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LC-ion trap electrospray MS-MS for the determination of cyclopiazonic acid in milk samples

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An LC-ESI-MS (negative ions) method was developed for the determination of cyclopiazonic acid (CPA), a mycotoxin produced by many *Aspergillus* and *Penicillium* genuses, in milk samples. First, the acquisition parameters of the ESI mass spectrometer were optimised for deprotonated CPA and MS-MS measurements were performed, giving a fragmentation pattern. After this stage, LC separation was applied to milk extracts (with or without CPA spikes) by means of an aminic column, using the selective reaction monitoring (SRM) acquisition mode for MS detection. The CPA response was linear over three decades of concentration and an LOD of 5 ng mL^{-1} was estimated; moreover, the extraction procedure produced almost quantitative recoveries of CPA from milk. Twenty different milk samples were analysed and three of them were found to be contaminated with CPA to various extents.

Introduction

Mycotoxins are toxic secondary metabolites produced by many species of moulds contaminating food, known for their mutagenicity, carcinogenicity and teratogenicity.[1] The main source of mycotoxins in the human and animal food chain is represented by the consumption of contaminated agricultural and animal products, and the presence of mycotoxins in such matrices has been demonstrated by several workers.[2–4]

Among mycotoxins, increasing attention has been devoted to cyclopiazonic acid (CPA), an indole tetraminic acid produced by many *Aspergillus* and *Penicillium* genuses. Although CPA toxicity data relative to humans are not available in the literature, several studies have been carried out on different animals. For instance, a number of toxicological investigations on rats, chickens and pigs have been reviewed,[5] neurochemical effects have been described in chickens[6] and mice[7] and inhibition of synthesis of proteins was found in broiler chickens.[8] Moreover, the accumulation of CPA in milk and eggs from lactating ewes and laying hens that had been given oral doses of mycotoxins[9] has been studied.

The risk of human exposure to CPA could arise from the consumption of a large range of contaminated food commodities, hence the determination of CPA in food samples represents an important aspect of food safety. Existing analytical methods for the determination of this mycotoxin are mainly based on thin-layer chromatography or high-performance liquid chromatography and have been applied to cheese,[10,11] corn[12,13] and peanuts.[14,15]

Recently, a highly sensitive method using solid-phase microextraction coupled with HPLC anion-exchange/UV detection for the determination of CPA in cheese has been developed in our laboratory.[16] In the present work, the combination of the separation capabilities of HPLC and the molecule identification power of electrospray ionization tandem mass spectrometry (ESI-MS-MS) was applied to the determination of CPA in milk samples. The procedure is simple and sensitive and the high specificity of the technique allows the use of a simpler sample pre-treatment compared with existing methods.

Experimental

Chemicals

Cyclopiazonic acid was obtained from Sigma (St. Louis, MO, USA) and used without further purification. Stock standard solutions (1000 ng μL^{-1}) were prepared in methanol and stored at 4 °C in the dark. More dilute solutions were prepared just before use with the HPLC mobile phase (see later) and filtered through a 0.2 μm Millex-HV type filter (Millipore). All organic solvents employed were purchased from Aldrich (Milwaukee, WI, USA) and were of HPLC grade. The HPLC mobile phase was filtered through a 0.2 μm nylon membrane (Whatman, Maidstone, UK) before use.

Apparatus

The LC-MS system consisted of a Waters (Milford, MA, USA) Model 600-MS multisolvent delivery system equipped with a Supelcosil LC-NH_2 column (250 × 2.1 mm id, particle diameter 5 μm) (Supelco, Bellefonte, PA, USA) coupled to an LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ionisation (ESI) source, a divert/inject valve (used as a loop injector with a 30 μL loop volume) and an electronically controlled, integrated dual syringe pump that delivers sample solution and/or sheath liquid from syringes into the ESI source.

Experimental conditions

Preliminary measurements were performed on CPA in order to optimise the operating parameters for ESI-MS acquisition. With this aim, CPA solutions (5 ng μL^{-1}) were infused into the spectrometer through its syringe pump at a flow rate of 20 μL min^{-1}.

The mobile phase for HPLC separations consisted of acetonitrile/ammonium acetate buffer (0.05 M, pH 5) (80 + 20 v/v). The flow rate was 0.2 mL min^{-1} and the column temperature was ambient. The mobile phase was degassed with helium.

The LCQ spectrometer was operated in the negative ion mode and the ESI-MS parameters were as follows: spray voltage, 5.5 kV; sheath gas (nitrogen), 1.2 L min^{-1}; capillary voltage, −31.0 V; capillary temperature, 190 °C; tube lens offset voltage, −49.0 V. Mass spectra were recorded using different acquisition modes (full scan, SIM, SRM) as specified in the text and figure captions. The collision energy adopted in the SRM mode was 25% of the maximum.

Pre-treatment of milk samples

Twenty samples of pasteurised cow's milk, produced in different regions of Italy were purchased at local supermarkets.

The extraction of CPA was performed by modifying the procedure reported by Prasongsidh *et al.*[17] In particular, 5 mL of a methanol/2% $NaHCO_3$ mixture (70 + 30 v/v) were added to each sample (5 mL); the resulting mixture was then defatted with hexane (10 mL) and centrifuged at 5000 rpm for 15 min. The organic phase was discarded and the defatted milk was acidified to pH 3 with 6 M HCl. A 10 mL volume of chloroform was added and the resulting mixture was shaken vigorously for 30 min and centrifuged at 5000 rpm for 15 min. The chloroform layer was collected and evaporated to dryness under a gentle stream of nitrogen. Finally, the residue was dissolved in 500 μL of methanol and filtered through a 0.2 μm nylon membrane (Whatman) and 30 μL were injected into the LC column.

In this case, the final purification on a small silica column was eliminated, thus making the extraction procedure faster.

Quantitative determinations

For quantitative determinations, a calibration curve was first obtained, in the range 5–1000 ng mL^{-1}, using CPA standard solutions in methanol. Another calibration was then performed in the same concentration range but using extracts of milk spiked with CPA, in order to check for possible matrix effects. In this case preliminary ESI-MS-MS measurements were made to ascertain that the extracts were CPA free (*i.e.*, contained levels of CPA below the limit of detection of the method).

The upper limit of the concentration range adopted for CPA calibrations was chosen to include the levels (200–600 ng mL^{-1}) determined in milk produced by lactating ewes which had been given high oral doses of CPA.[9] In fact, these values can provide an estimate of the highest CPA concentrations expected in milk, whose contamination arises mainly from using food containing mycotoxins to feed milk-producing animals.

CPA recoveries were calculated by spiking three milk samples with CPA standard solutions at concentrations of 5, 20 and 80 ng mL^{-1}.

Results and discussion

Direct infusion-ESI-MS

Preliminary experiments were performed by direct infusion of CPA standard solutions (5 ng μL^{-1}) into the mass spectrometer. When the optimization of the spectrometer parameters was completed, MS and MS-MS measurements were made. A full-scan ESI mass spectrum of CPA (negative ions), acquired in the range *m/z* 70–340, is reported in Fig. 1(A). As expected, only the $[M–H]^-$ (*m/z* 335) ion was observed in the spectrum, along with the corresponding isotope. A full-scan spectrum (not reported) was also recorded in the range *m/z* 50–1500 and no signal due to CPA adducts was observed.

The MS-MS spectrum of the deprotonated molecule ion at *m/z* 335 is shown in Fig. 1(B). Fragmentation of deprotonated CPA in the ion trap leads to three main product ions, two of which (those at *m/z* 180 and 140) can be further fragmented, whereas the ion at *m/z* 154 does not show any fragmentation, regardless of the collision energy adopted in the ion trap.

The fragments observed in CPA MS-MS spectra were assigned to the structures shown in Fig. 2; it is worth noting that

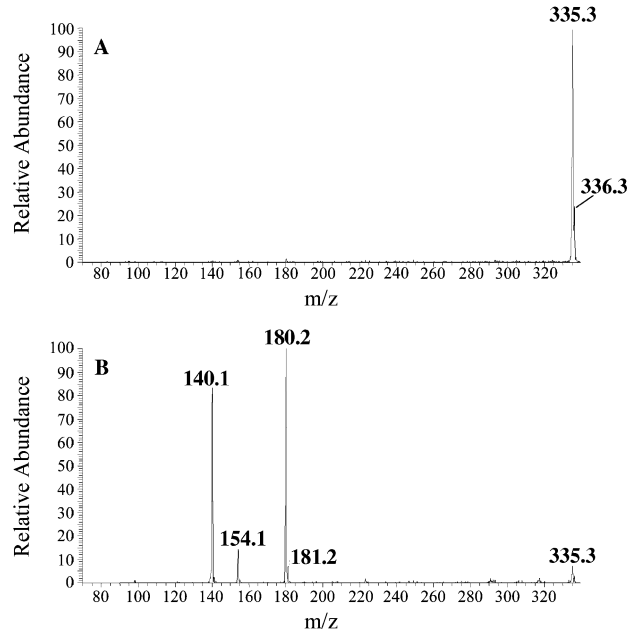

Fig. 1 (A) Full-scan ESI mass spectrum of CPA, acquired in the range *m/z* 70–340. (B) MS-MS spectrum of the CPA anion (*m/z* 335). For other conditions, see the Experimental section.

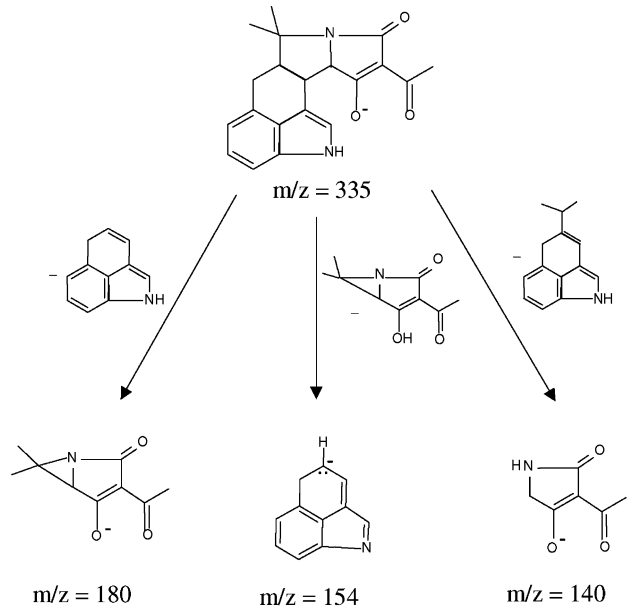

Fig. 2 Fragmentation pattern proposed for the CPA anion on the basis of the ions observed in the MS-MS spectrum.

they are similar to those observed in electron ionisation MS spectra of CPA.[18]

Selected reaction monitoring

The SRM acquisition mode was chosen for the quantitative determination of CPA, in order to minimize the interference due to other constituents of the milk samples and to enhance the sensitivity and the specificity of the technique. The $[M–H]^-$ ion (*m/z* 335) was isolated and fragmented inside the ion trap and the product ion at *m/z* 180 was then monitored during each chromatographic run.

An SRM chromatogram relevant to the injection of a methanol standard solution of CPA at a concentration level (10 ng mL^{-1}) close to its detection limit is shown in Fig. 3(A).

It should be pointed out that the extremely high signal-to-noise (S/N) ratio observed in the SRM chromatogram is only apparent. In fact, no other anion (due to solvent impurities) having, at the same time, an *m/z* value close to that of the CPA anion (so that it could be isolated in the ion trap) and a fragment ion with an *m/z* value close to 180 is eluted when standard solutions of CPA are injected. On the other hand, the electrical noise associated with the LCQ detector is automatically filtered, then a signal is observed only when CPA is eluted, whereas noise seems to be absent throughout the chromatogram.

A possible approach for estimating the limit of detection under these conditions is based on the standard deviation of the intercept of the calibration curve.[19] This method was adopted in the present work (see later).

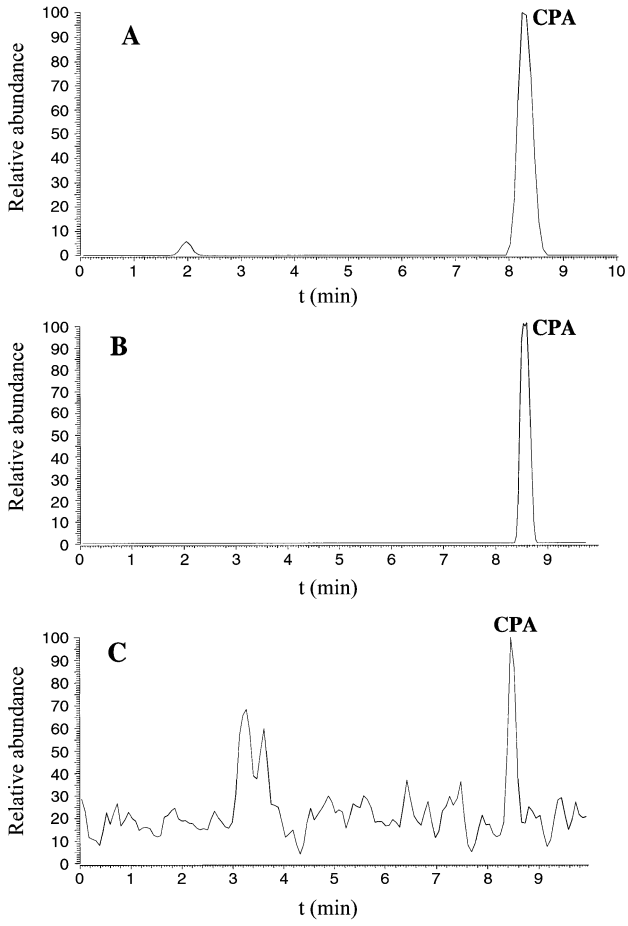

Fig. 3 (A) LC-ESI-SRM chromatogram relevant to the injection of a standard solution of CPA in methanol at a concentration level (10 ng mL^{-1}) close to its detection limit. (B) LC-ESI-SRM and (C) LC-ESI-SIM chromatograms relevant to a milk extract naturally contaminated with CPA. For other conditions, see the Experimental section.

Linear range, detection limit and precision

The calibration curve for CPA solutions in methanol in the SRM acquisition mode was linear in the range 20–1000 ng mL^{-1}, with a correlation coefficient better than 0.999 and an intercept not significantly different from zero at the 95% confidence level.

The LOD and LOQ were calculated, respectively, as the analyte concentrations for which the SRM peak area is equal to three and ten times the standard deviation of the intercept for the calibration regression line, and their values were 5 and 24 ng mL^{-1}.

The precision of the method was investigated on CPA standard solutions at a concentration level of 100 ng mL^{-1}. Replicate measurements (n = 8) were performed on the same day and the relative standard deviation was 4%.

Analysis of milk samples

Twenty different milk samples were examined for determination of CPA and in three cases the samples were found to be contaminated with CPA.

The LC-ESI-SRM results relevant to one of these samples are shown in Fig. 3(B); the LC-ESI-SIM chromatogram, obtained by monitoring the CPA anion at *m/z* 335, is also shown for comparison [Fig. 3(C)].

Owing to the complexity of the matrix (even after the extraction stage), other compounds in the milk extract are able to provide anions with *m/z* values close enough to that of CPA to be isolated in the ion trap (an isolation window of 4 *m/z* units, centred on the value 335, was adopted for SIM and SRM acquisitions), hence their peaks appear in the SIM chromatogram, at different retention times. However, when fragmented inside the ion trap (MS-MS), those species do not generate a product ion with an *m/z* value close to 180, so the CPA peak is the only one in the SRM chromatogram and can be easily integrated.

Even with this selectivity, the presence of special matrix effects, *e.g.*, intensity variations due to possible co-elutions with CPA of other anions having different *m/z* values (and hence not detected in the SIM mode or isolated in the SRM mode), cannot be excluded. Indeed, those species could compete with CPA for ionization in the electrospray interface, thus lowering its signal, although the peak shape is not affected.

A calibration curve was then constructed by spiking the extracts of CPA-free milk samples with the mycotoxin in the same concentration range as adopted for the calibration in methanol solution (5–1000 ng mL^{-1}). A linear dependence was found between the SRM signal and spiked CPA concentration, with a correlation coefficient of 0.9996 and intercept not significantly different from zero at the 95% confidence level. The LOD and LOQ obtained under these conditions were 6 and 36 ng mL^{-1}, respectively.

CPA-free milk was also used for recovery tests, performed by spiking the samples with CPA before extraction, as described in the Experimental section. The concentration in the extracts was then evaluated using the corresponding calibration curve and divided by a factor of 10, which takes into account the enrichment occurring during the extraction procedure. The comparison between these values and those used for the initial CPA spikes led to the recoveries reported in Table 1, which appear to be almost quantitative.

It is worth noting that analogous tests performed after using a purification step on a silica column at the end of the extraction procedure led to much lower CPA recoveries (47 ± 7%, n = 3). The SRM acquisition overcomes the need for this further purification of the milk extracts, leading to better recoveries and to a faster sample pre-treatment.

Table 1 Recoveries calculated by spiking CPA-free milk samples (n = 3) with CPA

CPA concentration spiked/ng mL^{-1}	Recovery (%)
5	96 ± 4
20	95 ± 4
80	95 ± 4

Owing to the 10-fold concentration enrichment occurring during the extraction stage and to the almost quantitative recovery, an LOQ of 4 ng mL^{-1} can be estimated for the initial milk samples. The concentrations of CPA in the three naturally contaminated milk samples, calculated by replicate analyses (n = 3), were found to be close to this value, *viz*., 4.5 ± 0.2, 6.2 ± 0.3 and 8.3 ± 0.3 ng mL^{-1}.

Conclusions

We have presented the first LC-ESI-MS-MS method for the determination of CPA in milk samples. The specificity of detection provided by the monitoring of a fragment of the analyte, using the SRM acquisition mode, permits the pre-treatment procedure to be shortened, eliminating purification of the milk extracts on silica columns. As a result, the whole procedure is faster and, more important, is characterised by almost quantitative extraction yields.

The method could be potentially applied to the determination of CPA in other food commodities and work in this direction is in progress.

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