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## Interference of ethoxyquin in the thin-layer chromatographic estimation of aflatoxin

Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) can be used as a chemical preservative (antioxidant) for the purpose of retarding oxidative destruction of carotenes, xanthophylls, and vitamins A and E in certain foods, forage crops, and animal feeds. We have lately been informed that it might give fluorescence on thin-layer chromatograms resembling that of aflatoxin[1]. Since we have been involved in aflatoxin methodology for almost three years this question seemed to be worthy of investigation. In order to find out to what extent the presence of ethoxyquin can confuse thin-layer chromatographic identification of aflatoxin $B_1$, a number of chromatographic systems developed for aflatoxin determinations were applied.

*Experimental*

*Samples.* Four samples of commercial-grade ethoxyquin, received from different sources were examined: EQ I = ethoxyquin, unidentified source; EQ II = ethoxyquin, Koch-Light Laboratories Ltd., Great Britain; EQ III = santoquin, Monsanto Company, U.S.A.; and EQ IV = kurasan, Východočeské chemické závody Synthesia, Czechoslovakia.

A standard of aflatoxin $B_1$ (3 $\mu$g/ml in chloroform) was used for comparison of the $R_F$ values and the intensity of fluorescence of the ethoxyquin spots. The ethoxyquin samples were dissolved in chloroform in concentrations giving an intensity of fluorescence of the same order as the aflatoxin standard.

*Chromatography.* To compare the $R_F$ values of aflatoxin $B_1$ and ethoxyquin three kinds of thin-layer chromatoplates were used: 0.4 mm and 0.25 mm thick layers of Silica Gel G (Merck), activated 2 h at 100°, and commercial Silufol 254 UV plates, Kavalier, Czechoslovakia. Aflatoxin $B_1$ (0.03 $\mu$g) and ethoxyquin solutions (10 $\mu$l) were spotted separately in alternate order and together in order to observe their resolution. Ten different solvent systems were employed:

S1: 3 % methanol in chloroform[2,10];
S1a: ethyl ether following the S1 system[3];
S2: 5 % methanol in chloroform[4];
S3: 7 % methanol in chloroform[5];
S4: benzene–95 % ethanol–water (46:35:19) two phase[5];
S5: benzene–95 % ethanol–water (46:35:19) upper phase;
S6: 10 % acetone in chloroform, equilibrated[6];
S7: 10 % acetone in chloroform, unequilibrated[7];
S8: 15 % acetone in chloroform[8];
S9: chloroform–acetone–*n*-hexane (85:15:20)[9].

Developed chromatoplates were examined under an analytical UV lamp (366 m$\mu$).

In order to determine whether fluorescence of ethoxyquin origin will also occur when its solution is run through a column such as is used in certain methods for a clean up procedure for aflatoxin extracts[10], columns consisting of silica gel 0.05–0.2 mm (Merck) and anhydrous sodium sulphate were prepared.

The ethoxyquin samples, alone or together with aflatoxin, were put on the columns, and subsequently eluted with 150 ml volumes of petroleum ether, ethyl ether, and 3% methanol in chloroform. The eluates were evaporated to dryness and dissolved in a small volume of chloroform and spotted on the TLC plates, and examined in UV light.

The following spray reagents were used: 5% nitric acid in water, and 1% 2,6-dibromoquinone-4-chloroimide in ethanol.

*Spectrophotometry.* The Unicam SP 800A Spectrophotometer, with 10-mm quartz cells was used. The UV absorption spectrum for four samples of ethoxyquin (concentration 0.15 mg/ml in 0.5 *N* HCl containing 1% of ethanol) was drawn up according to CHOY *et al.*[11]. The spectra of the fluorescent fractions recovered from the thin-layer plates, in concentrations corresponding to 0.15 mg/ml of ethoxyquin were also determined. Thin-layer plates were coated with Silica Gel H (Merck), 0.8 mm thick, and dried for 2 h at 100°. Ethoxyquin samples were dissolved in chloroform to a final concentration of 3 mg/ml, and 0.5 ml portions of these solutions were applied in a line across the chromatoplate; the plates were then developed twice, up to a distance of 15 cm from the base line, in 5% methanol in benzene. The blue fluorescent bands of ethoxyquin impurities were marked under UV light, scraped from the plates, and extracted five times with 1% ethanol in 0.5 *N* HCl up to 10 ml volume.

*Results and discussion*

The fluorescence of ethoxyquin solutions is known, and quantitative methods for it based on this property have been published[12,13]. In the literature available, however, no publication was found to describe its fluorescence on thin-layer plates.

Initially it was noted that the chloroform solution of EQ I, in a concentration of 0.2 mg/ml, gave fluorescent spots of the same colour and intensity as 3 $\mu$g/ml of aflatoxin $B_1$. At the start only the 0.4 mm Silica Gel G plates and the S1 system were involved. It soon turned out that in order to obtain similar spots from the remaining ethoxyquin samples, much higher concentrations were required: about 6 mg/ml for EQ II; 3 mg/ml for EQ III, and 1 mg/ml for EQ IV. This observation suggested it was not ethoxyquin itself which was responsible for this fluorescence. In fact, when the plates were sprayed with the 2,6-dibromoquinone-4-chloroimide reagent[14], the spots of ethoxyquin appeared high above the fluorescent spots, having $R_F$ values in different solvent systems varying from 0.75 to 0.95. Yet it was also possible to see the blue fluorescence emitted by true ethoxyquin, but only when the wet plates were inserted under the UV lamp. This was obtained when the plates were developed in the S4 and S5 solvent systems. The fluorescence disappeared swiftly as the plates dried off.

When the chloroform solutions of the four ethoxyquin samples were applied in the above concentrations on plates coated with an 0.25 mm layer of Silica Gel G or on Silufol sheets, the fluorescent spots corresponding to EQ II, EQ III, and EQ IV were not compact, and tailing occurred. The concentrations of ethoxyquin samples finally applied on 0.25 mm thin-layer plates and Silufol sheets were as follows: EQ I, 0.1 mg/ml; EQ II, 0.3 mg/ml; EQ III, 0.3 mg/ml and EQ IV, 0.2 mg/ml. The $R_F$ values in different solvent systems, for aflatoxin $B_1$ and fluorescing spots derived from ethoxyquin impurities are presented in Table I. In some solvent systems, particularly S8 and S9, the fluorescent spots of ethoxyquin origin tended to divide

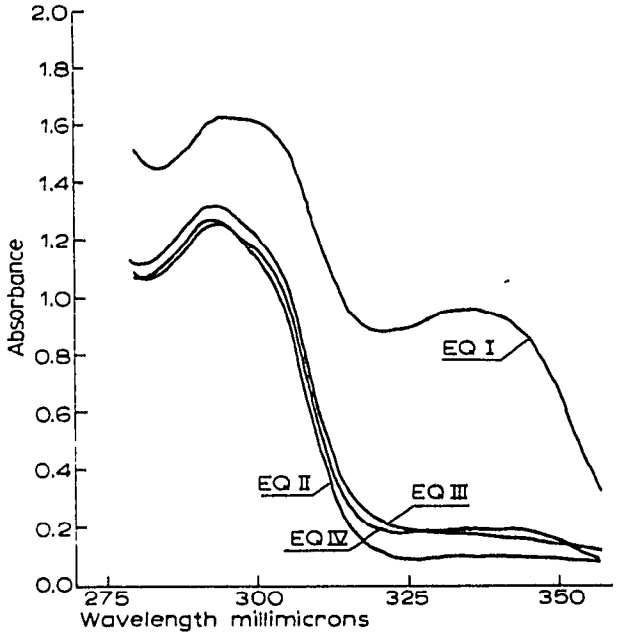
into two separate spots, suggesting that it was not just one chemical compound responsible for the fluorescence.

As is shown in Table I chromatograms developed in certain solvent systems exhibit similar $R_F$ values for the blue fluorescent spots of aflatoxin $B_1$ and impurities of ethoxyquin origin. As a result of this fact there is the possibility of mistake when the sample analysed for aflatoxin contains an additive of commercial ethoxyquin. In order to differentiate the aflatoxin fluorescence, and that from impurities accompanying ethoxyquin, a second development of the thin-layer plates in ethyl ether is to

TABLE I

COMPARISON OF THE $R_F$ VALUES FOR AFLATOXIN $B_1$ (Afl), AND THE FLUORESCENT SPOTS OF ETHOXYQUIN ORIGIN (EQ), SPOTTED ON DIFFERENT THIN-LAYER PLATES AND DEVELOPED IN A SERIES OF SOLVENT SYSTEMS

| *Solvent system* | *Silica Gel G 0.4 mm* | | *Silica Gel G 0.25 mm* | | *Silufol sheets* | |
|---|---|---|---|---|---|---|
| | *Afl* | *EQ* | *Afl* | *EQ* | *Afl* | *EQ* |
| S1 | 0.48 | 0.59 | 0.52 | 0.59 | 0.34 | 0.22 |
| S1a | 0.49 | 0.77 | 0.54 | 0.80 | 0.36 | 0.30 |
| S2 | 0.63 | 0.73 | 0.63 | 0.69 | 0.53 | 0.38 |
| S3 | 0.70 | 0.77 | 0.70 | 0.76 | 0.58 | 0.49 |
| S4 | 0.46 | 0.67 | 0.42 | 0.68 | 0.30 | 0.35 |
| S5 | 0.33 | 0.57 | 0.30 | 0.49 | 0.29 | 0.30 |
| S6 | 0.37 | 0.46 | 0.64 | 0.69 | 0.29 | 0.12 |
| S7 | 0.66 | 0.74 | 0.70 | 0.80 | 0.45 | 0.17 |
| S8 | 0.48 | 0.52 | 0.46 | 0.53 | 0.32 | 0.12 |
| S9 | 0.33 | 0.48 | 0.35 | 0.48 | 0.20 | 0.10 |



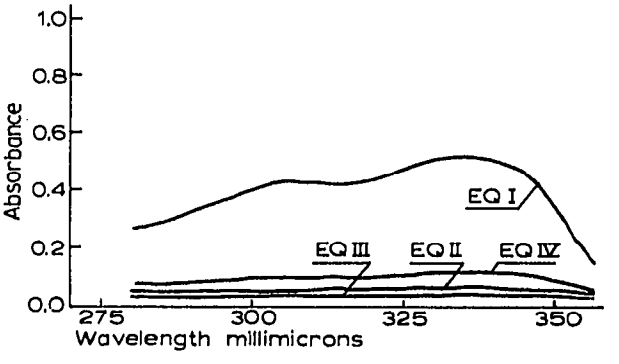




Fig. 1. The UV absorption spectra of the four ethoxyquin samples, concentration 0.15 mg/ml in 0.5 *N* HCl with 1 % ethanol.

Fig. 2. The UV absorption spectra of the fluorescent fractions recovered from the thin-layer plates, derived from the four ethoxyquin samples at the concentrations equal to 0.15 mg/ml, extracted with 1 % ethanol in 0.5 *N* HCl.

be recommended, as well as spraying the plates with 5 % nitric acid. After the application of this spray reagent, the fluorescence of aflatoxin $B_1$ changes to yellow, while the blue fluorescence of ethoxyquin impurities remains unchanged.

The behaviour of the blue fluorescence derived from ethoxyquin samples was also examined in the column chromatography process applied for aflatoxin purification. It was found that the fluorescent compounds present in ethoxyquin samples, were not eluted from the silica gel column with petroleum ether, but were quantitatively recovered by means of ethyl ether, and the third eluate methanol–chloroform was entirely free of them. On the other hand, aflatoxin $B_1$ remained on the column until the 3 % methanol in chloroform was applied, which eluted all the aflatoxin introduced to the column. This means that in case of the presence of ethoxyquin in a sample analysed for aflatoxin contamination, the run through the column for the clean up procedure will exclude the fluorescent compounds accompanying ethoxyquin from the final solution applied to the thin-layer plates.

Spectrophotometric estimations were also performed in order to furnish additional proof of the statement, concluded from the chromatographic examination, that it was not ethoxyquin which gave the permanent, blue fluorescence on the thin-layer plates. The UV absorption spectra for the four ethoxyquin samples, in dilute HCl solutions, show absorption in the range from 280 to 305 m$\mu$ as indicated in Fig. 1. The spectra of fluorescent fractions were definitely different in this range as is shown in Fig. 2. The concentration of the fluorescent compounds recovered from the thin-layer plates corresponded to the concentration of ethoxyquin samples introduced in Fig. 1. The blue fluorescent bands moved 5.5 to 7 cm from the base line, the width of them being different for the various samples: 1.5 cm for EQ I; 0.5 cm for EQ II; 0.8 cm for EQ III; and 1 cm for EQ IV.

The spectrophotometric evaluation of both ethoxyquin samples and the fluorescent fractions derived from these samples confirmed the results of the chromatographic findings that it was not ethoxyquin itself which exhibits aflatoxin-like blue fluorescence, but unidentified chemical impurities accompanying this compound.

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