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Analysis of cyclopiazonic acid in milk by capillary electrophoresis

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A Micellar Electrokinetic Capillary Chromatography (MEKC) method to detect cyclopiazonic acid (CPA) in milk and compare its quantifying efficiency to the Reverse Phase Liquid Chromatography (RPLC) method was evaluated. Alkaline milk samples were defatted, then acidified before being twice liquid–liquid extracted with chloroform. Bare fused-silica capillary-extended light path with 50 μm i.d. and Nova-pak C18-column, were used for the CPA separation in MEKC and RPLC respectively. The analytical response was linear from 40 ppb to 100 ppm CPA in MEKC (correlation coefficient, r = 0.99995). Recoveries of spiked CPA in milk were 78–81% over the range of 20 ppb to 500 ppb in MEKC and 71–80% in the range of 50 ppb to 500 ppb in RPLC. The detectable limit of CPA by MEKC was 0.27×10^{-07} pg ml^{-1}. Capillary electrophoresis (MEKC) is a better and rapid method for CPA detection in milk. © 1998 Elsevier Science Ltd. All right reserved

INTRODUCTION

Chemically known as indole tetramic acid, mycotoxin cyclopiazonic acid (CPA) is a potent neurotoxin, produced by *Penicillium* (Holzapfel, 1968) and *Aspergillus*. Fungi producing CPA are widespread in nature and are commercially utilised. Some produce both CPA and aflatoxins. CPA induces a neurological disturbance (Nishie *et al.*, 1984) and is acutely toxic in animals and humans (Rao & Husain, 1985). The toxin is detectable in agricultural commodities (Widiastuti *et al.*, 1988) and food (Le Bars, 1979). The appearance of CPA in animal feed (Dutton & Westlake, 1985) indicates the high probability of its presence in animal products (Norred *et al.*, 1985). After receiving the first dose, CPA was detected within one day in the milk of lactating ewes (Dorner *et al.*, 1994). Thus, human exposure to CPA through food including milk or dairy products is possible. However, CPA in milk and its exposure to humans is not well reported in the literature compared to that for aflatoxins. Lack of rapid, reproducible and low cost assays for screening of this mycotoxin in milk may contribute to this limitation.

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CPA detection in agricultural commodities and food has been done by thin-layer chromatographic (TLC) (Lansden, 1986), colorimetric (Rao & Husain, 1987) and spectrophotometry (Chang-Yen & Bidasee, 1990) techniques. Simultaneously, detection of CPA and aflatoxins was attained by gas chromatography (GC) (Goto *et al.*, 1990). Competitive enzyme-linked immunosorbent assay (ELISA) was developed (Hahnau & Weiler, 1991) with a variability of 14% of inter-assay between-day and 3.7% of intra-assay. Various normal and reverse phases of high performance liquid chromatography have been documented, however peaks obtained were too broad (Betina, 1993). Urano *et al.* (1992) recently published a sensitive RPLC method applied for corn and peanuts. In the meantime, a gel diffusion method (Simunek *et al.*, 1992) completed with the reported colorimetry (Rao & Husain, 1987) was used to detect CPA from dairy products.

Capillary electrophoresis (CE) is capable of separating several charged and water soluble molecules in a single analysis. In several applications, CE showed a greater efficiency compared to high performance liquid chromatography (Wätzig & Dette, 1993; Marina & Torre, 1994). With a wide range of methods, CE has been used to separate and quantify substances in

various fields including food analysis (Cancalon, 1995). Environmental pollutants including aflatoxins and six other mycotoxins were successfully separated by MEKC (Holland & Sepaniak, 1993). With the advantages of MEKC including simple method development, small sample volume requirement, less organic solvent utilised, high efficiency and short analysis time, this study assessed the efficiency of CE compared to RPLC (Urano *et al.*, 1992) in CPA detection, separation and quantification in milk.

MATERIALS AND METHODS

Equipment, reagents and samples

The liquid chromatography system consisted of two solvent delivery systems (Waters 501 & 510), injector Model U6K, Waters 484 tunable absorbance detector, Waters Baseline 810 Chromatography Workstation data handling system; LC column: Nova-pak, 150×3.9 mm i.d, packed with C18, 5 μm (Waters); Guard column: all-Guard GRP-2, Nova-Pak® C18 (Waters); 16-port vacuum manifold (Waters) and Silica gel cartridge Sep-Pak® plus silica (Waters). Capillary electrophoresis system HP^{3D}CE instrument equipped with a replenishment system, vial tray, diode array detector, ChemStation data software (Hewlett-Packard) and bare-fused-silica capillary-extended light path (50 μm i.d.×64.5 cm, 60 cm effective length & alignment interface) was used.

Analar grade solvents were used. Cyclopiazonic acid, tenuazonic acid, aflatoxins B_1, B_2, G_1, G_2 were obtained from Sigma chemical company. Milk samples were obtained from The Dairy Processing Plant, University of Western Sydney, Hawkesbury. N.S.W., Australia.

Standards

Stock solution: pure solid CPA (Sigma) was dissolved in methanol (liquid chromatography grade) at 1 mg ml^{-1} and stored at 0°C in the dark; working solution: dilute stock solution with methanol (liquid chromatography grade) with CPA concentration at 40, 100, 500 ng ml^{-1} and 1, 5, 10 μg ml^{-1}.

Extraction and clean-up

Twenty-five ml milk in triplicate was mixed with 25 ml methanol-2% sodium hydrogen carbonate mixture (7:3) (Urano *et al.*, 1992). The milk–methanol mixture was defatted with 100 ml hexane by 3 min shaking in wrist action (Flask shaker, Stuart Scientific). Centrifugation assisted the hexane layer separation. The sample layer was acidified to pH 3 with 6*N* HCl before being extracted twice for 30 min with 100 ml chloroform that was then dried with anhydrous sodium sulphate. The extract was evaporated at 40°C to dryness and redissolved in 5 ml chloroform before being cleaned in Sep-Pak plus silica gel cartridge by 10 ml diethyl ether and 5 ml chloroform–acetone mixture (1:1). CPA was eluted with 10 ml chloroform–methanol mixture (75:25), which was subsequently evaporated to dryness. The residue was redissolved again with 1.5 ml chloroform before being dried under nitrogen gas and kept in a dark screw-cap vial at 0°C until used.

Quantification

The residue was eluted in 400 μl of methanol (liquid chromatography grade) using a vortex mixer then 200 μl of it was confined to be directly injected into RPLC. The other 200 μl left was combined with 300 μl of MEKC mobile phase prior to injection onto the CE.

MEKC analysis

The temperature of the capillary's cartridge was set at 40°C. In between analysis the capillary was washed with 0.1 *M* sodium hydroxide and Milli-Q water before flushing with the mobile phase. Before sample injection, the capillary was conditioned by applying 20 kV of voltage for 10 s. The electric parameters of the CE were set with a positive polarity, 4.5 watt of power, 20 kV of voltage and a current of 50 μA. Similar to reported studies on aflatoxins (Holland & Sepaniak, 1993), the mobile phase in this study contained 0.05 *M* sodium deoxycholate, 0.01 M disodium hydrogen phosphate, and 0.006 M disodium tetraborate at a pH of 9.3 (adjusted with sodium hydroxide or phosphoric acid). The pressure applied during sample injection was 50 mbar in 7 s. The absorbance of CPA was recorded at 225 nm wavelength. A standard curve was developed from 40 ppb to 100 ppm with a correlation coefficient at 0.99995.

RPLC

The analysis was done according to the method of Urano *et al.* (1992). The chromatography, containing two pumps, was conditioned to give a linear gradient in 10 min from 100% A to 100% B, which was followed by an other 10 min 100% B. The mobile phases consisted of methanol–water (85:15) and 4 mM zinc sulphate–methanol–water (85:15). The flow rate was set at 1 ml min^{-1} and the injection volume was 20 μl. A standard curve was obtained from 500 ppb to 100 ppm with a correlation coefficient of 0.9999937.

The concentration of CPA detectable by MEKC was calculated with the following equation:

$$C_{cpa\,w}\ \text{ng ml}^{-1} = R_{MEKC}\ \text{ng}\ \mu\text{l}^{-1} \times Tv\mu\text{l} \times (1\ \text{V ml}^{-1}) \times 2.5$$

Based on this formula and the Hagen–Poiseuille equation, the detectable limit was calculated.

CPA analysed by RPLC was calculated with the equation:

$$C_{cpa\ w}\ ng\ ml^{-1} = R_{RPLC}\ ng/\mu^{-1} \times Tv\mu l \times (1\ V\ ml^{-1})$$

where $C_{cpa\ w}$ ($ng\,ml^{-1}$) = Concentration of CPA in test portion; Tv (μl) = Total concentrated extracted elute (400 μl); V (ml) = Total volume of the test portion; R_{MEKC} ($ng\,\mu l^{-1}$) = Result obtained and read in MEKC; R_{RPLC} ($ng\,\mu l^{-1}$) = Result obtained and read in RPLC; 2.5 = Coefficient of dilution of sample in MEKC system.

RESULTS AND DISCUSSION

The preliminary experiments involved optimising the set-up conditions of the CE, searching for an appropriate mobile phase by running pure CPA standards as samples and sodium dodecyl sulphate, sodium deoxycholate with different percentages of acetonitrile as part of the mobile phase. Sudan III was used as a marker to evaluate the efficiency of the CPA separation from some other mycotoxins. The optimum conditions (Fig. 1) found to detect and quantify CPA in milk are described in the method section. However, these conditions cannot ensure a detection of mixed mycotoxins such as aflatoxins B_1, G_1, tenuazonic acid and CPA in a single sample. Using a lower temperature and adding 7% acetonitrile to the MEKC mobile phase induces a good separation of CPA from the marker and the other mycotoxins including aflatoxins B_1, B_2, G_1, G_2 and tenuazonic acid (Fig. 2). Migration time and real time analysis maybe slightly longer than the one obtained by the method previously mentioned. Lower amounts of CPA standard are better detected by CE than RPLC. Thus, the calibration curve from 40 ppb to 100 ppm was obtained with the CE with a very good coefficient of correlation. The minimum quantifiable concentration of CPA was detected in spiked milk samples of 20 ppb in CE and of 50 ppb in RPLC. The sensitivity of RPLC was restricted when the signal: noise ratio was < 5:1. In contrast, while still maintaining high theoretical plates numbers and resolution, CE was capable of isolating the CPA peak at lower quantity, although the sample injection volume ($\approx$ 8.3 nl) was several times lower than that of RPLC (20 μl).

The ChemStation software in CE used in this study offered multiple time reference peaks, spectral analysis with a third dimension of analytical data and peak purity checking using a spectral library search, which allowed to filter out spectral noise and to quantify CPA

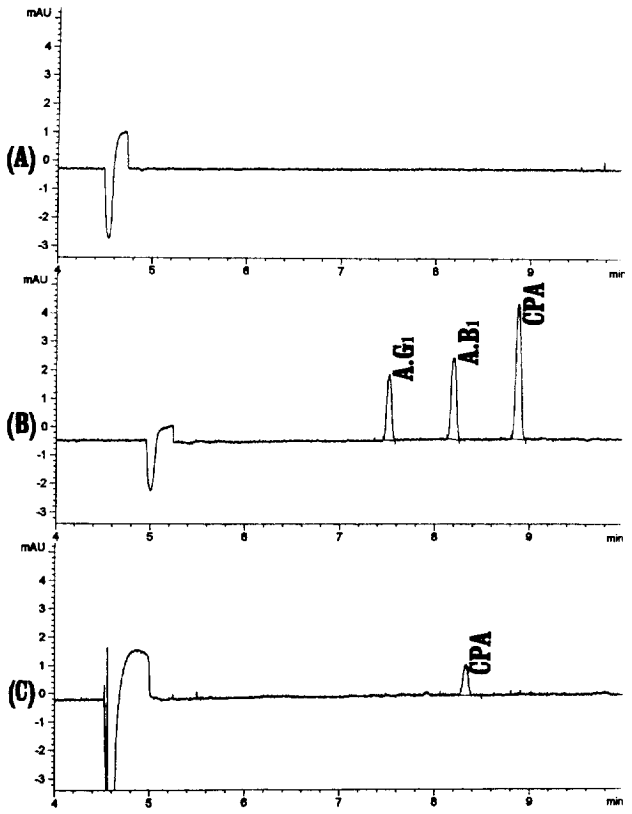

Fig. 1. Electropherograms of control milk (A), CPA (10 $\mu g\,ml^{-1}$), Aflatoxins B_1 and G_1 standards (5 $\mu g\,ml^{-1}$) (B) and spiked milk extracts (CPA at 1 $\mu g\,ml^{-1}$) (C). Conditions: bare fused-silica capillary-extended light path, 50 μm i.d. × 64.5 cm, 60 cm effective length; mobile phase composition, 0.05 M sodium deoxycholate, 0.01 M disodium hydrogen phosphate, and 0.006 M disodium tetra borate, pH 9.3; applied voltage 20 kV/50 μA and power 4.5 W; cassette temperature, 40°C and run time 10 min.

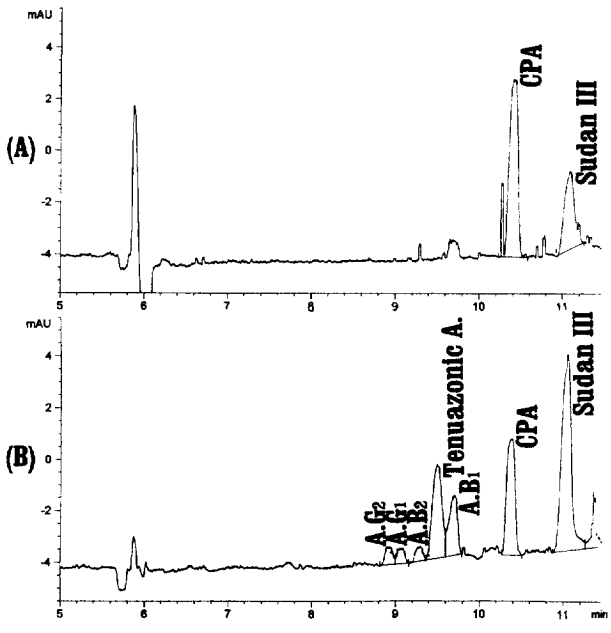

Fig. 2. Electropherograms of CPA (10 $\mu g\,ml^{-1}$) and marker (A) and CPA (5 $\mu g\,ml^{-1}$) with aflatoxins B_1(3 $\mu g\,ml^{-1}$) B_2 (1 $\mu g\,ml^{-1}$) G_1 (1 $\mu g\,ml^{-1}$) G_2 (1 $\mu g\,ml^{-1}$), Tenuazonic acid (5 $\mu g\,ml^{-1}$) and marker Sudan III (B). Conditions: bare fused silica capillary-extended light path, 50 μm i.d. × 64.5 cm, 60 cm effective length; mobile phase composition, 7% acetonitrile, 0.05 M sodium deoxycholate, 0.01 M disodium hydrogen phosphate, and 0.006 M sodium tetra borate, pH 9.3; applied voltage 20 kV/50 μA and power 4.5 W; cassette temperature, 30°C and run time 12 min.

Table 1. Recovery of CPA added to milk and quantified by RPLC and MEKC

CPA spiked (ng ml^{-1})	MEKC (%)					RPLC (%)				
	Avg.(n = 6)	Max.	min	SD	RSD	Avg. (n = 6)	Max.	min	SD	RSD
20	81.0[b]	89.9	66.7	8.3	6.7	nd[a]	nd	nd	nd	nd
50	80.5[b]	90.0	74.4	6.6	5.3	70.7[b]	80.0	62.3	5.8	4.1
100	79.6[b]	82.6	73.4	2.9	2.3	71.0[b]	83.2	67.7	5.7	4.1
200	80.7[c]	89.7	76.8	4.4	3.5	80.0[c]	85.6	69.8	7.2	5.8
500	77.9[c]	84.3	69.9	4.7	3.7	76.4[c]	96.0	70.4	8.9	6.8

[a]Not detectable.
[b]$P < 0.05$ significant difference between MEKC and RPLC.
[c]No significant difference.
SD- Standard deviation; RSD- Relative standard deviation.

at low level. The extended light path capillary used in this study permitted the accumulation of the sample three times bigger than the one in the inner diameter of the capillary at the bubble cell where light throughput. This allowed several folds increases in signal and thus improved the lower limit of detection as well as the linear of detection range in CE. The variability in CE in an inter-assay between-day and an intra-assay was 5.1% and 2.3%, respectively. However, to obtain the variability at such a percentage and to avoid buffer depletion, buffer replenishment was done at every fourth injection. Conditioning of the capillary before each analysis and using a unique batch of mobile phases ensured a low variability of interassay. Diode-array detector, peak purity examination by using spectra analysis and spectra libraries enhances the confirmation of peak identity even in a quantity as low as a picogram. Thus, CE is significantly more capable of detecting CPA in milk in low concentration than RPLC (Table 1). However, no difference was found at higher levels (200 ppb–500 ppb). The recoveries from milk spiked with 20 ppb to 500 ppb detectable by CE were 78–81% with a standard deviation varying from 2.9–8.3%. The relative standard deviation was between 2.3–6.7%. CPA spiked at 20 ppb was not detectable by HPLC. The recoveries from milk contaminated by 50 ppb to 1 ppm of CPA were 70–80% with the relative standard deviation between 4.1–6.8%. The results obtained in RPLC were consistent with the previous reported study done on peanuts and corn (Marina & Torre, 1994).

This study revealed that if parameters are optimised, including extraction and equipment, more than a dozen samples can be extracted per person in half a day. Real-time individual sample analysis for CPA with CE is about 10 min. Sequence analysis and the replenishment system provide the opportunity for multiple sample analysis. More than 40 samples can be loaded overnight on an automated CE with on-line injection. The result of this study indicates that CE can be employed in the surveillance system for CPA in milk, in feed or foods. Further studies on the method of CPA extraction in milk may reduce the sample extraction and preparation times.

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