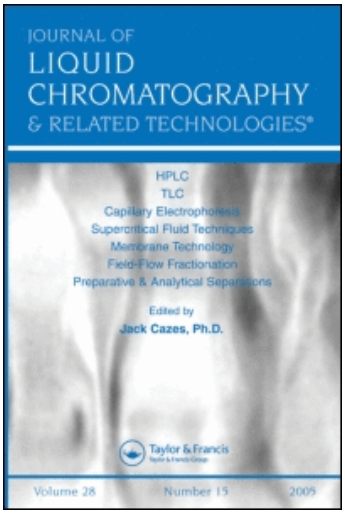


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# Instrumental HPTLC of Aflatoxins: Fluorescence Enhancement by Corn Free Fatty Acids

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# INSTRUMENTAL HPTLC OF AFLATOXINS: FLUORESCENCE ENHANCEMENT BY CORN FREE FATTY ACIDS

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## ABSTRACT

Greater than 100% recoveries using instrumental HPTLC were observed for aflatoxin (AFT) analysis in spiked corn samples. $I_2$ detection of spots over-laying AFT $B_1$ and $B_2$ were identified by GLC as $C_{16}$-$C_{18}$ free fatty acids (FFA). These FFA were found to enchance the fluorescence of AFT $B_1$ from 13.7% to 35.7% greater than controls resulting in >100% recoveries. The inclusion of glacial acetic acid in the TLC mobile phase resulted in an increased mobility of the FFA which eliminated the positive interference on AFT fluorescence. Recoveries using the modified developing solvent then gave values in acceptable ranges.

## INTRODUCTION

During the development of a method to analyze for aflatoxins in small samples of corn and corn dusts (1.0 g-0.01 g), a positive error in $B_1$ and $B_2$ values was consistently observed during aflatoxin (AFT) spiking and recovery studies using instrumental HPTLC with fluorescence detection.



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An actual example would consist of three 1.0 g samples of ground corn spiked with 50 ppb AFT $B_1$ and 10 ppb AFT $B_2$. After appropriate extraction and clean-up according to Zennie et al. (1), the AFT 's were quantified on HPTLC plates. Recovery would range from 56 to 83 ppb for $B_1$ and 12 and 14 ppb for $B_2$. These results show a 112 to 166% recovery for $B_1$ and a 120 to 140% recovery for $B_2$.

The most obvious cause for the increased fluorescence enhancement would be corn naturally contaminated with AFT; this however was ruled out by analyzing the unspiked corn and finding no discernable amounts of AFT 's. Inspection of the thin-layer plate under long-wave UV light revealed plainly visible AFT spots from both AFT standards and AFT spiked samples with no obvious overlapping or interferring fluorescent background material at or near the AFT 's. However, when the AFT areas were marked with pencil under UV light and then the plate placed in an $I_2$ tank, large areas which directly overlaid the AFT spots became visible. These were large tear shaped spots which had $R_f$ values slightly ahead of AFT $B_1$ and $B_2$, but which tailed over the AFT $B_1$ and $B_2$ areas. Removal of this interference for accurate AFT determination is warranted.



## EXPERIMENTAL

Column chromatography and preparatory TLC of corn extracts free of AFT resulted in the isolation of the interferring spot. The IR spectrum of the isolated material (neat on NaCl plates) gave absorptions at 1720 $cm^{-1}$ and a large broad band between 3400 $cm^{-1}$

and 2500 $cm^{-1}$ which are suggestive of a carboxylic acid. Additional strong absorptions at 2920 $cm^{-1}$ and 2850 $cm^{-1}$ and another at 720 $cm^{-1}$ denoted a long chain fatty acid. A stretching vibration at 3010 $cm^{-1}$ revealed unsaturation. The material was esterified using $BF_3$ in methanol and run on GLC and positively identified as a typical mixture of long chain fatty acids seen in corn, i.e., palmitic, stearic, oleic, linoleic, and linolenic acid.

All HPTLC experiments were performed using 10 cm x 10 cm. HPTLC plates (E. Merck) developed in an unlined tank. The developing solvents for all experiments were either solvent A (8% acetone in chloroform) or solvent B (chloroform, acetone, glacial acetic acid [92+8+1]). The fluorescence enhancement studies and the free fatty acid (FFA) $R_f$ determinations, were done by over-spotting the AFT on various concentrations of FFA. All AFT measurements were done on a Camag variable wave-length densitometer in fluorescence mode with a Hewlett Packard 3390A reporting integrator. Light source was a mercury lamp with excitation at 365 nm using a 400 nm cut off filter. Scanning slit width was 5 mm x 0.3 mm with a 0.5 mm/sec scan speed.

Samples of ground corn (1.0 g) were extracted with 25 ml $CHCL_3$, 1.0 g celite and 1 ml $H_2O$ in a 125 ml Erlenmeyer flask with 30 min of shaking. This method of extraction results in approximately 10 mg of extractables for 1.0 g corn.

For the AFT recovery experiment an extract from 1.0 g of AFT-free corn was cleaned-up by flash column chromatography (FCC) (3) using diethyl ether. The fraction which normally contains AFT is eluted off with 20% acetone in chloroform and brought to dryness

under $N_2$. To this residue 200 µl of benzene-acetonitrile (98+2) is added and then 5 µl of this solution is spotted on a HPTLC plate and then over-spotted with various concentrations of AFT $B_1$ and $B_2$. The plate was then developed with either solvent A or solvent B.

## RESULTS AND DISCUSSION

Corn oil contains 1.5 to 4.0% FFA (4). Assuming that none of the FFA are separated from the AFT during the clean-up procedure, then the final dilution used for spotting (here in our case 200 µl for 1.0 g samples) would contain 0.75-2.0 µg/µl of FFA. Thus a 5 µl spot could contain 3.75-10.0 µg of FFA; a concentration well within the range of interference.

Experiments with the isolated free fatty acid mixture showed that the $R_f$ values are strongly concentration dependent (Table 1). The higher $R_f$'s are produced by the greater concentration of the FFA's presumably because the material occurring in the front of the spot deactivates the silica gel as migration proceeds and the following material encounters a less polar absorbant (2).

Attempts to exclude completely the FFA during the clean up procedure with FCC by mobile phase modification were unsuccessful. However it was found that a decrease in the amount of FFA was observed in the AFT fraction if acetone-chloroform mixtures (i.e. 20% A/C) were used to remove the AFT from the column. The CB clean-up procedure (5) which uses chloroform-methanol (97+3) to remove AFT from the clean-up column had significantly higher FFA in the AFT fraction than the FCC clean-up procedure as revealed by $I_2$·

**TABLE 1**

$R_f$ Values of Corn Free Fatty Acids

of Differing Concentrations

| Concentration of FFA (μg/spot) | $R_f$ value[a] | $R_f$ value[b] |
|---|---|---|
| 1.4 | 0.35 | 0.49 |
| 3.7 | 0.35 | 0.49 |
| 7.4 | 0.39 | 0.50 |
| 22.0 | 0.44 | 0.54 |
| 36.6 | 0.48 | 0.56 |
| 73.3 | 0.54 | 0.60 |
| 220.0 | 0.63 | 0.68 |
| 366.5 | 0.66 | 0.69 |
| 1.5 ng - AFT $B_1$ | 0.35 | 0.33 |
| 0.3 ng - AFT $B_2$ | 0.26 | 0.28 |

[a] Mobile phase: 8% acetone in chloroform.

[b] Mobile phase: 8% acetone + 1% glacial acetic acid in chloroform.



However, the FCC clean-up procedure still gave recoveries of AFT greater than 100%. Using ammonia, diethylamine or other basic additiives to both the column mobile phase and the TLC eluent to retard migration of the FFA were rejected because of the possibility of AFT degradation (6).

The problem was solved by the addition of 1% glacial acetic acid (GAA) to the TLC solvent. Mobile phase B caused the FFA to migrate significantly ahead of the AFT $B_1$ and $B_2$ with no overlapping and streaking with $R_f$ values averaging 0.24 and 0.29 greater than AFT $B_1$ and $B_2$ respectively (Table 1). FFA in concentration ranges usually seen in AFT analysis gave an $R_f$

difference of 0.16 and 0.21 greater for AFT $B_1$ and $B_2$. The GAA addition also significantly reduced the FFA $R_f$ differences due to concentration. Presumably the GAA causes complete protonation of the carboxylic acid portion of the FFA and eliminates any partial ionization which would contribute to the streaking. It also would decrease the polarity of the FFA thus giving them greater mobility through the silica gel and consequently a higher $R_f$.

Fluorescence enchancement by the FFA was also concentration dependent (Table 2). Even though higher concentrations of the FFA have a greater $R_f$ difference compared to AFT-$B_1$ and $B_2$ as did the lower concentrations of FFA, they significantly enhanced the fluorescence to a greater degree than the lower concentrations of FFA. Apparently the tailing of the FFA in the higher concentrations was sufficient to over lay AFT $B_1$ and $B_2$. Using various concentrations of FFA's and over spotting with 1.5 ng of AFT $B_1$ and 0.3 ng $B_2$ mixture, the optimum concentration range of 1.33 μg to 7.33 μg of FFA was found to give the closest $R_f$'s to AFT $B_1$ and $B_2$ using mobile phase A as HPTLC developing solvent (Table 1). The fluorescent enhancement produced at these concentrations averaged about 13.7% for AFT $B_1$ and 16.1% for AFT $B_2$. However larger concentrations of the FFA's (36.5 to 366.5 μg) produced an average of 35.7% enhancement for AFT $B_1$ and 28.6% for $B_2$.

Recovery results using actual corn extracts with a FCC clean-up procedure using a HPTLC solvent with and without GAA are depicted in Table 3. The average recovery without the GAA addition for $B_1$ and $B_2$ are 126.5% and 120.3%. With GAA addition to the mobile phase the average recovery for AFT $B_1$ is 98.75% and for AFT

**TABLE 2**

% Enhancement of Fluorescence* of 1.5 ng AFT $B_1$ and 0.3 ng AFT $B_2$ by Corn FFA

| FFA Concentration μg/spot | AFT - $B_1$ | AFT - $B_2$ |
|---|---|---|
| 1.4 | 10% | 14.3% |
| 3.7 | 15% | 20.4 |
| 7.4 | 16.2% | 13.6 |
| 22.0 | 30.2% | 22.9 |
| 36.6 | 33.9% | 25.9 |
| 73.3 | 29.9% | 24.0 |
| 220.0 | 39.5% | 26.6 |
| 366.5 | 39.6 | 37.9 |

*Measured in triplicate using HPTLC plates.

**TABLE 3**

Recoveries* of AFT $B_1$ and $B_2$ in Spiked 1.0 g Corn Extracts

| Spiked AFT $B_1$, $B_2$ in ppb | Recovered[a] ppb $B_1$, $B_2$ | Recovered[b] ppb $B_1$, $B_2$ |
|---|---|---|
| 12, 2.4 | 15.6, 2.8 (130, 117) | 11.7, 2.3 (97.5, 95.8) |
| 20, 4.0 | 24.5, 4.3 (122.5, 107.5) | 19.5, 3.9 (97.5, 97.5) |
| 60, 12.0 | 74.9, 13.4 (125, 111.7) | 62.4, 12.2 (104, 101.7) |
| 100, 20.0 | 129.0, 29.4 (129, 147) | 88.4, 13.7 (88.4, 68.5) |
| 200, 40.0 | 259.2, 46.2 (129.6, 115.5) | 213, 34.7 (106.5, 86.7) |
| 500, 100 | 612.2, 123.0 (122.4, 123) | 493, 99.3 (98.6, 99.3) |

*Measured in triplicate and averaged. % Recoveries in parenthesis.

[a]Mobile phase: 8% acetone in chloroform.

[b]Mobile phase: Chloroform, acetone, glacial acetic acid (92+8+1).

$B_2$ 91.6%. If the unexplained low value for the 20 ppb $B_2$ is dropped then the recovery averages 96.2.

For the AFT range used (12-500 ppb for $B_1$ and 2.4-100 ppb for $B_2$) an average difference of 27.75% for $B_1$ and 28.7% for $B_2$ in recovery was observed for the 2 different mobile phases. Obviously mobile phase B gave recoveries much closer to the actual values.

The prevention of the FFA interference in HPTLC analysis becomes even more significant for mold infected corn and older stored corn ( 1 yr old). This is because an increased level of FFA is observed due to the hydrolysis of corn triglycerides by fungi, bacteria, heat, seed damage, and moisture (3,7). In particular, high levels of FFA have been attributed to Aspergillus flavus contamination of cotton seed, coconut oil, and chocolate seed (8,9,10). These results should be interpreted only for the HPTLC plates and the developing solvent used. However an inspection of any developed TLC chromatograms used for corn AFT analysis with over-lapping $I_2$ positive spots could reveal FFA not removed during the clean-up procedure.



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