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Determination of cyclopiazonic acid in cheese samples using solid-phase microextraction and high performance liquid chromatography

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Abstract

Solid phase microextraction (SPME), using a Carbowax/Templated Resin fiber, was optimized for the determination of the mycotoxin cyclopiazonic acid (CPA) and interfaced with HPLC-UV/DAD. All the parameters influencing SPME-HPLC, including extraction time and temperature, pH, ionic strength and desorption conditions, have been carefully examined. The method was successfully applied to the analysis of white surface cheese samples. SPME was capable of a selective extraction of CPA after a short sonication step in methanol. The whole extraction gave high recovery yields and was simpler and quicker than any other existing procedure for CPA extraction from cheese. A detection limit in cheese of 7 ppb was obtained.

Keywords: SPME; Cyclopiazonic acid; Cheese; Mycotoxins; HPLC

1. Introduction

Cyclopiazonic acid (CPA; Hozapfel, 1968), which represents a secondary metabolite produced by many species of moulds contaminating food, is able to cause pathological responses in human beings (Rao & Husain, 1985) and animals (Dorner, Cole, & Lomax, 1985). It has been detected in long and medium ripening cheese (Engel & Teuber, 1989) as a consequence of an accidental growth of moulds belonging to *Penicillium* and *Aspergillus* genus, which can bear the temperature and relative humidity occurring in the ripening process. Together with an accidental growth of moulds caused by environmental factors, it has also been reported an intentional contamination with specified strains of *Penicillium camemberti* in white surface mould-cheeses manufacturing (Le Bars, 1979). The presence of CPA in these kinds of cheeses is correlated to the toxigenic ability of starter fungal strain selected. Nevertheless toxicity data relative to human beings are not available in literature, cyclopiazonic acid doses eventually ingested by consumers (3–5 μg in a one-eighth of Camembert cheese; Le Bars, 1979) don't seem to be dangerous for human health and they are low compared with the 50% lethal dose in rats (36 mg/kg, per os; Purchase, 1971).

However, the aspects of cyclopiazonic acid toxicity in humans are not yet know and the determination of cyclopiazonic acid in food samples represents an important aspect for food safety. In fact, it has been the object of several analytical investigations using different techniques such as immunoassay (Hahnau & Weiler, 1991), capillary electrophoresis (Prasongsidh, Kailasapathy, Skurray, & Bryden, 1998) and chromatography (Goto, Shinshi, Tanaka, & Manabe, 1987; Landsen, 1984, 1986; Urano, Trucksess, & Matusik, 1993); a highly sensitive HPLC–UV method for the determination of cyclopiazonic acid in moulds has been recently (Monaci, Aresta, Palmisano, Visconti, & Zambonin, 2001) developed in our laboratory. However, existing techniques, usually, require complex isolation procedures (Le Bars, 1979) such as liquid–liquid extraction with azeotropic $CHCl_3$–MeOH; then the filtered and evaporated extracts are dissolved in a mixture of organic solvents and after defatting twice with hexane and acidification with HCl, they are back-extracted with $CHCl_3$, centrifuged, dried with Na_2SO_4, evaporated, dissolved in methanol and subjected to TLC or HPLC analysis.

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Solid phase microextraction (SPME) is a new and quite efficient extraction technique introduced by Arthur and Pawliszyn (1990), which can be applied to organic compounds. It allows simultaneous extraction and pre-concentration of analytes from sample matrix. Furthermore, SPME eliminates some disadvantages of conventional extraction techniques such as solid phase extraction (e.g. plugging of cartridges) and liquid–liquid extraction (e.g. use of toxic solvents).

SPME has been widely applied in combination with GC to monitor residues of chemicals in various environmental and food samples (Kataoka, Lord, & Pawliszyn, 2000). However, SPME/GC is generally limited to the analysis of volatile and thermally stable compounds; then, in order to widen its range of application, SPME has been interfaced with HPLC (Chen & Pawliszyn, 1995). Regardless its wide potentialities, SPME/LC applications are still restricted, probably due to the limited selection of commercially available fibre coatings.

In this study, solid phase microextraction has been optimised for the extraction of cyclopiazonic acid and interfaced with a HPLC–UV/diode array (DAD). The developed procedure has been applied to the analysis of several surface semi-soft cheeses in order to assess their possible contamination.

2. Experimental

2.1. Chemicals

Stock solution 0.1 mg/ml of cyclopiazonic acid was prepared in methanol and stored at 4 °C in the dark. Dilute solutions were prepared just before use.

All reagents and organic solvents used (Carlo Erba, Milan, Italy), were HPLC grade and filtered through a 0.45-μm membrane (Whatman Limited, Maidstone, UK) before use. Working solutions were prepared with phosphate buffer (5 mM, pH 2.8) and filtered through nylon filter ISO-DISC N-34 0.45-μm (Supelco, Bellefonte, PA) before use. Cyclopiazonic acid was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

2.2. Apparatus

The SPME–HPLC system consisted of a Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA), an SPME interface (Supelco), consisting of a standard six-port Rheodyne valve with a special fiber desorption chamber (total volume: 60 μl), installed in place of the sample loop, and a 5-μm Supelcosil LC–NH_2 column (250×2.1 mm i.d., Supelco). The detector was an HP 1040A photodiode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA) interfaced to an HP 85 computer equipped with an HP dual disk drive and HP 7470A plotter. A HP 3395 laboratory computing integrator directly connected to the photodiode array detector was also used.

2.3. Chromatographic and detection conditions

The mobile phase composition was as follows: acetonitrile/ammonium acetate buffer (50 mM, pH 5) mixture (80:20, v/v). The flow rate was 0.2 ml min^{-1} and the temperature was ambient. Mobile phase was degassed by an SCM 100 Vacuum Membrane Degasser (Thermo Separation Products). Detection wavelength was 285 nm (4 nm band-width) and reference signal was at 550 nm. Spectra were acquired in the 210–400 nm range at the apex and on the ascending or descending part of each peak. Peak purity could be checked by the technique of spectra overlaying, after normalisation.

2.4. Solid-phase microextraction

A silica fiber coated with 50-μm thick Carbowax/Templated Resin (CW/TPR-100, Supelco) film, was employed for this study. A manual SPME device (Supelco) was used to hold the fiber. The SPME device and procedure have been extensively described elsewhere (Arthur et al., 1990; Pawliszyn, 1997). Working solutions were prepared by spiking 4 ml of phosphate buffer (5 mM, pH 2.8) with different amounts of cyclopiazonic acid into 5 ml clear vials (Supelco). Then, the vials were sealed with hole caps and Teflon-faced silicone septa (Supelco). The extraction was carried out at 50 °C for 30 min under magnetic stirring in order to improve mass transfer from the aqueous sample into the fiber coating. CPA desorption was performed in static desorption mode by soaking the fiber in mobile phase directly into the desorption chamber of the interface for 5 min. Then, the valve was changed to the inject position for 20 s. In order to ensure a complete desorption from the fibre, a blank SPME-chromatogram (dynamic desorption mode, see "results and discussion") was performed before the next extraction.

2.4.1. Cheese samples

White surface cheese samples ($n=6$) were collected from different local markets in Southern Italy. Analyses were performed on a single representative aliquot of each sample including the crust and the peripheral zone. Samples (0.5 g) were previously crumbled in a vial, 3 ml of methanol were added and the resulting mixture sonicated for 5 min. Methanol was then separated from cheese by filtering twice through a nylon filter ISO-DISC N-34 0.45 μm (Supelco, Bellefonte, PA) and then evaporated under a gentle stream of N_2. The residue was dissolved in 4 ml of phosphate buffer (5 mM, pH 2.8) and then subjected to SPME.

Recoveries were calculated by spiking cheese samples with 10 μl of standard solutions corresponding to a

concentration of CPA in cheese of 0.1 and 1 ng/mg; after 12 h of equilibration time at room temperature samples were analysed as described before.

3. Results and discussion

3.1. Fibre coating material

The extraction was performed using a fused-silica fiber coated with a polar coating (Carbowax/TPR-100) made of various particles imbedded in partially cross-linked polymeric phases. The choice of this fiber was made according to the polarity of the analyte. Moreover, it had a further advantage due its high resistance to mechanical strength (Pawliszyn, 1997).

3.1.1. Extraction time and temperature

The extraction time profiles were established by plotting the area counts versus the extraction time. The equilibrium is reached when a further increase in the extraction time does not produce a significant increase in the response. Two different extraction temperatures were explored, as shown in Fig. 1, that reports the results obtained at 50 °C and room temperature. As apparent, the equilibrium was reached after about 60 min in both cases, while the absolute responses were higher at 50 °C even though an uptake decrease is generally reported with increasing temperatures (Grote & Levsen, 1999; Pawliszyn, 1999). Thus, 50 °C was chosen as working temperature. In any case, it is possible to obtain good extraction yields and reliable analysis in non-equilibrium conditions. In fact, the amount of the analyte adsorbed into the fiber is proportional to the initial concentration in the sample matrix, once the agitation conditions and the sampling time are held constant, and hence, SPME quantitation is feasible even before adsorption equilibrium is reached (Pawliszyn, 1997). Thus, in case of routine analysis this feature can be used to obtain a considerable time gain. In the present case, an extraction time of 30 min was chosen for further experiments.

3.1.2. Effect of ionic strength, organic solvent and pH

The effect of salt addition on SPME extraction was already studied by several authors (Grote et al., 1999; Muller, Fattore, & Benfenati, 1997). Even though salt addition usually increases the amount of analyte extracted (Muller et al., 1997; Bartak & Cap, 1997), the opposite behaviour was sometimes observed (Kataoka et al., 2000). In this case, the addition of sodium chloride (ranging from 0 to 100 mg/ml) did not show a positive effect so that it was considered no more.

As far as organic solvents are concerned, it was reported (Pawliszyn, 1999) for a series of aromatic compound, that the analyte uptake was not affected by methanol concentration up to 1%. On the other hand, it was also demonstrated that the effect of organic solvents was compound-dependent. In this case, the addition of small amounts of methanol in the range 1–10% resulted in a decrease of extraction yield.

On the contrary, a strong dependence of the extraction yield on the pH value was observed for acidic compounds (Bartak & Cap, 1997; Grote et al., 1999) since analytes in the neutral forms are more efficiently extracted by non-ionic polymeric coatings. Thus, the effect of pH on the extraction efficiency of CPA was examined by using different pH buffers and the relevant

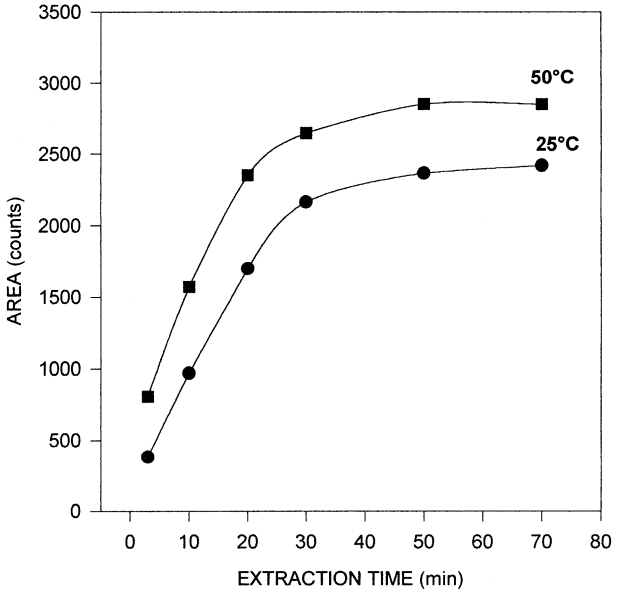

Fig. 1. Area counts versus extraction time obtained at different temperatures.

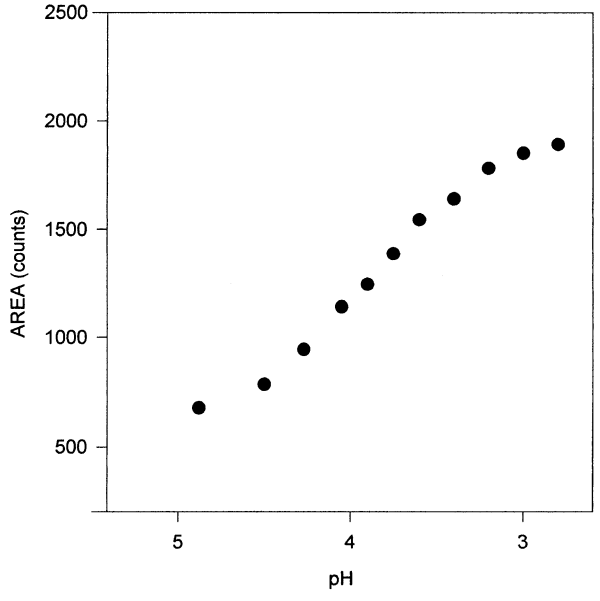

Fig. 2. Effect of pH on CPA extraction.

results are shown in Fig. 2. As expected, a noticeable response increase in the extraction efficiency was observed by varying pH in the range 2.8–4.9.

3.1.3. Desorption conditions and "carry-over"

Two different desorption modes were compared in this study: dynamic desorption and static desorption.

In the first case, when the fiber is inserted into the desorption chamber the injector is immediately changed to the "inject" position. Then, the analytes are desorbed by the moving stream of mobile phase. However, a very broad peak was obtained in this mode, probably due to a strong adsorption of CPA on the fiber. Therefore, a static desorption was performed: the fiber was soaked in mobile phase for a variable period of time before injection onto the HPLC column. The best conditions were reached after 5 min of static desorption; then, the fiber was exposed for 20 s to the mobile phase stream. This was found to be a critical step to optimize the chromatographic efficiency; generally speaking, a fiber exposition to the mobile phase stream for more then 20 s produced a broadening of the chromatographic peak.

Fig. 3 shows a comparison between two HPLC–UV chromatograms relevant to (1) the direct injection of a standard solution of CPA (injected volume, 60 μl) and (2) the SPME of the same solution, respectively. As apparent, the enrichment factor provided by SPME was about three.

3.2. Linear range, detection limit and precision

The response of the developed SPME–HPLC procedure was linear from the lowest concentration at which CPA was detected over at least two concentration decades (0.5–100 ng/ml) with correlation coefficients better than 0.997 and an intercept not significantly different from zero at 95% confidence level.

The estimated LOD and LOQ obtained in this study on standard solutions were 0.56 and 1.35 ppb, and were calculated according to IUPAC (Long & Winefordner, 1983). Fig. 4 reports an SPME–HPLC chromatogram relevant to a standard solution of CPA at its LOD concentration.

The precision of the method was investigated by using standard solutions of CPA. Replicate measurements ($n=3$) in standard solution of CPA (at 20 ng/ml) were performed daily, for 5 days in order to estimate the within-day and between-days precision. The two estimates do not differ significantly according to an F-test. The reproducibility (calculated as relative standard deviation of the entire set of data) was 3.4%.

3.2.1. Cheese samples analysis

Cheese samples were sonicated with methanol, filtered and the evaporated extracts were dissolved in mobile phase. As a first approach, the resulting solution was directly injected in the system; the relevant chromatogram is reported in Fig. 5a. As apparent, CPA was not separated by interfering substances in this case. Thus, the solution was subjected to SPME as described in the Section 2. Fig. 5b shows an SPME–HPLC–UV chromatogram relevant to the analysis of a cheese extract. As clearly shown, the peak relevant to cyclopiazonic acid appears adequately separated by matrix components, highlighting the advantage of this fast and selective technique. All analysed samples were found to be contaminated with variable quantities of CPA.

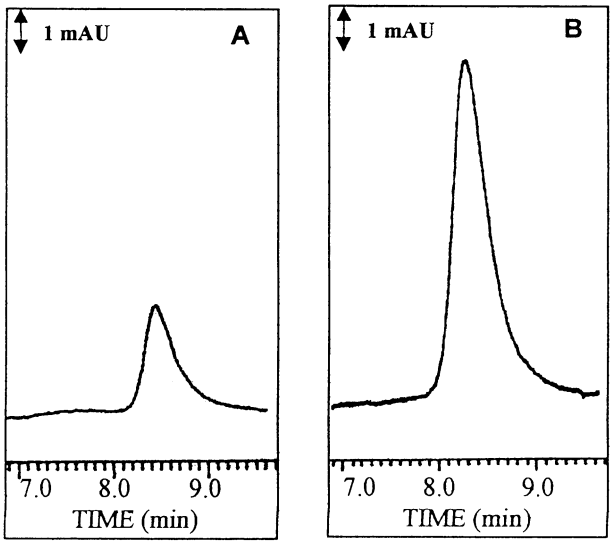

Fig. 3. (A) HPLC–UV chromatogram relevant to the direct injection of a standard solution (25 ng/ml) of CPA. (B) HPLC–UV chromatogram relevant to the SPME of the same solution. Attenuation, 10 mAU full scale. Chromatographic conditions and spectral acquisition as specified in Section 2; detection at 285 nm.

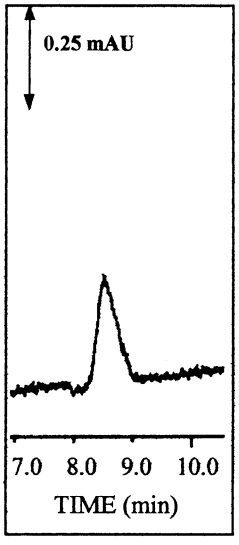

Fig. 4. SPME-HPLC chromatogram relevant to a standard solution of CPA at its LOD concentration (0.5 ng/ml). Attenuation, 1 mAU full scale. Chromatographic conditions and spectral acquisition as specified in Section 2; detection at 285 nm.

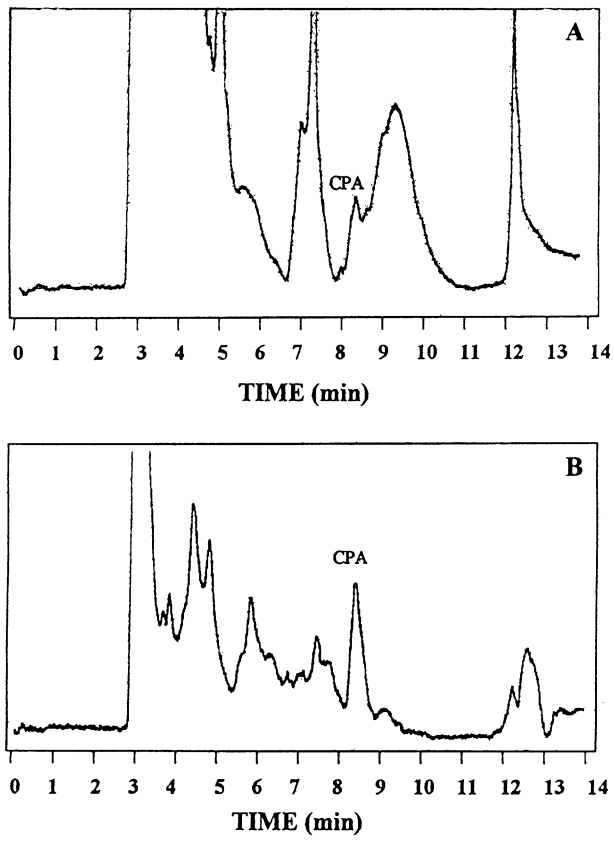

Fig. 5. (A) HPLC–UV chromatogram relevant to the direct injection of a cheese extract. (B) HPLC–UV chromatogram relevant to SPME of the same cheese extract. Attenuation, 5 mAU full scale. Chromatographic conditions and spectral acquisition as specified in Section 2; detection at 285 nm.

Percentage recoveries were calculated by spiking cheese samples ($n=4$) with standard solution of CPA as described in Section 2; the values obtained were 82 (RSD% 3.9) and 86 (RSD% 5.2) at 0.1 and 1 ng/mg, respectively.

The estimated concentration of CPA in the white surface cheeses analysed in the present study ranged from 20 to 80 ppb. The RSD% calculated by replicating analysis ($n=3$) on the same cheese sample was 4.5. The detection limit obtained in cheese (calculated as three-fold the standard deviation of a cheese sample at a contamination level of 20 ppb) was 7 ppb.

4. Conclusions

In this paper, an SPME procedure, using a Carbowax/Templated Resin fiber, for the determination of cyclopiazonic acid has been developed for the first time and interfaced with HPLC–UV/DAD.

The method was then successfully applied to the analysis of white surface cheese samples. A short sonication step in methanol was necessary to extract CPA from cheese; than SPME was capable of a selective extraction of CPA. The whole extraction gave high recovery yields and results far simpler and faster than any other existing procedure for CPA extraction from cheese.

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