*Journal of Chromatography*, 595 (1992) 143–148
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 926

# Fluorescence detection of trichothecene mycotoxins as coumarin-3-carbonyl chloride derivatives by high-performance liquid chromatography

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(First received September 9th, 1991; revised manuscript received November 28th, 1991)

ABSTRACT

A new and simple procedure was developed for the determination of trichothecenes mycotoxins by high-performance liquid chromatography using fluorescence detection. The procedure involves the synthesis of the derivatizing reagent, coumarin-3-carbonyl chloride, and its use in the esterification of T-2, HT-2, T-3 and T-4. A clean-up procedure was used using silica cartridge to remove the excess reagent peaks. The esterified toxins were simultaneously separated on a reversed-phase column using acetonitrile–water containing acetic acid as the mobile phase and detected by fluorescence. The minimum detectable amounts of T-2 and its metabolites were 2.0 ng and 0.83 ng, respectively, at a signal-to-noise ratio of 2 and injection volume of 10 $\mu$l.

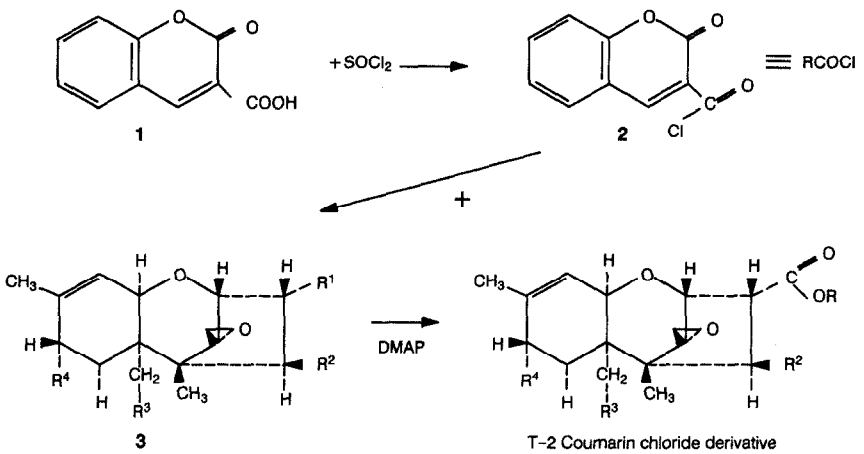
## INTRODUCTION

Trichothecene mycotoxins are mold metabolites produced by various strains of Fusarium, Trichoderma, Myrothecium, and other species of imperfect fungi. The group is characterized by the 12,13-epoxy-trichothec-9-ene ring system. In blood T-2 and HT-2 are easily interconverted and hydrolyzed to T-3 and T-4 toxins (Fig. 1, compound 3). T-2 and its derivatives belong to the group that does not contain a carbonyl function at C-8 conjugated with a double bond in 9–10 [1]. The absence of this $\delta$-$\beta$-enone feature prevents these compounds from being detected by UV absorption.

Numerous analytical methods have been published. The most frequently employed methods are thin-layer chromatography (TLC) [2,3], enzyme-linked immunoassay (ELISA) [4–7], gas chromatography [8–10] combined with mass spectrometry [11], or with tandem mass spectrometry [12], or thermospray liquid chromatography–mass spectrometry [13,14]. The use of the coumarin-3-carbonyl chloride as derivatization reagent permits the fluorescence detection of the hydroxyl function of the trichothecenes. Acylation is commonly used for labelling hydroxy compounds and permits rapid and quantitative derivatization. A pre-column derivatization has been reported to form a chromophore with *p*-nitrobenzoyl chloride with an absorption maximum at 254 nm, the method has the disadvantages that the reagent gives an interfering peak and also a large number of organic compounds absorb at this low wavelength [15]. A more recent post-column derivatization technique involves an alkaline decomposition to form formaldehyde and a modified Hantzch reaction with methylacetoacetate and ammonium acetate [16]. This procedure was suitable for deoxynivalenol (DON), nivalenol, fusarenone trichothecenes that contain a carbonyl function at C-8, but not for T-2 and its derivatives. Polycyclic aromatics such as anthracene-3-carboxylic chloride have been employed for the determination T-2 toxin and DON.

Coumarins as fluorophore possess a high molecular extinction coefficient and quantum yields. For trace analyses, derivatization of the target com-

0021-9673/92/$05.00 




| *Trichothecene* | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| HT-2 toxin | OH | OH | OAC | $OCOCH_2CH(CH_3)_2$ |
| T-2 toxin | OH | OAC | OAC | $OCOCH_2CH(CH_3)_2$ |
| T-3 toxin | OH | OH | OH | $OCOCH_2CH(CH_3)_2$ |
| T-4 toxin | OH | OH | OH | OH |

Fig. 1. Structures of trichothecenes.

pounds offers significant advantages such as improving selectivity. A coumarin-3-carbonyl chloride derivative has been successfully applied to hydroxy compounds such as hydroxy-steroids and prostaglandins [17]. In the present work, we have sucessfully empolyed coumarin-3-carboxylic acid chloride to derivativze T-2 and its "major metabolites" for analyses by high-performance liquid chromatography (HPLC). The reaction is represented in Fig. 1 below.

## EXPERIMENTAL

### *Chemicals and materials*

Coumarin-3-carboxylic acid, 4-dimethylamino pyridine (DMAP), thionyl chloride (99%) were obtained from Aldrich (Milwaukee, WI, USA). Trichothecenes were obtained from Sigma St. Louis, MO, USA). Silica gel cartridges were purchased from BDH (Toronto, Canada). LDX filters were purchased from Chromatographic Sciences (Montreal, Canada). Water was purified using a Millipore Milli-Q system. All other solvents were glass distilled or HPLC grade from BDH (Toronto, Canada).

### *Apparatus*

A Perkin-Elmer LS-4 liquid chromatograph equipped with microprocessor control delivery system and an auto-injector ISS-sampling system was employed. An RP-18, 5-$\mu$m column (250 × 4.6 mm I.D.) protected with a RP-18, 7-$\mu$m guard column (15 × 3.2 mm I.D.) was used. Mobile phase was acetonitrile-water (65:35, v/v) containing 0.75% acetic acid and flow-rate of 1 ml/min. Fluorescence detection conditions were excitation at 292 nm and emission at 425 nm, both with 10 nm slit widths.

Electron impact mass spectra were obtained with Kratos MS 25 or VG 15-250 spectrometers set at 70 eV.

### *Methods*

*Synthesis of coumarin-3-carbonyl chloride.* Coumarin-3-carboxylic acid (5 g) was suspended in dry dichloromethane (30 ml) and refluxed while adding thionyl chloride (10 ml) dropwise. A drying tube packed with calcium chloride was placed in the outlet of the condenser. The mixture was refluxed for 40 min. Some dichloromethane was added to prevent dryness. On completion of the reaction the solvent was evaporated under vacuum at 50°C. The residues were dissolved in anhydrous chloroform and the solution was heated with careful addition of hexane until the solution turned cloudy. Yellow pale crystals (3.83 g) were obtained on cooling,

yield 70%; m.p.: 143°C (uncorrected). The mass spectrum had peaks at $m/z$ 208 ($M^+$, 8%, agrees for $C_{10}H_5ClO_3$), 173 ($M^+ - Cl$), 145 ($M^+ - COCl$).

*Preparation of standard solution.* (i) Trichothecene standards T-2: 25 mg, dissolved in 100 ml toluene to obtain a concentration of 0.25 $\mu g/\mu l$; HT-2: 5 mg; T-3: 5 mg; T-4: 5 mg, three standard solutions at concentration of 50 $ng/\mu l$ in 100 ml toluene solution were prepared. These standard solutions were stored at 4°C and brought to room temperature before use. (ii) Coumarin-3-carbonyl chloride 65 mg was dissolved in 20-ml volumetric flask, to obtain a concentration of 3.25 $\mu g/\mu l$. (iii) 4-Dimethylaminopyridine (DMAP) 130 mg was dissolved in volumetric flask (20 ml) to obtain a concentration of 6.5 $\mu g/\mu l$.

*Derivatization conditions.* All glassware were rinsed with toluene before use. In 15-ml screw cap conical tube, 10 $\mu l$ of each trichothecene standard solution was evaporated to near dryness under slow stream of nitrogen. To each tube, 10 $\mu l$ of DMAP 6.5 $\mu g/\mu l$ solution was added, followed by the addition of 10 $\mu l$ of the coumarin reagent. The tube was closed and the mixture incubated at 80°C for 20 min. The mixture was then cooled in ice water and then purification procedure followed as detailed below.

*Preparation of the standard curves.* To obtain the calibration curve, a series of standard solution containing each of the four toxins was used at concentration between 0.01 and 0.5 $\mu M$. DMAP was added at concentrations between 0.13 and 3.98 $\mu M$ and the carbonyl chloride between 0.037 and 1.125 $\mu M$. Derivatization was carried out as mentioned in the derivatization procedure.

*Purification of the reaction mixture.* The silica gel cartridge was pre-conditioned first with 5 ml of chloroform–methanol (70:30) and then 5 ml of benzene. The cooled reaction mixture was then quantitively transferred to the silica gel column with benzene (5 × 1 ml). The benzene fractions were discarded, then a mixture ethyl acetate–hexane (80:20, 5 × 2 ml) was added to the column and discarded. The derivatized trichothecenes were eluted with ethyl acetate–hexane (90:10, 2 × 7 ml). The eluate was collected and evaporated to dryness under a gentle flow of nitrogen. To the residues 1 ml of the HPLC mobile phase: acetonitrile–water (65:35) containing 0.75% acetic acid was added, the solution filtered through LXD filter (4.5 $\mu m$) and 10 $\mu l$ injected into an HPLC system.

## RESULTS AND DISCUSSIONS

Reaction conditions were optimised by investigating variables such as solvent, reagent ratio, temperature and reaction times. The effect of benzene and methylene chloride as solvent was investigated. This later solvent gave an additional peak in the chromatogram. The optimum molar ratio of the derivatizing reagent–base–sample was 1:3:0.5. No improvement in peak height was obtained when the relative porportion of the derivatizing reagent was greater than 1. The unreacted reagents eluted near the solvent front and their peaks show little tailing, also the peaks of interest eluted far from the solvent front.

Each toxin was tested for temperature effect, the derivatization of T-2 and HT-2 was least affected by temperature. The derivatization was optimal at room temperature and remained unchanged at higher temperatures. However, the best peak response for T-3 and T-4 was obtained when a reaction temperature of 80°C and a reaction time of 20 min was used. A longer time didn't change the peak height.

The limit of detection which was defined as the lowest instrumental signal was obtained at 0.83 ng/10 $\mu l$ injection for HT-2, T-3, T-4 and at 2.0 ng/10 $\mu l$ injection for T-2, when the signal to noise ratio ($S/N$) was 2. It appears that the detection limit for HT-2, T-3 and T-4 was lower than for T-2 because T-2 has only one hydroxyl, HT-2 two hydroxyl, T-3 three and T-4 four for derivatization. The fluorescence properties of the toxins increase as the number of the chromophores undergoing esterification increases.

The HPLC separation was performed with acetonitrile–water (65:35), and 0.75% of acetic acid was added to the HPLC mobile phase, in order to separate all four derivatives. No reaction was observed between the mobile phase and the derivatizing agent. The purification using preconditioned silicagel cartridge gave very good recoveries. Table I summarizes the recoveries obtained for various trichothecenes. The recoveries ranged from 80–100%. In the HPLC mobile phase, the derivatives were stable at room temperature up to 10 days.

TABLE I

PERCENT RECOVERY OF MIXTURE OF T-2, HT-2, T-3, T-4 AFTER SILICA GEL PURIFICATION

| Trichothecenes | Concentration (ng/μl) | Recovery ± R.S.D. (%)[a] |
|---|---|---|
| T-2 | 125 | 100 ± 8.09 |
| HT-2 | 25 | 102 ± 3.78 |
| T-3 | 25 | 91 ± 9.22 |
| T-4 | 25 | 80 ± 4.77 |

[a] Relative standard deviation ($n = 3$).

Fig. 2 displays the chromatogram of a single injection of T-2, HT-2, T-3, T-4; (a) before and (b) after clean up on silica Sep-pak column; (c) a blank of the reaction mixture containing only reagents. As shown, most of the excess reagent peaks are eliminated in 2b. Fig. 3 displays the standard curve for the four trichothecenes. Excellent linearities with correlation co-efficient over 0.996 were obtained for all toxins (Table II). The highest relative standard deviation (R.S.D.) obtained for all trichothecene standards is $\leqslant 5\%$ (Table III), except for T-4 which

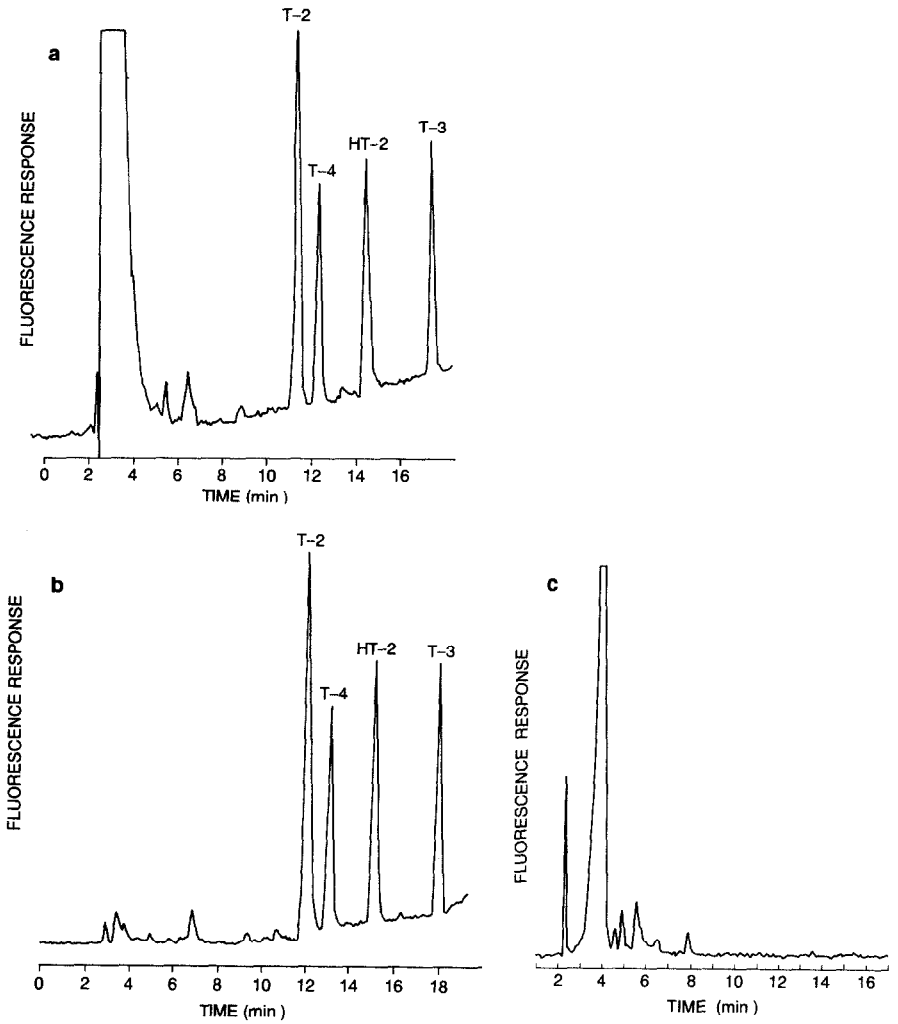



Fig. 2. Chromatogram of derivatization reaction mixtures of T-2, T-4, HT-2, T-3: (a) Before clean-up on a silica gel cartridge; (b) after clean-up on a silica cartridge; (c) a blank of the reaction mixture containing only reagents. HPLC conditions: reversed-phase chromatography on RP-18, 5 μm, 250 × 4.9 mm I.D. Mobile phase, acetonitrile–water (65:35 v/v) containing 0.75% acetic acid at 1 ml/min. Detection, excitation and emission at 292 nm and 425 nm respectively, same conditions for a, b and c.

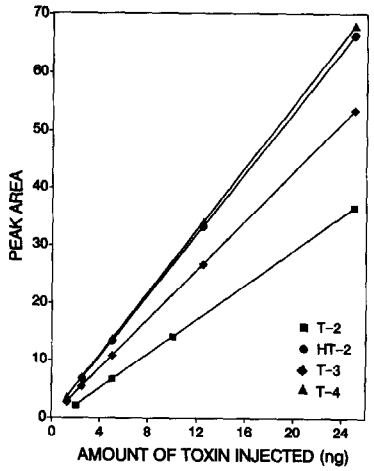



Fig. 3. A plot of concentration *vs.* peak area of derivatives of various mycotoxins.

TABLE II

LINEAR REGRESSION DATA AND CORRELATION COEFFICIENTS ($r$) FOR DERIVATIZED TRICHOTHECENES

$y = mx + b$

| Mycotoxins | Slope $m$ ($ng^{-1}$) | Intercept $b$ | Correlation coefficient of calibration graphs $r$ |
|---|---|---|---|
| T-2 | $1.5 \cdot 10^3$ | $-8.48 \cdot 10^2$ | 0.9961 |
| HT-2 | $2.2 \cdot 10^3$ | $-4.1 \cdot 10^3$ | 0.9960 |
| T-3 | $2.14 \cdot 10^3$ | $-4.88 \cdot 10^2$ | 0.9963 |
| T-4 | $2.88 \cdot 10^3$ | $-5.70 \cdot 10^3$ | 0.9902 |

TABLE III

CHROMATOGRAPHIC DATA FOR TRICHOTHECENE MYCOTOXINS

| Mycotoxins | Retention time mean ± S.D.[a] (min) | Detection limit (ng/10 μl) | R.S.D.[c] (% Peak Area) |
|---|---|---|---|
| T-2 | 12.5 ± 0.46 | 2.0 | 3.65 (12.5)[b] |
| HT-2 | 15.1 ± 0.79 | 0.83 | 5.78 (12.5) |
| T-3 | 17.9 ± 0.70 | 0.83 | 5.31 (12.5) |
| T-4 | 13.6 ± 0.63 | 0.83 | 10.05 (12.5) |

[a] $n = 4$.
[b] The numbers in parentheses are the relevant concentrations in ng/10 μl.
[c] $n = 4$.

was 10%. The reason for the large deviation has not been determined.

## CONCLUSION

The synthesis of the coumarin-3-carboxyl chloride is simple and straight forward and its use as a derivatization reagent has proven quite efficient because of its high reactivity with hydroxyl group(s). The derivatization must be carried out in aprotic solvent to prevent hydrolysis to the parent acid. The proposed method for the determination of tricothecenes is very sensitive and rapid, it allows the simultaneous separation, detection and quantification of more than one mycotoxin in a short time. Therefore determination of at least 20 samples in 8 hours was possible. The detection limits are quite low for all trichothecenes. Although the applicability of the method has not yet been assessed, it offers a good scheme for separating solvent as well as the interfering peaks from the peaks of desired compounds. The toxins are very well separated from each other for easy detection and quantification. Futhermore this method can be used for the selective determination of other tricothecenes with similar functional groups.

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