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Simple and sensitive high-performance liquid chromatography–fluorescence method for the determination of citrinin Application to the analysis of fungal cultures and cheese extracts ☆

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Abstract

A new and highly sensitive method for the detection of the important mycotoxin, citrinin, has been developed. Spectroscopic studies demonstrate that the fluorescence of this metabolite is influenced by the pH of the environment. This fact was exploited in the chromatographic determination of citrinin with fluorescence detection. The proposed method, based on the addition of 1 *M* hydrochloric acid as an acidic post-column reagent, has a limit of detection of $0.9 \cdot 10^{-7}$ *M*. Analytical validation shows that linearity can be assumed from $2 \cdot 10^{-7}$ to 10^{-4} *M* citrinin. The repeatability and reproducibility are satisfactory, with R.S.D. = 5.1% ($n = 9$, $c = 10^{-5}$ *M*) and R.S.D. = 7.2% ($n = 9$, $c = 10^{-5}$ *M*). The method was also applied to the determination of this mycotoxin produced by mould cultures isolated from soft cheese and also from soft cheese and also from cheese extracts spiked with citrinin. The specificity of the method is demonstrated and the necessity for post-column acidification is illustrated on real samples.

Keywords: Fungal cultures; Cheese; Citrinin; Mycotoxins

1. Introduction

Citrinin [IUPAC: (3*R*,4*S*)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid; C.A. 518-75-2], a fungal metabolite first isolated in 1931 from *Penicillium citrinum* [1] is produced by several species of the genera *Aspergillus* and *Penicillium*. This mycotoxin (Fig. 1) has antibiotic, bacteriostatic, antifungal and antiprotozoal properties, but it may be a cause of nephrotoxicity in several animal species. Likewise, citrinin may be present in naturally and artificially produced mouldy cheese

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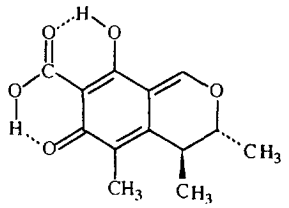

Fig. 1. Chemical structure of citrinin.

[2–4], but it is important to indicate that the presence of the fungus in cheeses is not always associated with the presence of mycotoxin. In addition, trace amounts of citrinin may be produced at earlier stages of the fungal growth [5]. These facts explain the need for a sensitive analytical procedure for the determination of citrinin in various foodstuffs and especially in cheese.

Several TLC methods for the determination of this mycotoxin have been published, but, as reported in recent reviews [6], they exhibit relatively low sensitivities. Several other methods have recently been published which use reversed-phase high-performance liquid chromatography (RP-HPLC) with acidic mobile phases and fluorescence detection [5,7,8]. In these studies, fluorescence detection has been shown to be much more sensitive than UV absorbance detection. Acidification of the eluent is mandatory for chromatography because of the acidic character of the citrinin. This acidification reduces the interactions between the analyte and the free silanol groups of the stationary phase. On the other hand, the weak fluorescence properties of citrinin in comparison with other mycotoxins such as zearalenone and ochratoxin, prompted us to perform a brief spectroscopic study to select the optimum detection conditions which would be compatible with a satisfactory elution of citrinin. Post-column addition of diluted mineral acid lowers the pH and improves the signal. In this paper, we report on an improved post-column fluorimetric detection of citrinin coupled with ion pairing RP-HPLC and on the use of this method in real samples (fungal cultures and cheese extracts).

2. Experimental

2.1. Chemicals

Citrinin was obtained from Sigma (St. Louis, MO, USA). For HPLC, a citrinin (10^{-4} *M*) working standard methanolic solution was used for both chromatographic and spectroscopic studies. All solvents used in chromatography were of HPLC grade; solvents used for spectroscopy were of spectroscopic grade. Tetrabutylammonium hydroxide (TBA) was obtained from Sigma. All other reagents used were of analytical grade.

2.2. Apparatus

A LS 50B Model luminiscence spectrometer (Perkin-Elmer, Norwalk, CT, USA) was used for spectroscopic studies of citrinin. Both excitation (ex) and emission (em) slits were set at 5 nm. All measurements were performed in triplicate in a 10-mm quartz cuvette at 20 ± 2°C.

The chromatographic system consisted of two Model LC-9A metering pumps (Shimadzu, Kyoto, Japan), one for the eluent, equipped with a 20-μl loop injector (Rheodyne Model 7125) and the other for introducing the acidic post-column reagent. The mobile phase and the post-column reagent were mixed with a Tee mixer (Supelco, USA). Fluorescence detection was performed with a LC 240 fluorescence detector (Perkin-Elmer) with $\lambda_{ex} = 331$ nm and $\lambda_{em} = 500$ nm. The chromatographic separation was achieved on a C_{18} Nucleosil, 5 μm column (250×4.6 mm I.D., Interchrom, France). The temperature of the column was maintained at 21 ± 0.1°C with a column oven (Crococil®, France). Results were recorded with a C-R5A Chromatopac integrator (Shimadzu).

2.3. Chromatographic conditions

The mobile phase consisted of a solution of tetrabutylammonium hydroxide ($5.7 \cdot 10^{-4}$ *M*) in methanol, adjusted to pH 5.5 by addition of aqueous hydrochloric acid (1 *M*). The eluent was carefully degassed and filtered prior to use, and a

flow-rate of 0.8 ml/min was applied, with the column oven set to 21°C. The post-column reagent was 1 *M* aqueous hydrochloric acid pumped at a flow-rate of 0.2 ml/min. The retention time of citrinin under these conditions was 11.5 min.

2.4. Fungal strains

The fungal strains were isolated from soft cheese. All colonies were seeded as many times as necessary on 2% malt agar (Difco) until pure cultures were obtained. The fungi were identified on the basis of their macroscopic and microscopic characteristics according to the keys of Von Arx [9], Raper and Thom [10] and Raper and Fennell [11]. The works of Barnett and Hunter [12] and Ainsworth and Sussman [13] were also consulted.

2.5. Preparation of extracts from fungal cultures and cheese

The moulds were examined for toxin production by inoculation in yeast extract agar and the contents of the Petri dishes were extracted according to El-Banna et al. [14]. Extraction of cheese was performed according to the method of Taniwaki and Van Dender [4], and the removal of lipid from the samples according to Siriwardana and Lafont [15]. If necessary, methanolic cheese extracts were spiked with citrinin stock solution in order to obtain a level of 0.1 mg/l ($4 \cdot 10^{-6}$ *M*).

3. Results and discussion

3.1. Spectroscopic investigations

Most of the HPLC techniques for determination of citrinin employed fluorescence detection instead of UV absorbance because of the conjugated, planar structure of the compound studied [16]. Because citrinin is acidic, a brief spectroscopic study was performed in order to explore the relationship between the fluorescence emission and the pH. As no change in the excitation and emission maxima (331 and 500 nm respectively) was noted from pH 0 to pH 7, the emission maximum (500 nm) was recorded in this pH range (adjusted with dilute HCl and citrate/HCl buffer), with a fixed concentration of citrinin (10^{-4} *M*) (Fig. 2). The fluorescence signal is at a maximum around pH 2.5. The shape of this graph suggests that this pH zone corresponds to the non-ionized form of citrinin. Hence, non-ionized citrinin exhibits a high fluorescence quantum yield because of the intramolecular hydrogen bonds linking the phenol and keto functions to the carboxyl group of the citrinin nucleus. In contrast, the ionized species (at lower and higher pHs) seem non-fluorescent or poorly fluorescent. When the pH increases, the progressive dissociation of the acidic functions leads to a disruption of the pseudo-pentaheterocyclic moiety of citrinin and thus to a decrease in conjugation and in fluorescence properties. At high proton concentration, a decrease of emission is also noted although not well understood. This could be related to the progressive protonation of the oxygen atoms of the citrinin molecule giving a charge-transfer complex with reduced

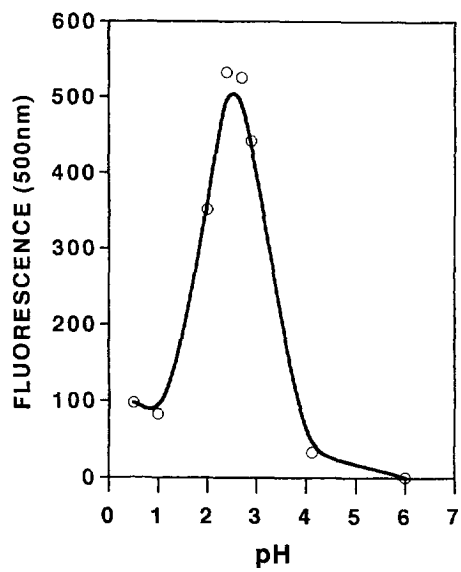

Fig. 2. Graph of the intensity of fluorescence ($\lambda_{em} = 500$ nm; $\lambda_{ex} = 331$ nm) of an aqueous citrinin solution (10^{-4} *M*) vs. pH.

conjugation, as already reported with coumarinic compounds in strong acids [17]. In a second stage, as acidification was shown to be necessary to improve the signal, the nature of the acidic reagent was investigated.

First, buffered solutions were discarded in order to simplify the chromatographic system. Then, different acids were tested. Assays demonstrated that strong mineral acids (HNO_3, H_2SO_4, $HCLO_4$, HCl) were preferable to weak organic acids such as acetic or formic. HCl was chosen for simplicity, as no influence of the anions had been demonstrated.

Because citrinin is determined by RP-HPLC, some spectra of 10^{-4} *M* citrinin were also recorded in pure water, methanol and acetonitrile. As no difference in fluorescence has been observed between the three types of solutions, it was presumed that the pH effect would be the same for our mobile phase. Consequently, a pH of 2.5 was selected for the optimum fluorescence detection of citrinin.

3.2. Chromatography

Although normal-phase liquid chromatography has been used by some authors for citrinin determination [8], most of the analytical methods reported in literature use RP-HPLC with an acidic eluent due to the acidic character of the citrinin molecule [18–20]. The use of cationic ion-pairing reagents such as tetrabutylammonium hydroxide (TBA) has also been reported [5] but we noted a decrease in signal when fluorescence detection is performed, in agreement with Ref. [8].

As our goal was to develop a method suitable for many different samples: mould strains cultures and foods. The use of an ion-pairing reagent was "first" envisaged in order to achieve the best possible selectivity in complex samples. Moreover, it was checked that the chromatography of citrinin was not reproducible in the absence of TBA. In fact, for a fixed pH, a symmetry factor >140% and a time retention variation of about 20% were noted. Experiments showed that a minimum concentration of $5.7 \cdot 10^{-4}$ *M* of TBA was necessary for a reproducible elution of citrinin. We suppose that this was due to the interaction of the hydroxyl and carbonyl functions of the citrinin molecule with the free silanols of the reversed-phase support.

On the other hand, as no difference was noted between the use of methanol or acetonitrile on the fluorescence of citrinin, methanol was chosen as an organic modifier of a binary eluent because it promoted a strong solvent–solute interaction. Thus, a mobile-phase pH variation study between pH 4 and 6.5 was performed because ion-pairing with TBA needs, at least, a partial ionization of the citrinin. Experiments showed that a pH of 5.5 gives the best results (with the best symmetry factor and the highest number of plates). In agreement with the spectroscopic data, a constant decrease in the signal was noted, and a constant decrease of the capacity factor k' (3.27 at pH 4.5; 1.92 at pH 6) was also noted for chromatography. This latter point seems to be in contradiction with the occurrence of ion-pairing in the mobile phase since the pH increase must be associated with a progressive ionization of the acidic functions of citrinin and thus with an increase in ions-pair formation. This suggests that the amount of TBA in the mobile phase is certainly too low for complete ion-pairing with the two ionizable acidic functions of citrinin and for the partitioning of the ion-pairing reagent between the stationary and the mobile phase. Thus, the decrease of citrinin k' could be simply related to the progressive ionization of a not totally paired molecule. Nevertheless, it should be pointed out that the TBA level is sufficient to render the elution reproducible.

As already noted, even if pH 5.5 is suitable for the elution, it does not allow optimum fluorescence detection. Consequently a post-column acidification is mandatory in order to improve the detection of citrinin. It should be noted that, from a chromatographic viewpoint, a post-column device was preferable to the direct acidification of the eluent in order to prevent accelerated damage to the reversed-phase column due to the use of extreme pHs.

3.3. Optimization of the post-column reagent

HCl was chosen for the post-column acidification and its concentration was selected with the

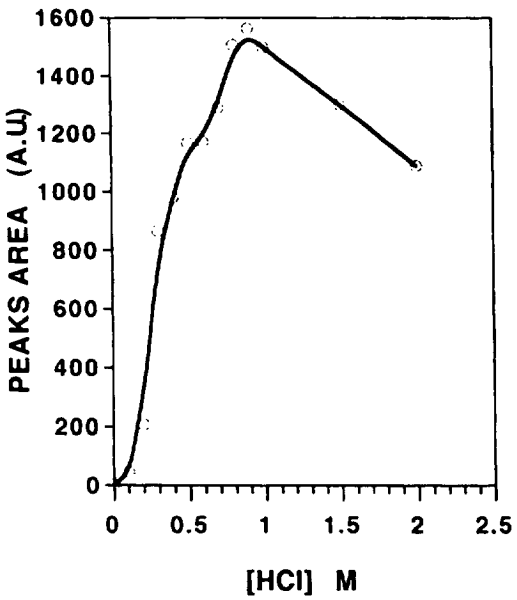

Fig. 3. influence of the concentration of HCl used as post-column reagent (flow-rate = 0.2 ml/min) on the fluorescence signal of citrinin (λ_{em} = 500 nm). For other chromatographic conditions: see text.

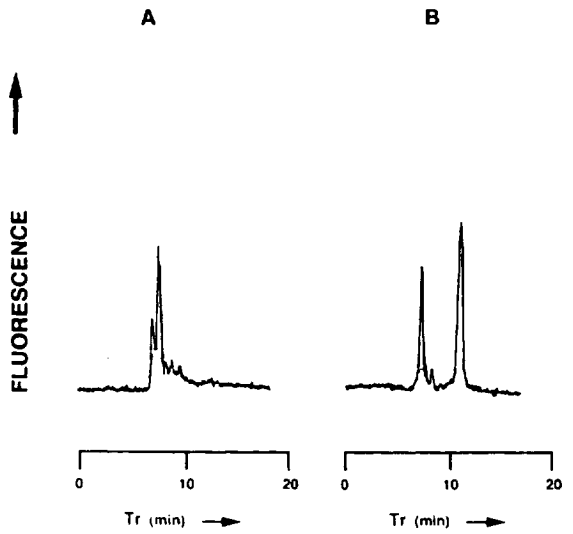

Fig. 4. Effect of post-column acidification on the fluorescence signal of citrinin. Comparison of the different chromatograms. (A) mobile phase used as post-column reagent; [citrinin] = 10^{-3} *M*. (B) 1 *M* HCl used as post-column reagent; [citrinin] = 10^{-5} *M*. In each case: post-column flow-rate = 0.2 ml/min. For other chromatographic conditions: see text.

help of the graph in Fig. 3. An optimum fluorescence response is given by 1 *M* HCl. Likewise, it should be pointed out that the apparent post-column pH was ca. 2.4 and thus corresponds to the previously established optimum of fluorescence. This pH value is caused by the dilution effect of the post-column device (0.2 ml/min for the post-column flow-rate compared to 0.8 ml/min for the eluent flow-rate) and from the dissociation and polarity characteristics of the fluid obtained from the mixing of the eluent and the HCl solution. The post-column flow-rate was varied from 0.025 ml/min to 0.8 ml/min and finally set at 0.2 ml/min. In fact, at flow-rates <0.05 ml/min the pumping, almost without back pressure, is not very constant as shown by the variations of the fluorescence signal on several injections of a citrinin working solution (i.e. R.S.D. = 29.2% for 0.025 ml/min, $n = 6$). Thus, as no improvement of the signal was noted at 0.1 ml/min; a 0.2 ml/min flow-rate was selected as a compromise between the need to limit the post-column dilution effect and optimization of detection (i.e. R.S.D. = 5.02% for 0.2 ml/min, $n = 6$). Lastly, and as expected, the use of a torroidal coil placed after the mixing tee was shown to be useless. Fig. 4 illustrates the post-column enhancement effect due to the addition of the dilute HCl solution. The enhancement factor almost reaches 1200. It is important to note that such a considerable enhancement can not be obtained by direct acidification of the mobile phase because of the presence of TBA which quenches the citrinin fluorescence [5]. In fact, the post-column dilution effect reduced the TBA concentration and thus, limited the fluorescence quenching.

3.4. Analytical figures of merits and real samples testing

The method developed in this study was linear from $2 \cdot 10^{-7}$ *M* to 10^{-4} *M* ($r = 0.999$, linearity test highly significant with $p < 0.001$). The repeatability and the reproducibility ($c = 10^{-5}$ *M*; $n = 9$ for both measurements) are satisfactory with R.S.D.s of 5.1 and 7.2%, respectively. The limit of detection was established as $0.9 \cdot 10^{-7}$ *M* (signal-to-noise ratio = 3) and the limit of quanti-

fication as $2 \cdot 10^{-7}$ *M*. The specificity was evaluated first by simultaneous injection of some important mycotoxins often found with citrinin, e.g. zearalenone and ochratoxin A and secondly by the analysis of real samples. In the first case, the selectivity of the chromatographic system (due in part to the use of TBA) demonstrated the absence of interfering peaks. The second case is illustrated by Fig. 5 which shows the analysis of a cheese extract free of citrinin and extracted according to Taniwaki and Van Dender [4] (Fig. 5a). This cheese is frequently polluted by *Aspergillus* and *Penicillium* strains which are possible producers of citrinin [21]. Figs. 5b and 5c show the same extract spiked with $4 \cdot 10^{-6}$ *M* of citrinin, detected in absence and in presence of post-column acidification. This example clearly underlines both the specificity (Fig. 5a) of the method and the importance of the post-column acidification (Fig. 6) in comparison with the direct fluorescence detection mode using the same spectroscopic settings and with the mobile phase used as post-column reagent (Fig. 5b).

As another illustration of the practical interest of the proposed method, assays with mould cultures have been performed. A total of ten mould strains isolated from cheese have been studied, three were identificated as *P. viridicatum*, two were *P. roqueforti*, two *P. citrinum*, two *P. expansum*, and finally one *P. claviforme*. All the strains were confirmed previously as producers of citrinin with TLC, by Lee et al. [22]. All the culture extracts were evaporated and redissolved in methanolic solution (0.5 ml). Citrinin was detected in all the extracts. The highest producer of citrinin was the *P. claviforme* strain followed by one of the *P. roqueforti* strains. In all extracts citrinin could easily be detected even when diluted ten-fold, and in several extracts even when diluted one hundred-fold.

Fig. 6 shows the quantification of citrinin in an extract of a *P. citrinum* culture. As seen, the post-column acidification allows the detection of a trace amount of citrinin, in this case $4 \cdot 10^{-7}$ *M* (Fig. 6b), whereas detection at this level is rarely possible when no acidification is performed.

As a consequence of the present work, some other chromatographic developments are in pro-

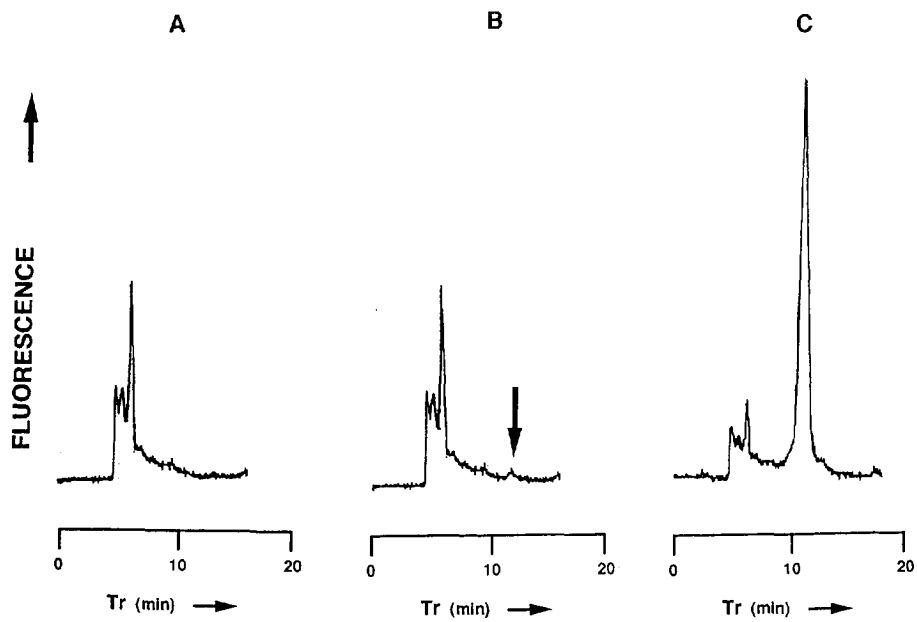

Fig. 5. Chromatograms of cheese extract. (A) non spiked extract. (B) spiked extract ($4 \cdot 10^{-6}$ *M* citrinin), without post-column acidification. (C) spiked extract ($4 \cdot 10^{-6}$ *M* citrinin), with post-column acidification (1 *M* HCl). For chromatographic conditions: see text.

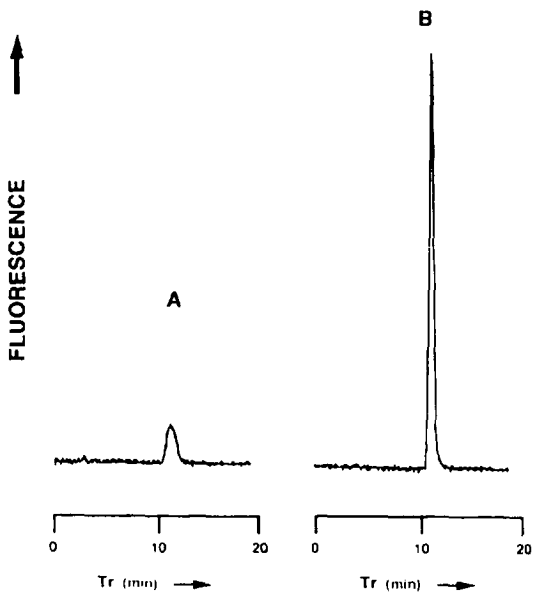

Fig. 6. Strain culture extract from *Penicillium citrinum* containing $4 \cdot 10^{-7}$ *M* citrinin. (A) without PC acidification. (B) with PC acidification. For chromatographic conditions: see text.

gress in order to adapt the proposed method to the simultaneous HPLC–fluorimetric determination of citrinin, ochratoxin, zearalenone and aflatoxins in various fungal strains.

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