by ERNST *et al.*[16]. Although for some single pesticides other enzymatic sources (*e.g.* honeybee brains[16]) seem to give a higher sensitivity, we preferred to use rat liver because rats are frequently used in biological laboratories. Experiments made with beef liver gave no better results.

Layer thickness

In contrast to what has been suggested in literature[3,7,14–16] we have found no remarkable advantage by using 450-μ instead of 250-μ-thick silica gel layers; thus we preferred to use the traditional 250 μ layer.

"Pesticide free" extracts

We had some problems in obtaining "pesticide free" extracts. Although the vegetables we used were not directly treated with pesticides, an unintentional contamination was present in apples and cabbages. Fortunately, a comparison with fortified samples showed that the contamination level was about 10% of the tolerance limits and so, nevertheless, we used them as "pesticide free" extracts.

Quantitative approach

By direct visual comparison, spots differing in size by 30% from the reference are easily distinguished. Therefore for fruit and vegetable samples, a decision (accepted or not accepted) can be taken if the contamination level is far enough from the tolerance limits. In doubtful cases, a GC or specific colorimetric determination has to be made.

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