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Note

Quantitative determination of organophosphorus pesticides by thin-layer densitometry

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Thin-layer chromatography (TLC) is frequently used for the determination of organophosphorus pesticides in the study of environmental contamination of soil[1], air[2], water[2-5] and plant samples[6], as well as in the control of animal[7] and human food, fruits and vegetables[8,9]. The determinations for forensic and toxicological purposes[10-12] have also been described. This widespread application of TLC has resulted in numerous data being produced on the efficacy of separation on different layers[13,14] and with different solvent systems[15]. The sensitivity and selectivity of detection with a series of reagents for making the spots visible has also been studied[2,7,8,15-17], the best results being obtained by the detection of spots based on enzyme inhibition[4,6].

Evaluation of thin-layer chromatograms made so far, by comparison of the colour intensity and/or spot area with those obtained with standard solutions[1,7,8], is claimed by the authors to be semi-quantitative.

In the present work on the determination of organophosphorus pesticides, thin-layer chromatograms are evaluated quantitatively by direct densitometric measurements of the difference in the colour or fluorescence of spots and the layer after making the spots visible with silver nitrate or by enzyme inhibition.

EXPERIMENTAL

Materials

The organophosphorus standards used were as follows: dichlorvos (DDVP) (purity 93%), obtained from World Health Organisation (Geneva, Switzerland; trichlorfon, the product of Bayer (Elberfeld, G.F.R.); malathion (purity 96%), the reference standard Cat. No. 2576, obtained from EPA (Perrine, Fla., U.S.A.); and parathion (purity 99%), purchased from Bayer (Leverkusen, G.F.R.). Stock solutions were prepared by weighing the pure substances and dissolving them in ethanol. The same solvent was used for further dilution.

Plates were coated with a 0.5-mm thick layer of Kieselgel G nach Stahl (Typ 60, E. Merck, Darmstadt, G.F.R.). Thin-layer chromatograms were developed with the solvent system chloroform–acetone (1:1)[11]. For the separation of malathion and parathion, if necessary, the solvent system acetone–benzene–*n*-hexane (5:2:13)[18] was

used. The solvents were purified in the usual way and freshly distilled fractions were taken daily for the detection by enzyme inhibition.

The silver nitrate reagent used for detection[11] consisted of a solution of 2.0 g of AgNO$_3$ in 25 ml of double-distilled water that was made up to 100 ml with acetone. This solution was left to stand for 10–15 days in the refrigerator before use. The enzyme solution[19] was a 10% aqueous solution of rat plasma diluted with Sörensen's buffer (pH 8) in the ratio 1:1 v/v, the substrate solution A[19], a 0.5% solution of N-methylindoxyl acetate in the solvent system acetone–water (2:3) and the substrate solution B[20], a 1 mg/ml solution of indophenyl acetate in absolute ethanol.

Instrument

A Camag T-scanner was used for densitometric measurements.

Procedures

In all experiments the volume applied on the thin-layer plate was 10 μl. A series of standard solutions, including the stock solution, was prepared daily for the determination of trichlorfon.

Detection with silver nitrate. After development and evaporation of the solvents, the chromatograms were sprayed with the reagent for detection. Uniformly coloured spots, suitable for quantitative evaluation, appeared after the plates had been heated
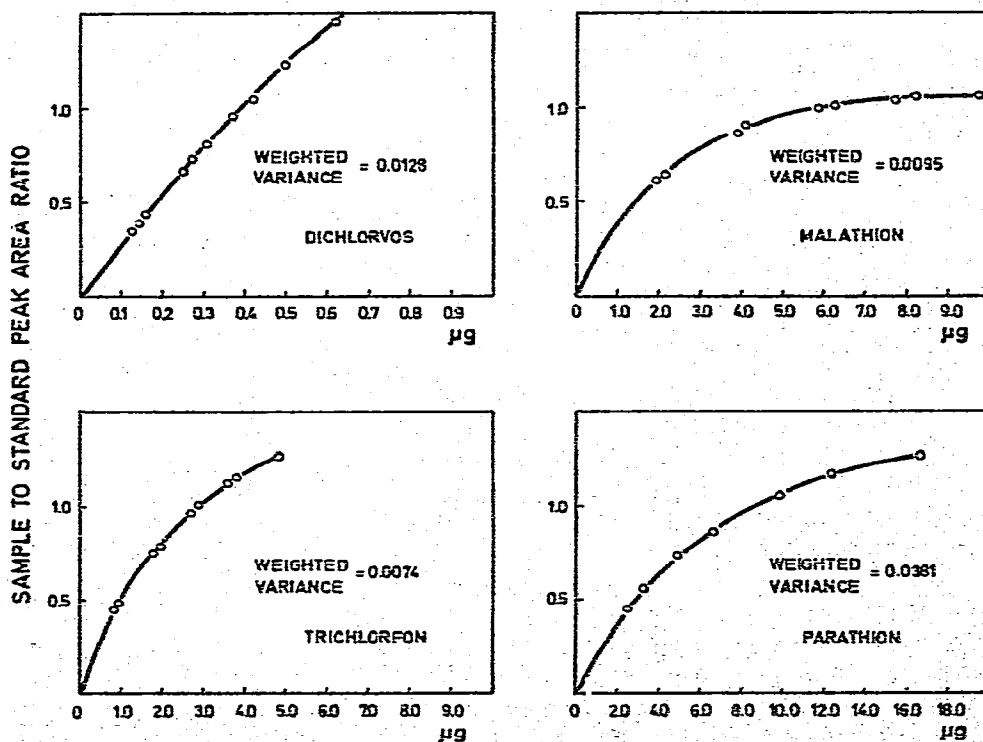

Fig. 1. Calibration curves for quantitative TLC analysis of organophosphorus pesticides, with AgNO$_3$ as reagent for spot detection. (Plots of the fitted functions.)

for 30 min at 135°. The spots were brown for dichlorvos, yellow-brown for trichlorfon, dark yellow for malathion and pale yellow for parathion. Densitometric measurements were performed with a VIS light source, using only a primary filter (460–470 nm).

Detection by enzyme inhibition. The developed and dried chromatograms with dichlorvos and/or trichlorfon spots were uniformly sprayed with the enzyme solution and placed in a moisture-saturated atmosphere for 30 min. The wet plates were then cautiously sprayed with substrate solution A. After a few minutes, dark violet spots became visible in UV light (366 nm) against the intensively fluorescent background. After spraying with the substrate solution, the chromatograms were placed in a dark place and the optimum values measured after 30 min.

Densitometric measurements were performed with a VIS light source, using both a primary (360 nm) and a secondary (415 nm) filter.

Bromination[21]. Parathion and malathion were oxidized by exposing the chro-
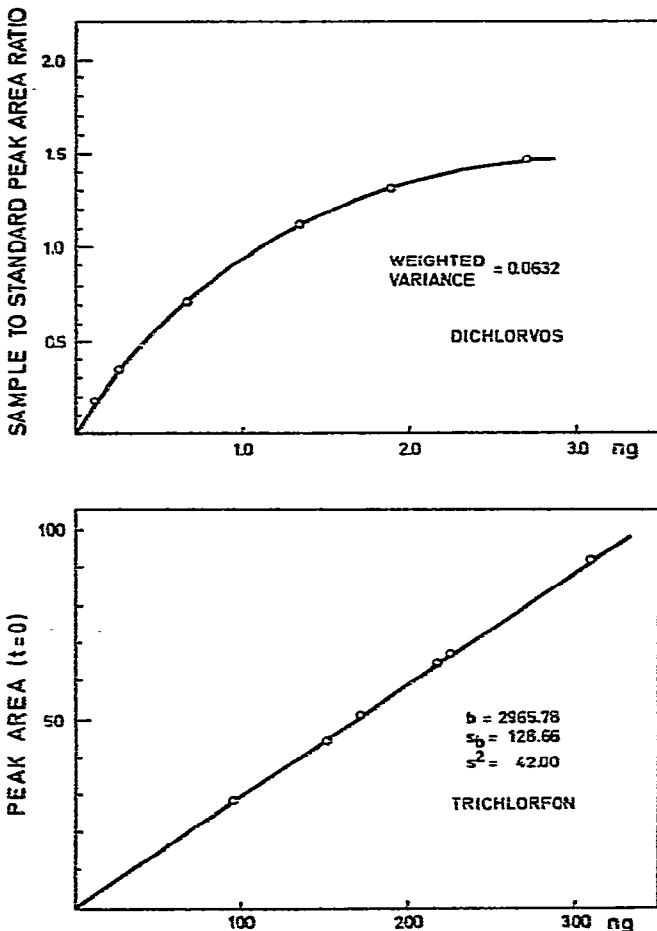

Fig. 2. Calibration curves for quantitative TLC analysis of dichlorvos or trichlorfon. The detection of spots was performed by inhibition of acetylcholinesterase activity with indoxyl acetate as fluorogenic substrate. (Plot of the fitted function for dichlorvos.)

matograms to bromine vapour during 5 min. The adhering bromine was then expelled by flushing the chromatograms with air for 20 min.

The plates with a 0.25-mm thick layer were sprayed with the enzyme solution and placed for 30 min in a tank saturated with water vapour. The wet chromatograms were then sprayed with substrate solution B and dried at 37° for 20 min. Light yellow spots on a pink background were measured with a VIS light source using only the secondary filter (510 nm).

RESULTS AND DISCUSSION

The calibration curves for the quantitative determination of organophosphorus pesticides were constructed by taking the ratio of sample peak area to standard peak area as the ordinate so as to eliminate possible variations in experimental conditions. The peak areas were determined by the Monte Carlo method[22]. The ranges of linearity
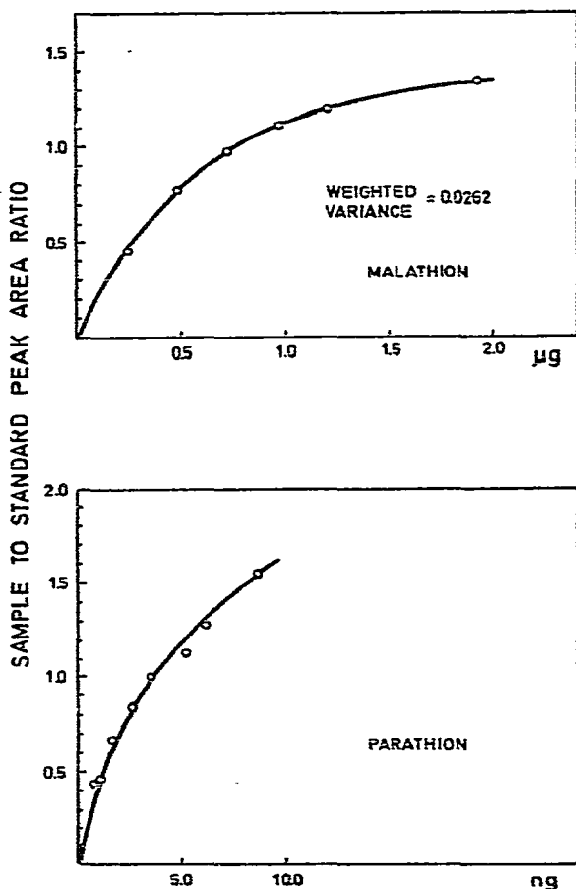

Fig. 3. Calibration curves for quantitative TLC analysis of malathion and parathion. The detection of spots was performed by inhibition of acetylcholinesterase activity with indophenyl acetate as chromogenic substrate. (Plot of the fitted function for malathion.)
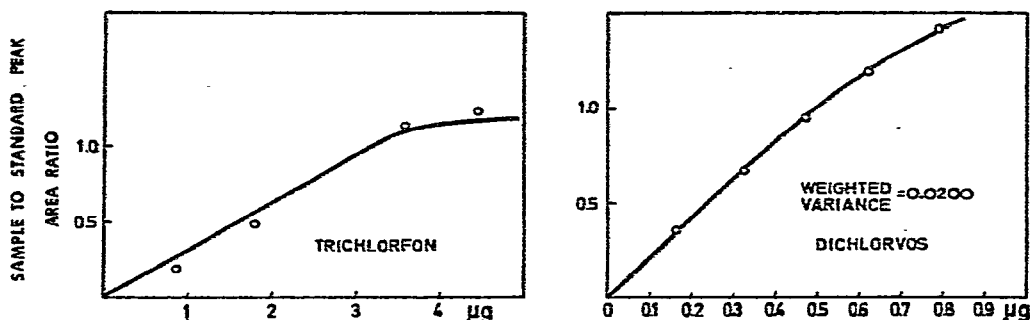
Fig. 4. Calibration curves for quantitative TLC analysis of dichlorvos and trichlorfon present together, with AgNO₃ as reagent for spot detection. (Plot of the fitted function for dichlorvos.)

and reproducibilities obtained for the studied pesticides with the procedures described are shown in Figs. 1–3.

As the conversion of trichlorfon into dichlorvos is a spontaneous process the assessment of its extent at a given time has been the objective of numerous studies. Considering the different toxicities of trichlorfon (LD_{50} for female rats, 560 mg/kg) and dichlorvos (LD_{50} for female rats, 56 mg/kg) on the one hand, and the readiness for conversion under biological conditions on the other, the usefulness of the procedure, which permits the simultaneous determination of these pesticides, becomes evident.

In the experiments with silver nitrate as the reagent for spot detection, no evidence was found to indicate that the conversion of trichlorfon into dichlorvos takes place during the analysis. The reproducibility of results for the two compounds present together is acceptable for amounts of 0.1–1 μg and 1–4 μg for dichlorvos and trichlorfon, respectively (Fig. 4). However, the much higher sensitivity obtained by spot detection based on acetylcholinesterase inhibition by organophosphorus pesticides proves that the conversion proceeds continuously.

The thin-layer chromatographic assay of trichlorfon, with spot detection based on enzyme inhibition, yielded an intense spot with an R_F value characteristic of dichlorvos and a slightly visible spot with the R_F value of trichlorfon (Table I). The determinations of trichlorfon performed at fixed time intervals from the start of the experiment and extrapolation to zero time make is potsible to assess directly, but only approximately, the amount of trichlorfon initially present.

TABLE I

R_F VALUES OF ORGANOPHOSPHORUS PESTICIDES

Solvent systems: I, chloroform–acetone (1:1); II, benzene–acetone–n-hexane (10:25:65).

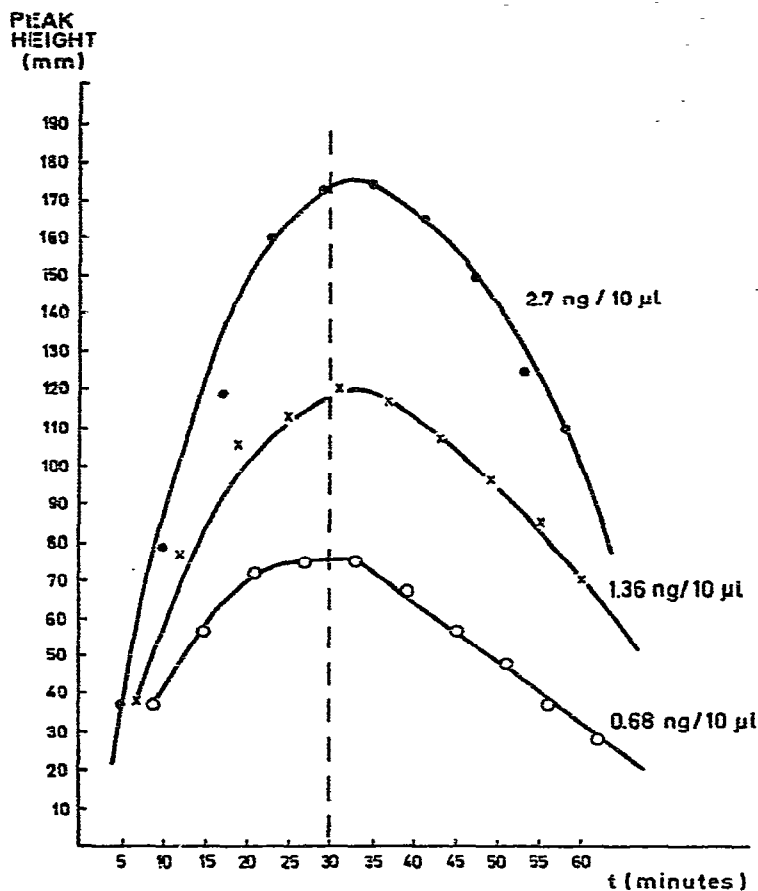| Compound	R_F value	
	I	II
Dichlorvos	0.52	0.38
Trichlorfon	0.36	
Malathion	0.62	0.53
Parathion	0.62	0.64

Fig. 5. Dependence of the fluorescence quenching intensity on time elapsed from the moment of spot provocation.

The measurement of fluorescence quenching intensity as a function of time elapsed between the spraying with the substrate and the densitometric measurement showed optimum and reproducible values within the interval 5–65 min. As the maximum value is reached after about 30 min (Fig. 5), the spots of the standard were measured at the 28th and 34th minutes and those of the sample at the 30th and 32nd minutes.

The bromination procedure was applied in order to convert the parathion and malathion into their corresponding oxidation products, which are suitable for detection by enzyme inhibition. It was shown in a series of experiments that for malathion the sensitivity of detection does not depend on the time of bromination. However, the best results for parathion were obtained with a bromination time of 5 min (Fig. 6).

The analytical procedures with the carefully defined experimental conditions described above were successfully applied to the determination of organophosphorus pesticides in an aqueous environment and in toxicological research.

The detection with silver nitrate although neither as selective nor as sensitive
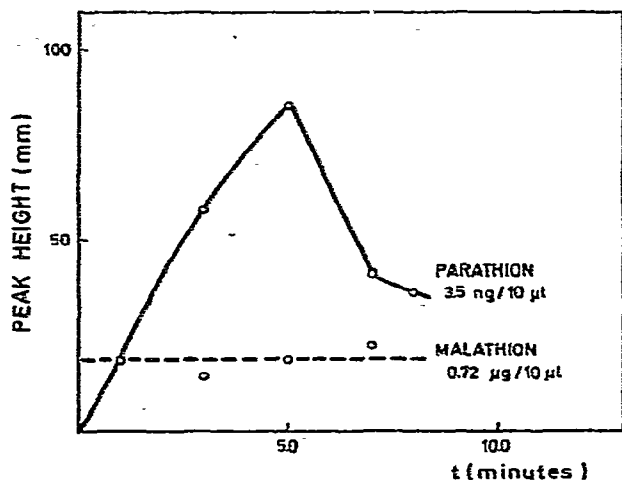
Fig. 6. Dependence of spot intensity of malathion and parathion on the bromination time.

as the detection by enzymic inhibition, proved to be useful for the determination of dichlorvos and trichlorfon present simultaneously in amounts of 0.1–1 $\mu g/\mu l$ and 1–4 $\mu g/\mu l$ respectively.

ACKNOWLEDGEMENTS

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