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Amino-bonded silica as stationary phase for liquid chromatographic determination of cyclopiazonic acid in fungal extracts

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Abstract

A new high-performance liquid (HPLC) chromatographic method is described for cyclopiazonic acid (CPA) determination in fungal cultures on a propylamino-bonded stationary phase with a CH_3CN/CH_3COONH_4 buffer as mobile phase. Retention of CPA on propylamino modified silica under acidic conditions (protonated amino groups and deprotonated CPA) is governed by a mixed ion-exchange-reversed-phase mechanism. In addition to non-polar (hydrophobic) interactions, polar interactions with the surface silanols are also possible and become important as the polarity of the mobile phase decreases. A detection limit of 25 pg of CPA standard is obtained that represents an improvement of more than two orders of magnitude compared to existing HPLC procedures. UV-detector response was linear to 200 ng of CPA. Fungal extracts can be analysed after a simple dilution step with UV diode array detection that provides peak identity/purity assessment. The suitability of the proposed method as a rapid confirmatory test to assess the toxigenic potential of different *Aspergillus* and *Penicillium* strains is demonstrated by the analysis of 54 fungal extracts.

Keywords: Chiral stationary phases, LC; Cyclopiazonic acid; Mycotoxins

1. Introduction

Cyclopiazonic acid (CPA) is a mycotoxin with an indole tetramic acid structure (Fig. 1) that has been isolated from numerous species of *Aspergillus* and *Penicillium* [1,2]. The toxin has been found as a natural contaminant of corn and peanuts [3–5] as well as of cheese colonized with *P. camemberti* [6,7] or other *Penicillium* species [8]. Studies on animals revealed that CPA may be distributed in different organs and tissues, e.g. poultry meat [9], and toxin residues are suspected to occur in milk and eggs [10] as a consequence of contaminated feedstuff consumption by dairy cattle and chickens. Thus exposure to CPA through the food chain is of potential

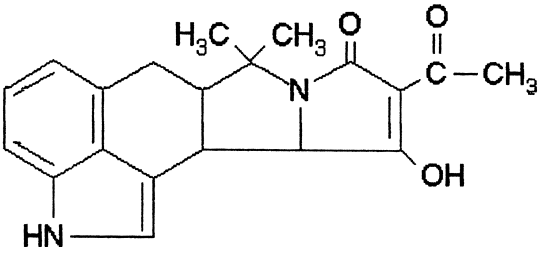

Fig. 1. Structure (one of the possible tautomeric forms) of cyclopiazonic acid.

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concern for human health; indeed, CPA has already been implicated in a human intoxication event involving contaminated kodo millet seeds [11].

Fast, sensitive and selective analytical methods for CPA detection in a variety of fungal cultures, agricultural commodities and dairy products are then highly desirable.

In addition to colorimetric [12] and immunoassay [13] methods, several chromatographic procedures have been developed so far. Among these, thin-layer chromatography (TLC) is the most widely used method [5–7,14] though it suffers from plate-to-plate variability (due to the requirement of oxalic acid impregnation of each plate) and from inaccuracy due to inconsistent colour development by reaction with the Ehrlich reagent (4-dimethylaminobenzaldehyde/HCl). Detection limits of ca. 125 ng/g for peanuts and corn can be obtained [14]. Accurate quantitation at lower concentration levels can be afforded by high-performance liquid chromatography (HPLC). The most common reversed-phase (RP) systems, however, were found useless [15] for compounds containing the tetramic acid moiety such as tenuazonic and cyclopiazonic acids. Gradient elution RP-HPLC also failed to solve problems originating from unacceptably high capacity factors, severe peak tailing and dependence of retention time on CPA concentration. Ligand exchange chromatography on a C_{18} stationary phase loaded with a hydrophobic chelating agent, such as 4-dodecyldiethylentriamine (C_{12}-trien), has been proposed by Landsen [16]. Reasonable retention times and fairly symmetric peak shape were obtained; overnight equilibration with a mobile phase composed of C_{12}-trien, zinc acetate, ammonium acetate, 2-propanol and acetonitrile was required to achieve a stable column modification. Normal phase liquid chromatography on silica gel column was proposed by Goto et al. [17]; a significant improvement in detection limits was claimed but the solvent system used (ethyl acetate/2-propanol/aqueous ammonia) may shorten the useful life of the column by dissolving the silica gel. Normal phase ion-pair partition LC on silica gel has been recently proposed [18] for simultaneous determination of major secondary metabolites (including CPA) produced by *Aspergillus* species. The method was applied to chloroform extracts of fungal cultures without a preliminary clean-up step. Metal complexation chromatography based on a linear gradient of $ZnSO_4$ in methanol/water on a reversed-phase C_{18} column has been developed by Urano et al. [4] for CPA determination in corn and peanuts. More recently a similar approach has been used for simultaneous determination of CPA and tenuazonic acid in tomato products [19].

In the present paper, a new chromatographic approach based on the use of propylamino-bonded silica gel stationary phase operated with CH_3CN/CH_3COONH_4 buffer system is presented. The optimisation of the various chromatographic conditions permitted to achieve high column efficiency with a significant improvement of the detection limit.

2. Experimental

2.1. Apparatus

The chromatographic system consisted of a low-pressure mixing binary pump (P2000 Spectra-Physics) equipped with a Rheodyne 7125 injection valve (20-μl injection loop), a SCM1000 on-line solvent degasser (Thermo Separation Products) and a 250×4.6 mm column with an aminopropyl-bonded silica gel 5 μm packing (Supelcosil LC-NH_2, Supelco).

UV detection was accomplished by a Hewlett-Packard model 1040A diode array detector (DAD) controlled by an HP85 computer and a HP 7470A printer–plotter. The analog output of the DAD was also fed to an HP model 3395 integrator.

2.2. Chemicals

CPA standard was obtained from Sigma. A stock CPA solution (1 mg/ml) was prepared in methanol and stored at 4 °C in the dark. Working solutions were prepared immediately before use by serial dilution with mobile phase. Solvents used were HPLC grade (Aldrich); all the other chemicals were analytical reagent-grade.

Different strains of *Penicillium* and *Aspergillus* genus, isolated from dairy products produced in southern Italy, were grown on Czapek yeast autolysate agar (CYA) and yeast extract sucrose agar (YES) and extracted following the protocol of

Frisvad and Thrane [20]. The content of eight Petri dishes was extracted first with 150 ml of chloroform–methanol (2:1, v/v) and after an acidification step extracted again with 150 ml of acetone–ethyl acetate (1:1, v/v). The combined extracts were then evaporated to dryness using a rotary evaporator in 40 °C water bath. The residue was reconstituted in 4 ml of methanol, filtered and diluted as necessary with mobile phase before injection into the HPLC system.

Undiluted extract (5 μl) was also applied to a TLC plate and analysed according to the method of Landsen et al. [14].

Milk samples were extracted according to the procedure described by Urano et al. [4] slightly modified by omitting the clean-up step on silica cartridge; briefly, 2.5 ml of cow milk were added to an equal volume of methanol–2% $NaHCO_3$ solution (7+3) and then defatted with hexane. The aqueous extract was then acidified with 5 *M* HCl and CPA was partitioned into 5 ml chloroform. The organic phase was evaporated to dryness under a gentle stream of N_2 and reconstituted in 500 μl of methanol.

2.3. Chromatographic conditions

Unless otherwise specified, a mobile phase composed of CH_3CN–50 m*M* CH_3COONH_4 pH 5 (80:20, v/v) was used at a flow-rate of 1 ml/min. Temperature was ambient. UV detection was carried out at 285 nm (4 nm band-width) using a reference signal at 550 nm (50 nm band-width). Spectra were acquired in the 210–400 nm range and peak purity checked by spectra overlaying after normalization. Column dead volume was measured by injecting 5 μl methanol. The buffer pH was adjusted by acetic acid or ammonia addition.

3. Results and discussion

3.1. Chromatographic behaviour of CPA on propylamino-bonded silica

The most widely used stationary phases for CPA separation/detection are unmodified silica and octadecylsilane (C_{18})-bonded phase loaded with a chelating agent (ligand exchange chromatography) or metal ions (complexation chromatography). As far as we know, the potential of silica gel modified by polar functional groups, e.g. aminopropyl, has not yet been explored. Amino stationary phases bonded to silica gel by means of a short hydrocarbonaceous spacer, are less polar than non-modified silica. The presence of the spacer (C_3 in the case of aminopropyl silica) is not only responsible for the mobility of the bonded groups with respect to the surface of silica gel [21,22], but because of the non-polar character it leads to possible retention of the solute by hydrophobic (lipophilic) mechanism especially in strongly polar mobile phases. In addition residual silanols may participate in polar interactions with sample solutes and contribute to retention in non aqueous mobile phases. As far as the functional group itself is considered, it is responsible for dipole–dipole interactions, H-bonding, and anion-exchange with dissociated species under acidic conditions. Then propylamino-bonded silica gel may operate under adsorption, partition or ion-exchange mode or under mixed mechanisms depending on the composition of the mobile phase and the nature of the solute. These characteristics may be explored to devise a simple, sensitive and highly efficient chromatographic method for CPA that possibly overcomes the drawbacks of the existing ones.

Preliminary attempts to operate under reversed-phase conditions with aqueous mobile phases containing various amounts of organic modifiers (e.g. acetonitrile, methanol, 2-propanol) proved unsuccessful because of heavily tailed chromatographic peaks and poor column efficiency. Eluents were then buffered at proper pH values in order to operate under anion-exchange mechanism; CPA is a monobasic acid whose pK_a is not reported in the literature; the pK_a of CPA, however, can be reasonably assumed equal to that ($\cong 3.5$) of tenuazonic acid [23] that possesses the same enolised β-diketone system (Fig. 1). Ammonium acetate was chosen as a buffer due to its favourable solubility in mobile phases highly enriched with organic solvents.

Fig. 2 shows the influence of the buffer's ionic strength on the solute retention at a constant pH value. As can be seen, the capacity factor k' increases as the ionic strength decreases. For anion exchange mechanism, a plot of k' versus the re-

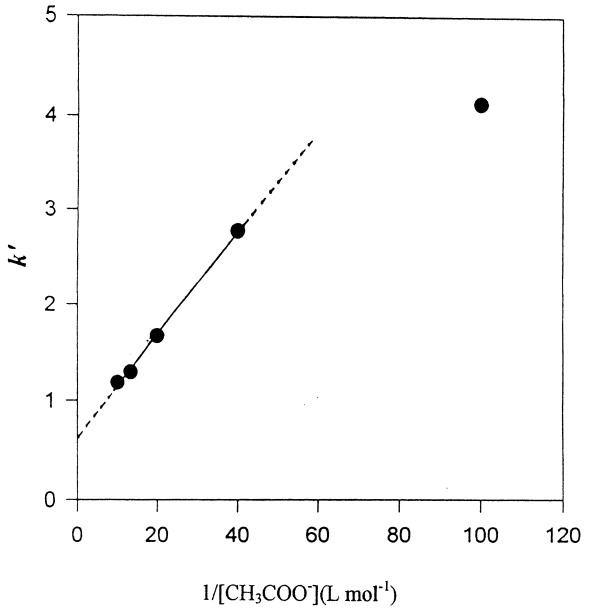

Fig. 2. Plot of k' against 1/buffer concentration. Mobile phase: CH_3CN–CH_3COONH_4, pH 5 (80+20, v/v).

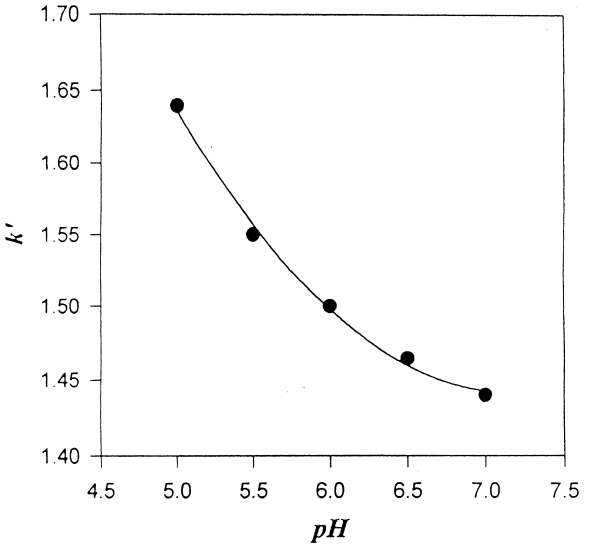

Fig. 3. Effect of eluent pH on the retention of CPA at constant ionic strength. Mobile phase: CH_3CN–50 m*M* CH_3COONH_4 (80+20, v/v). The pH values refer to the aqueous buffer before mixing with the organic solvent.

ciprocal of the concentration of the competing ion should be linear with a zero intercept [24,25]. The non-zero intercept on the *y*-axis (infinite ionic strength) shown in Fig. 2, should be indicative of the contribution of mechanism(s) other than ion exchange (vide infra) to retention.

The influence of the eluent pH on the retention of CPA at constant ionic strength is shown in Fig. 3; increasing the pH of the buffer from 5 to 7 causes a slight decrease in retention (k'), which is apparently unexpected under the assumption that an anion-exchange mechanism is operating (for an acidic solute, retention should increase on increasing pH). Since it is unlike that the fraction of protonated surface amino groups on silica can significantly change in the pH range explored, the above finding could be simply explained invoking an increase in the concentration of the competing ion (acetate) passing from pH 5 to pH 7 (this should agree with the results of Fig. 2). However the possibility of an electrostatic interaction (repulsion) with residual surface silanols, whose ionization increases on increasing pH [25], as well as an internal masking of amino groups by surface silanols [26,27] cannot be ruled out.

It is worth noting that efficiency and peak symmetry were found dependent on ionic strength and pH and that a mobile phase acetonitrile/50 m*M* buffer pH 5 (80+20, v/v) gave the best results in term of plate counts (ca. 40 000 m^{-1}) and peak asymmetry (1.2) measured according to Foley and Dorsey [28].

The influence of the organic modifier content in the mobile phase has also been investigated in order to confirm whether retention is at least partly controlled by mechanisms (e.g. reversed-phase) other than ion exchange. Indeed stationary phases with polar groups bonded to silica via a non-polar spacer generally exhibit reversed-phase behaviour in aqueous–organic mobile phases (where the retention is controlled mainly by hydrophobic interactions) and normal-phase behaviour in organic mobile phases where the polar interactions between solutes and stationary phase are most important. Fig. 4 shows the change caused by increasing (at constant pH and ionic strength) the volume fraction φ of acetonitrile (the less polar solvent) in the aqueous–organic mobile phase; as can be seen, a curvature of the k' versus φ plot is observed.

When a combination of polar and non-polar mechanisms occurs, Fisher and Jandera [29] have shown that for polar-bonded phases used with aque-

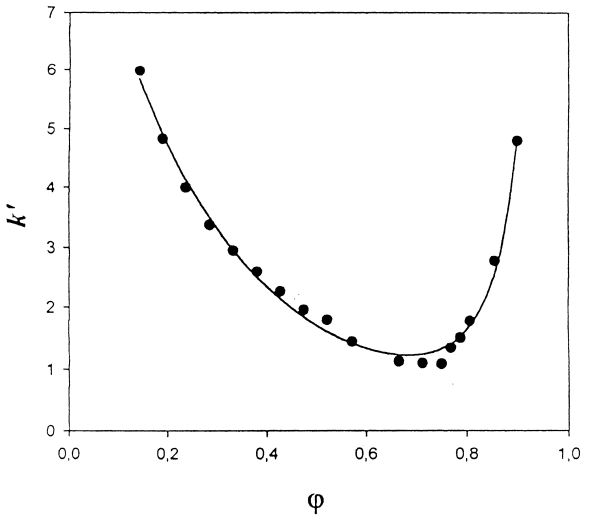

Fig. 4. Dependence of the capacity factor k' of CPA on the volume fraction φ of CH_3CN at constant pH and ionic strength. Mobile phase: CH_3CN–50 m*M* CH_3COONH_4, pH 5. Solid line represents the best fit of Eq. (1) modified by the addition of the K_{iex} term (see text).

ous–organic mobile phases, retention can be described by the following equation:

$$k' = a10^{-b\varphi} + k'_0(1-\varphi)^{-n} \quad (1)$$

where φ is the volume fraction of the less polar (organic) solvent. The first and the second term in Eq. (1) represent the non-polar (hydrophobic) and polar contribution, respectively; k'_0 represents the polar contribution to the capacity factor in pure water as mobile phase. When polar interactions contribute significantly to the retention, k' starts to increase (i.e. non-linear dependence of log k' vs. φ) and even a minimum can be observed in the k' (or log k') versus φ plot. As can be seen in Fig. 4 for a volume fraction of CH_3CN near 0.75 there is a minimum in retention. Indeed data in Fig. 4 can be fitted by Eq. (1) modified by the inclusion of a third constant term k_{iex} to account for the contribution of ion-exchange mechanism to retention; fitting parameters ($R=$ 0.9796) in Eq. (1) are: $a=11.44$, $b=2.51$, $k'_0=$ 0.034, $n=2.00$ and $k_{iex}=1.00$. The polar contribution to retention, calculated as the %ratio of the second term in Eq. (1) to the value of k', can be estimated around 43% at $\varphi=0.8$; it decreases to negligible values at lower φ whilst it predominates at $\varphi=0.9$ (as the polarity of the mobile phase is decreased, polar interaction with the silanols becomes increasingly important).

Further control of retention can be achieved through the addition of a second organic modifier such as CH_3OH. Fig. 5 shows changes in retention when CH_3CN in the mobile phase is progressively replaced by the less lipophilic CH_3OH at constant pH, ionic strength, and organic modifier/water ratio (80+20, v/v). A linear dependence of log k' on φ is observed indicating that the predominant contribution arises from non-polar interactions. It is worth noting that methanol concentrations higher than 5% also result in a deterioration of the column efficiency and peak shape.

In conclusion, the above findings seem to indicate that retention of CPA on aminopropyl-modified silica under acidic conditions (protonated amino groups and deprotonated CPA) is governed by a mixed mechanism. Ion exchange operates simultaneously to a reversed-phase mechanism; in addition to non-polar (hydrophobic) interactions, polar interactions with the surface silanols are also possible and become important as the polarity of the mobile phase

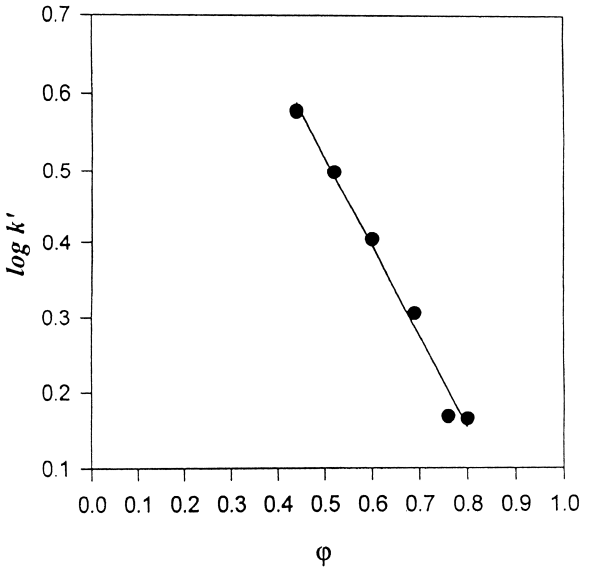

Fig. 5. Dependence of retention (log k') on the CH_3CN volume fraction φ. Mobile phase: (CH_3CN+CH_3OH)–50 m*M* CH_3COONH_4 buffer, pH 7 (80+20, v/v). The organic modifier composition was varied, by methanol addition, from 80% CH_3CN to (44% CH_3CN+36% CH_3OH).

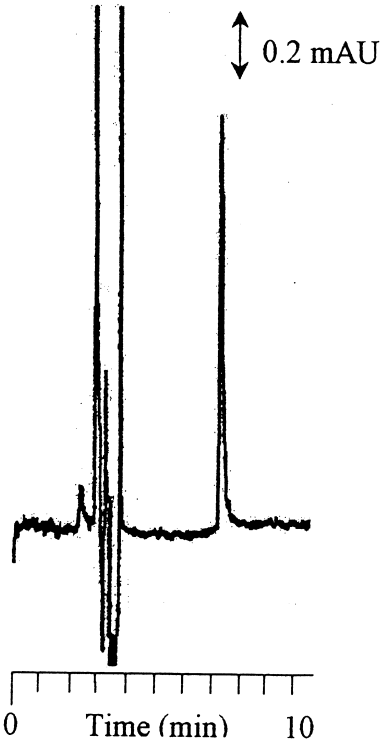

Fig. 6. Chromatogram relevant to 125 pg CPA injected. Mobile phase: CH_3CN–50 m*M* CH_3COONH_4, pH 5 (80+20, v/v).

decreases. Ionic strength, pH, nature and concentration of the organic modifier(s) can be manipulated to adjust retention, column efficiency and peak shape.

3.2. Applications

Fig. 6 shows the chromatogram obtained by injecting 125 pg of CPA standard; the response is linear up to at least 200 ng injected. Unweighted linear regression gave the following equation: peak area (a.u.)=(5.83±1.86)+(8.45±0.06) CPA (ng), $R^2=0.9996$. The detection limit calculated at a signal-to-noise ratio of 3 (noise taken [30] as the standard deviation of the intercept of the calibration plot) was of 25 pg/injection. This is significantly lower (Table 1) than that of previously reported chromatographic methods.

Finally, Fig. 7 shows the chromatogram obtained on a crude extract of *P. camemberti* cultured on a suitable synthetic medium; no preliminary clean-up was required since the extract could be directly injected after a proper dilution with mobile phase. Peak purity/identity could be easily assessed (inset of Fig. 7) by the technique of spectra overlaying after normalization, which is helpful in analysis of

Table 1
Comparison of detection limits (CPA standards) for various chromatographic and non-chromatographic methods

	Detection limits	Ref.
Chromatographic methods		
Ligand exchange	4 ng (100 μl injected)	[16]
Metal complexation	20 ng (20 μl injected)	[4]
Normal phase on silica gel	0.2 ng	[17]
Normal phase ion-pair partition on silica gel	5 ng (20 μl injected)	[18]
Ion exchange on aminopropyl silica gel	25 pg (20 μl injected)	This work
Thin layer chromatography	25 ng per spot	[14]
Non-chromatographic methods		
Capillary electrophoresis	0.27×10^{-7} g/ml (8.3 nl injected)	[32]
Immunoassay	30 pg/well	[13]
Immunoassay	0.2 ng/ml	[33]

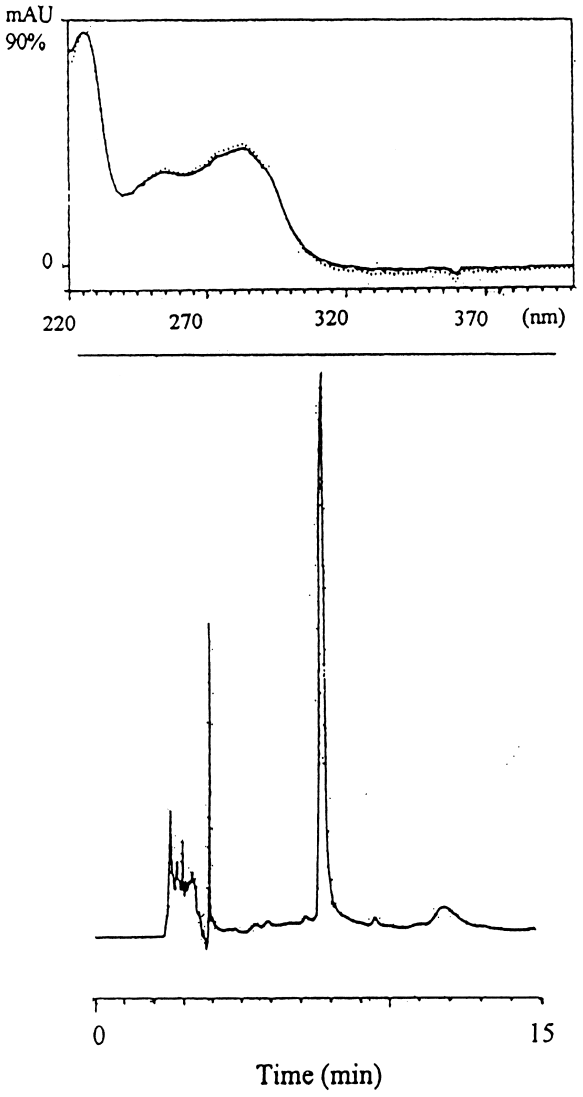

Fig. 7. Chromatogram obtained on a fungal extract of *P. camemberti*. Before injection, the crude extract was diluted (1:400) with mobile phase. Chromatographic conditions as in Fig. 6. The inset shows the overlay of normalised spectra acquired on the apex (dotted line), the ascending (solid line) and descending part (dashed line) of the peak at a retention time of 7.78 min. Absorbance axis: 60 mAU full-scale.

naturally contaminated samples. More than 50 different fungal extracts of *Aspergillus* and *Penicillium* strains have been analysed (Table 2). The results (Table 2) demonstrated the potential of the method as a rapid confirmatory test to assess their toxigenic potential [31]. Work is in progress to extend the proposed method to contaminated dairy products analysis, where the high sensitivity of the technique can be fruitfully explored. A preliminary result relevant to a milk sample spiked with CPA at 80 ng/ml level and extracted as described in the Experimental section is shown in Fig. 8.

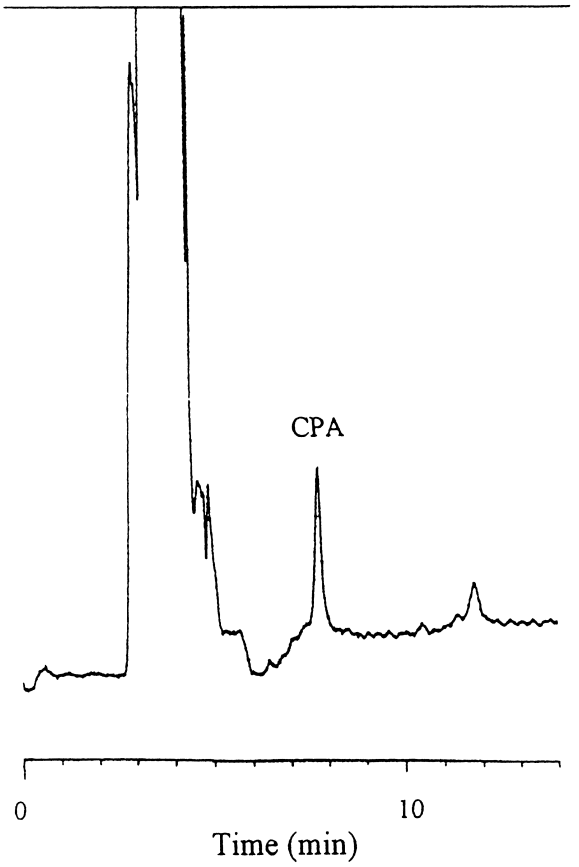

Fig. 8. Chromatogram of an extract of a milk sample spiked with CPA at 80 ng/ml level. Absorbance axis: 8 mAU full-scale. Other conditions as in Fig. 6.

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Table 2
Production of CPA by different fungal strains

Penicillium and *Aspergillus* species	Number of tested strains	Number of CPA-producing strains	Concentration range (μg/ml)
P. aurantiogriseum	3	1	2100
P. brevicompactum	9	0	–
P. camemberti	4	3	1500–3000
P. chrysogenum	2	0	–
P. commune	16	9	530–1800
P. crustosum	5	2	1350–1500
P. hirsutum	1	1	800
P. roqueforti	6	0	–
P. verrucosum	2	2	3100–6500
A. versicolor[a]	6	6	26–140
Total	54	24	

[a] *A. versicolor* extracts with CPA content near to the lower limit of the concentration gave no or very poor colour development when analysed by TLC probably due to the inadequate sensitivity.

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