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Kinetic study of cyclopiazonic acid during the heat-processing of milk

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Heat-stability of cyclopiazonic acid (CPA) incorporated in milk was assessed under different conditions. The CPA residue in milk was analysed by micellar capillary electrophoresis. Three batches of 1 μg CPA/ml contaminated milk were heated at 60, 80 and 100°C for 15–60 min to simulate heat processing employed in the dairy industry. Only 3–9%, 14–18% and 25–30% of CPA were degraded, respectively, and degradation followed a pattern of a first-order reaction. Heating the milk for 2 h at 60, 80 and 100°C decreased the CPA level by 9–17%, 20–34% and 49–50%, respectively. Storage of the heated milk overnight at 4°C induced a further reduction of CPA. Autoclaving the milk or heating canned milk in a retort for 30 min at 120°C led to a loss of 33–36% of CPA. Simulation of heat-treatments used by the dairy industry induced no significant degradation of CPA. The inability of the heat-treatment during milk processing to eliminate the mycotoxin emphasises the serious potentiality of CPA exposure for liquid milk consumers.

INTRODUCTION

Cyclopiazonic acid (CPA) is an indole tetramic acid mycotoxin, produced by several genera of *Aspergillus* (Dorner *et al.*, 1983) and *Penicillium* (Engel, 1981; Hermansen *et al.*, 1984). Co-production of this neurotoxic mycotoxin and the notorious aflatoxin by many genera of moulds *Aspergillus* (Dorner *et al.*, 1984; Blaney *et al.*, 1989; Cvetnic, 1994; Gqualeni *et al.*, 1996; Goto *et al.*, 1996) initiated scientific interest. A retrospective study on the "Turkey 'X' disease", which occurred in the 1960s, revealed an association of CPA and aflatoxin (Cole, 1986), whose actions were additive in animals (Morrissey *et al.*, 1987; Pier *et al.*, 1989). CPA was linked to animal neurological signs including catalepsy (Purchase, 1971; Nishie *et al.*, 1985) and opisthotonus (Stoltz *et al.*, 1988) and had several target organs such as liver, kidney and the digestive system (Lomax *et al.*, 1984; Nuehring *et al.*, 1985). Oral LD_{50} in rat was 36 mg/kg in males and 63 mg/kg in females. The potential hazard of CPA for humans has not been completely determined; however, incidence (Rao and Husain, 1985) and symptoms in humans including sleepiness, tremors and giddiness have been reported (Bhide, 1962). Many CPA-producing fungi are commercially utilized (Orth, 1977; Le Bars *et al.*, 1988) and commonly found in agricultural commodities (Lee and Hagler, 1991). Hence the natural occurrence of this mycotoxin in animal feedstuffs has been well documented (Balachandran and Parthasarathy, 1996). Feeding of CPA to a lactating animal induced carry-over into the milk within 1 day followed by a twofold increase to 568 ng/g after a second dose (Dorner *et al.*, 1994). Withdrawal of CPA feeding to animals did not interrupt its presence and it persisted at 262 ng/g in milk for several days. This finding has indicated a high potential human exposure to CPA through consumption of contaminated liquid milk and dairy products. However, incidence of CPA in milk and its exposure to humans is still not well documented in the literature compared to that for aflatoxins.

To render milk safe and to extend its shelf life, raw milk is commonly processed prior to human consumption. It undergoes several treatments including heat-processing to eliminate micro-organisms, inactivate enzymes and alter chemical and/or physical properties for further processing. The thermal effect on the stability of CPA in milk may be similar to that of aflatoxins (Polzhofer, 1977; Gelosa and Buzzetti, 1994). Several models of study of heat-processing of the aflatoxin M have been documented (Purchase *et al.*, 1972; Wiseman and Marth, 1983).

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Due to public health concern and the economic viability of dairy products, the potentiality of thermal reduction and the level of CPA residues in milk following heat-processing should be established. The overall objective of this study was to assess the CPA stability to heat treatment similar to that used in the dairy processing industry and to determine the resistance of this mycotoxin in milk to thermal destruction.

MATERIALS AND METHODS

Artificially contaminated milk

Whole raw milk was obtained from the Dairy Processing Plant, University of Western Sydney, Hawkesbury, NSW, Australia. Pure solid cyclopiazonic acid (Sigma) in methanol was stored at 0°C in the dark until use. Artificially contaminated milk was prepared similar to the method of Wiseman and Marth (1983) by adding CPA in methanol into milk at 4°C constantly agitated by a magnetic stirrer.

Thermo-kinetics of CPA in milk heated at 60, 80 and 100°C for 1 h

The heating of milk in this study was done as in the model of Stoloff *et al.* (1975) to study aflatoxin M. Five hundred millilitres of whole raw milk containing 1 μg of CPA/ml with a magnetic stirrer was placed in a 1 l pyrex bottle, hermetically closed with a screw cap, which was pierced through by a thermocoupole probe line connecting the milk in the bottle to a squirrel data logger (model 1200, Grant Instruments, UK). This squirrel permitted constant monitoring and recording of temperature in the milk in the bottle initially warmed to a required temperature by a hot plate stirrer (Cimarec 3, Barnstead/Termolyne, USA) before being transferred into a shaking water bath (Tecator 1024, Linbrook, Sweden) set at the same temperature. Pressure was released at regular intervals. Control milk was extracted for CPA analysis prior to any heat-treatment. Three contaminated groups of milk samples were heat-treated to 60, 80 and 100°C and three trials for each group of milk samples were conducted. When the desired temperature reached after 15, 30, 45 and 60 min, triplicate samples of 30 ml heated milk were taken and gradually cooled in water, then in an ice water bath, before being extracted for CPA.

Thermo-resistance of CPA in milk after 2 h heat-treatment

Using the same method and conditions, three groups of similar milk samples were heated for 2 h; three trials for each group of milk samples were conducted. The temperature of the milk was monitored and recorded. One group within three trials of heated milk samples was extracted for CPA analysis after cooling in an ice water bath. Further degradation of CPA after high heat-treatment was assessed by leaving another set of treated milk samples at 4°C overnight before being extracted for CPA.

Stability of CPA in milk heated at 120°C

Three samples of raw contaminated milk (500 ml) containing 1 μg CPA/ml were canned before being heated to 120°C in a 10001 vertical retort (Ronald J. Murray and Son, Boiler makers and Engineers, Australia). The temperature was monitored and recorded using a squirrel data logger. Pressure control in the retort permitted a rapid heat and cooling process.

Another set of similar milk samples placed in Pyrex bottles were heated to 120°C for 30 min in an autoclave (Atherton Equipment, Australia). The milk sample was left for 30 min to cool in the autoclave before the CPA was extracted. An autoclave tape and a thermometer indicated the desired temperature and the duration when reached.

CPA behaviour in milk under the simulation of heat-treatments used by the dairy industry

Identical contaminated raw milk (500 ml) in 1 l pyrex bottles was heated similarly to study the thermo-kinetics of CPA under the following conditions: 60°C for 30 min, 70°C or 90°C or 100°C for 15 s. The temperature was monitored and recorded. All samples were cooled in an ice water bath before extraction of CPA.

Analysis of cyclopiazonic acid by capillary electrophoresis (CE)

Extraction and preparation of milk samples were done as previously reported (Prasongsidh *et al.*, 1998). Samples were treated with methanol–sodium hydrogen carbonate before being defatted by hexane, acidified with hydrochloric acid, then extracted with chloroform. Centrifuging after defatting by hexane and chloroform extractions enhances CPA recovery. The samples were filtered by 0.45 μm filter (Millipore) then cleaned in a silica gel cartridge Sep-Pak® plus silica (Waters) using diethyl ether, chloroform–acetone and chloroform–methanol. After evaporation to dryness, the chloroform–methanol residue was eluted with liquid chromatography grade methanol then with the mobile phase of the capillary electrophoresis prior to injection. The mobile phase of CE and sample diluent were prepared as previously reported (Prasongsidh *et al.*, 1998; Prasongsidh and Skurray, 1998). They consisted of a mixture of sodium deoxycholate, disodium hydrogen phosphate, and disodium tetraborate at a pH of 9.3. Capillary electrophoresis with diode array detector (Hewlett-Packard) and a bare fused silica capillary-extended

light path (50 μm i.d. × 64.5 cm and 150 μm i.d. bubble, 60 cm effective length and alignment interface) was used. Temperature of the capillary cassette was set at 40°C and the capillary was washed between analyses with 0.1 M sodium hydroxide and Milli-Q water before flushing with CE mobile phase. The polarity was set at positive mode. Applied voltage, current and power were set at limits of 20 kV, 50 μA and 4.5 W, respectively. The absorbance was recorded at 220 nm wavelength.

RESULTS AND DISCUSSION

Thermo-kinetics of CPA in milk

Data obtained from the sets of milk heated at 60, 80 and 100°C for an hour are shown in Table 1 and represent the residue from the initial level of CPA in artificially contaminated milk, which was kept at 4°C before any heat-treatment. The original level of CPA in each lot of contaminated milk varied without statistically significant difference from 1 to 0.98 μg/ml. Samples centrifuged and filtered, prior to the cleaning by Sep-Pak®, enhanced CPA recovery. Every sample of three trials, taken at 15 min intervals from the milk heated at 100°C, had the amount of CPA significantly decreased ($p < 0.05$). In the first 15 min of heat at 100°C, more than 12% decrease was observed. However, degradation of the mycotoxin was less than 31% even after 1 h of heating at this temperature. Heating of milk, for a similar length of time at 80°C, significantly ($p < 0.05$) reduced the amount of CPA up to 14–18% (Table 1). In contrast, no significant decrease of CPA in milk heated at 60°C was found in the first 15 min. Significant degradation was observed after 30 min of heating in trials 2 and 3. No more than 3–9% of CPA original level was reduced after an hour of heating at 60°C. Even though any heat treatment failed to totally eliminate the mycotoxin from milk in an hour, the degradation of CPA depended on time and temperature. Using semilogarithmic coordinates, Fig. 1 was obtained, which indicates that CPA in milk exposed to heat-treatments at 60, 80 and 100°C induces kinetics which follow a first-order reaction.

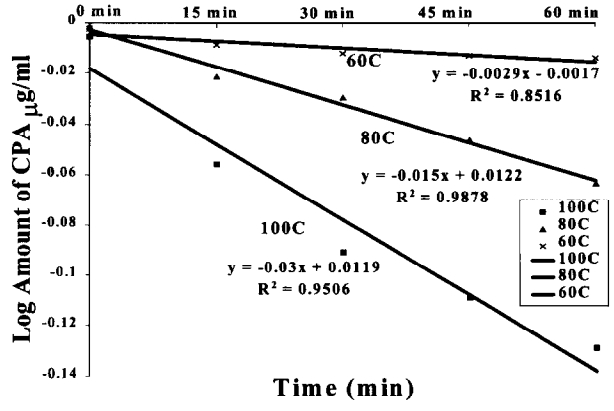

Fig. 1. Kinetics of reaction of CPA in milk heated at 60, 80 and 100°C.

Table 1. Residue of CPA in contaminated milk at 1 μg/ml heated at 60, 80 and 100°C from 15 min to 1 h

	CPA residual (μg/ml)[a]	SD[e]	%[d]	CPA residual (μg/ml)[a]	SD[e]	%[d]	CPA residual (μg/ml)[a]	SD[e]	%[d]
100°C[c]	Trial 1			Trial 2			Trial 3		
0 min	0.99	0.03	0.73	0.99	0.05	1.28	0.98	0.08	1.95
15 min	0.88[b]	0.04	12.08	0.88[b]	0.02	12.10	0.88	0.11	12.34
30 min	0.77[b]	0.05	23.43	0.8[b]	0.11	18.92	0.75[b]	0.10	24.75
45 min	0.74[b]	0.01	26.30	0.78[b]	0.03	22.25	0.74[b]	0.04	25.89
60 min	0.69[b]	0.05	30.67	0.74[b]	0.12	25.72	0.73[b]	0.10	27.07
80°C[c]									
0 min	1.00	0.00	0.34	1.00	0.00	0.23	0.98	0.02	1.99
15 min	0.95[b]	0.02	4.86	0.95[b]	0.02	4.91	0.93	0.03	6.79
30 min	0.93[b]	0.04	6.64	0.93[b]	0.04	6.59	0.92[b]	0.03	7.58
45 min	0.90[b]	0.01	10.17	0.88[b]	0.02	11.66	0.87[b]	0.07	12.93
60 min	0.86[b]	0.03	13.67	0.83[b]	0.01	16.74	0.82[b]	0.00	18.22
60°C[c]									
0 min	0.99	0.03	0.54	0.99	0.02	0.74	1.00	0.00	0.20
15 min	0.98	0.01	2.04	0.98	0.04	2.30	0.99	0.01	1.44
30 min	0.97	0.04	2.84	0.96	0.01	3.70	0.96	0.04	4.34
45 min	0.97	0.02	3.06	0.96[b]	0.03	3.73	0.93[b]	0.02	6.67
60 min	0.97	0.03	3.28	0.95	0.08	4.52	0.91[b]	0.09	8.70

[a]Triplicate samples.
[b]Significant difference with the original level $p < 0.05$.
[c]Milk heated at 60, 80 or 100°C.
[d]Percentage degradation of CPA in milk.
[e]Relative standard deviation equal to 0.00.
SD = Standard deviation.

Table 2. Residue of CPA in contaminated milk at 1 μg/ml heated at 60, 80 and 100°C for 2 h and left overnight at 4°C

	CPA residual (μg/ml)[a]	SD[e]	%[d]	CPA residual (μg/ml)[a]	SD[e]	%[d]	CPA residual (μg/ml)[a]	SD[e]	%[d]
100°C[c]	Trial 1			Trial 2			Trial 3		
Original level	0.99	0.03	0.73	0.99	0.05	1.28	0.98	0.08	1.95
2 h of heating	0.50[b]	0.15	50.44	0.51[b]	0.03	49.20	0.50[b]	0.05	49.68
2 h of heating and 4°C overnight	0.38[b]	0.01	61.91	0.45[b]	0.03	54.98	0.46[b]	0.02	53.98
80°C[c]									
Original level	1.00	0.00	0.34	1.00	0.00	0.23	0.98	0.02	1.99
2 h of heating	0.78[b]	0.02	22.06	0.83[b]	0.01	19.40	0.66[b]	0.04	34.22
2 h of heating and 4°C overnight	0.56[b]	0.04	44.05	0.70[b]	0.03	29.71	0.57[b]	0.07	42.96
60°C[c]									
Original level	0.99	0.03	0.54	0.99	0.02	0.74	1.00	0.00	0.20
2 h of heating	0.91[b]	0.03	9.29	0.83[b]	0.01	17.32	0.89[b]	0.01	11.00
2 h of heating and 4°C overnight	0.80[b]	0.06	19.63	0.77[b]	0.05	22.62	0.81[b]	0.00	18.68

[a]Triplicate samples.
[b]Significant difference with the original level $p < 0.05$.
[c]Milk heated at 60, 80 or 100°C.
[d]Percentage degradation of CPA in milk.
[e]Relative standard deviation equal to 0.00.
SD = Standard deviation.

Effect of storage (4°C) after a long heat-treatment (2 h) on CPA levels

After 2 h, an apparent decrease (50%) of CPA in milk heated at 100°C was observed (Table 2). Leaving overnight at 4°C induced a further loss of the mycotoxin up to 54–62%. Heating the milk at a lower temperature, 60°C and 80°C for 2 h, resulted in greater retention of CPA. Only 20–34% of CPA was degraded at 80°C but further loss of 30–44% of the original level was observed after storage overnight at 4°C. Two hours of heat at 60°C reduced CPA content to no more than 9–17% of the original value. A substantial further loss of 19–23% of CPA was observed after standing overnight at 4°C. The loss of CPA in milk during long time exposure to heat at 60, 80 and 100°C showed a degradation and temperature dependence (Fig. 2). Storage at an even lower temperature still led to a substantial further loss of the mycotoxin. However, this prolongation of heat-processing at high temperature, which normally affects milk quality and denatures certain milk components still, failed to eliminate the toxin from milk.

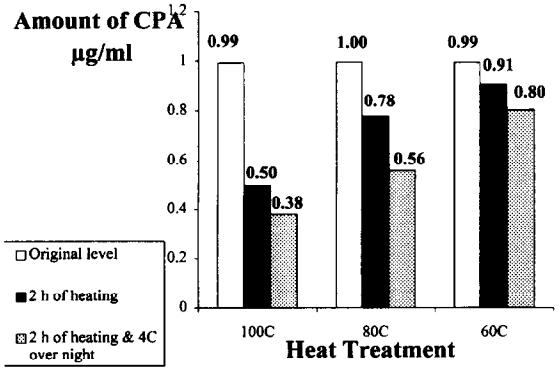

Fig. 2. Residues of CPA in contaminated milk at 1 μg/ml heated at 60, 80 and 100°C for 2 h and left at 4°C overnight.

Resistance of CPA in milk heated at 120°C

Heating of milk at 120°C in a retort for 30 min achieved a reduction of CPA to 36.5% of its original content (Table 3). No significant differences were found in three trials of experiments after the heat-treatment. Hermetically closed cans during the whole period of heating in the retort may explain this finding. In contrast, the milk placed in pyrex bottles, autoclaved at 120°C, showed significant differences between trial 1 and trial 2 or trial 3. Non-uniform heating and a slow process of heating or cooling in the autoclave may affect the level of CPA in trial 1. However, after 30 min of autoclaving at 120°C, the amount of CPA residue was not significantly different from that in the milk heated in the retort (Table 4).

Using a temperature and duration of heat-treatment similar to that employed in the dairy industry failed to decrease CPA by more than 7% of its original content. Table 5 indicates that the same temperature and duration as milk pasteurization (60°C for 30 min) can only reduce the CPA content by 3.5%. Higher temperature–short time processes, such as 70, 90 and 100°C for 15 s, reduced only of 0.39, 2.1 and 6.6%, respectively, of mycotoxin.

The observation that heat-processing of milk at 60–120°C from 15 s to 2 h did not destroy all of the CPA indicates a high probability of industrial processing conditions not destroying CPA from contaminated milk. Some temperatures used in this project were similar to those normally used in the dairy industry. However, significant residues of CPA persisted even after an hour of over-processing milk (Table 1). Half an hour of

Table 3. Residue of CPA in canned milk contaminated by 1 μg/ml heated at 120°C for 30 min in a retort

	CPA residual (μg/ml)[a]	SD	RSD	% denaturation
CPA at original content	1.00	0.00	0.00	0.10
Trial 1	0.64**	0.00	0.00	36.19*
Trial 2	0.64**	0.05	0.00	36.29*
Trial 3	0.65**	0.03	0.00	35.11*

[a]Triplicate samples.
*Significant difference with the original level $p < 0.05$.
**No significant difference between trials $p > 0.05$.
SD = Standard deviation.
RSD = Relative standard deviation.

Table 4. Residue of CPA in milk contaminated by 1 μg/ml autoclaved at 120°C for 30 min

	CPA residual (μg/ml)[a]	SD	RSD	% degradation
CPA at original content	1.00	0.00	0.00	0.46
Trial 1	0.66[b]	0.01	0.00	33.87*
Trial 2	0.62**	0.02	0.00	37.63*
Trial 3	0.64**	0.02	0.00	36.24*

[a]Triplicate samples.
[b]Significant difference between trials.
*Significant difference with the original level $p < 0.05$.
**No significant difference between trials $p > 0.05$.
SD = Standard deviation.
RSD = Relative standard deviation.

Table 5. Residue of CPA in milk containing 1 μg CPA/ml heated at 60, 70, 90 and 100°C

Temperature and duration of treatment	Residue of CPA (μg/ml)[a]	SD	RSD	% degradation
CPA at original content	1.00	0.02	0.00	0.00
60°C for 30 min	0.97*	0.01	0.00	3.49
70°C for 15 s	1.00**	0.02	0.00	0.39
100°C for 15 s	0.98**	0.03	0.00	2.14
100°C for 15 s	0.93*	0.00	0.00	6.57

[a]Triplicate samples.
*Significant difference with the original level $p < 0.05$.
**No significant difference with the original level $p > 0.05$.
SD = Standard deviation.
RSD = Relative standard deviation.

heat treatment at 120°C still left more than 60% of the original amount of the mycotoxin in the milk (Tables 3 and 4). Short time treatments (1 s) but with a higher temperature (133°C), such as ultra high temperature processing, may not cause greater loss of CPA.

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