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AN INVESTIGATION OF FLAVONES AS FLUOROGENIC SPRAY REAGENTS FOR ORGANIC COMPOUNDS ON A CELLULOSE MATRIX*

PART I. GENERAL DISCUSSION OF THE METHOD

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SUMMARY

Ten flavones were investigated in this study as possible fluorogenic sprays for polar organic compounds separated on cellulose thin-layer chromatograms. Baygon (2-isopropoxyphenyl-N-methylcarbamate) was chosen as the test system since the authors are primarily interested in pesticide analysis. Intensely yellow spots were observed on slightly yellow fluorescing backgrounds with solutions of fisetin, robinetin and flavonol, which are all flavonols with unsubstituted 5-positions. An attempt has been made to explain this fact.

The fluorescence phenomenon is believed to be attributable to an enhancement effect on the fluorescence of the flavones caused by the relatively high polarity of the adsorbed organic molecule in the chromatographic spot.

The fluorescence spectra of fisetin and background are shown and visual observation of the high stability of the fluorescence suggests the possible use of flavones for quantitative studies of pesticides.

INTRODUCTION

Only few methods for the analysis of pesticides by *in situ* fluorometry on thin-layer and paper chromatograms have appeared in the literature. Chlorinated insecticide spots, for instance, can be rendered fluorescent after the chromatogram is sprayed with N-methylcarbazole or Rhodamine B[1]. Buquinolate (ethyl 4-hydroxy-6,7-diisobutoxy-3-quinoline carboxylate) was determined in chicken feeds by BORFITZ *et al.*[2] who used a chromatographic–fluorometric method. Fluorescent whiteners, known as Calcofluors, have been applied successfully to the visualisation of carbamates, uracils and ureas[3].

A procedure for the fluorescent determination of phosphate esters on paper chromatograms was suggested by GORDON *et al.*[4] and later applied by RAGAB[5] to pesticides on silica gel layers. This method involved bromination followed by spraying with ferric chloride and finally with 2-(*o*-hydroxyphenyl)benzoxazole to produce

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fluorescent spots for a number of organophosphate insecticides. The method was later improved by FREI and co-workers[6] who made use of a combination of new reagents and salt solutions. Recently, an *in situ* fluorometric method for the quantitative determination of Sevin was developed[7]. In this method Sevin was hydrolysed with sodium hydroxide directly on the plate to the highly fluorescent anion of α-naphthol.

In the present study, flavones were investigated as fluorogenic sprays for the visualisation of pesticides on cellulose layers. The chemistry of flavones has been extensively studied and is well presented in a book by GEISSMAN[8]. These compounds have been used as analytical chelating agents for the determination of cations and some anions[9]. Many of the chelates were observed to be fluorescent. The only instance where a flavone has been applied to the detection of organic materials was reported by TADEMA AND BATELAAN for phenols and nitrophenols[10]. Fluorescence quenching was used.

Flavones were chosen for this investigation because of their ability to be almost non-fluorescent in a non-polar medium and extremely fluorescent in a polar one. It was thought initially that if such compounds were sprayed on a surface of low polarity such as cellulose, the background would remain weakly or non-fluorescent. The presence of a polar substance on the surface would enhance the fluorescence of the spray, hence enabling its detection as a spot.

In this particular work on pesticides, the insecticidal carbamate Baygon was chosen as the test sample since carbamates are polar compounds and also because of the great need for a fluorescent method suitable for their analysis.

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 3, N.Y., U.S.A.

Cellulose powder (MN-300, Macherey, Nagel and Co., Duren, G.F.R.) was used. A sample of Baygon was provided by Chemagro Corporation, Kansas City, Mo., U.S.A. It was recrystallised before use from 50 % ethanol. All the chemicals utilised in this study were of reagent grade quality and the solvents were distilled before use.

The fluorescence spectra were recorded with an Aminco-Bowman Spectrophotofluorometer with TLC attachment. A 230-W mercury UV lamp, Hanovia type 16106, was employed for visual observation of the fluorescence.

General procedure

Prior to use, the cellulose powder was washed twice in a mixture of isopropanol–ammonium hydroxide–water (6:3:1) and once in isopropanol alone, then dried in an oven at 105° for 8 h. The plates were then prepared (250 μ thickness) with a Desaga TLC applicator. A mixture of 15 g of cellulose in 85 ml of water homogenised in a blender was applied. After drying in air, the plates were eluted in ether to ensure the removal of any remaining organic impurities and dried in air immediately before use.

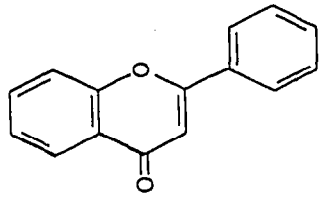
A standard solution of Baygon was prepared 1000 p.p.m. (1 mg/ml) in isopropanol and 1 μl of the pesticide solution was spotted with a 10-μl Hamilton microsyringe. Then the plate was sprayed lightly with a 0.05 % solution of the flavone in isopropanol, dried in air and observed under a long wavelength UV light source.

A TLC separation of Baygon was also carried out following a procedure proposed by RAGAB[11]. A cellulose plate was spotted with 1 μg of the pesticide, then sprayed with a 5 % solution of mineral oil in hexane (v/v), and eluted 10 cm in a solution of acetone–water, (50:50). The plate was dried and sprayed lightly with a 0.05 % solution of fisetin in isopropanol.

For the recording of fluorescence spectra, the plate was placed face down on the TLC attachment of the instrument. The spot was positioned visually in the light path and excitation and emission peaks recorded in the usual fashion.

RESULTS AND DISCUSSION

The structure of flavone is shown below (I). The flavone molecule may contain hydroxy groups at all the positions in the rings, except 1- and 4-, and is highly conjugated; a condition necessary for fluorescence.



(I)

The flavones utilised in this study are listed in Table I. Flavonol, fisetin, and robinetin are 3-hydroxyflavones; kaempferol, quercetin, and morin are 3,5-dihydroxyflavones; chrysin, apigenin, and acacetin are 5-hydroxyflavones; and rutin is a 3-O-glucoside of quercetin.

TABLE I

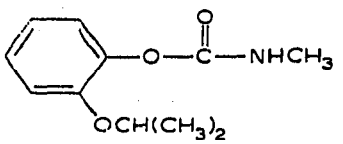
EVALUATION OF FLAVONES

Common name	*Chemical name*	*Fluorescence of background*	*Fluorescence of pesticide spot*[a]
Flavonol	3-hydroxyflavone	slightly yellow	yellow
Fisetin	3,3′,4′,7-tetrahydroxyflavone	slightly yellow	yellow
Robinetin	3,3′,4′,5′,7-pentahydroxyflavone	slightly yellow	yellow
Kaempferol	3,4′,5,7-tetrahydroxyflavone	none[b]	none
Quercetin	3,3′,4′,5,7-pentahydroxyflavone	none[b]	none
Morin	2′,3,4′,5,7-pentahydroxyflavone	none[b]	none
Chrysin	5,7-dihydroxyflavone	none	none
Apigenin	4′,5,7-trihydroxyflavone	none	none
Acacetin	5,7-dihydroxy-4′-methoxyflavone	none	none
Rutin	quercetin-3-rutinoside	none	none

[a] Baygon (1 μg) was spotted on a cellulose plate, sprayed with a 0.05% solution (isopropanol) of the flavone.

[b] In the cases of morin, quercetin, and kaempferol, the background becomes slightly fluorescent with a large excess of the spray.

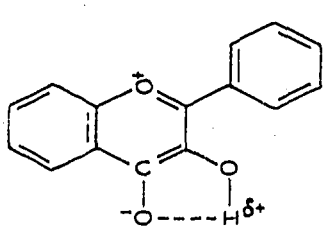
Table I summarizes the results obtained when a 0.05 % solution of the flavone is sprayed on a cellulose thin-layer plate. Only the 3-hydroxyflavones gave fluorescence of the background. The 3,5-dihydroxyflavones and the nonflavonols did not produce any fluorescence when sprayed lightly on the plate, although kaempferol, quercetin, and morin showed a slight fluorescence when sprayed in excess. In the cases of flavonol, robinetin, and fisetin, an intense yellow fluorescence developed on the plate where 1 μg of Baygon (II) had been spotted. Thus, this phenomenon is specific to the three 3-hydroxyflavones with the unsubstituted 5-position.



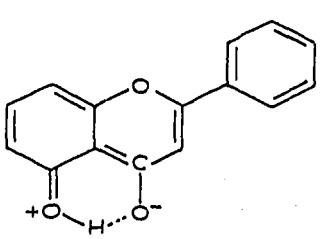
(II)

It is well known that flavonols (3-hydroxyflavones) are extremely fluorescent in a medium of high dielectric constant ($HClO_4$) or mild basicity[12] (NH_4OH), while others that do not possess the 3-hydroxy group, are not. A basic difference exists between 3-, and 5-hydroxyflavones. A number of studies by partition chromatography[13,14], IR[15], UV, and visible absorption[16], and NMR[12] have been carried out on the nature of the hydroxyl groups in the molecule and they all indicate that the 5-hydroxyflavone is much more hydrogen bonded than the 3-hydroxy species. According to SIMPSON AND GARDEN[13], the hydrogen bonding between the carbonyl and the 3-hydroxy group differs from the chelation of the 5-hydroxy group in that there is no mechanism in the former structure for the transfer of electrons from the hydroxyl to the carbonyl group.

It was suggested that for 3-hydroxyflavones (III) an electrostatic attraction is established between the hydrogen atom carrying a fractional positive charge, arising from the -I effect of the hydroxy-oxygen atom, and the carbonyl-oxygen atom. In the case of 5-hydroxyflavones (IV), resonance throughout the molecule permits the complete separation of charges between the carbonyl and hydroxyl groups thus strengthening the hydrogen bonding.



III



(IV)

From the IR studies[14] it was shown that introduction of a 5-hydroxy group into a 3-hydroxyflavone causes the absorption due to the O–H stretching to be intensified and shifted indicating a decrease in the overall hydrogen bonding. The fact that structures (III) and (IV) are cross-conjugated, *i.e.*, the two chelate systems are formed by mutually opposing mechanisms, would explain that the simultaneous chelation of 3- and 5-hydroxy groups is weaker than expected.

These resonance structures (III and IV) help to explain why 5-hydroxyflavones are not fluorescent while 3-hydroxyflavones are. In structure (IV), the "*n*" electrons of the oxygen atom in the pyrone ring are not delocalised as is the case for structure (III) and it is well known that these electrons can cause quenching or at least a reduction of the total fluorescence when present in a molecule[17]. Therefore it is not surprising that when both 3- and 5-hydroxy groups are present in the flavone molecule, as is the case with morin, the total fluorescence observed is less than that given when only the 3-hydroxy group is present. This is supported by the fact that only fisetin, robinetin, and flavonol gave positive results (Table I).

Although 3,5-dihydroxyflavones can be considered non-fluorescent on cellulose which is a weakly polar adsorbent, it seems logical to assume that they would become increasingly fluorescent on more polar surfaces with a shift in equilibrium to the five-membered ring structure (III). This has been partly confirmed in another study where significant fluorescence was observed on silica gel and alumina layers after spraying lightly with morin or other similar compounds.

Other effects which may be of importance are the pH of the plate, which differs somewhat on various chromatographic adsorbents, and also possibly a special kind of interaction (chemisorption) between substrate and spray reagent. Some fluorescence has been observed, for example, on silica gel plates after exposure to ammonia or bromine vapours and subsequent spraying. This new aspect is currently being investigated. The other factor of importance may be the difference in the hydrogen bonding between the spray reagent and the cellulose, which contains many hydroxyl groups, as compared to silica gel or alumina. Not much is known about these interactions and they are generally classified under the term chemisorption.

It was observed that 1 μg of Baygon could also very easily be detected on the plate with the fisetin spray after it had been sprayed with mineral oil and eluted in 50 % acetone[11], by reversed-phase chromatography. It was noticed, however, that in order to keep the background fluorescence to a minimum, freshly prepared cellulose plates had to be used and precleaning of the cellulose powder was essential. A number of eluting solvents, such as chloroform, hexane, carbon tetrachloride, pyridine, *n*-butanol, *n*-octanol, benzene, ethanol, 25 % acetic acid, ether, acetone, water, hydrochloric acid (1 %), and ammonia (1 %) were also investigated and no interference with the actual fluorescence phenomenon was observed.

The fluorescence spectrum of the fisetin background and that of the highly fluorescent Baygon spot are shown in Fig. 1. The excitation and emission wavelengths for the fluorescent spot are identical with those of the background, but of considerably increased intensity. Thus as had been expected, a fluorescence enhancement of the flavone is brought about by the pesticide. A similar phenomenon is also observed in solution if a few drops of fisetin are added to about 10 ml of a 1000 p.p.m. solution of Baygon. An intensive fluorescence results in comparison with the blank.

The fluorescence spectra of flavonol and robinetin were also recorded, with excitation and emission maxima for the flavonol background and spot being at 360 and 528 nm, and for the robinetin background and spot at 375 and 535 nm, respectively. In the case of flavonol, the background of the plate is strongly fluorescent, thus not very practical for analytical use, while robinetin behaves similarly to fisetin.

The neutral pH and the low polarity of the cellulose surface seem important criteria for reducing the fluorescence of the background. With further investigations

it may be possible to reduce background fluorescence by using a more purified form of cellulose or another adsorbent. Fluorescing agents other than flavones could also be investigated.

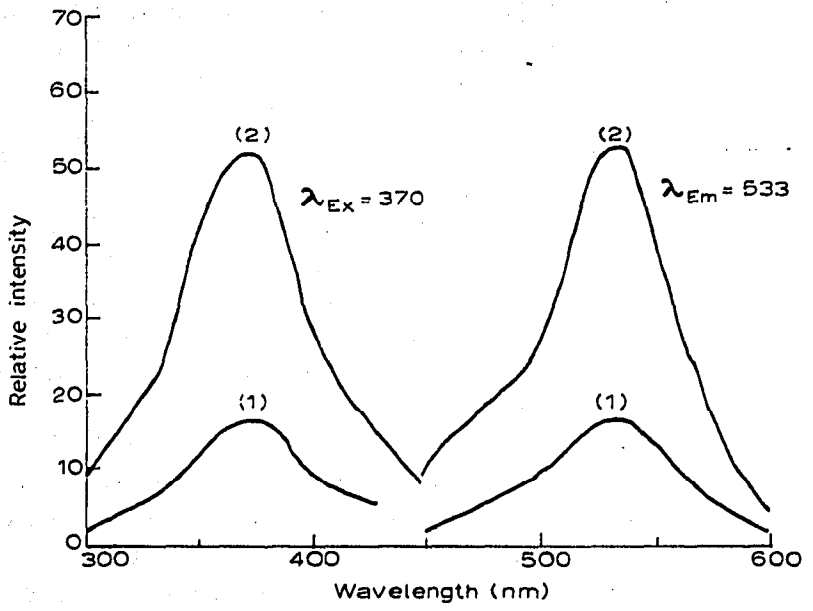


Fig. 1. Emission and excitation spectra of fisetin on cellulose layers. Conditions: (1) background; Meter Multiplier — 0.03; (2) Baygon spot; Meter Multiplier — 0.01.

CONCLUSIONS

The principle on the basis of which flavones are functioning as fluorogenic reagents, namely, the fluorescence enhancement of certain reagents due to an increase in polarity of the surrounding medium, opens a wide new field of investigation. Obviously, there should be a large number of other reagents that may behave similar to flavones and variations in the choice of chromatographic adsorbents will also yield interesting results as is shown by a similar study currently being carried out on silica gel.

While pesticides are the main interest to us in this investigation, further studies of the method are suggesting that the number of organic compounds that could be determined by this same approach is unlimited. The limitations of the resulting analytical methods will rest primarily with the efficiency of the chromatographic separation procedures.

The visually observed stability of the fluorescence produced depends solely on the reagent and is usually such that *in situ* quantitative determination of compounds on chromatograms seems feasible. Quantitative work is also supported by the fact that all the compounds would fluoresce at the same wavelength which would facilitate scanning procedures. Studies along those lines are currently in progress.

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