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FLAVONES AS FLUOROGENIC SPRAY REAGENTS FOR ORGANOTHIOPHOSPHORUS PESTICIDES ON SILICA GEL LAYERS

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SUMMARY

A method is discussed for the detection and quantitative determination of organothiophosphorus pesticides by *in situ* fluorometry after separation on silica gel layers. Yellowish-green fluorescent spots are obtained when the plate is sprayed with a 3-hydroxyflavone, such as robinetin, after bromination. An attempt is made to explain this phenomenon. Linear calibration curves up to 4 μg per spot of the pesticide have been obtained and a relative standard deviation of approximately 4% can be expected at the 1.0-μg level. Visual and instrumental detection limits are around 0.04 μg per spot for certain pesticides.

INTRODUCTION

The use of flavones as fluorogenic spray reagents is relatively new. ČERNÝ *et al.*[1] used alumina layers impregnated with morin for the detection of steroids. Substances containing α,β-unsaturated keto groups formed dark spots on a yellow fluorescent background under UV light. Other compounds were visible as bright yellow spots on a darker background. In all cases 100 μg or more of the steroids had been spotted.

SCHELLENBERG[2] detected N-protected amino acids and peptide derivatives on silica gel thin-layer chromatograms after spraying the layer with a 0.05% solution of morin and heating at 100° for 2 min. Yellow fluorescent or dark absorption spots were observed under UV light. The limit of detection for most compounds was around 2 μg per spot. Morin has also been employed for the detection of phenols and nitrophenols[3].

Recently it was found[4,5] that quantities as low as 0.02 μg of some organic pesticides could be detected on cellulose layers sprayed with 3-hydroxyflavones such as flavonol, fisetin and robinetin. Yellow fluorescent spots were observed on a relatively non-fluorescent background.

In this study, the use of flavones as spray reagents on silica gel layers is investigated, since most of the thin-layer chromatographic (TLC) methods that have been reported for organophosphorus pesticides make use of this type of adsorbent.

EXPERIMENTAL

Chemicals and apparatus

The flavones fisetin, kaempferol, quercetin, morin, and rutin were purchased from Fluka A.G., Chemische Fabrik, Buchs S.G., Switzerland; chrysin, apigenin and acacetin from Aldrich Chemical Co., Inc., Milwaukee, Wisc., U.S.A.; robinetin-aglucone from Koch-Light Laboratories, Colnbrook, Bucks., Great Britain; and flavonol from Eastman Organic Chemicals, Distillation Product Industries, Rochester, N.Y., U.S.A. Unless otherwise stated, 0.05% solutions of the flavones were prepared in ethanol.

The pesticides were supplied as analytical standards by several manufacturers as listed by KENAGA AND ALLISON[6]. Stock solutions of the pesticides (spotted by means of 1-μl Microcaps) were prepared 1000 p.p.m. in *n*-hexane from which dilution series were made. All the solvents were redistilled.

Fluorescence spectra were measured with the Aminco-Bowman Spectrophotofluorometer with TLC attachment. Quantitative work was carried out with the Zeiss Chromatogram-Spectrophotometer.

Chromatography and detection

The chromatoplates (20 × 20 cm) were coated 250 μ thick with a mixture of 30 g of Silica Gel N (Macherey, Nagel and Co., Duren, G.F.R.) in 80 ml of water by means of a Desaga TLC applicator. After drying in air the plates were placed in an oven at 105° for 30 min before use. For chromatographic separation the pesticides were spotted 2 cm from the bottom of the plates and developed 10 cm in *n*-hexane–acetone (5:1)[7].

After removal from the chromatographic chamber the plate was dried for 5 min at 105°. While the plate was still hot it was brominated for 10 sec in a chromatographic tank containing a 10% solution of bromine in carbon tetrachloride; after a few minutes of cooling it was sprayed in excess with the particular flavone. The plate was then activated in the oven for another 5 min to produce the fluorescence.

In situ qualitative and quantitative studies

The procedure for recording the fluorescence spectra with the Aminco-Bowman spectrophotofluorometer has already been described[8].

To investigate the fading behaviour with the flavone robinetin, a 1-μg sample of Trithion was spotted, eluted, brominated, and sprayed, and the disappearance of the fluorescence with time was monitored instrumentally. For reproducibility studies, nine spots were developed simultaneously on one plate and scanned in the direction of elution. In all studies the M-365 excitation filter and the emission monochromator set at 505 nm were used for optimum conditions with the Zeiss Chromatogram-Spectrophotometer. For quantitative work a 0.1% solution of robinetin in ethanol was employed.

RESULTS AND DISCUSSION

The flavones used in this study are listed in Table I. In previous work[4,5], only

TABLE I

EVALUATION OF FLAVONES ON SILICA GEL

Common name	*Chemical name*	*Fluorescence of background*
Flavonol	3-hydroxyflavone	bluish-green
Fisetin	3,3′,4′,7-tetrahydroxyflavone	light yellow
Robinetin	3,3′,4′,5′,7-pentahydroxyflavone	dark yellow
Kaempferol	3,4′,5,7-tetrahydroxyflavone	light yellow
Quercetin	3,3′,4′,5,7-pentahydroxyflavone	slightly yellow
Morin	2′,3,4′,5,7-pentahydroxyflavone	yellowish-green
Chrysin	5,7-dihydroxyflavone	none
Apigenin	4′,5,7-trihydroxyflavone	none
Acacetin	5,7-dihydroxy-4′-methoxyflavone	none
Rutin	quercetin-3-rutinoside	light yellow

3-hydroxyflavones showed a slight yellow fluorescence on cellulose layers. However, on silica gel, which is a much more polar medium, both 3-hydroxy- and 3,5-dihydroxyflavones were fluorescent, whereas, as expected, the 5-hydroxyflavones were not. Heating in the oven for 5 min at 105° resulted in fading of the background fluorescence with the 3-hydroxyflavones in the order flavonol < fisetin < robinetin. Without heating the fading would take several hours. No change was observed for the other flavones.

Direct spraying with robinetin of silica gel plates spotted with the pesticides DDT, Trithion, Proban, Malathion, prometryne, CIPC, Sevin, and Baygon resulted in yellow fluorescent spots against a relatively quenched background after heating

TABLE II

DETECTION LIMITS OF ORGANOTHIOPHOSPHORUS PESTICIDES

Pesticide	*No. of sulphur atoms*[a]	*Visual limit of detection (μg)*
Trithion	3	0.04
Guthion	2	0.06
Parathion	1	0.1
Proban	1	0.1

[a] The influence of type and number of sulphur atoms on the fluorescence has been discussed (see ref. 8).

in the oven for 5 min at 105°. However, the method was not sensitive (~ 5 μg per spot). Considerable improvement in the detection limit was noticed with organothiophosphorus pesticides if the plate was brominated before spraying with the flavone (see Table II). As in previously discussed work[4], the fluorescence phenomenon in this method was specific to 3-hydroxyflavones with the 5-position unsubstituted. Robinetin was preferred over fisetin and flavonol, due to its lower background fluorescence.

The fluorescence spectral data for the three 3-hydroxyflavones on silica gel are shown in Table III. The spectra obtained for both Guthion and Trithion sprayed with robinetin after bromination are identical, suggesting that the fluorescence is

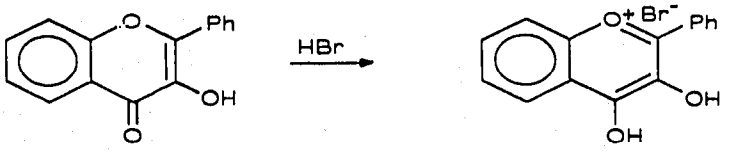
TABLE III

FLUORESCENCE SPECTRAL DATA ON SILICA GEL LAYERS

Fluorescent spot	*Wavelength (nm)*	
	Excitation	*Emission*
Flavonol	410	480
Fisetin	425	500
Robinetin	430	510
Robinetin + Guthion + Br_2	370	505
Robinetin + Trithion + Br_2	370	505
Robinetin + HBr	370	505

independent of the pesticide. Furthermore, these spectra are markedly different from those of the flavone robinetin. It is believed that upon bromination of the organothiophosphorus pesticides, hydrobromic acid is formed and eventually causes the fluorescence. The spectrum of robinetin sprayed on HBr supports this.

Fluorescence emission is caused by a change of the electronic arrangement in the ring formed through 3-hydroxyl and 4-carbonyl[9]. The effect of HBr on robinetin or any other 3-hydroxyflavone is assumed to be a protonation of the carbonyl oxygen to form a highly fluorescent species of the type shown below. Such salts are known to exist and have been isolated[10]. The phenomenon that occurs upon spraying of the

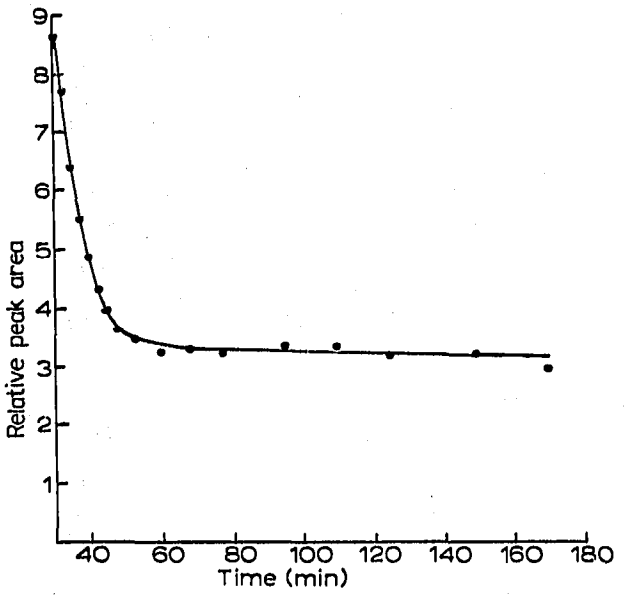


robinetin on silica gel layers (*i.e.*, the background is highly fluorescent but disappears on standing or with the influence of heat) is probably due to strong intermolecular forces, such as hydrogen bonding, which can cause quenching of the fluorescence[11]. This type of interaction is quite important on silica gel layers.

Fig. 1 illustrates the fading characteristics of a fluorescent spot obtained by spraying 1 μg of brominated Trithion with robinetin. A 62% decrease in fluorescence is observed for the first hour, followed by a zone of reasonable stability. It seems that the spot is extremely fluorescent under anhydrous conditions, but as the plate cools off and moisture is re-adsorbed the fluorescence intensity decreases until a saturation point is reached. Reproducibility studies were therefore carried out after 1-h standing in the dark. An average value of 4.7% relative standard deviation was obtained for 1-μg spots ($n = 9$) of Trithion.

Calibration plots of fluorescence *vs.* concentration for the pesticide Trithion sprayed with robinetin are shown in Fig. 2. The plots are linear up to about 4 μg per spot. The curvature above 4 μg can be attributed to concentration quenching effects which have been reported previously[8]. Below 0.1 μg the reproducibility of the method becomes rather poor and the use of standard addition techniques is recommended. Similar results were obtained with Parathion.

Various types of silica gel, such as Silica Gel H, Silica Gel HR, Silica Gel G, and Silica Gel S (all Merck reagents), and also MN Silica Gel N from Macherey, Nagel



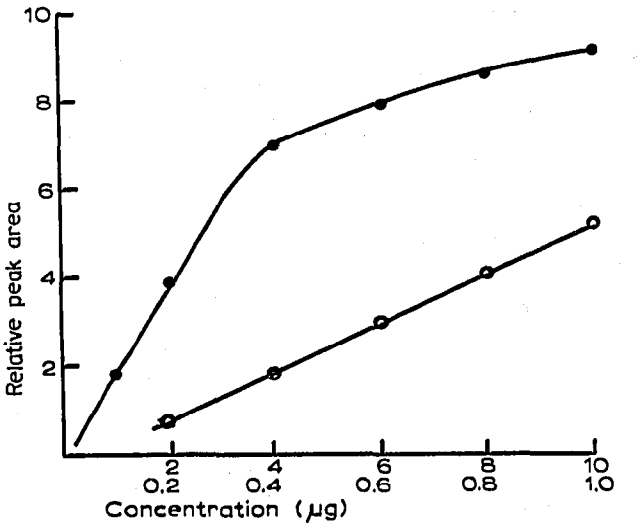


Fig. 1. Study of the fluorescent fading of a spot of brominated Trithion sprayed with robinetin.

Fig. 2. Calibration curves for Trithion. ●, 1.0–10 µg; ○, 0.2–1 µg.

and Co., have been tested. The best results were obtained with silica gel without binder. For the bromination step, the best results were obtained by brominating for 5–10 sec. Longer bromination causes an increase in background fluorescence. The influence of varying the concentration of the spray reagent was studied also. Quantitative work was feasible with 0.025, 0.05, and 0.1% solutions in ethanol (w/v), but reproducibility as well as fluorescence intensity were best at 0.1%.

CONCLUSIONS

The method described offers another approach to the determination of organothiophosphorus pesticides by *in situ* fluorometry. The possibility of being able to use flavones as spray reagents on silica gel layers is advantageous, since many separation procedures have been reported on this matrix. Even though it is slightly less sensitive and precise when compared to the SAQH–Mn procedure* described earlier[8], it is equally simple, and the reagents are readily available from commercial sources. Since linear calibration curves going through the origin are obtained below 4 µg per spot, it should be possible to apply standard addition techniques and thus increase the practical concentration range of the method. Since other parameters are the same as in the SAQH–Mn method[8], the procedure should be adaptable to the quantization of subparts per billion levels of organothiophosphorus pesticides in water samples without major clean-up and to more complex biological samples in conjunction with suitable clean-up methods. It should offer a valuable alternative to gas–liquid chromatography and serve as a check method.

* This method is similar to that published previously[12] except that the chelating agent is salicyl-2-aldehyde-2-quinolylhydrazone (SAQH).

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REFERENCES

1 V. ČERNÝ, J. JOSKA AND L. LÁBLER, *Collect. Czech. Chem. Commun.*, 26 (1961) 658.
2 P. SCHELLENBERG, *Angew. Chem., Int. Ed. Engl.*, 1 (1962) 114.
3 G. TADEMA AND P. A. BATELAAN, *J. Chromatogr.*, 33 (1968) 460.
4 V. MALLET AND R. W. FREI, *J. Chromatogr.*, 54 (1971) 257.
5 V. MALLET AND R. W. FREI, *J. Chromatogr.*, 56 (1971) 69.
6 E. E. KENAGA AND W. E. ALLISON, *Bull. Entomol. Soc. Amer.*, 15 (1969) 85.
7 J. ASKEW, J. H. RUZICKA AND B. B. WHEALS, *Analyst*, 94 (1969) 275.
8 R. W. FREI AND V. MALLET, *Environ. Anal. Chem.*, in press.
9 T. HAYASHI, K. HARA, S. KAWAI AND T. OHNO, *Chem. Pharm. Bull. (Tokyo)*, 18 (1970) 1112.
10 W. C. BLASDALE, *J. Amer. Chem. Soc.*, 67 (1945) 491.
11 B. L. VAN DUUREN, *Chem. Rev.*, 63 (1963) 325.
12 P. E. BELLIVEAU, V. MALLET AND R. W. FREI, *J. Chromatogr.*, 48 (1970) 478.