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Simultaneous liquid chromatography–fluorescence analysis of type A and type B trichothecenes as fluorescent derivatives via reaction with coumarin-3-carbonyl chloride

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Abstract

A method for the simultaneous LC–fluorescence detection (FLD) determination of eight trichothecenes A and B by pre-column derivatization with coumarin-3-carbonyl chloride, a highly fluorescent fluorophore, has been developed. The reaction conditions (temperature, reaction time, reactant ratios) were optimized to give a reproducible quantitative conversion. All derivatives were characterized by LC–MS. The chromatographic parameters were optimized (column, eluent) to give a very good separation of three type A (diacetoxyscirpenol, T-2 toxin, HT-2 toxin) and five type B trichothecenes [deoxynivalenol (DON), nivalenol, fusarenon-X, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol]. The best conditions were obtained on a narrow-bore C_{18} column with a water–methanol gradient. The detection limits (S/N = 3:1) in grain samples, with an injected volume of 5 μl, were 0.2–1 ng/g for all trichothecenes. These values are more than one order of magnitude lower than those of other LC–FLD and LC–MS methods and are similar to those obtained by GC–MS. The calibration curves were linear between 100 and 2500 ng/g. The method was successfully applied to the analysis of a certified wheat reference material, after solvent extraction and clean-up on a Mycosep column, obtaining a good recovery (89% for DON) and a high accuracy (*z*-score value: 0.67).
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Keywords: Derivatization, LC; Food analysis; Trichothecenes; Coumarin-3-carbonyl chloride; Mycotoxins

1. Introduction

Trichothecenes constitute the largest group of *Fusarium* mycotoxins. At present, over 170 trichothecenes have been isolated and characterized, which may be divided into four groups: non-macrocyclic (A–C) and macrocyclic compounds (D) [1,2]. Trichothecenes belonging to groups A and B are frequently found as contaminants in several foods, in particular in cereals (wheat, maize, barley, and oat) and several derived products, such as corn flakes, flour, infant foods, malt, and beer. The general structures of trichothecenes A and B are reported in Fig. 1.

Both type A and type B trichothecenes exhibit acute toxicity, causing vomiting and feed refusal [specially deoxynivalenol (DON)]; type B trichothecenes are implicated in chronic toxicoses, resulting in extensive hemorrhage, a general inflammatory response, and hematological toxicities. Furthermore, some of them, including the most toxic T-2 toxin, inhibit both protein synthesis and mitochondrial function in vitro and in vivo and show immunosuppressing

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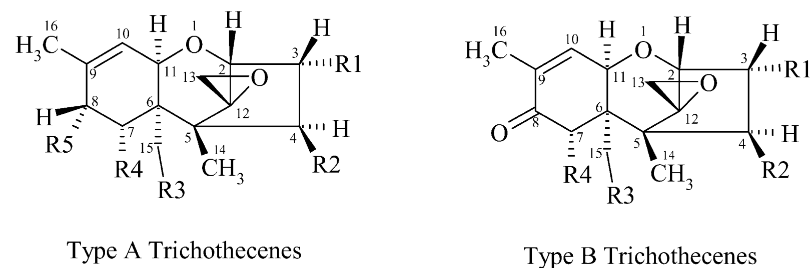

		MW	R_1	R_2	R_3	R_4	R_5
Type A Trichothecenes	*T-2 toxin*	466	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
	HT-2 toxin	424	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
	Diacetoxyscirpenol (DAS)	366	OH	OAc	OAc	H	H
Type B Trichothecenes	*Nivalenol (NIV)*	312	OH	OH	OH	OH	-
	Deoxynivalenol (DON)	296	OH	H	OH	OH	-
	Fusarenon X (FUSX)	354	OH	OAc	OH	OH	-
	3-acetyl-deoxynivalenol (3-ac-DON)	338	OAc	H	OH	OH	
	15-acetyl-deoxynivalenol (15-ac-DON)	338	OH	H	OAc	OH	-

Fig. 1. Structures of the main type A and type B trichothecenes.

effects at low concentrations. The T-2 toxin is also a suspected carcinogenic compound [3–5].

Different toxins are often simultaneously present in contaminated foods: thus, new methods are needed for the sensitive and accurate determinations of these toxins and, preferentially, for the simultaneous determination of both type A and type B trichothecenes.

On account of the different polarities of trichothecenes A and B, the existing analytical procedures usually differ in extraction [6–9], clean up [6,9–13] and chromatographic conditions [13,14], depending on which group is to be analyzed.

Gas chromatography with capillary columns and electron-capture (ECD) or mass spectrometric (MS) detection is the most widely used chromatographic technique for the determination of trichothecenes in foods [15–20]. GC methods are based on derivatization of the hydroxyl groups as trimethylsilyl, trifluoroacetyl, pentafluoropropionyl or heptafluorobutiryl derivatives: the choice depends on the type of trichothecene and the method of detection. Moreover, since a mixture of derivatizing agents has to be employed in order to assure complete derivatization, elimination of excess reagent is necessary before GC analysis [13].

LC with UV detection cannot be used for type A trichothecenes since they do not have a chromophore absorbing in the UV–vis range. Pre-column derivatization with *p*-nitrobenzoyl chloride and post-column derivatization involving alkaline degradation were reported, although the latter is suitable only for type B trichothecenes [13,21,22].

Since LC with fluorescence detection (FLD) generally allows for a high sensitivity and a good selectivity, several fluorophores have been proposed as hydroxyl derivatizing reagents, such as 1-ethoxy-4-(dichloro-1,3,5-triazinyl) naphtalene, 9-fluorenylmethyl chloroformate, anthracene-9-carbonyl chloride and 1-anthroylnitrile [8,23,24].

More recently, a good method for the determination of type A trichothecenes in cereal grains by LC–FLD was proposed by Jimenez et al. [25–27], who used coumarin-3-carbonyl chloride as derivatizing agent, for its high reactivity and high quantum yield. However, the method gave worst results with type B trichothecenes, on account of the presence of a higher number of hydroxyl groups which gave rise to incomplete or unreproducible derivatization [26,27].

The aim of this work is to provide a method for the simultaneous determination of both type A and type B trichothecenes by LC with fluorescence detection using coumarin-3-carbonyl chloride as derivatizing reagent. The first aim was to optimize the derivatization reaction by carefully controlling the reaction conditions (temperature, reaction time and reactant molar ratio) and its reproducibility and to identify all the derivatives by LC–MS analysis. Then, the LC–FLD method was developed by evaluating the performances of different C_{18} columns and mobile phases. Finally, the method was calibrated with trichothecene standards and applied to the analysis of real grain samples.

2. Experimental

2.1. Chemicals

Trichothecene standards (99% purity, 1 mg) deoxynivalenol (DON), nivalenol (NIV), T-2 toxin (T-2),

fusarenon-X (FUSX), diacetoxyscirpenol (DAS), HT-2 toxin (HT-2), 3-acetyldeoxynivalenol (3-ac-DON) and 15-acetyldeoxynivalenol (15-ac-DON) were obtained from Sigma–Aldrich (Steinheim, Germany). Stock solutions of trichothecene standards were prepared in acetonitrile from the commercial standards (500 μg/ml). Work solutions containing 3 μg/ml of each standard were prepared in acetonitrile. Certified reference material T2204 (wheat flour containing incurred deoxynivalenol residues, 894 ng/g), tested under the supervision of the Food Analysis Performance Assessment Scheme (FAPAS) (DEFRA, Central Science Labs., York, UK), was used.

All solvents used were of LC grade from Carlo Erba (Milan, Italy); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore, Marlborough, MA, USA). Dimethylaminopyridine (DMAP) was obtained from Sigma (Steinheim, Germany); coumarin-3-carboxylic acid and thionyl chloride were from Fluka (Buchs, Switzerland).

2.2. Sample extraction and clean up

Aliquots (25 g) of naturally contaminated samples of finely ground cereals were blended for 5 min in a high-speed blender (Ultraturrax T25, IKA, Stauffen, Germany) with 100 ml of water–acetonitrile (20:80, v/v). After filtering through Whatman No. 4 filter papers, 8 ml of filtrate were eluted through a Mycosep 225 column (Romer Labs., Union, MO, USA). A 4 ml volume of the purified extract was transferred in a vial. The solvent was evaporated to dryness under nitrogen and the residue was dissolved in 1 ml of methanol.

2.3. Synthesis of coumarin-3-carbonyl chloride [25]

Coumarin-3-carboxylic acid was suspended in dry dichloromethane (30 ml) and thionyl chloride (10 ml) was added dropwise for 40 min under reflux. Then, the solvent was evaporated under vacuum at 40 °C. The residue was dissolved in anhydrous chloroform and the solution was heated at 40 °C. By careful addition of hexane, the solution turned cloudy and pale yellow crystals of coumarin-3-carbonyl chloride (C3CC) were obtained on cooling. The precipitate was filtered, crystallized from $CHCl_3$–hexane and stored in an amber-colored bottle. The product is quite stable and may be maintained for more than a month (refrigerated, under nitrogen). The melting point of the solid was 143 °C. The yield of the reaction was 89%.

2.4. Derivatization with coumarin-3-carbonyl chloride

The reaction was carried out at different temperatures (80, 100 °C), with various reactant ratios (toxin–C3CC–DMAP = 1:50:50, 1:100:100 or 1:200:200) for several minutes (40, 60, 80 min). The best derivatization conditions turned out to be the following: 80 °C for 60 min, using CH_3CN as solvent and DMAP as base at a reactant molar ratio toxin–C3CC–DMAP = 1:100:100.

To a vial containing 100 μl of a methanol solution of the standard (3 μg/ml) or of the sample, 85 μl of DMAP in acetonitrile (10 mg/ml) were added, followed by the addition of 150 μl of a solution of coumarin-3-carbonyl chloride (C3CC) in acetonitrile (10 mg/ml). The mixture was evaporated under nitrogen and re-dissolved in 500 μl of CH_3CN, then it was heated at 80 °C for 60 min in a heater block. After derivatization, the vial was cooled in ice water, evaporated to dryness under nitrogen and the residue dissolved in 1 ml of CH_3CN.

2.5. LC–MS

The LC–MS system consisted of a 2695 Alliance Separation Module equipped with a 996 photodiode array detector (all from Waters, Milford, MA, USA) and a ZMD single quadrupole mass spectrometer with an electrospray ionization (ESI) source (Micromass, Manchester, UK). The electrospray probe was operated in the positive ion mode; cone voltage 30 V, capillary voltage 3.1 kV, total ion current scan 150–1500 *m/z*, scan duration 3.95 s, nitrogen flow in spray phase 86 l/h and nitrogen flow in desolvation phase 474 l/h. Water–CH_3CN (48:52, v/v) acidified with 1% formic acid was used as eluent. A C_{18} Spherisorb S3ODS2 column (250 mm × 4.6 mm, 5 μm) was used and the flow rate was 1 ml/min. The column flow (95%) was splitted by a T-tube before the ESI probe.

2.6. LC–FLD

Analyses were performed with an Alliance Waters 2695 Separation Module equipped with a Waters 474 fluorescence detector (λ_{ex} = 292 nm and λ_{em} = 425 nm) (Waters). Three different columns were tested: Spherisorb S3ODS2 (250 mm × 4.6 mm, 5 μm), Spherisorb S3ODS2 (150 mm × 4.6 mm, 5 μm) and Spherisorb S3ODS2 (250 mm × 2.1 mm, 3 μm) columns (Waters) with different gradients. Preliminary experiments and LC–MS analyses were performed using the conditions reported by Jimenez et al. [25] (Spherisorb S3ODS2, 250 mm × 4.6 mm, 5 μm; flow rate: 1 ml/min, eluent: CH_3CN–water (65:35); FLD: λ_{ex} = 292 nm, λ_{em} = 425 nm).

The best separation of the eight trichothecenes was obtained on the narrow-bore column using a methanol–water gradient: 0–10 min, methanol–water (60:40); 10–40 min, linear to methanol–water (80:20); 40–50 min, methanol–water (60:40) for column reconditioning.

The linearity was tested in the range 10–5000 ng/ml by injecting standard solutions of each trichothecene at nine concentration levels. Calibration curves were based on the analysis of spiked wheat samples at six concentration levels (2500–100 ng/g): the proper amount of toxin was added to each wheat sample prior to extraction and clean up (n = 3 at each level).

The significance of the linear regression and the intercept of each calibration curve were calculated by SPSS 8.0 statistical software with the analysis of variance (ANOVA) and the Student *t*-test, respectively.

The detection (LOD: $S/N = 3:1$) and quantification (LOQ: $S/N = 10:1$) limits were obtained by injection of wheat samples spiked at proper levels. The intra-day precision was determined by performing three replicate extractions, derivatization and injection of three different samples: a blank wheat sample was used spiked at three different concentration levels with the eight toxins (levels of contamination: 1500–300 ng/g), since no reference materials contaminated with both type A and type B trichothecenes are commercially available. The inter-day precision of the procedure was obtained from standard deviations and relative standard deviations (R.S.D.s), by performing the same procedure (extraction, derivatization and analysis) five times within 2 weeks.

3. Results and discussion

3.1. Optimization of the derivatization reaction

The derivatization conditions were optimized with special attention for temperature, reaction time and molar ratios of the reagents.

Preliminary reactions between coumarin-3-carbonyl chloride and DAS (type A) and DON (type B) were performed, in order to evaluate the reactivity of each group of toxins toward the fluorophore using different derivatization conditions. Differently from Jimenez et al. [25], the derivatization was performed using acetonitrile as solvent, which, being an aprotic polar solvent, is more suitable than toluene for solubilizing the more polar trichothecenes B.

The first parameter studied was the reaction time: the experiment was performed for DON and DAS, at 80 °C and at a toxin–C3CC molar ratio = 1:50, taking samples after 20, 40, 60, and 80 min. The mixture was evaporated under nitrogen, the residue was dissolved in the eluent and analyzed by LC–MS, using the conditions reported in the Section 2.5. The conversion was evaluated by measuring the ratio between the peak area of the underivatized toxin before and after the derivatization reaction (Fig. 2a and b).

After 20 min (the conditions applied by Jimenez et al.), only about 45% conversion was obtained, which explains the unsatisfactory repeatability observed for type B trichothecenes, as small differences in the experimental conditions may induce significant variations in the derivatization yield. A total derivatization was not achieved even after 80 min. In the case of DON, selectivity was also unsatisfactory as a mixture of different derivatized product (mono- and biderivatized) was obtained.

In order to improve the derivatization efficiency, we increased the temperature to 100 °C, taking successive samples after 20, 40, and 60 min. On the basis of the results obtained, it appeared that higher temperatures do not speed up the derivatization kinetics, but rather lead to degradation of the derivatives if the duration time exceeds 40 min.

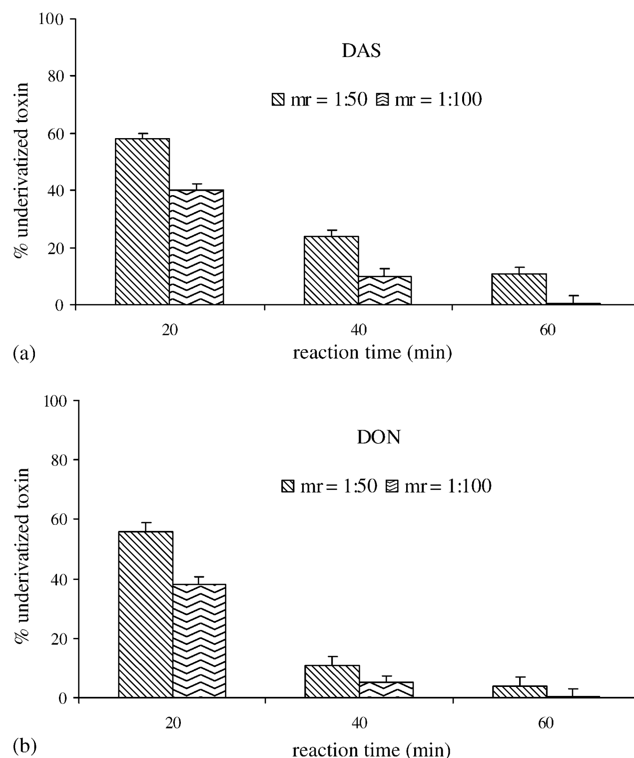

Fig. 2. Percentage of underivatized toxin vs. time for DAS (a) and DON (b) by reaction with coumarin-3-carbonyl chloride in acetonitrile at 80 °C at different toxin–C3CC molar ratios (mr = 1:50, 1:100).

Finally, we evaluated the effect of the molar ratio between the reactants: three different reactions were performed at 80 °C for 60 min, using a molar ratio of toxin–C3CC of 1:50, 1:100, and 1:200. The chromatographic analyses showed that, using a molar ratio toxin–C3CC of 1:100, the peak of the underivatized toxin totally disappeared after 60 min (Fig. 2a and b). No significant changes were observed at a higher molar ratio (1:200). Under these conditions, the selectivity was also greatly improved: in the case of DON (three hydroxyl groups) only the biderivatized product was obtained. The optimized reaction conditions for type A and type B trichothecenes were: acetonitrile as solvent, DMAP as the base, molar ratio toxin–C3CC–DMAP = 1:100:100, at 80 °C for 60 min.

The derivatization repeatability was verified by both LC–FLD and LC–MS. The reaction was performed in triplicate on the same (R.S.D. = 2.8%) and different days (R.S.D. = 4.2%).

An important parameter is the C3CC purity: the recristallization step is the critical step to obtain a pure fluorophore and to avoid the presence of interfering peaks during LC–FLD. Moreover, the complete dryness of the glassware is fundamental to avoid hydrolysis of coumarin-3-carbonyl chloride.

Table 1
Summary of the derivatization pattern of trichothecenes under the optimized conditions

Trichothecene	Nr. hydroxyl groups	Main product	Underivatized toxin	Secondary products
3-ac-DON	2	Monoderivatized	n.d.	n.d.
15-ac-DON	2	Monoderivatized	n.d.	n.d.
DAS	1	Monoderivatized	n.d.	–
FUSX	3	Biderivatized	<0.1%	n.d.
DON	3	Biderivatized	<0.1%	n.d.
T-2	1	Monoderivatized	n.d.	–
NIV	4	Triderivatized	n.d.	<0.1%
HT-2	2	Monoderivatized	n.d.	n.d.

n.d. = not detected.

3.2. LC–MS characterization of the derivatives of the eight trichothecenes

The derivatization reaction between C3CC and the eight trichothecenes was performed using the optimized conditions and the derivatives were analyzed by LC–MS. T-2 and DAS have only one hydroxyl group, whereas HT-2 and type B trichothecenes have from two to four hydroxyl groups. Therefore, it was necessary to study the nature of the derivatives and the reproducibility of the reaction. These experiments allowed to characterize in an univocal way the derivatives obtained and to evaluate at the same time the conversion percentage. T-2, DAS, 3-ac-DON and 15-ac-DON showed only the mono-functionalized product, with a conversion of 95% (ratio between the peak area of the underivatized toxin before and after the derivatization reaction). For HT-2, DON, NIV and FUSX the derivatization gave a main product (conversion: >90%) and only traces of secondary products. The results obtained in the optimized conditions are summarized in Table 1.

Now, the presence of the monoderivatized products for T-2 and DAS was expected since they have only one hydroxyl group at the 3 position, but also 3-ac-DON and 15-ac-DON gave the monoderivatized product, although both have two hydroxyl groups in the more hindered positions 7, 15, and 3, 7, respectively. It is feasible that the proximity of an acylated group (either the natural acetoxy or the coumaroyl group) prevents a second derivatization. Accordingly, also for HT-2, which bears two hydroxyl groups in the 3 and the 4 positions, the main product was the monoderivatized one with only traces of the biderivatized product. With DON and FUSX, which have three hydroxyl groups in the same positions (3, 7, and 15), the main products were the biderivatized ones, whereas with NIV, which has four hydroxyl groups (3, 4, 7, and 15), the main product was the trisubstituted one. Although it is not possible with these experiments to identify unequivocally the substitution positions, we think that the derivatization may occur preferentially at the positions 3 and 15, with the former being more accessible to the reagent and the latter being a primary alcohol.

In order to evaluate the inter-day precision, the reaction was repeated five times using a blank sample spiked with a mixture of the analytes and obtaining a R.S.D. < 3.2% for each tested mycotoxin. Thus, the reaction is highly reproducible.

3.3. Optimization of LC–FLD analysis

The LC procedure for type A trichothecenes [25] involved the use of a C_{18} standard-bore column and an isocratic elution with water–CH_3CN (42:58, v/v) as mobile phase. Under these conditions, a mixture of eight type A and type B trichothecenes (DAS, DON, T-2, HT-2, NIV, FUSX, 3-ac-DON, 15-ac-DON), after reaction with C3CC, were well separated; only DON and FUSX showed a partial overlapping. In order to improve the separation and to avoid the DON and FUSX coelution, a gradient was performed. Different acetonitrile–water and methanol–water mobile phases were tested, using two different C_{18} standard bore columns of different lengths (250 and 150 mm) with identical diameter and granulometry and a C_{18} narrow-bore column (250 mm). The best results were obtained with the narrow-bore column in terms of both chromatographic separation and sensitivity with a methanol–water gradient. The chromatogram obtained for a wheat sample spiked with a mixture of the eight functionalized trichothecenes is reported in Fig. 3.

The chromatogram obtained for a blank sample showed only the peak of the derivatizing reagent within the first 5 min,

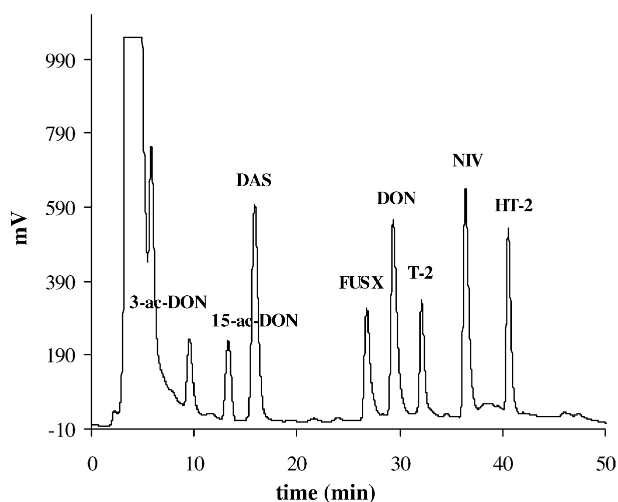

Fig. 3. Chromatogram of a standard mixture of the coumaroyl derivatives (amount of each trichothecene before derivatization: 3 ng/μl). Chromatographic conditions: column, Spherisorb S3ODS2 narrow-bore (250 mm × 2.1 mm, 3 μm); eluent, water–CH_3OH; gradient (see Section 2.5); flow rate: 0.2 ml/min.

Table 2
Linear calibration parameters obtained for each toxin

Trichothecene	Retention time (min)	Slope	Intercept	r^2	LOD (ng/g)
3-ac-DON	9	$(1.39 \pm 0.02) \times 10^3$	$(1.11 \pm 0.01) \times 10^5$	0.9967	1.0
15-ac-DON	12	$(2.58 \pm 0.03) \times 10^3$	$(7.29 \pm 0.03) \times 10^5$	0.9979	1.0
DAS	16	$(5.09 \pm 0.01) \times 10^3$	$(2.53 \pm 0.02) \times 10^5$	0.9979	0.2
FUSX	27	$(2.38 \pm 0.02) \times 10^3$	$(1.43 \pm 0.03) \times 10^5$	0.9983	0.6
DON	29	$(4.34 \pm 0.03) \times 10^3$	$(2.67 \pm 0.04) \times 10^5$	0.9986	0.4
T-2	32	$(2.02 \pm 0.04) \times 10^3$	$(4.03 \pm 0.02) \times 10^4$	0.9973	0.6
NIV	38	$(4.31 \pm 0.01) \times 10^3$	$(1.73 \pm 0.05) \times 10^5$	0.9993	0.2
HT-2	41	$(2.93 \pm 0.04) \times 10^3$	$(1.98 \pm 0.02) \times 10^5$	0.9966	0.4

Chromatographic conditions: C_{18} Spherisorb S3ODS2 narrow-bore column (250 mm × 2.1 mm, 3 μm); water–CH_3OH mobile phase, gradient (see Section 2); flow rate: 0.2 ml/min; fluorescence detector (λ_{ex} = 292 nm, λ_{em} = 425 nm).

perfectly matching the initial chromatographic profile of the spiked sample.

The linearity was tested in the concentration range 10–5000 ng/ml. Calibration curves were obtained by adding the proper amount of each toxin to a blank wheat sample (six concentration levels). The LOD ($S/N = 3/1$) and the LOQ ($S/N = 10/1$) were lower than 1 ng/g and than 3.5 ng/g, respectively, for all the trichothecenes (Table 2).

The obtained limits are more than two orders of magnitude lower than those obtained by LC–UV [2,16] and one order of magnitude lower than those obtained by LC–atmospheric pressure chemical ionization (APCI) MS [2]. They are also one order of magnitude lower than those previously obtained by Jimenez et al. [25–27] for type A trichothecenes by derivatization with coumarin-3-carbonyl chloride. Although LODs are comparable with those obtained with GC–MS, our method allows a more precise quantification with less sample pretreatment: indeed, for GC–MS analysis the excess of derivatizing agents should be accurately removed, multiple ion monitoring of at least three ions should be used for identity confirmation and addition of an internal standard is generally recommended for quantification [13].

3.4. Application to naturally contaminated samples

The procedure was applied to a certified wheat reference material (contamination level: 894 ng/g; satisfactory range: 603–1185 ng/g). The wheat sample was extracted using the procedure normally used for the analysis of trichothecenes in cereals, then derivatization and LC analysis were performed, as described in the Section 2. The chromatogram obtained for the reference material is reported in Fig. 4.

In order to correctly identify the toxin, the sample was spiked with an authentic standard of DON.

The procedure for the analysis of the reference material was repeated three times and the measured value for DON was corrected for the recovery. The assigned (894 ng/g) and the detected (795 ng/g) DON concentrations were consistent with the satisfactory range, with an accuracy value $E = 11\%$ and a satisfactory z-score value ($z = 0.67$). The recovery calculated for the certified reference material was 89% at the contamination level.

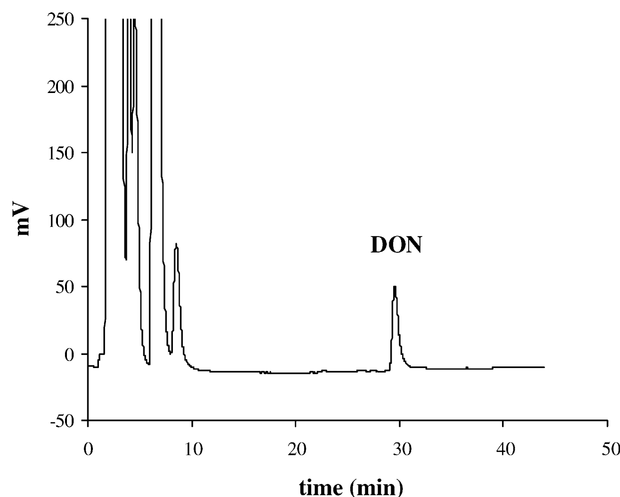

Fig. 4. Chromatogram of a wheat sample containing 795 ng/g of DON (FAPAS certified reference material).

4. Conclusions

The method here proposed for the simultaneous detection of type A and type B trichothecenes is very satisfactory. The study of the parameters for the derivatization of trichothecenes with coumarin-3-carbonyl chloride (molar ratio, temperature, reaction time) allowed not only the derivatization of type A but also of type B trichothecenes in a nearly quantitative yield for both classes. The derivatization is reproducible and very selective with only one main product being formed, although every toxin shows a different derivatization pattern, according to the number of the hydroxyl groups present in the molecule. All the fluorescent derivatives were characterized by LC–MS analysis.

The LC–FLD analysis of the cumaroyl fluorescent derivatives obtained according to the optimized procedure is simple, sensitive and allows the simultaneous determination of three type A (DAS, T-2, HT-2) and five type B trichothecenes (3-ac-DON, 15-ac-DON, DON, NIV, FUSX) and it can be applied successfully to other trichothecenes of both classes. Both LOD and LOQ ($0.2 < \text{LOD} < 1$ ng/g and $0.9 < \text{LOQ} < 3.5$ ng/g, respectively) are lower than those reported for the detection of type A and type B trichothecenes using

either LC–UV or LC–APCI–MS and are of the same order of magnitude of those obtained by GC–MS.

The method was successfully applied to the analysis of certified wheat reference materials, obtaining a good accuracy with a recovery of 89%.

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