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Esterase inhibition technique for the detection of organophosphorus pesticides on thin-layer chromatograms

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The determination of traces of organophosphorus insecticides in foodstuffs often presents some difficulties where large numbers of samples have to be analysed. The need for a rapid, low cost screening procedure is especially felt by those laboratories which have to provide results for the enforcement of pesticide residue legislations. Gasliquid chromatography with the phosphorus specific thermionic detector (1) is often difficult to adapt as a routine method, because of the capricious nature of many commercial available devices. Thin-layer chromatography yields excellent separations of a great number of organophosphorus pesticides, but the sample extracts need to be cleaned-up thoroughly when chemical chromogenic sprays (2, 3) are used. Even when the clean-up requirements are met, no chemical chromogenic reagent is selective and sensitive enough to be applicable in all cases. Moreover, organophosphorus pesticides are already degraded shortly after application. As a result of exposure to sun, air and enzymes, derivatives are formed which may be present in the final foodstuffs as toxic substances. Most of the existing official analytical methods, however, will only detect the parent compounds, which may already have been reduced to a permissible level. These difficulties may be overcome by using enzymatic methods, because nearly all organophosphorus insecticides are, or can be converted into, potent inhibitors of the group of enzymes which are called cholinesterases. These enzymes are widespread in nature and their classification has been found to be very difficult. Strictly speaking, a cholinesterase is an enzyme which specifically catalyses the hydrolysis of acetylcholine. This substrate specificity is however, by no means absolute. Many organophosphorus compounds inhibit also enzymes which are able to hydrolyze noncholine esters. In residue analysis, where various substrates are used, the term esterase inhibition is, therefore, somewhat more appropriate. Considerable work has been reported concerning the detection of cholinesterase inhibitors on paper chromatograms (4, 5). Enzymatic detection on thin-layer chromatograms proved to be more difficult, because all early attemps to obtain inhibition directly on the plates were unsuccessful (6). It is only during the last years that workers as *El Rafai* (7), *Ackermann* (8), *Mendoza* (9) and some others have coupled thin-layer chromatography with suitable enzymatic detection techniques. However the routine application of these procedures has proved to be rather difficult because it has not always been possible to reproduce the reported results in other laboratories. The purpose of this paper is to propose some simple methods of enzymatic detection which have proved to be reproducible in our laboratory on a routine scale. The sensitivity and specificity achieved will permit

analysis of smaller quantities of samples and will lower substantially the requirements imposed on clean-up procedures.

The methods here described can be divided in four steps:

1. Thin-layer chromatography of the sample extracts.
2. Oxydation of the thiophosphates to active esterase inhibitors with bromine.
3. Inhibition of the esterases from a spray containing a suitable enzyme source, in the areas on the layer occupied by the pesticides.
4. Application of the substrate and the development of a background colour by revealing the hydrolysis product.

1. Thin-layer chromatography of the sample extracts

The adsorbant of choice is silica-gel G: Plates of 20×20 cm can be coated, dried and activated in the usual way. Very practical is the use of ready made plates: for example those of Merck, SiO_2G, catalogue number 5554/0025. These plates are very tightly coated which makes them easier to handle. An additional advantage is the better reproducibility of the Rf-values, although these are generally lower than those observed on hand-layered plates. Suitable quantities of sample extracts and reference solutions are spotted at 1,5 cm intervals. Sample extracts of fruits and vegetables in chloroform or methylene chloride rarely need any cleanup (10). The quantity to be spotted depends of course on the limit of detection of the pesticide(s) sought for. See table II. The plates are developed by ascending chromatography over a distance of 15 cm. For the mobile phases to be used we recommend three different types:

a) Chloroform-acetone, 9 : 1 v/v for very polar insecticides and for the oxygen analogues of the thiophosphates.

b) Pentane-ethylacetate, 4 : 1 v/v for the less polar thiophosphates.

c) Pentane-methylene chloride-ethyl ether, 8 : 2 : 2 v/v for special separations, for example in case of simultaneous presence of parathion, methylparathion and malathion.

The Rf-values of 13 organophosphorus compounds + some of their primary derivatives in the three separatory systems are listed in table I.

Table I

Pesticide	$R_f \times 100$ in system		
	a	b	c
Parathion	83	62	72
Paraoxon	62	6	—
Methylparathion	78	43	64
Methylparaoxon	51	3	—
Malathion	78	39	53

Malaoxon	55	3	—
Fenitrothion	79	48	67
Fenitro-oxon	56	4	—
Guthion (Azinphos)	70	12	27
Guto-Oxon	40	0	—
Diazinon	71	46	47
Diazoxon	35	2	—
Dursban	87	73	79
Dimethoate	19	0	2
Phosdrin (mevinphos)	35, 24	3	7,4
Dichlorvos (DDVP)	52	9	16
Dibrom	60	21	23
Dipterex (trichlorphon)	8	0	0
Co-Ral (Coumaphos)	81	35	58
Coro-oxon	58	—	—

2. Oxydation of the thiophosphates to active esterase inhibitors

Conversion of thionophosphates and dithiophosphates to their esterase inhibiting oxygen analogues is carried out by exposing the plate during 30 seconds to bromine vapour. This is carried out by placing the chromatogram in an all glass tank, for example, a large desiccator, previously saturated with bromine vapour. The plate is then positioned in a strong draft of air for about 20 minutes to remove the excess bromine.

The organophosphates DDVP, phosdrin and dibrom do not need bromine treatment, because they possess already the P = O functional group associated with esterase inhibition.

Diptese insecticide is a special case: it does only inhibit esterase enzyme after partial dehydrochlorination to dichlorvos.

If this substance has to be detected, the chromatogram is sprayed with 5 percent aqueous ammonia solution and the hydrolyses is allowed to take place for 15 minutes at room temperature.

3. Inhibition of the esterases: application of the enzyme

It is generally known that the organophosphorus insecticides differ greatly in their ability to bring out significant esterase inhibition. Their inhibitory power depends to a large extent on the enzyme source. Malathion, for instance, inhibits very strongly the esterase present in the erythrocytes of some mammalian species, but it is quite ineffective in its ability to inhibit bovine serum esterase. There is no doubt that this dissimilarity is due to variations in the esterase composition.

Already mentioned in the literature is the use of homogenates of bovine (8) or rabbit liver (5) and saline extracts of fly-heads (10) or honeybee brains (11).

Evidently, these somewhat unusual reagents may be difficult to obtain, especially for small laboratories. We have looked, therefore, for commercially avai-

lable enzyme sources with a more or less constant esterase activity. Among the several possibilities we have found that lyophilized human and horse serum from the Nutritional Biochemical Corp. (Cleveland Ohio 44128, USA) were the most suitable. These chemicals keep almost indefinitely in the freezing compartment of a refrigerator.

The chromatogram is sprayed thoroughly with a 0,5 % aqueous solution of lyophilized horse serum and the reaction between pesticide and esterase is allowed to proceed for about 30 minutes at 37 ° C.

4. Substrate application and revelation of the hydrolysis product

The substrate solution is prepared just prior to spraying by dissolving 5 mg of beta-naphthyl acetate in a 2 ml of absolute ethanol. The quality of the naphthyl acetate is very important: it must not be too old and should not contain any free naphthol. For the final revelation a solution of 20 mg Fast Blue salt (di-o-anisidine-tetrazoliumchloride) in 20 ml of distilled water is added. The solutions are mixed and sprayed on the chromatogram. Hydrolysis of the substrate is best carried out by incubating the plate in a humid atmosphere at 37 ° C. Within 15 minutes the areas occupied by the pesticides are revealed as white or yellowish-white spots on a reddish-violet background due to the reaction between the liberated naphthol and the Fast Blue salt.

This revelation method is very sensitive: nanogram quantities of most organophosphorus pesticides can be observed without difficulty. The limits of detection for the individual compounds are given in Table II.

Alternative detection method

For a few pesticides, like malathion and dimethoate, the sensitivity achieved in the above described procedure is not sufficient.

There is also the possibility of interference: phenolic compounds present in some fruit extracts may yield coloured reaction products with the diazonium reagent which sometimes mask the inhibition zones of the pesticides sought for. In those cases, we recommend the use of another detection system based on the reaction:

$$\underset{\text{acetylcholine}}{CH_3COOCH_2CH_2N^+(CH_3)_3} + H_2O \longrightarrow \underset{\text{choline}}{HOCH_2CH_2N^+(CH_3)_3} + \underset{\text{acetic acid}}{CH_3COOH}$$

Unfortunately, we could not perform this reaction directly on silica-gel plates. It proved, however, very easy to transfer the pesticides from the chromatogram to a layer of agar wherein the reaction proceeded without difficulty.

The agar layer is prepared by dissolving 5 g of Bacto-agar in 320 ml of boiling water; 35 mg of bromothymol blue, dissolved in 0,5 ml of 0,1 N aqueous sodium hydroxide are added and the solution is allowed to cool. When the temperature has dropped to approximately 50 ° C, the pH is adjusted to 7,9 and the agar solution is mixed with 75 ml of 1 % aqueous solution of lyophilized human serum. The gel mixture is evenly poured on a plexiglass plate of 44×24

cm with an edge of 8 mm high. The final layer of solidified agar is about 4 mm thick and can be used for the simultaneous revelation of 2 thin-layer chromatograms.

The chromatogram is developed and exposed to bromine vapour in the way already described. Before performing the revelation, the silica-gel layer thould be neutralized completely by spraying the chromatogram with 0,5 N aqueous sodium hydroxide containing bromothymol blue as an indicator. The spraying is continued until the plate has an even blue-ish appearance. Subsequently, the plate is carefully pressed on the agar medium. Complete contact is ensured by placing a heavy glass plate on the back side of the chromatogram.

Transfer of the pesticides from the silica-gel to the agar is allowed to take place for at least one hour. The chromatogram is then removed and the agar layer is flooded with a freshly prepared 0,4 % aqueous solution of acetylcholine iodide.

This substrate diffuses into the agar and the acetic acid split off by the enzyme changes the colour of the indicator from blue to yellow. After about 30 minutes at room temperature the esterase inhibiting pesticides appear clearly as dark blue spots against the yellowing opaque background.

In this way a «negative» picture of the chromatogram is obtained which shows only the spots of the pesticides present. Other substances from the sample extract like chlorophyl, carotenoids etc. remain on the silica-gel. This technique is especially suitable for the analysis of extracts containing much extraneous material. Its sensitivity is often better than that obtained in the naphthyl acetate method as is shown in Table II.

Table II

Limits of detection achieved by the enzymatic revelation techniques expressed in nanograms

Pesticide	Contact agar method Acethylcholine / human serum	Direct method Naphthyl acetate / horse serum
Parathion	0,5	1
Methylparathion	5	10
Malathion	50	> 500
Fenitrothion	5	10
Guthion (Azinphos)	5	10
Diazinon	2	5
Dursban	not determined	0,1
Dimethoate	200	> 1000
Phosdrin (mevinphos)	0,5	5
Dichlorvos (DDVP)	1	1
Dibrom	10	0,5
Dipterex	2	2
Co-Ral	10	20

NB. The oxygen analogues of the thiophosphates generally have a detection limit close to that of their parent compound.

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Summary

1. Using the described method, reproducible results were obtained in the detection an identification of 13 organophosphorus pesticides and 7 oxidation products separated by thin layer chromatography on silica gel.
2. Three mobile phases of different polarity are proposed and the corresponding R_f values are indicated.
3. For the relevation of the chromatograms two esterase inhibition techniques are described:
   — by direct application of the reagents and the enzyme on the thin layer (direct method)
   — by transfer of the pesticides from the silica gel plate to an agar layer (contact agar method) containing the enzyme and an indicator.
4. The commercially available lyophilized horse and human serum were found to be the most suitable sources of esterase enzyme.
5. For the majority of the pesticides examined the limits of detection achieved vary between 1 and 10 nanograms.
6. The proposed method can be used as a screening procedure for routine analyses.

Résumé

1. La méthode décrite ci-dessus, nous a permis de détecter et d'identifier de façon reproductible 13 pesticides organophosphorés et 7 produits d'oxydation, séparés par chromatographie sur couche mince de silica gel.
2. Trois phases mobiles de polarité différente sont proposées avec les R_f correspondants.
3. Deux techniques de révélation par inhibition de l'esterase sont décrites:
   — la première par application directe des réactifs et de l'enzyme sur la couche mince (méthode directe),
   — la seconde par transfert des pesticides du chromatogramme sur une couche d'agar (méthode de transfert par contact) contenant l'enzyme et un indicateur.
4. Les sources d'enzyme (esterase) sont le serum de cheval et le serum humain lyophilisés, tous deux offerts couramment sur le marché des réactifs.
5. La limite de détection oscille entre 1 et 10 nanogrammes pour la majorité des pesticides examinés.
6. La méthode proposée se prête aux analyses d'orientation et de routine.

Zusammenfassung

1. Die beschriebene chromatographische Methode mittels Kieselgel-Dünnschichtplatten hat uns den Nachweis und die Identifizierung von 13 Phosphorsäureestern und 7 ihrer Oxydationsprodukte auf zuverlässige Weise erlaubt.
2. Drei mobile Phasen verschiedener Polarität werden vorgeschlagen; die entsprechenden R_f-Werte sind angeführt.

3. Für die Entwicklung der Chromatogramme sind zwei enzymatische Verfahren vorgeschlagen, beruhend auf der Hemmung der Esteraseaktivität:
   — Direkte Methode durch Auftragen der Reagentien und des Enzyms direkt auf die Dünnschichtplatte,
   — «Transfer»-Methode durch Ueberführung (Kontaktabdruck) der chromatographierten Pestizide auf eine Agarschicht, welche das Enzym und einen Indicator enthält.
4. Als Enzymquelle (Esterase) wurden die im Handel leicht erhältlichen lyophilisierten Pferde- und Humansera verwendet.
5. Die mit der Methode erfaßbaren minimalen Mengen Pestizid schwanken zwischen 1 und 10 Nanogramm für die Mehrzahl der untersuchten Substanzen.
6. Die vorgeschlagene Methode eignet sich für orientierende Untersuchungen und für Routineanalysen.

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