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Simultaneous high-performance liquid chromatographic determination of ochratoxin A and citrinin in cheese by time-resolved luminescence using terbium

B.I. Vazquez[a,*], C. Fente[a], C. Franco[a], A. Cepeda[a], P. Prognon[b], G. Mahuzier[b]

[a]*Laboratorio de Higiene e Inspección de Alimentos, Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain*

[b]*Laboratoire de Chimie Analytique II, Faculté de Pharmacie, 5 rue J.B Clément, 92290 Châtenay-Malabry, France*

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Abstract

A simultaneous reversed-phase HPLC determination of two major mycotoxins, ochratoxin A and citrinin, in soft cheese is proposed. Both mycotoxins are eluted on a C_{18} RP support (25 × 4.6 mm I.D.) using an isocratic eluent consisting of methanol–water (70:30, v/v) containing tetrabutylammonium hydroxide (10^{-3} *M*), acidified to pH 5.5 with HCl, and pumped at a flow-rate of 0.8 ml/min. Prior to detection, a butanolic solution of $5 \cdot 10^{-3}$ *M* terbium–$5 \cdot 10^{-4}$ *M* trioctylphosphine oxide (TOPO)–$2.5 \cdot 10^{-2}$ *M* triethylamine (TEA) was pumped in a postcolumn mode at a flow-rate of 0.2 ml/min to perform time-resolved luminescence (TRL) detection of the corresponding terbium chelates (λ_{ex} = 331 nm/λ_{em} = 545 nm). The method is linear from $3.5 \cdot 10^{-6}$ to $2 \cdot 10^{-5}$ *M* for citrinin and from $1 \cdot 10^{-5}$ to $5 \cdot 10^{-5}$ *M* for ochratoxin A. The repeatability and reproducibility (R.S.D.) are 1.9 and 2.4% for citrinin ($c = 3.5 \cdot 10^{-6}$ *M*; $n = 10$), and 7.2 and 8.3% for ochratoxin A ($c = 1.0 \cdot 10^{-5}$ *M*; $n = 10$). The limits of detection, for a signal-to-background ratio of 3, are $2 \cdot 10^{-6}$ and $3 \cdot 10^{-6}$ *M* for citrinin and ochratoxin A, respectively. With the proposed method, ochratoxin A and citrinin are easily determined in soft cheeses, with a significative increase in selectivity in comparison with direct fluorescence detection.

Keywords: Food analysis; Luminescence; Time-resolved luminescence detection; Detectors, LC; Ochratoxin A; Citrinin; Mycotoxins; Toxins; Terbium

1. Introduction

Since the discovery, in the early 1960s, that the severe toxic outbreak of "turkey X disease" was caused by aflatoxins [1], other mycotoxins were subsequently shown to be involved in the aetiology of acute and chronic diseases both for men and animals [2].

Data from several workers have proved that the amount of different products subject to spoilage by moulds during distribution or storage is not negligible, and due to mycotoxin-producing moulds. These toxins are reported to be ubiquitous, and thus recognized as a real public health problem [3–7].

* Corresponding author.

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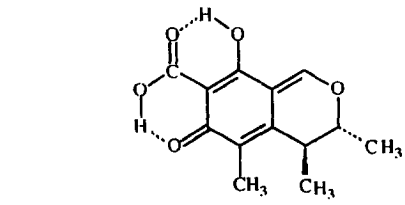
Among them, citrinin and ochratoxin A are secondary metabolites produced by fungal species included in the genera *Aspergillus* and *Penicillium* and their simultaneous production by strains of *Penicillium verrucosum*, which has also been reported as a very active colonizer on dairy products [3,7–10].

Owing to their nephrotoxic effects on animal and human health, these toxins have been studied in a large variety of foodstuffs. The consumption of contaminated feed by domestic animals can result in the contamination of food such as meat, milk and other dairy products, especially soft cheese [11–15]. Hence the determination of citrinin and ochratoxin A appears to be of major importance.

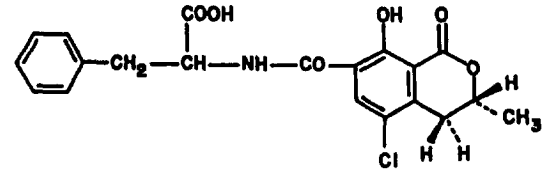
Several thin-layer chromatographic (TLC) multi-mycotoxin methods have been proposed [16–20]. Reversed-phase high-performance liquid chromatographic (RP-HPLC) methods with absorbance detection have also been applied [21–23]. However, in recent years, fluorescence detection has been almost universally adopted for citrinin, ochratoxin A and other acidic mycotoxins, taking advantage of the natural fluorescence of these compounds [3,16,20,22,24,25].

Nevertheless, in practice, the application of all these methods to different complex matrices could become tedious and time consuming and sometimes lacking in specificity. This especially implies a great concern with interferences from contaminated samples which diminish the specificity and selectivity of analyses for some mycotoxins such as citrinin and ochratoxin A even when using fluorescence detection, especially as the fluorescence ratio between ochratoxin A and citrinin is about 1700 when they are simultaneously detected in a weakly acidic medium.

To overcome these severe limitations, we explored a more specific method for the detection of these two compounds, i.e., time-resolved luminescence (TRL) of their lanthanide chelates. Their chemical structures show, in common, acidic functions (carboxylic and phenolic for citrinin and ochratoxin A, respectively) close to donor groups (α-phenoxy and α-keto for citrinin and α-keto for ochratoxin A), which make them good candidates for the chelation of metal ions (Fig. 1). The six-sided pseudocycles formed in this way exhibit a stability which largely depends on the surrounding substituents attached to the aromatic moiety. On the other hand, the lanthanide chelates exhibit a long-life emission (over 500 μs depending on the experimental conditions) that allows a TRL measurement with an emission maximum located at 545 nm for terbium chelates ($^5D_4 \rightarrow {}^7F_5$ transition), thus minimizing the background signal and increasing the selectivity of the detection [26–28].



CITRININ



OCHRATOXIN A

Fig. 1. Structures of citrinin and ochratoxin A.

Owing to the spectroscopic characteristics of the lanthanide ions, a brief spectroscopic study of the complex formation between these mycotoxins and the three major emitting species in solution, europium, samarium and terbium, was performed. Finally, terbium (Tb^{3+}) was selected owing to its higher efficiency in the energy transfer process.

Optimization of the TRL terbium signal was carried out during the study of the influence of the solvent, by selecting the alkalinising agent (triethylamine) and adjusting the concentration of the synergistic agent trioctylphosphine oxide (TOPO).

In this paper, we report a simultaneous method for the determination of citrinin and ochratoxin A in soft cheese using TRL detection and

postcolumn (PC) addition of terbium. The analytical interest of lanthanide chelate formation for the determination of mycotoxins in this complex matrix is also emphasized.

2. Experimental

2.1. Chemicals

Citrinin and ochratoxin A, tetrabutylammonium hydroxide (TBA) and trioctylphosphine oxide (TOPO) were purchased from Sigma (St. Louis, MO, USA), and triethylamine (TEA) from Merck. Samarium(III) chloride hexahydrate ($SmCl_3 \cdot 6H_2O$) (>99.999%), terbium(III) chloride hexahydrate ($TbCl_3 \cdot 6H_2O$) (>99.999%) and europium(III) chloride hexahydrate ($EuCl_3 \cdot 6H_2O$) (>99.999%) were purchased from Aldrich (Darmstadt, Germany). All other chemicals were of analytical-reagent grade.

2.2. Solutions

Methanolic $1 \cdot 10^{-2}$ *M* stock standard solutions were prepared for each mycotoxin and stored protected from light at 4°C for 1 week. Working solutions were prepared daily with the selected solvent by appropriate dilution of the stock solutions.

Stock methanolic solutions of lanthanide salts and TOPO ($1 \cdot 10^{-2}$ *M*) were kept at 4°C protected from light, and diluted to the desired concentration with the appropriate solvent as needed.

Tris buffer was adjusted at pH 8 with HCl [29,30].

2.3. Apparatus

An LS 50B luminescence spectrometer (Perkin-Elmer, Norwalk, CT, USA) was used for spectroscopic studies. Excitation and emission slits were both set at 5 nm. All the measurements were performed at room temperature, in triplicate, in a 10 mm quartz cuvette at 20 ± 2°C.

The chromatographic system consisted of two LC-9A metering pumps (Shimadzu, Kyoto, Japan), one for the eluent, equipped with a 20-μl loop injector (Rheodyne Model 7125), and the second for introducing the lanthanide postcolumn reagent. The mixing of the mobile phase and the postcolumn reagent was performed with a Tee mixer (Supelco, Bellefonte, PA, USA). Luminescence detection was performed with an LC 240 detector (Perkin-Elmer) and UV measurements at 331 nm with an SPD-6A detector (Shimadzu). Chromatographic separation was achieved on a C_{18} Inertsyl OSD-2 5-μm column (250 × 4.6 mm I.D.) (Interchrom, France). The temperature of the column was maintained constant at 21 ± 0.1°C with a column oven (Crococil, France). Chromatograms were recorded with a C-R5A integrator (Shimadzu).

2.4. Chromatographic conditions

The mobile phase was methanol–water (70:30, v/v) containing tetrabutylammonium hydroxide (10^{-3} *M*) and acidified at pH 5.5 with hydrochloric acid. Careful degassing and filtration (Waters–Millipore HA filters) were performed prior to use. The eluent was pumped at a flow-rate of 0.8 ml/min. The postcolumn reagent was a butanolic solution of $5 \cdot 10^{-3}$ *M* Tb^{3+}–$5 \cdot 10^{-4}$ *M* TOPO–3.5 μl/ml ($2.5 \cdot 10^{-2}$ *M*) TEA, pumped at a flow-rate of 0.2 ml/min.

Time-resolved detection was performed setting the excitation at 331 nm and the emission at 545 nm. The delay time (t_d) and the gating time (t_g) were optimized to eliminate the luminescence background; they were set at 0.03 and 1 ms, respectively.

2.5. Preparation of cheese extracts

Extraction of cheese was performed according to Taniwaki and Van Dender [11] and Wei-Yun et al. [31]. When necessary, methanolic cheese extracts were spiked with convenient amounts of citrinin and ochratoxin A.

3. Results and discussion

One of the main problems associated with the simultaneous determination of the two studied

mycotoxins in soft cheese comes from the lack of specificity of the extraction step. This is due to the different physico-chemical features of these mycotoxins which imply the co-extraction of numerous endogenous substances, especially aromatic and coloured compounds, whatever the extraction procedure used [11,31]. Our efforts to make this step more selective were unsuccessful, maybe due to the difference in maturation (i.e., composition) between the cheese samples to be assayed. In that context, the selectivity of the detection mode used after the chromatographic separation appears to be crucial. The UV absorption, with a maximum located around 331 nm for both compounds, appears to be insufficiently selective, as shown on a blank chromatogram (Fig. 2) showing heavy polluted profiles, with interfering peaks eluted under the compounds of interest as seen on the corresponding spiked sample [see Fig. 7 (I)], despite the convenient sensitivity obtained (Table 1). HPLC with fluorescence detection has been widely preferred [21–23]. Nevertheless, the matrix variability frequently observed in soft cheeses led to the occurrence of unexpected disturbing peaks with various retention times. This prompted us to seek a more specific method of detection than direct fluorescence, i.e., time-resolved luminescence (TRL).

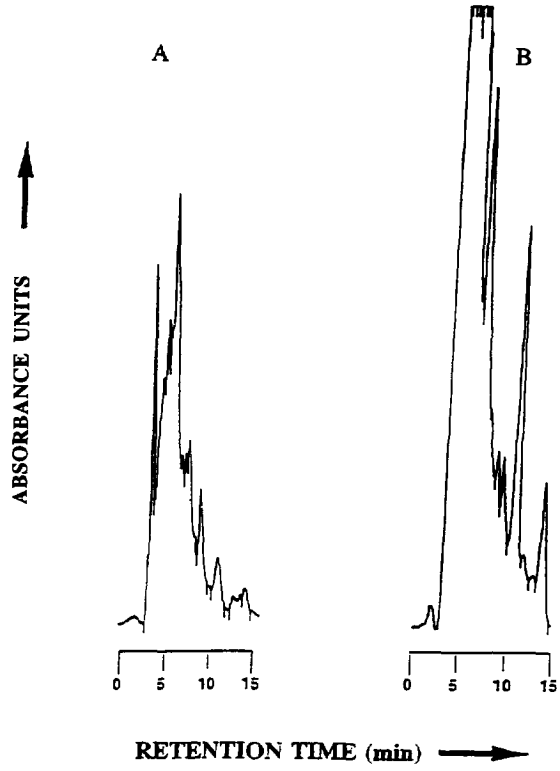


Fig. 2. Chromatograms of cheese extract blanks with UV detection ($\lambda = 331$ nm). (A) Extraction according to Taniwaki and Van Dender [11]; (B) Extraction according to Wei-Yun et al. [31].

3.1. Chromatography

Normal-phase HPLC is commonly applied for the determination of citrinin [32], but reversed-phase HPLC is almost universally used for ochratoxin A. Since our aim was to develop a simultaneous method for the determination of these two toxins, and because the most recently reported HPLC methods for citrinin and ochratoxin A are reversed-phase procedures [16,20,22,25], an isocratic mobile phase of methanol–water was envisaged. Methanol was chosen in preference to acetonitrile because it has been demonstrated to act as a better enhancer for the terbium luminescence signal [33].

A cationic ion-pairing reagent was added to the mobile phase to make the chromatography reproducible and to ensure a convenient resolution ($R_s = 1.4$) between citrinin and ochratoxin A. Owing to the acidic character of both toxins and to their partial ionization at the pH of the mobile phase, tetrabutylammonium hydroxide (TBA) was used, according to data previously reported in the literature [34]. For our experiments, a concentration of $1 \cdot 10^{-3}$ M TBA was necessary. The elution conditions for full optimization (i.e., pH, temperature and MeOH concentration), are displayed in Fig. 3.

It should be noted that the separation of citrinin and ochratoxin A was the key point of the chromatography (see above), and a satisfactory resolution is only achieved with careful control of (1) column temperature (21 ± 0.5°C), (2) methanol content (70 ± 2%, v/v) and (3) apparent pH of the eluent (5.5 ± 0.1) (Fig. 4).

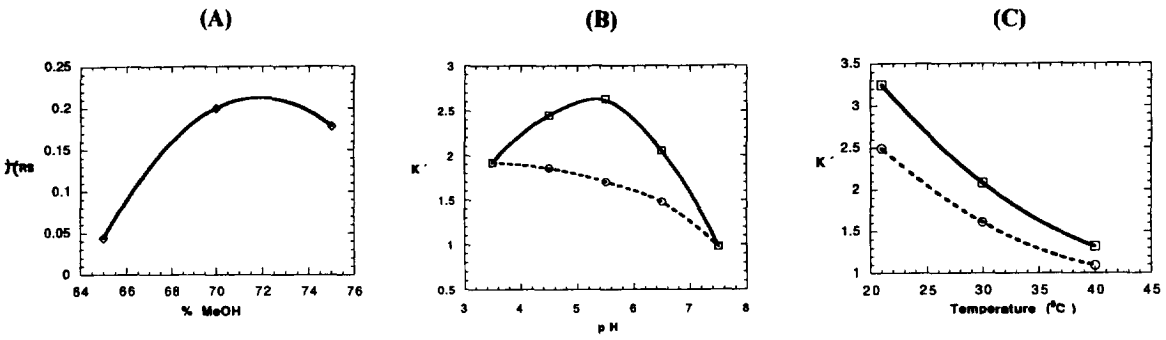


Fig. 3. Mobile-phase optimization for (○) citrinin ($8 \cdot 10^{-4}$ *M*) and (□) ochratoxin A ($8 \cdot 10^{-6}$ *M*). Each point is the mean of three measurements. (A) Influence of the methanol content (Π_{R_s} versus MeOH concentration (%, v/v); (B) influence of pH; (C) influence of column temperature.

Optimization of the elution conditions was carried out using the Π_{R_s} algorithm according to Schoenmakers [35].

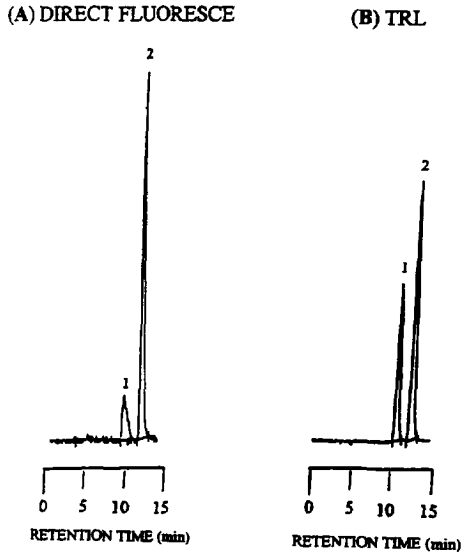


Fig. 4. Typical chromatograms of (1) citrinin and (2) ochratoxin A. Two solutions (A and B), with different concentrations, are represented in order to show clean and nearly similar chromatograms. (A) $9 \cdot 10^{-4}$ *M* citrinin and $6 \cdot 10^{-7}$ *M* ochratoxin A. Mobile phase, methanol–water (70:30, v/v) containing 10^{-3} *M* TBA with direct fluorescence detection ($\lambda_{ex} = 331$ nm and $\lambda_{em} = 472$ nm). (B) $7 \cdot 10^{-6}$ *M* citrinin and $2 \cdot 10^{-5}$ *M* ochratoxin A. Same mobile phase as in (A), with a postcolumn eluent consisting of $5 \cdot 10^{-3}$ *M* Tb^{3+}–$5 \cdot 10^{-4}$ *M* TOPO–$2.5 \cdot 10^{-2}$ *M* TEA in butanol with TRL detection ($\lambda_{ex} = 331$ nm and $\lambda_{em} = 545$ nm; $t_d = 0.03$ and $t_g = 1$ ms).

3.2. Optimization of the postcolumn (PC) reagent

As citrinin and ochratoxin A exhibit native fluorescence, a common direct fluorescence detection system ($\lambda_{ex} = 331$ nm and $\lambda_{em} = 472$ nm) was used as a reference detection mode for the study. This set-up was chosen in part owing to the very close absorbance maxima of the two compounds of interest (330–332 nm under our experimental conditions) and in part owing to the convenient averaged emission maximum (472 nm) valid for the two compounds.

First, the formation of chelates with different lanthanide ions, samarium, europium and terbium, was studied. The best response was obtained for ochratoxin A and citrinin with terbium. Consequently, the pair of wavelengths chosen was (see above) $\lambda_{ex} = 331$ nm and $\lambda_{em} = 545$ nm. After optimization, the delay and the gating time were set at 0.03 and 1.00 ms, respectively.

An alkaline pH is the most favourable for the formation of chelates, owing to the ionization of the acidic mycotoxins [33]. However, at pH > 8.5, terbium hydroxide starts to precipitate. Therefore, we first selected alcohols instead of water as postcolumn solvents, and second TEA, which is miscible in alcohols and acts as a base strong enough to increase the ionization of the acidic functions of the two toxins. In practice, 3.5 μl/ml ($2.5 \cdot 10^{-2}$ *M*) of TEA were added to the PC reagent to obtain a suitable final apparent pH

between 6.5 and 7.5, and in order to avoid the precipitation of the solution which occurs with higher concentrations of TEA.

As mentioned above, the PC solutions were prepared in an alcoholic solvent (first in methanol) instead of water. The inhibitory effects of water on the luminescence of the lanthanide chelates has been reported previously [33,36]. To control this drawback, the addition of TOPO is often used, since the molecule (via its oxygen atom) creates a shield around the lanthanide complex. Fig. 5 shows, as an example, the luminescence of the terbium chelate of citrinin versus different concentrations of TOPO and TEA in methanol.

As already mentioned, experiments with a Tris-buffered solution (pH 8) and several alcohols were attempted to enhance the TRL signal. Among the alcoholic solvents tested, butanol gave the best results. Its higher viscosity compared with methanol limits the non-radioactive deactivation of the chelate by a reduction in the number of molecular collisions [37] (Fig. 6). Moreover, it should be noted that the choice of butanol was facilitated because terbium is no longer soluble in higher alcohols such as pentanol and hexanol. Fig. 4B shows the final chromatographic separation of citrinin and ochratoxin A with the optimized postcolumn eluent reagent in comparison with direct fluorescence detection (Fig. 4A).

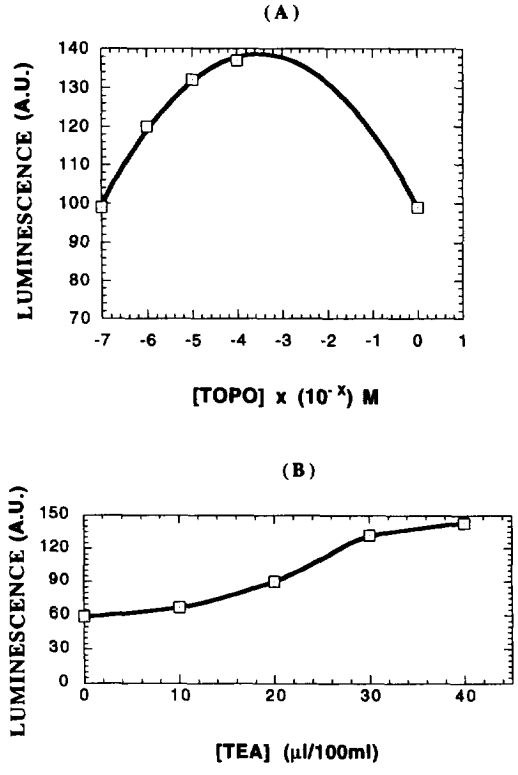


Fig. 5. Study of the luminescence response of citrinin ($5 \cdot 10^{-4}$ *M*) with terbium ($1 \cdot 10^{-3}$ *M*) in the postcolumn eluent, (A) as a function of TOPO concentration and (B) as a function of TEA concentration. For TRL conditions, see Experimental.

3.3. Analytical figures of merit and analysis of real samples

The proposed method was demonstrated to be linear from $3.5 \cdot 10^{-6}$ to $2 \cdot 10^{-5}$ *M* ($r = 0.982$ and linearity test highly significant with $p < 0.001$) for citrinin and from $1 \cdot 10^{-5}$ to $5 \cdot 10^{-5}$ *M* ($r = 0.993$ and linearity test highly significant with $p < 0.001$) for ochratoxin A. The repeatability and the reproducibility ($c = 3.5 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ *M* for citrinin and ochratoxin A, respectively; $n = 10$ in each case) seem convenient with R.S.D.s of 1.9 and 2.4% and 7.2 and 8.3%, respectively, for the two mycotoxins. It seems that, although not clearly understood, at the limit of quantification

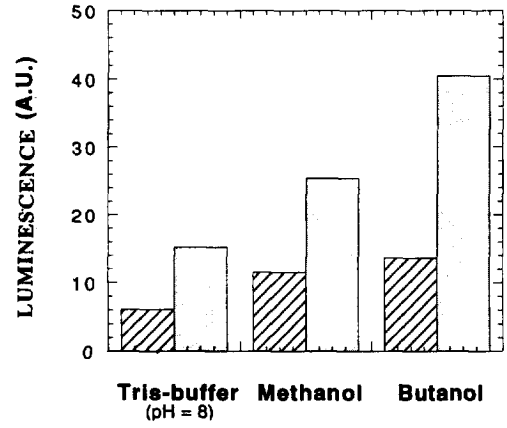


Fig. 6. Luminescence signal of (hatched boxes) citrinin ($4 \cdot 10^{-6}$ *M*) and (shaded boxes) ochratoxin A ($3 \cdot 10^{-5}$ *M*) as a function of the nature of the solvent of the PC reagent. For the composition of the PC reagent, see text.

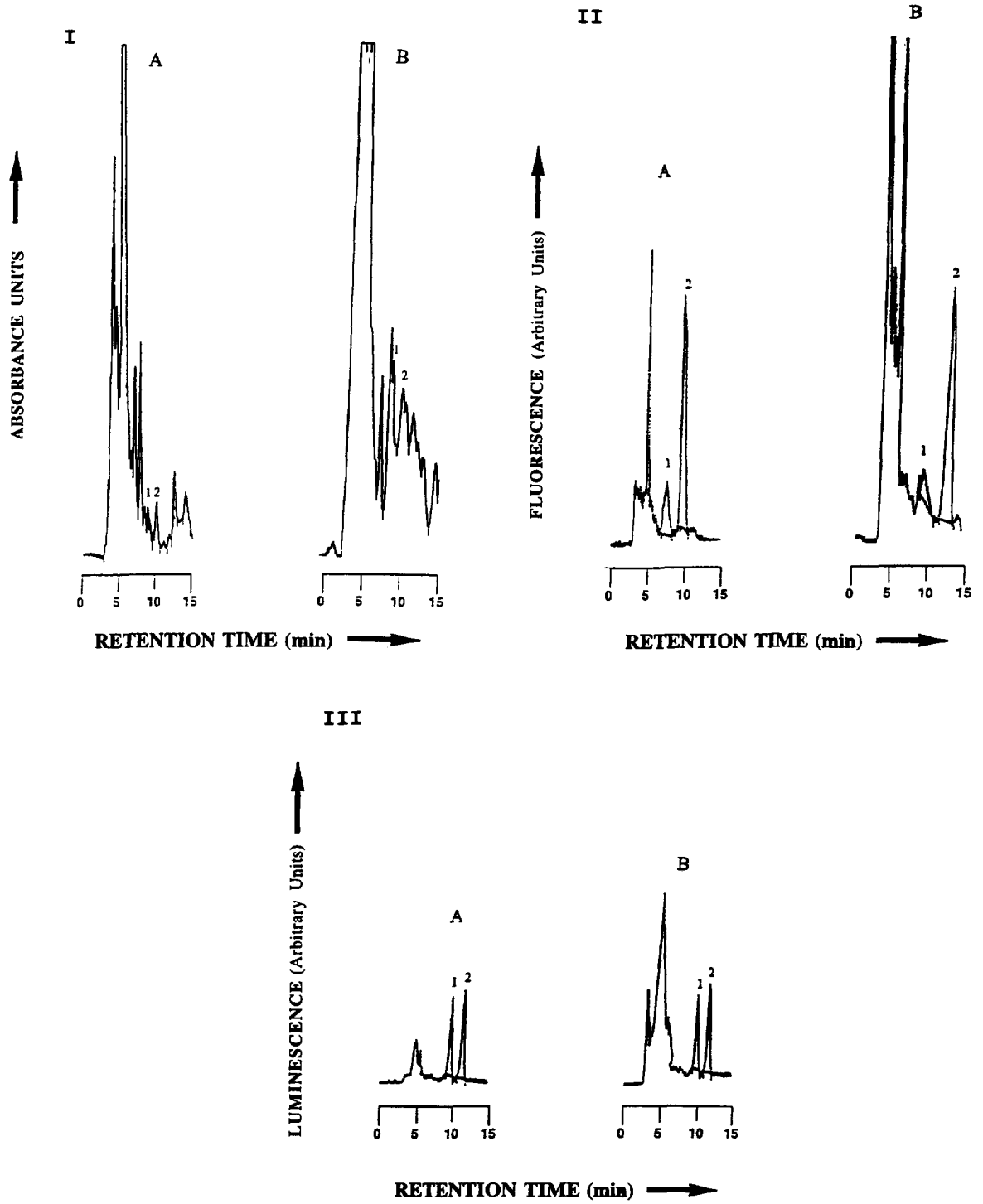


Fig. 7. (I) Chromatograms of cheese extracts (the same as in Fig. 2) spiked with (1) citrinin and (2) ochratoxin A, with UV detection ($\lambda = 331$ nm). Extracts spiked with $3 \cdot 10^{-6}$ *M* citrinin and $5 \cdot 10^{-6}$ *M* ochratoxin A. (II) Chromatograms of cheese extracts spiked with (1) citrinin and (2) ochratoxin A, with direct fluorescence detection ($\lambda_{ex} = 331$ nm/$\lambda_{em} = 472$ nm). Extracts spiked with $2 \cdot 10^{-3}$ *M* citrinin and $1 \cdot 10^{-6}$ *M* ochratoxin A. (III) Chromatograms of cheese extracts spiked with (1) citrinin and (2) ochratoxin A, with TRL detection. For conditions, see text. Extracts spiked with $4.5 \cdot 10^{-6}$ *M* citrinin and $1 \cdot 10^{-5}$ *M* ochratoxin A.

Table 1
Comparison of limits of detection (M) between direct fluorescence, TRL and UV detection modes for citrinin and ochratoxin A (signal-to-background ratio = 3)

Mycotoxin	Direct fluorescence (λ_{ex} = 331 nm/λ_{em} = 472 nm)	TRL (λ_{ex} = 331 nm/λ_{em} = 545 nm)	UV (λ = 331 nm)
Citrinin	$1 \cdot 10^{-4}$	$2 \cdot 10^{-6}$	$2 \cdot 10^{-6}$
Ochratoxin A	$8 \cdot 10^{-8}$	$3 \cdot 10^{-6}$	$3 \cdot 10^{-6}$

level the accuracy is lower for ochratoxin A than for citrinin.

Table 1 shows the detection limits (signal-to-background ratio = 3) of UV, direct fluorescence and TRL detection. It can be observed that the terbium chelate substantially enhances the detection of citrinin from $1 \cdot 10^{-4}$ to $2 \cdot 10^{-6}$ M, i.e., more than 70-fold, yielding a performance similar to that with UV detection. In contrast, the TRL and UV detection of ochratoxin A are significantly weaker than in the direct fluorescence mode. For ochratoxin A this is certainly due to a lower association constant with Tb^{3+} than with citrinin.

The limits of detection (LODs) obtained with the proposed method appear modest in comparison with those of other published HPLC–TRL techniques performed in complex matrices. As an example, serum and urine analyses using lanthanide chelates of tetracyclines [38,39], oroate [40], fluoroquinolones [33] and theophylline [41] exhibit lower LODs (ranging around 10^{-8} M) than ochratoxin and citrinin. In contrast, some antibiotics such as bleomycin are detected similarly to the studied mycotoxins (LOD = $3 \cdot 10^{-6}$ M) [42]. Such differences could be attributed, in part, to the respective performances of the HPLC devices used, but especially to the difference in energy transfer efficiency between the chelating agent and the ligand, that is, the difference in magnitude of the association constants of the complexes. In our case, the limited available amounts of the toxins involved in the present study make this constant determination too uncertain. Consequently, further discussion concerning the TRL performance established here with respect to the points raised above is unfortunately not possible.

Lastly, and as an example, Fig. 7 shows chromatograms corresponding to the analysis of two cheese extracts extracted according to Taniwaki and Van Dender [11] and Wei-Yun et al. [31], spiked with citrinin and ochratoxin A, analysed first with UV absorption detection (1) as a comparison, then with direct fluorescence detection (2) and finally with TRL detection (3). As can be seen, a decrease in the background noise, associated with better selectivity, is evident and confirms the interest of the proposed method.

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