FATE OF THE NEUROTOXIC MYCOTOXIN, CYCLOPIAZONIC ACID IN DAIRY PRODUCTS

A thesis submitted to The University of Western Sydney for the degree of Doctor of Philosophy

by

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September 1998.
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
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DEDICATION

In memory of my stepfather Pheuy Chitranondh whose will and resolution gave me the best education that he could afford and his love.

In memory of my parents Khampheng and Chansouk Boupha who gave me life and love.

Thanks to my uncle, Khamphay Boupha for his precious support.

I wish to gratefully acknowledge Ministers of Health and all members of the Ministry of Health for their support.

I gratefully acknowledge and thank Tony and Orawan Liu, Director and Managing Director of Jaiara Pty. Ltd. for their warm and constant support for my family and myself during my study.

To Anothai and Somchan Prathoumvan for their warm support and assistance.

To my wife Vandara, my son Sithivorada and my stepmother Bouachine for their support, love and suffering during my study.
STATEMENT

The studies presented in this thesis were undertaken by the author in the Centre for Advanced Food Research, The University of Western Sydney, Hawkesbury, New South Wales, Australia under the supervision of Associate Professor Dr. G. R. Skurray from the Centre for Advanced Food Research, The University of Western Sydney, Hawkesbury and Associate Professor Dr. W. L. Bryden from the Department of Animal Science, The University of Sydney, New South Wales, Australia.

To the best of my knowledge and belief, this thesis contains no material that has been previously published, or written by other person, except where due reference is made in the content of the text.

Prasongsidh Chitranondh Boupha

September 1998.
ACKNOWLEDGMENTS

I wish to thank sincerely my major supervisor, Associate Professor Dr. Geoffrey R. Skurray for accepting and allowing me to work on this project under his supervision in the Centre for Advanced Food Research, Faculty of Science, and Technology, University of Western Sydney, Hawkesbury (UWS). I am most grateful for his constant enthusiasm, his guidance, advice, constant support, assistance in the technical aspects of this projects, use of the HPLC and CE and in the writing of manuscripts for publication and this thesis.

I wish to gratefully acknowledge my panel supervisor, Associate Professor Dr. Wayne Bryden for his support and guidance and his original suggestion of this project.

I also wish to gratefully acknowledge Dr. James Bergan and Associate Professor Dr. Jim Hourigan for their advice, support and encouragement.

I also wish to gratefully acknowledge Dr. Alfio Comis, my preceding panel supervisor in my study for M.Sc. (Research) for his advice on thesis writing and presentations.

I wish to gratefully acknowledge Mr. Robert Sturgess, Laboratory Manager of the School of Food Science, for his warm and constant support, encouragement and his help in editing manuscripts for publication.

Thanks to Dr. Kaila Kailasapathy for his assistance in manufacturing ice cream and in editing the manuscript for publication.
The assistance rendered by the Faculty of Science and Technology members and staff of the Dairy Processing Plant, University of Western Sydney, Hawkesbury, especially Mr. Mel O’Rourke, Mr. Jon Boersma, Mr. Neville Snowden, Mrs. Kim Brown and Mrs. Helen Crowley is acknowledged.

Thanks to my fellow postgraduates especially Mrs. Rosalie Durham and Ms. Marica Solina in appreciation of their support, help and encouragement.

Financial support from Ausaid during my study in Australia is gratefully acknowledged. Additional financial support by the Jaiara Pty Ltd. who employ me as part time research staff is also gratefully acknowledged.

And of course, not forgetting my family including brothers and sister who supported my family and myself throughout the long period that I have been away from home.
PUBLICATIONS

The studies presented in this thesis have been published or submitted for publication in the following communications:


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LIST OF ABBREVIATIONS

β- CPA : β- Cyclopiazonic acid  
α-CPA : α- Cyclopiazonic acid  
µg : Microgram  
µM : Micromole  
AFB1 : Aflatoxin B1  
AFM1 : Aflatoxin M1  
CE : Capillary electrophoresis  
Cfu : Colony forming unit  
CGE : Capillary gel electrophoresis  
CIEF : Capillary isoelectric focusing  
CPA- imine : Cyclopiazonic acid imine  
CPA : Cyclopiazonic acid  
CTTP : Capillary isotachophoresis  
CZE : Capillary zone electrophoresis  
DAD : Diode-array detection  
ELISA : Enzyme-linked immunosorbent assay  
EOF : Electroosmotic flow  
GC : Gas chromatography  
GC-MS : Gas chromatography- Mass spectracing  
HIV : Human immunodeficiency virus  
HPLC : Liquid chromatography  
HPTLC : High performance thin layer chromatography  
ip : Intraperitoneal  
LD50 : Lethal Dose 50%  
MEKC : Micellar electrokinetic capillary chromatography  
MEC : Micellar electrokinetic chromatography  
MS : Mass spectracing system  
PAGE : Polyacrylamide gel electrophoresis  
op : Per oral  
RPLC : Reverse phases liquid chromatography  
RSD : Relative standard deviation  
SD : Standard deviation of the mean  
SDS : Surfactant anionic  
TLC : Thin layer chromatography
SUMMARY

The aim of the study in this thesis was to assess the stability of the mycotoxin, cyclopiazonic acid (CPA) in milk and dairy products processed from contaminated milk. A method was developed to detect CPA in milk and milk products using micellar electrokinetic capillary chromatography (MEKC), a technique of capillary electrophoresis (CE), which was rapid and non-labour-intensive. The quantifying efficiency of CE in detecting CPA was compared to Reverse Phase Liquid Chromatography. The analytical response was linear from 40 ppb to 100 ppm CPA in CE (correlation coefficient, $r = 0.99995$) and recovery of spiked CPA in milk was 78-81% over the range of 20 ppb to 500 ppb. The detectable limit of CPA by CE was $2.7 \times 10^{-8}$ pg/mL.

Heat-stability of CPA in milk was assessed under different conditions. To mimic the heat processing that is used in the dairy industry, CPA artificially contaminated milk at $1\,\mu g/mL$ was heated to $60^\circ$, $80^\circ$ and $100^\circ$C for 15 to 60 minutes. After one hour of heating milk to $60^\circ$, $80^\circ$ and $100^\circ$C, the concentration of CPA decreased by 3 - 9%, 14-18% and 25 - 30% respectively. The decrease of CPA during the heating of milk followed a pattern similar to that of first-order reactions. Contaminated milk at the same temperature for 2 hours induced a decrease of the CPA level of 9 - 17%, 20 - 34% and 49 - 50% respectively. Further CPA reductions were observed with milk stored at 4°C overnight. Autoclaving milk for 30 minutes at 120°C lead to a CPA decrease of 33 - 36%. Similar decreases were found in canned milk, which had been retorted for 30 minutes at 120°C. No significant degradation of CPA ($p>0.05$) was induced by simulation of heat-treatments used by the dairy industry at $70^\circ$ and $90^\circ$C for 15 seconds. A longer heat treatment of $60^\circ$C for 30 minute led to a 10% decrease in the level of CPA.

CPA decreased by 1.4% following 4 days of simulating collecting, storing and transporting of contaminated (1μg CPA/mL) raw milk. Storage at 4°C for 21 days, simulating retail milk, moderately reduced the CPA level by 5.8%. CPA decreased in frozen and freeze dried milk stored at -18°C, indicating a similar trend.
However, in both products, a decrease of less than 12% CPA was noted in spite of a storage period of 140 days.

Simulating unsweetened condensed milk production by preheating 4L contaminated milk to 100°C and concentrating under steam injection to 1.5L led to a decrease of CPA by 39.7%. In contrast, in order to remove water from milk, evaporation of the milk under vacuum at low temperature (60°C) induced only a modest decrease of CPA (0.7%). CPA was stable in both concentrated and evaporated milks throughout an 8 week storage period at 4°C. CPA levels did not decrease during the manufacture of milk powder by spray drying.

The influence of basic and acidic pH on 1μg CPA/mL in McIlvaine buffers at 4°C was studied for 30 days. CPA was more stable in a neutral pH than in a basic or acidic environment. By the end of the 30 day period, the concentration of CPA was reduced to 70% and 64% at pH 4 and 2 respectively. In contrast, yogurt made with artificially contaminated whole milk (1μg CPA/mL), showed a reduction of more than 70% CPA on the first day of storage. However, more than 12% of the mycotoxin was still detectable in yogurt on the 21st day of storage. Heating milk to 85°C for 20 min before the inoculation of starter culture reduced the CPA content. A similar decrease in CPA content was observed in chemically acidified milk although the decrease was less than in yogurt.

Hot and cold treatments during the manufacture of ice cream, using artificially contaminated raw whole milk (1μg CPA/mL), did not greatly affect the toxin levels in the mix. A reduction of nearly 12% CPA was observed from mixing until ageing. However, following pasteurisation, ageing at 4°C overnight had a slight effect on CPA levels (2% decrease). Storage of 9 weeks at -20°C appeared to adequately limit the loss of CPA. However, a decrease of the CPA level was observed in ice cream after 4 weeks (5%) and 9 weeks (12%) of storage.

Stability and partition behaviour of 1μg CPA/mL milk, carried-over into Cheddar cheese, was studied. CPA content in cheese and whey varied from 1.7 - 2.3μg/g and 0.6 - 0.7μg/mL respectively. The average CPA increase in the final curd was 1.95 fold over the contaminated milk. The percentage of CPA carry-over from
milk into cheese-curd and the enrichment factor of CPA in the cheesemaking varied from 24.7 to 34.1% and 2.3 to 3.4 respectively.

Five per cent of CPA was carried-over into butterfat following manufacture of the butter with contaminated milk. Spiking a similar amount of CPA to an equal volume of skim milk-cream system confirmed that cream retained CPA between 50 to 59% of the toxin previously spiked in cream alone.

The individual or combined effect of UV-visible radiation, hydrogen peroxide, riboflavin and temperature on 2μg CPA/mL in an aqueous solution or in milk was assessed. A slight CPA decrease in aqueous solution (9.1%) and in milk (7.6%) occurred after 1h exposure to UV-visible radiation at 4°C. UV radiation also enhanced CPA reduction in acidic conditions. One per cent hydrogen peroxide induced a slight initial CPA decrease then a 60% decrease after 1h in aqueous solution. A similar trend was observed in milk but the effect of hydrogen peroxide was less intense. A dramatic CPA decrease (86%) occurred in aqueous solutions treated with 6% hydrogen peroxide. At a higher temperature (60°C), hydrogen peroxide further decreased CPA levels. The reduction in CPA correlated with the concentration of riboflavin with or without hydrogen peroxide treatment. Complete elimination of CPA occurred after 30 min with a 3.2mM riboflavin and 6% hydrogen peroxide treatment. A combination of 0.5mM riboflavin with 1% hydrogen peroxide decreased CPA in aqueous solutions at 4°C but less effect was observed in milk. No significant difference (p>0.05) in CPA concentration was established in UV-visible radiated, heated and non-heated milk. This demonstrated that there was no interference by indigenous enzymes to CPA reduction.

CPA in an aqueous solution decreased 30% from the original concentration (2μg CPA/mL) when it was treated at 4°C for 16h with 1% acidic food additives such as hydrochloric acid (HCl) and sulfuric acid (H₂SO₄). CPA in the similar aqueous solution decreased nearly 39% after exposure to alkaline food additive (sodium bicarbonate NaHCO₃, or sodium carbonate Na₂CO₃). Only 50% CPA in the identical solution was recovered when treated with sodium hydroxide (NaOH) or sodium sulfite (Na₂SO₃). In contrast, complete elimination of CPA from the similar
aqueous solution occurred when treated with sodium hypochlorite (NaOCl). Sodium dithionite (Na₂S₂O₄) was less effective among neutral food additives compared to potassium nitrate (KNO₃), sodium nitrite (NaNO₂) and hydrogen peroxide (H₂O₂) in eliminating CPA from the similar aqueous solution. CPA levels were reduced 79 - 85% when treated with sodium metabisulfite (Na₂S₂O₅) and ammonium peroxodisulfate [(NH₄)₂S₂O₈]. Complete elimination of CPA in the aqueous solution was also obtained by treating with sodium chlorite (NaClO₂), potassium bromate (KBrO₃) and sorbic acid. In contrast, CPA levels in the aqueous solution were reduced to 80 and 72% when exposed to benzoic acid or ammonia. A study on treatment times revealed that several food additives completely eliminated CPA in the aqueous solution in less than 15 min. Food additives used in this study at low concentrations still reduced CPA levels. A further reduction of CPA in the aqueous solution was obtained when exposed to higher temperatures and treated with food additives tested.

The results from this thesis demonstrate that CPA in milk at concentrations found in naturally contaminated milk could not be eliminated by the heat-treatment during milk processing, storage, processing and manufacture of dairy products. Occurrence of CPA in cheese curd, butter or cream following manufacture with contaminated milk was demonstrated. CPA is left in milk despite UV-visible radiation treatment with or without hydrogen peroxide and/or riboflavin. Chemical treatment, which is capable of completely eliminating CPA, is prohibited and impractical to use for milk treatment. Stability of CPA in milk and milk products confirms the potential of the toxin to reach consumers of dairy products.
GENERAL INTRODUCTION

Mycotoxins are secondary metabolites of fungi capable of inducing mutagenicity, carcinogenicity, teratogenicity, oestrogenicity and toxicity to humans and animals when the toxins are consumed. Published on the incidence of mycotoxins involving food and feedstuffs reveal the potential risk of exposure to humans and animals (CAST, 1989; Blunden et al., 1991; Truksess, 1994, 1995 and 1997). Before the outbreak in 1960 of “Turkey X Disease” in the Great Britain, despite the incidence of diseases in man, mycotoxicoses had been the “neglected diseases” (Forgacs and Carll, 1962; Van Egmond, 1989). Fungal toxins attracted scientific attention following this mycotoxicosis of turkeys and the discovery of aflatoxins. It was subsequently shown that among aflatoxins, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) was the most toxic and carcinogenic. Following the supply of feedstuffs containing AFB<sub>1</sub> to lactating cattle, carry-over of the mycotoxin occurs in milk in the form of metabolite aflatoxin M<sub>1</sub> (AFM<sub>1</sub>). The latter toxin subsequently occurs in dairy products and persists despite milk processing and treatment.

Retrospective studies on the Turkey “X” Disease demonstrated that aflatoxins alone could not induce all the clinical signs occurring in the turkeys, especially neurotoxicity. Only the association of another mycotoxin, Cyclopiazonic acid (CPA) with aflatoxin could explain completely the symptoms in the Turkey “X” Disease (Cole, 1986a).

CPA is an indole tetramic and a lipophilic monobasic acid initially reported by Holzapfel (1968). Originally, CPA did not attract much scientific attention because it was not found highly toxic compared to other secondary metabolites of fungi. Gradually, the focus changed since CPA was found to be associated with aflatoxin and linked to the food chain. Moreover, the capacity of CPA to induce disease in all species of animals studied and occasionally in humans, reveals its risk potential (Harrison, 1971; Rao and Husain, 1985; Cole, 1986a). Depending on the animal species, CPA exhibits a relatively strong acute toxicity, neurological signs and enteritis (Cole and Cox, 1981). The occurrence of fungal producers of CPA and CPA in a wide range of food and feed highlights their high human potential risk.
Evidence of CPA association in cases of disease due to naturally occurring toxicosis has been reported. The evidence emphasises that CPA needs to be studied in more depth (Polster et al., 1990; Trucksess, 1994; Balachandran and Parthasarathy, 1996; Trucksess, 1995, 1997).

CPA is often associated with aflatoxin. The actions of the two mycotoxins are additive (Lansden and Davidson, 1983). Co-production of CPA with aflatoxin by the same genera of fungi and their involvement in natural outbreaks of aflatoxicosis were reported (Cole, 1986b; Gqualeni et al., 1996a). Fungi co-producers of CPA and aflatoxin are ubiquitous and occur in numerous agricultural commodities. CPA has a high possibility of occurring in the same foods aflatoxins (Bryden, 1987).

Studies with animals reveal CPA distribution in organs and tissues. Indirect contamination or carry-over of mycotoxins in milk is a consequence of the occurrence of the toxins in feedstuff consumed by dairy cattle. The carry-over of CPA into milk and eggs has been reported (Elrington, 1991; Dörner et al., 1994).

The few reports in the literature on CPA in dairy products are due to the direct occurrence of the toxin in cheese resulting from fungi used for fermentation or unintentional fungal growth (Engel and Teuber, 1989). The effect of milk processing on CPA carry-over in contaminated milk or dairy products has not been reported.

Reports on CPA in milk and its potential exposure risk to humans are lacking. Given the widespread distribution of fungi CPA-aflatoxin producers and compared to the high incidence of aflatoxin M1 in dairy products, the incidence of CPA may be widespread.

A sensitive method of CPA analysis using capillary electrophoresis was developed in this thesis and then used to study the behaviour of CPA in milk and milk products. The stability of CPA in milk from farm to factory was assessed. A study on the effect of milk processing with or without milk component separation on CPA in contaminated milk was also conducted. Assessments on CPA behaviour in milk under different pHs, temperatures, UV energies and duration of treatment were also performed. The rate of CPA carry-over from milk into dairy products and the partition behaviour of the toxin between fractions of whey, curd or buttermilk were studied. A treatment to eliminate CPA from products was also assessed.
CHAPTER I:

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SECTION (1): MYCOTOXINS AND CYCLOPIAZONIC ACID

1.1. MYCOTOXINS

Mycotoxins are secondary metabolite of fungi, which grow and proliferate within or outside food or feed commodities.

Fungi may cause in humans or animals, three types of illness:

- **Infection** due to mycoses,
- **Allergies** following spore inhalation or contact with fungi,
- **Toxicoses** subsequent to ingestion of food contaminated by fungal metabolites.

Toxicoses are different from mycoses and can be determined by distinguishing the specific character of the fungi. Fungi responsible for toxicoses are neither contagious nor infectious (Moreau, 1979). They develop various toxic substances that may be poisonous to animals (zootoxic), to plants (phytotoxic) and to microorganisms (antibiotic).

Mycotoxins induce diseases in humans and animals following consumption of toxin contaminated food or feed (Austwick, 1968; Moreau, 1979). “MYCOTOXIN” comes from two words in Greek such as ‘ΜΥΚΗΣ’ and ‘ΤΟΞΙΚΟΝ’, which mean ‘fungus’ and ‘arrow-poison’ respectively (Cheeke and Shull, 1985; Van Egmond, 1989). Word for word, mycotoxin signifies ‘poison from fungi’. Mycotoxin is neither infectious nor contagious (Wren, 1994). Historically, danger due to fungi was established early. However, danger from toxins was not recognised.

1.1.A. History

Fungal poisoning has been recognised since the Renaissance. Legend indicates that illness caused by fungi had been occurring before the age of ancient Greco-Romans. However, until the end of the nineteenth century, few observations were made. It was found that consumption of bread made from grain contaminated by *Fusarium* induced dizziness and headaches in man (Woronin, 1891). Intoxication
of cattle following consumption of “forage poison”, due to mould growth on feed, was also noted in 1850 (Dinwiddie, 1893).

The experimental work of Turesson (1916) demonstrated the relationship between the accumulation of the spores of fungi and harm caused by toxin production. Subsequently, the ingestion of moulds of certain species of *Aspergillus* or *Penicillium* was known to induce convulsions and even death.

According to Goldblatt (1972), ingestion of rye infested with mould *Claviceps purpurea*, which induced ergotism, was probably the first mycotoxicosis known and recorded in history. It was suspected to be the cause of periodic outbreaks of “St. Anthony’s Fire” that in feudal Europe killed several thousand people. Similar outbreaks also occurred in the 1930’s in England and in Russia. Another serious mycotoxicosis epidemic in humans was alimentary toxic aleukia. This disease ravaged Russia from late last century to the end of the Second World War. Due to these outbreaks, special organisations were established in the former USSR to study mycotoxicoses (Bilai, 1953; Sarkisov, 1954; Spesivtseva, 1960). However, in general, the effects of mycotoxicose were largely ignored in the West where mycotoxicoses later became known as “The Neglected Disease.”

Following the development of an apparently new disease during 1960 in Great Britain, mycotoxins attracted much more attention. The unknown disease caused more that 100 000 deaths in turkey poult's and ducklings. These animals had consumed feed made with Brazilian groundnut meal unloaded from the cargo ship “S. S. Rosetti” (Cole, 1986a). Turkey poult's were suffering initially from the unknown illness was referred to as “Turkey’s X disease” and was thought to be a virus disease of unusual actiology (Blount, 1960; 1961). The incident was not limited to Great Britain. Similar diseases also occurred in Kenya and Uganda (Asplin and Carnaghan, 1961). The magnitude of the incidents and mortality focused attention and stimulated interest in the scientific community resulting in multidisciplinary intervention to solve Turkey ‘X’ disease. Subsequently studies revealed that the real aetiological agent was a second metabolite of a fungus identified as *Aspergillus flavus* Link ex Fries (Sargeant et al., 1961). The toxin was named, aflatoxin, according to an abbreviation of *Aspergillus flavus* and toxin. Intensive research on
aflatoxin formed the origin of the modern-day science of mycotoxicology. Meanwhile, papers and studies on aflatoxin or other mycotoxins were published in around the world (Moreau, 1979).

However, retrospective studies during the 1980’s, on the Turkey “X” Disease revealed that aflatoxin alone could not induce all signs occurring in the turkeys, especially neurotoxic signs. Only the association of Cyclopiazonic acid (CPA) with aflatoxin could explain completely the symptoms in Turkey “X” Disease (Cole, 1986a).

It had previously been shown that co-production of cyclopiazonic acid (CPA) with aflatoxin and their involvement in natural outbreaks of aflatoxicosis had been occurred (Cole, 1986b; Gqualeni et al., 1996a). The capability of CPA to induce diseases in animals or humans was also documented (Harrison, 1971; Rao and Husain, 1985).

I.1.B. Origin of Mycotoxins

Between three and four hundred mycotoxins are currently known to be produced by recognised fungi (Jones et al., 1994). Based on the variations within species of fungi, 10 000 mycotoxin producers are estimated to threaten foodstuffs (Betina, 1989). The production of mycotoxins, including many other mould secondary metabolites, requires previous mould development. However, the presence of a fungus does not mean that a specific toxin or secondary metabolite is present or produced. The same toxin may be produced by a range of different fungi. A fungus may co-produce several different mycotoxins (Betina, 1989). Biosynthesis of secondary metabolites depends on several influential factors including the species and strain of mould (Moss, 1991).

Table 1.1 lists fungal genera known to be responsible for toxicoses and examples of mycotoxicosis with certain of their causative mycotoxins are shown in Table 1.2. Strains of a single species may vary in the quantity of toxin produced. Some strain may degenerate in culture and have their capacity to produce toxin altered.

The growth and activity of fungi are greatly influenced by the availability of nutrients, pH, radiation, temperature and water activity interaction. Moreover,
biological interaction and chemical agents such as acid treatment, pH change due to lactic fermentation and agricultural biocides affect development of mould producers and interfere in mycotoxin production.

<table>
<thead>
<tr>
<th>Absidia</th>
<th>Gibberella</th>
<th>Rhizoctonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria</td>
<td>Gliocladium</td>
<td>Rhizopus</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>Gloeotinia</td>
<td>Sclerotium</td>
</tr>
<tr>
<td>Byssochlamys</td>
<td>Hemispora</td>
<td>Scopulariopsis</td>
</tr>
<tr>
<td>Cephalosporium</td>
<td>Mucor</td>
<td>Sporidesmium</td>
</tr>
<tr>
<td>Chaetomium</td>
<td>Myrothecium</td>
<td>Stachybotrys</td>
</tr>
<tr>
<td>Cladosporium</td>
<td>Neurospora</td>
<td>Stemphylium</td>
</tr>
<tr>
<td>Curvularia</td>
<td>Oospora</td>
<td>Thamnidium</td>
</tr>
<tr>
<td>Dendrodochium</td>
<td>Paecilomyces</td>
<td>Trichoderma</td>
</tr>
<tr>
<td>Diplodia</td>
<td>Penicillium</td>
<td>Trichotheicum</td>
</tr>
<tr>
<td>Epicoccum</td>
<td>Periconia</td>
<td>Verticillium</td>
</tr>
<tr>
<td>Fusarium</td>
<td>Pithomyces</td>
<td>Wallemia</td>
</tr>
</tbody>
</table>

(Moreau, 1979).
I.1.C. Occurrence in Food, Feed and Potential Health Implications

The fact that fungal producers of mycotoxin no longer remain in food or feed, does not imply that no toxin exists (Betina, 1989). The main sources of mycotoxins in the animal and human food chains are contaminated agricultural products. Toxigenic fungi can grow and produce mycotoxin on growing crops, seed, leaves, stems, fruits, plants and feed. The contamination of mycotoxins can take place before, during and following harvest including during transportation, shipment and storage. Apart from agricultural products, animal products may contain mycotoxins resulting from the direct contamination by toxigenic fungi or the consumption of contaminated feed by animals resulting in secondary mycotoxicose. Figure 1.1 proposed by Smith and Moss (1985) clearly describes primary or secondary mycotoxicoses.

The carry-over of toxin into animal products especially milk is a cause of concern despite the amounts of mycotoxin residues being several times lower compared to that in feed. Since milk is a part of nearly all diets in most countries of the world, there is a possibility that mycotoxins may be consumed directly through milk, whey-based products and dairy products (Betina, 1989). Moreover, certain moulds used in cheese and food fermentation are also capable of producing mycotoxins (Le Bars, 1979; Pitt et al., 1986; Engel and Teuber, 1989).

Other than in natural products, mycotoxins are also found in work environments as air-born dust particles or even misused as possible warfare agents (Betina, 1989).

Mycotoxins induce adverse health effects in both farm and laboratory animals and humans (Malloy and Marr, 1997). Depending on dosage or host species susceptibility, mycotoxins can induce acute or chronic toxicity. Mycotoxins cause different effects in different organs and may ultimately result in death.
<table>
<thead>
<tr>
<th>Mycotoxicosis/Causative mycotoxins</th>
<th>Animal species</th>
<th>Main toxigenic fungi</th>
<th>Main susceptible crops</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxicosis, B₁, B₂, G₁, G₂</td>
<td>Poultry, Swine, Cattle</td>
<td><em>Aspergillus flavus</em>, <em>A. parasiticus</em></td>
<td>Corn, small grains, peanuts, cottonseeds, cassava, copra, most nut crops, mixed feeds.</td>
</tr>
<tr>
<td>Ergotism</td>
<td>Poultry, cattle, sheep, swine</td>
<td><em>Claviceps purpurea</em></td>
<td>Rye, wheat, barley, rice, millet, sorghum, triticale, rye grass</td>
</tr>
<tr>
<td>Ergotamine, ergotoxin and ergometrine alkaloids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zearalenone toxicosis</td>
<td>Swine</td>
<td><em>Fusarium spp.</em></td>
<td>Corn, wheat, barley, sorghum, oats</td>
</tr>
<tr>
<td>Trichothecen toxicoses</td>
<td>Swine, cattle, poultry</td>
<td><em>Fusarium spp.</em></td>
<td>Corn, wheat, barley, oats, millet, rye, mixed feeds</td>
</tr>
<tr>
<td>T-2 toxin, diacetoxyscirpenol, vomitoxin and others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucoencephatomalacia</td>
<td>Horse</td>
<td><em>Fusarium moniliforme</em></td>
<td>Corn</td>
</tr>
<tr>
<td>Ochratoxidosis</td>
<td>Swine, poultry</td>
<td><em>Penicillium viridicatum</em>, <em>Aspergillus ochraceus</em></td>
<td>Corn, oats, barley, beans, peanuts, mixed feeds</td>
</tr>
<tr>
<td>Ochratoxin A, B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrinin toxicosis</td>
<td>Swine, poultry</td>
<td><em>Penicillium spp</em></td>
<td>Wheat, rye, oats, barley</td>
</tr>
<tr>
<td>Cyclopiazonic acid *</td>
<td></td>
<td></td>
<td>Corn, peanuts, soil, Grain, pepper, cacao, pitachios cheese, meat, peanuts, cured ham, fermented sausage, raw ham.</td>
</tr>
</tbody>
</table>
I.1.D. Economic Significance of Mycotoxins and Treatment

To express the effect of mycotoxin on health and economy, Betina (1989) cited the view of Smith and Hacking: "..... mycotoxins are exceptionally important in assembled groups of animals such as dairy and feedlot cattle, pigs and poultry because of their high intake of stored concentrated plant-derived feeds. The economic losses due to mycotoxins are likened to an iceberg as we see only what is on the surface..... The major impact of mycotoxins remains beneath the surface......" (Smith and Hacking, 1983)
Economic and trade problems due to mycotoxin occur at all levels between producers and consumers (Dawson, 1991). Categorical information on the effect of mycotoxins on the economy is still rather limited. However, economic loss may occur in levels of production of food, feed, crops or animals. Possible loss also occurs at the distribution and processing level. The loss of crops, livestock and products could adversely affect national economies that already have to bear other national expenses in mycotoxin analysis, control programs, research, law suits and hospitals due to mycotoxin effects. As much as 25% of the world’s food crops are affected according to the estimation conducted by The Food and Agriculture Organisation (FAO). The size of losses depends on several factors including the type of grain, animal, animal products and other variables of national economies. However, the effects and concentrations of mycotoxin in products, treatment and decontamination have been studied and some methods show promising prospects for commercial applications (Charmley et al., 1995).

I.1.E. Aflatoxin: The Most Notorious Mycotoxin

Among the known mycotoxins, aflatoxin is the most studied and significant as a potentially hazardous substance to man and animal. Aflatoxin is a secondary metabolite of several fungi including Aspergillus flavus and Aspergillus parasiticus. Aflatoxin has a great impact on mycotoxicology (Sargeant et al., 1961). To date, ten different aflatoxins have been identified such as aflatoxins B₁, B₂, B₂ₐ, G₂, G₂ₐ, M₁, M₂, P₁, Q₁ and R₀.

Compared to other mycotoxins, aflatoxin is the most mutagenic and carcinogenic (Jesenskà, 1993). Of the aflatoxins, aflatoxin B₁ is the most toxic and toxicity and carcinogenic. Aflatoxin producers are able to produce toxins on practically every foodstuff or feedstuff that support fungal growth. Aflatoxin has been found in most agricultural commodities including rice, wheat, sorghum, groundnuts, oil seeds, cottonseed and corn. Consequently, the presence of aflatoxin in feedstuffs is regularly reported (Scott; 1978; Afzal et al., 1979; Bryden et al., 1980; Dutton and Westlake, 1985; Jelinek et al., 1989; Rustom, 1997).
I.1.F. Aflatoxin Occurrence in Milk and Milk Products

The occurrence of aflatoxin B₁ in dairy products can be due to direct or indirect contamination. Direct contamination is a consequence of the fungal production in dairy products. Indirect contamination occurs when lactating dairy cattle are fed aflatoxin B₁ (AFB₁) contaminated feed. The hydroxylated derivative of AFB₁, aflatoxin M₁ (AFM₁) is carried-over into the milk and subsequently in dairy products (Van Egmond, 1989). A survey in human breast milk revealed the presence of aflatoxin proving that carry-over has occurred as well in human milk (El Nezami et al., 1995).

The possibility of other mycotoxin being carried-over into milk has also been studied during the 1980’s. Among them, ochratoxin A and zearalenone (Shreeve et al., 1979). T-2 toxin, a trichothecene mycotoxin, which was suspected of being misused in chemical warfare in Laos, Kampuchea and Afghanistan, can also be transmitted in milk (Robinson et al., 1979; Mirocha et al., 1983; Joffè, 1986). Sterigmatocystin and deoxynivalenol are also carried-over into milk (Kraus, 1978; Prelusky et al., 1984). Due to the rarity of these mycotoxins and the outcomes of these studies, this seemed to show no reason for concern. There has been no need to further study these mycotoxins (Van Egmond, 1989). However, recent findings in human breast milk of ochratoxin A and aflatoxin, indicate that these mycotoxins pose a high potential risk for reaching consumers and therefore, further study is essential (Jonsyn et al., 1995).

Cyclopiazonic acid (CPA), a neurotoxic mycotoxin, has been shown to be transmitted into milk and eggs (Elrington, 1991; Dorner et al., 1994). The frequent occurrence of CPA in foods and feed incites numerous organisations including the Committee on natural toxins of the Journal of AOAC International to emphasise that CPA needs to be studied in more depth (Polster et al., 1990; Truckses, 1994; Balachandran and Parthasarathy, 1996; Truckses, 1995; 1997).

I.1.G. Relationship of Aflatoxin and Cyclopiazonic Acid

The co-production of CPA and aflatoxin by the same source of fungi, namely, strains of Aspergillus is well known. Frequent reports of this co-production indicate a close association of the two mycotoxins including their frequency of
occurrence (Gallagher et al., 1978; Domer et al., 1984; Blaney et al., 1989; Polster et al., 1990; Lee and Hagler, 1991; Cvetnic, 1994; Gqualeni et al., 1996a; Goto et al., 1996). The occurrence of CPA in the same types of food or feedstuffs with aflatoxin or other mycotoxins has been reported (Widiastuti et al., 1988). In addition to this information on the relationship between CPA and aflatoxin, specific information on CPA will be discussed in relation to its occurrence in milk and in association with aflatoxin in Section (2) of this chapter.

I.2. CYCLOPIAZONIC ACID

Cyclopiazonic acid was originally discovered by Holzapfel (1968) as a metabolite of Penicillium cyclopium Westling (P. griseofulvum) during routine toxicity screening of fungi from groundnuts. The potential risk of CPA to animals and humans was initially considered low because it is less toxic compared to other secondary metabolites of fungi. Consequently, CPA did not attract the attention of the scientific community. Gradually, the focus changed as the toxin was found to be associated with the occurrence of aflatoxin in the food and feed chain. Moreover, the capacity of CPA to induce disease in all species of animals studied and occasionally in humans, reveals its risk potential (Harrison, 1971; Rao and Husain, 1985; Cole, 1986a).

I.2.A. Chemical and Physical Properties

1.2.A.1. Chemical properties

Cyclopiazonic acid is an indole tetracarboxylic acid and a lipophilic monobasic acid. The structure of α-Cyclopiazonic acid or cyclopiazonic acid was initially reported by Holzapfel (1968). The toxin has a structural resemblance to lysergic acid with the absolute configuration of C_{20}H_{20}N_{2}O_{3} (Figure 1.2) (Holzapfel, 1971). CPA was formulated as an indole tautomer of acyltetramic acid with an L-configuration (Holzapfel and Schabert, 1977). The presence of an indole system unsubstituted at either the α or β position of the toxin suggested by the occurrence of a purple-blue colour under Ehrlich spray reagent colouration. Ehrlich spray reagent was described by Lansden (1986) as a solution of 1g 4-dimethylaminobenzaldehyde in 75 ml ethanol and 25 ml of concentrated HCl. The reagent was used to spray on air-dry
TLC plate containing cyclopiazonic acid, which gives an appearance of blue spots in 10 min without heating.

CPA has two indole derivatives Bissecodehydro cyclopiazonic acid (β-CPA) and cyclopiazonic acid-imine (CPA-imine) also produced by *Penicillium cyclopium*. The two derivatives were discovered during a study of the CPA biosynthetic pathway (Holzapfel *et al.*, 1970). Subsequent studies on the two intermediates revealed that they are relatively non toxic compared to CPA. β-CPA was found to be a direct precursor of α-CPA (Holzapfel, and Wilkins, 1971; Steyn *et al.*, 1975; de Jesus *et al.*, 1981). The structural configurations of β-CPA and CPA imine are shown in Figure 1.2.

L2.A.b. Physico-chemical properties

1. α-Cyclopiazonic acid (α-CPA) *(Pohland *et al.*, 1982)*

   a) Synonyms : none.

   b) Chemical name : 10 - acetyl - 2, 6, 6a, 7, 11a, 11b - hexahydror - 11 - hydroxy - 7, 7 - dimethyl - (6α, 11αβ, 11βα) - 9H - Pyrrolo [1', 2': 2, 3] iso indolo [4, 5, 6-cd] indol-9-one.

   c) Empirical formula : C_{20}H_{20}N_{2}O_{3}.

   d) Molecular weight : 336.4.

   e) Description : α-CPA is an optically active, colourless, odourless, crystalline metabolite which is characterised by indole and tetramic acid moieties.

   f) Characterisation data :

      1. Melting range : 214-218°C (after drying for 1 hour at 60°C).

      2. Specific rotation : \([\alpha]_D^{19}= -109.4^\circ\]

         concentration : 2973 \(\mu\)M/L.

         solvent : chloroform.

      3. Circular dichroism : \(\Delta\varepsilon (\lambda_{365})^0; \Delta\varepsilon (\lambda_{301})^{+1.8}; \Delta\varepsilon (\lambda_{292})^0;\)

         \(\Delta\varepsilon (\lambda_{269})^{-4.56}; \Delta\varepsilon (\lambda_{253})^0.\)

         concentration : 351.1 \(\mu\)M/L.
\[ \alpha - \text{Cyclopiazonic Acid} \quad \beta - \text{Cyclopiazonic Acid} \]

Cyclopiazonic Acid Imine

Figure 1.2. The structure of Cyclopiazonic acid and its derivatives.
(Cole, 1984; Dictionary of Natural Products).
solvent : methanol

temperature : 22°C

cell length : 2mm.

5. CPA behaves as a monobasic acid. Like other tetramic acids, CPA forms a stable complex with a metal cation.

6. CPA gives a purple-blue to Ehrlich colour reaction and orange-red colour to FeCl₃ reaction (Purchase, 1974)

7. CPA is a chelating agent and a lipophilic monobasic acid similar to two other mycotoxins Tenuazonic acid and Erythroskyrine (Holzapfel, 1968; Holzapfel and Wilkins, 1971; Holzapfel, 1980). CPA forms a stable complex with iron, metal cations and divalent cations (Steyn and Rabie, 1976, Gallagher et al., 1978)

2) β-Cyclopiazonic acid (β-CPA)

a) Empirical formula : C₂₀H₂₂N₂O₅.

b) Molecular weight : 338.405

c) Characterisation data

1. Melting range : 168-169°C.

2. Specific rotation : [α]₀° = - 186° (c, 0.42 in Py) (Pohland et al., 1982)

3. Crystallised from chloroform-diethyl ether and methanol.

4. β-CPA Gives similar colour reaction with FeCl₃ as the one of α-CPA but has no reaction with Ehrlich’s reagent (Purchase, 1974).

3) Cyclopiazonic acid imine (CPA-imine):

a) Empirical formula : C₂₀H₂₁N₂O₂.

b) Molecular weight : 335.405

c) Characterisation data

1. Melting range : 277-278°C.


3. Crystallised from methanol solution.
1.2.B. **Biosynthesis of Cyclopiazonic Acid**

The biosynthesis of α-CPA was studied by culturing *Penicillium cyclopium* in Czapek medium at 25°C for 7 days (Holzapfel and Wilkins, 1971). On the basis of structure analysis, carbon skeletons of CPA appeared to be derived from tryptophan, a C₅ unit formed from a molecule of mevalonic acid and two molecules of acetic acid. The discovery of β-CPA as an immediate precursor of CPA occurred in this study. Labeled substrates including sodium [1-¹⁴C] acetate; [2-¹⁴C] mevalonic acid and DL-tryptophan were added to the culture and incorporated in CPA (Holzapfel, 1980). The productions of β-CPA and CPA imine were also initiated under similar conditions in identical Czapek medium. The amount of the two precursors augmented, then decreased when CPA levels increased (Betina, 1989). β-CPA was found directly incorporated into CPA. α-acetyl-γ-(β-indolyl)-methyltetramic acid was established to be a precursor for both β-CPA and CPA (McGrath *et al.*, 1973). Therefore, CPA biosynthesis is done from tryptophan via α-acetyl-γ-(β-indolyl)-methyltetramic acid and β-CPA.

1.2.C. **Biological Activities of Cyclopiazonic Acid**

Cyclopiazonic acid has three main biological activities including alteration of calcium homeostasis, electric charge alteration in plasma membranes and mitochondria and antioxidant functions.

1.2.C.a. **Alterations in calcium homeostasis**

According to Goeger and Riley (1989), CPA has two methods of altering calcium homeostasis.

CPA is specific inhibitor of Ca²⁺-ATPase in cardiac, skeletal muscle sarcoplasmic reticulum and endoplasmic reticulum. CPA has been also used to study the role of kinetic mechanisms including the excitation and contraction of the sarcoplasmic reticulum (Seidler *et al.*, 1989). Various types of muscles from different types of animals were used in the study including rat skeletal muscle (Goeger *et al.*, 1988), intact smooth muscle of dog (Bourreau *et al.*, 1991), skinned muscle fibres (Kurebayashi, and Ogawa, 1991), skinned ileal smooth muscle
(Ueyama et al., 1992), and cardiac muscle in adult rat (Agata et al., 1993). The inhibitive action of CPA on sarcoplasmic reticulum has negative inotropic effects on the myocardium of adult rats. In contrast, only minimal inhibition effects of CPA were obtained on neonatal myocardium.

CPA can also alter calcium permeability of vesicle and the kinetics of calcium uptake by cells (Riley et al., 1990). CPA alteration can be either passive or active in calcium-loaded sarcoplasmic reticulum vesicles (Goeger and Riley, 1989). It appears that the reaction of CPA is non-specific. As a selective inhibitors of Ca²⁺-ATPase, CPA inhibits Ca²⁺ uptake and refilling in the reticulum vesicles (Shima, and Blaustein, 1992). CPA was also used as a tool in separating various cellular mechanisms that intensified cardiac muscle relaxation (Balke and Wier, 1991; Pery-Man et al., 1993).

L.2.C.b. Electric charge alterations

CPA increases the accumulation of tetraphenylphosphonium in treated cells (Riley and Goeger, 1992). Tetraphenylphosphonium is a lipophilic quaternary cation usually used to measure the potentiality of the trans-membrane of vesicles and mitochondria. The toxin has been used as a probe for charge alterations in lipids and proteins in membranes (Riley et al., 1986).

L.2.C.c. Antioxidant activity

The prevention of lipid peroxidation affirms CPA’s ability to act as an antioxidant. CPA and CPA imine are the only tetramic acids that can prevent another mycotoxin such as patulin. Moreover, they also induce lipid peroxidation in animal cells (Riley and Showker, 1991). However, the mechanism by which CPA prevents lipid peroxidation is not fully understood (Halliwell, 1990). Moreover, CPA acts as a chelating agent, but a comprehensive knowledge of the mechanism of action has not yet been elucidated (Holzapfel, 1968).

L.2.C.d. Other biological activities observed

CPA significantly increases the relative weight of the proventriculus, kidneys and liver in broiler chickens, circulating levels of uric acid and cholesterol are also
elevated. In contrast, serum phosphorus is decreased under the effect of CPA (Smith et al., 1992).

The relationship of CPA to HIV was also reported. CPA produced by the strain *Penicillium duclauxii* possesses HIV-1 protease inhibitor (Drombrowski et al., undated).

### 1.2.D. Toxicological Properties of Cyclopiazonic Acid

#### 1.2.D.a. Toxicity effects

CPA exhibits a relatively strong acute toxicity (Cole and Cox, 1981). Depending on the animal species, the toxicity effects of CPA are exhibited differently in various internal organs (Nishie et al., 1985). Extensive studies reveal that CPA is toxic in pigs, rats, chicks, turkey poults, ducklings, dogs and rabbits (Dorner et al., 1983; Lomax et al., 1984; Nishie et al., 1984; Van Rensburg, 1984; Morrissey et al., 1985; Nuehring et al., 1985; Wilson et al., 1989 and 1990; Voss et al., 1990). The liver is the most affected organ. The LD$_{50}$ of CPA in certain animal species is shown in Table I.3.

The toxic effects of CPA manifest rapidly when the toxin is injected (Purchase, 1971). Rats showed reluctance to move 5 min after having CPA intraperitoneally administrated. In contrast, oral administration of CPA induced a delayed effect. The insolubility of CPA in acidic stomach contents reduces absorption of CPA. Consequently, strong nervous signs were not observed.

However, the outcome was different when CPA was orally administrated to ducks. Nervous signs of the ducks were similar to that in rats intraperitoneally injected. This may indicate that the degree of CPA absorption varies with different animal species (Purchase, 1971).

The toxic effects of CPA are modified by several factors including the method of experimentation, the dose of the toxin, the length of exposure and the species and gender of the experimental animal. LD$_{50}$ of CPA in male animals was between 12 and 36 mg/Kg of body weight compared to 63 mg/Kg of body weight in females (Terao and Ohtsubo, 1991). A LD$_{50}$ dose of CPA (36 mg/Kg or higher) in
male mice provokes focal necrosis in the liver, kidneys, heart, pancreas and spleen. Lower doses only induce effects on epithelial cells (Dorner et al., 1985).

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Route</th>
<th>$LD_{50}$ (mg/Kg body weight)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>male</td>
<td>ip</td>
<td>13.0</td>
<td>Nishie et al., 1985</td>
</tr>
<tr>
<td>Mice</td>
<td>female</td>
<td>po</td>
<td>63.0</td>
<td>Nishie et al., 1987</td>
</tr>
<tr>
<td>Rat</td>
<td>male</td>
<td>ip</td>
<td>2.3</td>
<td>Purchase, 1971</td>
</tr>
<tr>
<td>Rat</td>
<td>male</td>
<td>po</td>
<td>36.0</td>
<td>Purchase, 1971</td>
</tr>
<tr>
<td>Rat</td>
<td>female</td>
<td>po</td>
<td>63.0</td>
<td>Purchase, 1971</td>
</tr>
<tr>
<td>Chick</td>
<td>male</td>
<td>po</td>
<td>12.5</td>
<td>Wilson et al., 1989</td>
</tr>
<tr>
<td>Chick</td>
<td>female</td>
<td>po</td>
<td>13.1</td>
<td>Wilson et al., 1989</td>
</tr>
<tr>
<td>Poult</td>
<td>male</td>
<td>po</td>
<td>19.0</td>
<td>Wilson et al., 1989</td>
</tr>
<tr>
<td>Poult</td>
<td>female</td>
<td>po</td>
<td>17.9</td>
<td>Wilson et al., 1989</td>
</tr>
<tr>
<td>Duckling</td>
<td>mixed</td>
<td>po</td>
<td>38.6</td>
<td>Wilson et al., 1989</td>
</tr>
<tr>
<td>Quail</td>
<td>male</td>
<td>po</td>
<td>69.6</td>
<td>Wilson et al., 1989</td>
</tr>
</tbody>
</table>

I.2.D.b. Acute and chronic effects

1) Intoxication clinical signs

CPA induces the following signs in animals after injection or oral administration: dehydration, diarrhea, weakness, depression, inappetence, opisthotonos, hyperaesthesia, hypokinesis, failure to gain weight, loss of weight, vomiting, upper gastrointestinal tract ulceration, neurological disturbance, convulsion, and death (Lomax et al., 1984; Nishie et al., 1984; Norred et al., 1985; Wilson et al., 1989 and 1990). CPA is found in the skeletal muscles following feeding to animals. This mode of distribution and necrotic effect on muscles elucidate the reason the animal has abnormal posture or convulsions after ingestion of CPA (Norred et al., 1985).

2) Accumulation and lesions caused by chronic effects

The chronic effects of CPA are lesions in saliva glands, gastro-intestinal tract, pancreas, liver, spleen, heart and skeletal muscles. Norred et al., (1985) reported that the accumulation of CPA was not selective in specific tissue following oral or parenteral administration. However, the same author found in a subsequent study that CPA accumulated to a significant degree in skeletal muscle tissue of chickens (Norred et al., 1988). Typical lesions caused by CPA have the appearance of pyknotic nuclei in hepatocyte or coagulating necrosis of cells. CPA also induces eosinophilic necrotic fibers in skeletal muscles, in germinal centers of mesenteric lymph nodes and in white pulpa of the spleen (Morrissey et al., 1985).

3) Excretion and elimination

The main elimination of CPA is faecal excretion and urine. Entero-hepatic recirculation of CPA is also considered as a process of elimination: biliary excretion is an important mode of eliminating CPA (Norred et al., 1985).

4) Neurotoxic and tremorgenic effects

The biological activities of CPA enable the toxin to function as a potent neurotoxin. The intraperitoneal injection of 8 mg CPA/Kg into rats induced nervous symptoms within 2 hours (Purchase, 1971). Van Rensburg (1984) consistently found a similar effect from CPA. Neuropharmacological effects of CPA were also found in male mice CF BR (Nishie et al., 1985). Pharmacological activities of CPA are
similar to that of antipsychotic drugs such as chlorpromazine and reserpine. CPA also induces catalepsy, convulsions with hypokinesia, hypothermia, tremor, dyspnoea and prolonged sleep.

5) **Hepatotoxic and nephrotoxic effects**

The kidneys and liver are major toxic targets in pigs fed daily for two weeks with CPA (Lomax et al., 1984). Similar effects were found in other animals including birds, chickens and rats (Purchase, 1971; Dorner, 1982 and 1983). However, manifestation of CPA toxicity is dependent on the animal species. For instance, CPA behaves primarily as an entero-nephrotoxin in pigs but acts as a hepatotoxin in rats.

6) **Cytotoxicity and effect on immune system**

Yates et al., (1987) discovered that CPA was more cytotoxic than aflatoxins when *Photobacterium phosphoreum* strain NRRLB-1177 was used in cytotoxicity tests. Subsequent studies claimed that cytotoxic effects of CPA was dose related (Reddy et al., 1989; Smith et al., 1992).

Studies on the effect of CPA on immune systems revealed that the toxin is capable of overcoming the suppression of delayed cutaneous hypersensitivity response caused by aflatoxin. However, CPA itself has no effect on delayed hypersensitivity to *Mycobacterium tuberculosis* and on T cells (Hill, et al., 1986; Richard et al., 1986; Pier et al., 1989). However, studies on certain animal found that CPA can be immunosuppressive (Nuehring et al., 1985).

7) **Carcinogenicity, mutagenicity and teratogenicity**

Besides aflatoxin and sterigmatocystin, CPA is classified as a very low carcinogenic mycotoxin. Aflatoxin can produce and extend hepato-cellular necrosis even with an insignificant dose, while CPA even at higher doses can only provoke necrosis at focal hepato-cellular and bile ducts (Purchase, 1971). CPA is thus unlikely to become hepato-carcinogenic even if fed in acute lethal doses to rats. In contrast, Frank et al., (1977) found CPA acted as a carcinogenic toxin in their studies.

Reports on mutagenic effects of CPA were conflicting. Wehner et al., (1978) failed to find CPA to be mutagenic, both with or without metabolic activation when *Salmonella typhimurium* was used in the test. In contrast, Pier (1991) claimed that
CPA appeared to be mitogenic. Moreover, Sorenso et al., (1984) demonstrated that CPA was mutagenic to Salmonella typhimurium TA98. It was suggested that conflicting reports were due to different doses of CPA being used in each study.

Using Fischer rats, Morrissey et al., (1984) found that the teratogenic potential of CPA was very weak. However, a slowdown of skeletal development in foetuses was observed. Subsequent study on embryotoxicity of CPA was also reported (Khera et al., 1985)

1.2.E. Human Potential threat of CPA.

The occurrence of mycotoxigenic fungi and mycotoxins in a wide range of food and feed highlights the human potential risk. Evidence of CPA association in naturally occurring toxicosis is shown in Table 1.4.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Species</th>
<th>Feedstuff</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey &quot;X&quot; disease</td>
<td>Pouls</td>
<td>Peanuts</td>
<td>Cole, 1986a</td>
</tr>
<tr>
<td>Kodua poisoning</td>
<td>Man, cattle</td>
<td>Millet</td>
<td>Rao and Husain, 1985</td>
</tr>
<tr>
<td>Mortality</td>
<td>Quail</td>
<td>Mixed feed</td>
<td>Stoltz et al., 1988</td>
</tr>
<tr>
<td>Reproductive failure</td>
<td>Pigs</td>
<td>Sunflower</td>
<td>Ross et al., 1991</td>
</tr>
<tr>
<td>Mortality</td>
<td>Cattle</td>
<td>Barley</td>
<td>Harrison, 1971</td>
</tr>
<tr>
<td>Staggers</td>
<td>Cattle</td>
<td>Mixed feed</td>
<td>Hacking and Harrison, 1976</td>
</tr>
</tbody>
</table>

*(Bryden, 1992)*
Detection of CPA is often associated with aflatoxin, which is harmful to humans. Moreover, the actions of the two mycotoxins are additive (Lansden and Davidson, 1983).

Reports on the significance of CPA to humans are conflicting. Van Rensburg (1984) observed relatively mild pathological effects of CPA following weekly sublethal doses in rats and concluded that low levels of CPA were not a significant threat to humans. Other scientists claimed that the potential risk of CPA relied on conditions used in food processing and CPA production by fungi. Moreover, they claimed that the potential food contamination of CPA was low (Still et al., 1978a; Goto et al., 1990).

In contrast, Morrissey et al., (1985) and Norred et al., (1985) confirmed that the toxicity of CPA was cumulative and therefore of concern with regard to human exposure. Furthermore, CPA accumulation in meat, milk and egg was confirmed (Norred et al., 1985; Suksupath, 1993; Dorner et al., 1994). According to these authors, CPA is likely to be a candidate for toxicity problems in the food chain. Consequently, CPA has been placed in the category of potentially serious mycotoxins (Lansden and Davidson, 1983; Cole, 1984; Khera et al., 1985; Norred et al., 1988). Cole (1986a) and Dorner et al., (1983) demonstrated that the first outbreak of "Turkey X Disease" was the result of synergism between CPA and aflatoxins. Bradburn et al., (1994) strongly supported this finding by establishing that the turkey poult had ingested groundnut cake containing CPA as high as 31 µg/Kg. Several other outbreaks of disease due to ingestion of food contaminated by CPA were reported (Wilson et al., 1968). CPA has been suspected of initiating poisoning in humans in regions where kodo millet seeds are consumed (Rao and Husain, 1985). Symptoms caused by CPA encountered in humans are sleepiness, tremors, nausea and giddiness (Bhide, 1962).

Due to the ubiquitous character of fungi producing CPA, it is apparent that these fungi are a significant potential risk as a food and dairy product contaminant. Moreover, the potential risk from CPA becomes more visible apparent as the number of countries reportedly occurrence of the toxin increases (Gallagher et al., 1978).
1.2.F. Cycloiazonic Acid Producing Fungi

Reports of fungi producing CPA have been conflicting due to many mycotoxins being named after the fungi presumed to be their creators. In reality, some of them did not produce the particular toxin. Moreover, some fungi were eventually incorrectly identified (Pitt, 1979; Frisvad, 1989). Initially CPA was isolated from *Penicillium cyclopium* Westling, which was later named *Penicillium griseofulvum* Diercky (Holzapfel, 1968; de Jesus *et al.*, 1981). The latter, in fact, was originally called *Penicillium urticae* strain G 391. Ducklings had an acute toxicity when fed with corn containing these fungi (De Scott, 1965).

Studies on production of CPA by *Aspergillus* and *Penicillium* is still in current scientific reports (Lee and Hagler, 1991; Cvetnic, 1994; Gqualeni *et al.*, 1996b).

Reports on species of *Aspergillus* and *Penicillium* that produce CPA (Suksupath, 1993) with more updated information are shown in Tables 1.5a and 1.5b.

Similar to other mycotoxins, the factors that cause synthesis of the toxin are not yet known, but it is understood that specific media and suitable environmental conditions are required (Diener *et al.*, 1987). Cole (1984) confirmed that other than media, CPA production depends on temperature and incubation time. However, these factors also depend on the genera of fungi producing CPA.

1.2.G. Co-production of Cycloiazonic Acid and Aflatoxin.

CPA can be simultaneously co-produced with some mycotoxins including aflatoxin by several species of *Aspergillus* (Gallagher *et al.*, 1978; Gqualeni *et al.*, 1996a). It is known that CPA has a high possibility of occurring in the same food as aflatoxin (Bryden, 1987). Fungi co-producers of CPA and aflatoxin are ubiquitous and occur in numerous agricultural commodities. This had been confirmed by the report of Widiastuti *et al.*, (1988). The report revealed that the incidence of CPA in corn samples from Indonesia was very high. Moreover, all the corn samples contaminated by CPA also contained aflatoxin.

Another study in Australia revealed that more than 71% of 38 fungi samples of genera *Aspergillus flavus* isolated from Queensland were capable of co-producing
both aflatoxin and CPA (Blaney et al., 1989). Lee and Hagler, (1991) isolated *Aspergillus flavus* from contaminated maize, then incubated into the sample liquid medium. Among 19 isolated fungi, 11 of them co-produced CPA and aflatoxin.

<table>
<thead>
<tr>
<th><em>Aspergillus</em> spp.</th>
<th>Medium source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus flavus</em> Link</td>
<td>Red wheat</td>
<td>Luk et al., 1977</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Wheat, rice, corn</td>
<td>Gallagher et al., 1978</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Culture broth</td>
<td>Yokota et al., 1981</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Submerged cultures</td>
<td>Hermansen et al., 1984</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Groundnut, corn</td>
<td>Cole, 1984</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Kodo millet seed</td>
<td>Rao and Husain, 1985</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Dried bean, corn, meal, macaroni, pecans</td>
<td>Trucksess et al., 1987</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Maize</td>
<td>Lee et al., 1991</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Dried beans and maize</td>
<td>Cvetnic, 1994</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Feed</td>
<td>Polster et al., 1990</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Culture</td>
<td>Blaney et al., 1989</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>Culture</td>
<td>Cole, 1984</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>Culture</td>
<td>Orth, 1977</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>Culture</td>
<td>Yokota et al., 1981</td>
</tr>
<tr>
<td><em>Aspergillus tamarii</em> Kita</td>
<td>Peanut</td>
<td>Dorner, 1983</td>
</tr>
<tr>
<td><em>Aspergillus tamarii</em></td>
<td>Kodo millet seed</td>
<td>Rao and Husain, 1985</td>
</tr>
</tbody>
</table>

*Polster et al., (1990); Lee et al., (1991); Suksupath (1993); Cvetnic, (1994).*
Table 1.5b. List of *Penicillium spp* capable of producing cyclopiazonic acid a

<table>
<thead>
<tr>
<th><em>Penicillium spp</em></th>
<th>Medium source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Penicillium cyclosporum</em> Westling</td>
<td>Groundnut</td>
<td>Holzapfel, 1968</td>
</tr>
<tr>
<td><em>Penicillium cyclosporum</em></td>
<td>Groundnut</td>
<td>Purchase, 1971</td>
</tr>
<tr>
<td><em>Penicillium patulum</em></td>
<td>Groundnut</td>
<td>Leistner and Pitt, 1977</td>
</tr>
<tr>
<td><em>Penicillium camembertii</em> Thom</td>
<td>Meat, cheese</td>
<td>Still <em>et al.</em>, 1978a</td>
</tr>
<tr>
<td><em>Penicillium camembertii</em></td>
<td>Cheese</td>
<td>Le Bars, 1979, 1988</td>
</tr>
<tr>
<td><em>Penicillium camembertii</em></td>
<td>Submerged cultures</td>
<td>Hermansen <em>et al.</em>, 1984</td>
</tr>
<tr>
<td><em>Penicillium caseicolum</em></td>
<td></td>
<td>Engel, 1981</td>
</tr>
<tr>
<td><em>Penicillium viridicatum</em></td>
<td>Country cured ham</td>
<td>Cole, 1984</td>
</tr>
<tr>
<td><em>Penicillium crustosum</em></td>
<td>Raw ham</td>
<td>Cole, 1984</td>
</tr>
<tr>
<td><em>Penicillium crustosum</em></td>
<td></td>
<td>El-Banna <em>et al.</em>, 1987</td>
</tr>
<tr>
<td><em>Penicillium puberulum</em></td>
<td>Fermented sausage</td>
<td>Cole, 1984</td>
</tr>
<tr>
<td><em>Penicillium camembertii</em> Thom</td>
<td>White cheese</td>
<td>Pitt <em>et al.</em>, 1986</td>
</tr>
<tr>
<td><em>Penicillium commune</em></td>
<td>Culture</td>
<td>Pitt and Leistner, 1989</td>
</tr>
<tr>
<td><em>Penicillium commune</em></td>
<td></td>
<td>Gualeni, <em>et al.</em>, 1996b</td>
</tr>
<tr>
<td><em>Penicillium urticae</em></td>
<td>Dried bean, macaroni</td>
<td>Truckess <em>et al.</em>, 1987</td>
</tr>
<tr>
<td><em>Penicillium commune</em> Thom</td>
<td></td>
<td>Pitt and Leistner, 1989</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td></td>
<td>El-Banna <em>et al.</em>, 1987</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em> Thom</td>
<td></td>
<td>Pitt and Leistner, 1989</td>
</tr>
<tr>
<td><em>Penicillium griseofulvum</em> Submerged cultures</td>
<td></td>
<td>Hermansen <em>et al.</em>, 1984</td>
</tr>
<tr>
<td><em>Penicillium griseofulvum</em> Dierckx</td>
<td></td>
<td>Pitt and Leistner, 1989</td>
</tr>
<tr>
<td><em>Penicillium griseofulvum</em> Maize</td>
<td></td>
<td>Reddy <em>et al.</em>, 1989;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>El-Banna <em>et al.</em>, 1987</td>
</tr>
<tr>
<td><em>Penicillium hirsutum</em></td>
<td></td>
<td>Pitt and Leistner, 1989</td>
</tr>
<tr>
<td><em>Penicillium hirsutum</em> Dierckx</td>
<td></td>
<td>Malik <em>et al.</em>, 1986</td>
</tr>
<tr>
<td><em>Penicillium verrucosum</em> Var. cyclosporum</td>
<td></td>
<td>El-Banna <em>et al.</em>, 1987</td>
</tr>
<tr>
<td><em>Penicillium viridicatum</em></td>
<td></td>
<td>Pitt and Leistner, 1989</td>
</tr>
<tr>
<td><em>Penicillium viridicatum</em> Westling</td>
<td></td>
<td>El-Banna <em>et al.</em>, 1987</td>
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</tbody>
</table>

Reports of another study revealed that *Aspergillus flavus* isolated from contaminated rice also co-produced the two mycotoxins (Richard et al., 1990). Urano et al., (1992b) reported that peanuts and corn samples from USA contained *Aspergillus flavus* producing CPA and aflatoxin. A recent study found that other than *Aspergillus flavus, Aspergillus tamarii* also co-produces CPA and aflatoxin (Goto et al., 1996).

Several other mycotoxins including sterigmatocystin, aflatrem, aspergillic acid, kojic acid and β-nitropropionic acid are also secondary metabolites of *Aspergillus flavus*, along with CPA and aflatoxin (Cole and Cox, 1981). Moreover, several other mycotoxins, secondary metabolites of fungi *Aspergillus, Penicillium* or *Fusarium* have been found in the same commodities as CPA. The occurrence of CPA in the same food or feedstuff with zearalenone, patulin and ochratoxin A have been reported (Trucksess et al., 1987; Widiastuti et al., 1988). Currently, no studies have yet been reported on the synergistic or additive toxicity of CPA with these mycotoxins (Bryden, 1992).

Despite the fact that CPA has been reported to co-occur with aflatoxin, the report on their toxicity interaction has been contradicting. Some reports have claimed that no interaction occurs (Morrissey et al., 1987; Richard et al., 1990). However, several other reports confirmed significant interaction between the two toxins in animal model studies (Pier et al., 1989; Smith et al., 1992).

1.2.H. Occurrence and Incidence of Cyclopiazonic Acid

The rate of production of CPA, on average, is much greater than that of aflatoxin by fungal co-producers of the toxin (Lee and Hagler, 1991). The majority of *Aspergillus flavus* isolated can produce CPA, but there is no correlation between production of CPA and aflatoxin (Luk et al., 1977; Gallagher et al., 1978; Blaney et al., 1989).

1.2.H.a. Cyclopiazonic Acid in Agricultural Commodities

The occurrence of CPA in agricultural products can possibly occur in the field before their storage (Gallagher et al., 1978). Among agricultural commodities studied, peanuts are the most frequently reported in the detection of CPA at preliminary screening (Gallagher et al., 1978; Lansden and Davidson, 1983; Reddy
and Reddy, 1992). Maize is another agricultural product likely to be highly contaminated by CPA. Widiastuti et al., (1988) reported that more than 50% of maize samples collected in Indonesia contained high levels of CPA (Table 1.6). Household stored grains were found to be contaminated at a very high level by the toxin (Cvetnic, 1994). Cereals, legumes, kodo millet, sunflower grains, beans, and dried figs were also contaminated by CPA (De Scott; 1965; Rao and Husain, 1985; Ross et al., 1991; Steiner et al., 1993).

**L2.H.b. Fungi Producer of Cyclopiazonic Acid in Food and Feedstuffs.**

Incidence of fungi producers of CPA in certain types of foods demonstrates the possibility of direct contamination by CPA. These fungi have been isolated from cheese, corn, sunflowers, macaroni, pecans animal rations, feeds and several other kinds of foods (Gallagher et al., 1978; Still et al., 1978a; Le Bars, 1979; Frevel, 1981; Dutton and Westlake, 1985; Truckses et al., 1987; Ostry and Polster, 1989; Ross et al., 1991). Fermented foods including ham, sausage and frankfurter were also contaminated by the fungi producer of CPA (Leistner and Pitt, 1977; Leistner and Eckardt, 1979; Leistner et al., 1989). The presence of CPA in commercial cheese samples provides evidence of natural occurrence of the toxin in human food (Table 1.6).

**L2.H.c. Cyclopiazonic Acid in Animals and Animal Products.**

The occurrence of CPA in feed to be consumed by animals that would be used for meat and milk, increases the possibility of CPA being “carried-over” into animal products (Norred et al., 1985). Carry-over of CPA into animal products is also demonstrated by the detection of the toxin in the milk of lactating ewes.

Feeding 5 mg CPA/g of live weight to a lactating animal induces milk carry-over within one day followed by an increase from 236 ng/g to 568 ng CPA/g after a second dose. Withdrawal of CPA from feeding to animals did not cease its presence in milk several days later (Dorner et al., 1994). The distribution of CPA in the body is not selective to specific tissues (Norred et al., 1985).

The occurrence of CPA in the body of animals destined for meat and in animal products including eggs and milk present a potential risk to consumers (Elrington, 1991; Suksupath et al., 1992; Dorner et al., 1994)
Table 1.6. Natural occurrence of cyclopiazonic acid in plant products and cheese.

(Bryden, 1992)

<table>
<thead>
<tr>
<th>Product</th>
<th>Location</th>
<th>Incidence</th>
<th>Concentration range (μg/Kg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>USA</td>
<td>6/6</td>
<td>Not stated</td>
<td>Gallagher et al., 1978</td>
</tr>
<tr>
<td></td>
<td>Indonesia</td>
<td>21/26</td>
<td>30 - 9,220</td>
<td>Widiastuti et al., 1988</td>
</tr>
<tr>
<td>Cheese</td>
<td>Germany</td>
<td>9/21</td>
<td>100 - 1,900 (^a)</td>
<td>Still et al., 1978a, b</td>
</tr>
<tr>
<td></td>
<td>France</td>
<td>11/20</td>
<td>50 - 1,500 (^b)</td>
<td>Le Bars, 1979</td>
</tr>
<tr>
<td></td>
<td>Germany</td>
<td>11/11</td>
<td>60 - 290 (^a)</td>
<td>Frevel, 1981</td>
</tr>
<tr>
<td></td>
<td>Germany</td>
<td>3/4</td>
<td>80 - 370 (^a)</td>
<td>Schoch et al., 1983</td>
</tr>
<tr>
<td></td>
<td>South Africa</td>
<td>Not stated</td>
<td>35 - 70 (^a)</td>
<td>Luck and Wehner, 1979</td>
</tr>
<tr>
<td>Millet</td>
<td>India</td>
<td>1/1</td>
<td>Not stated</td>
<td>Rao and Husain, 1985</td>
</tr>
<tr>
<td>Peanuts</td>
<td>USA</td>
<td>21/27</td>
<td>32-6,525</td>
<td>Lansden and Davidson, 1983</td>
</tr>
<tr>
<td>Sunflower</td>
<td>USA</td>
<td>1/1</td>
<td>10,000</td>
<td>Ross et al., 1991</td>
</tr>
<tr>
<td>Mixed *</td>
<td>South Africa</td>
<td>0/800</td>
<td>-</td>
<td>Dutton and Westlake, 1985</td>
</tr>
</tbody>
</table>

* Cereals and animal feedstuffs
\(^a\) In whole cheese.
\(^b\) In cheese rind.
I.2.1. Determination of Cyclopiazonic Acid

I.2.1.a. Biological and physicochemical

1) Toxicity Testing

The method used to study genotoxicity and cytotoxicity of CPA and aflatoxin B1 was proposed by Yates et al., (1987). The two toxins were analysed both separately and in combination using a comparison of their effect on luminescence in marine bacterium Photobacterium phosphoreum.

A cytotoxicity test capable of assessing the toxicity of several mycotoxins including CPA was proposed by Madhyastha et al., (1994). Bacillus brevis in disc diffusion assay was used as an indicator of CPA toxicity. Sensitivity of the method was 0.5μg CPA/disc. Recent methods of bioassay of CPA reported using Staphylococcus albus (Reddy and Reddy, 1995).

2) Enzyme-Linked Immunosorbent Assay (ELISA).

Hahnau and Weiler (1991) elaborated on a CPA competitive enzyme-linked immunosorbent assay, which used alkaline phosphatase as an enzyme tracer. No precleaning was required and the method had a sensitivity of 30pg - 2ng CPA. However, the variability of inter-assay between day and intra-assay was 14% and 3.7%, respectively. The same group of authors subsequently reported on production of a monoclonal antibody specific to CPA (Hahnau, and Weiler, 1993). Polyclonal and monoclonal antibodies against CPA were also studied by Xuan Huang and Fun Sun Chu (1993).

I.2.1.b. Extraction, clean up, separation and quantification of CPA.

1) Isolation and extraction

Similar to other relative indoles, CPA can be extracted by butanol, chloroform, chloroform-methanol, and dichloromethane. The best substrate for CPA isolation is an organic solvent such as chloroform following acidification (Cole, 1984). CPA can be re-extracted from the organic phase by using aqueous sodium bicarbonate solution. To obtain CPA, the aqueous phase needs to be acidified with HCl and then partitioned with chloroform.

Many mixtures of solvents have been used to extract CPA depending on the nature of the samples. Gorst-Allman and Steyn (1979) used methanol-chloroform
(1:1) to extract CPA from contaminated maize. Le Bars (1979) extracted the toxin from cheese with azeotropic chloroform-methanol. Benkhemmar et al. (1985) modified the method of Le Bars to use for CPA extraction from culture filtrates. The process was similar to that of Lansden (1986) used to extract CPA from peanut samples. A mixture of methanol-chloroform (20:80) was used in the extraction, which was followed by partitioning with aqueous sodium bicarbonate.

2) Cleaning and purification

To purify CPA from culture media, the method of column chromatography or thin layer chromatography has been used.

Holzapfel (1968) used column chromatography containing cellulose powder impregnated with formamide-oxalic acid (50:3) to clean CPA. Elution was done with a mixture of hexane-benzene (20:1 to 3:1). Collected eluents were combined and fractionated on a Dowex 1-x8 column, then washed with a gradient of 0-3.5 M formic acid in 1:1 aqueous methanol. Fractions collected were extracted by chloroform and crystallised from methanol solution.

Purification of CPA by using TLC was reported by Steyn (1969). The method used 0.4 N aqueous oxalic acid in a ratio of 1:2 to impregnate an activated silica gel G TLC plate at 100°C for 40 min. The plate was stored in a desiccator at room temperature before use. The presence of oxalic acid permits the migration of CPA in TLC plates. The system of solvents was a mixture of chloroform-isobutyl ketone (4:1). CPA can be detected ($R_f \approx 0.65$) under UV light (dark spot) or spraying with concentrated sulphuric acid or 1% ethanolic iron III chlorine (red-brown spot) or Ehrlich reagent (blue-purple) followed by heating at 110°C for a 10 min period. CPA has a violet-red colour if held on a plate with a prolonged standing time without being sprayed by any solution. This method (Steyn, 1969) used two dimensional TLC to quantify CPA. The following solvent systems were used: Acetate-isopropanol-ammonia solution con. (20:15:10) and chloroform-acetic acid concentrated (10:1). Following a spray with Ehrlich reagent, the amount of CPA was measured by chromatogram scanner.
3) Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC)

The detection of CPA by TLC has been extensively used (Gallagher et al., 1978; Le Bars, 1979; Lansden and Davidson, 1983). The methods of analysing CPA in agricultural commodities using TLC are presented in Table 1.7 (Steyn et al., 1991). It was recommended that oxalic acid-impregnated plates be used to secure the best results (Gorst-Allman and Steyn, 1979).

<table>
<thead>
<tr>
<th>Commodity analysed</th>
<th>Extraction solvent</th>
<th>Cleanup procedure</th>
<th>Analytical technique</th>
<th>Detection limit(ng/g)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>CHCl₃</td>
<td>Solvent partitioning</td>
<td>TLC-UV</td>
<td>-</td>
<td>Gallagher et al., 1978</td>
</tr>
<tr>
<td>Cheese</td>
<td>CHCl₃:MeOH azeotrope</td>
<td>Precipitation, solvent partitioning</td>
<td>TLC-UV</td>
<td>-</td>
<td>Le Bars, 1979</td>
</tr>
<tr>
<td>Cereals, etc.</td>
<td>CH₂CN :4% KCl (9:1)</td>
<td>Solvent partitioning</td>
<td>TLC-Chromophore spraying</td>
<td>-</td>
<td>Popken and Dose, 1983</td>
</tr>
<tr>
<td>Peanuts</td>
<td>CHCl₃:MeOH: H₃PO₄ (400:100:1)</td>
<td>Defatting, solvent partitioning</td>
<td>C₁₈ HPLC - UV</td>
<td>-</td>
<td>Lansden, 1984</td>
</tr>
<tr>
<td>Maize and peanuts</td>
<td>CHCl₃:MeOH: H₃PO₄</td>
<td>Defatting, solvent partitioning</td>
<td>TLC-Chromophore spraying</td>
<td>125</td>
<td>Lansden, 1986</td>
</tr>
</tbody>
</table>

* Steyn et al., (1991)
The sensitivity of the methods depends on several factors such as the type of plate, method of activation, atmosphere, humidity and mobile phase system, etc. To improve the method of CPA detection in cereals, the use of dimethylamino-benzaldehyde spray as a chromophore following the separation of CPA by two dimensional TLC was recommended (Popken and Dose, 1983). The modified method of silica gel TLC by adding ethylenediamine tetracetic acid (EDTA) or oxalic acid was recommended for the analysis of multiple acidic mycotoxins including CPA (Steyn, 1969; Steyn et al., 1991).

The various solvent systems used for CPA separation by TLC are as follows: chloroform-methyl isobutyl ketone (4:1), chloroform-acetone (9:1) or (95:5), chloroform-methanol (98:2), ethyl acetate-2-propanol-ammonia solution (20:15:10) and toluene-ethyl acetate-formic acid (5:4:1).

Despite abundant screening methods for CPA detection in agricultural commodities being reported, the recovery levels of the reported methods were not high. Therefore, newly developed analytical methods for CPA-aflatoxin in food and feedstuffs are required (Steyn et al., 1991).

4) Colorimetric method

The colorimetric method reported by Rathinavelu and Shanmugasundaram (1984) was similar to TLC in that it was based on a colour reaction with Ehrlich's reagent, which reacted with the indole group. The method was used to detect CPA in food, feed, rice, corn, and wheat. The technique relies on the reaction between p-dimethylaminobenzaldehyde with α and β unsaturated indole in the presence of concentrated HCl. A purple cation resulting from condensation at the α-position, is measured at the wavelength at 560 nm. The occurrence of colour follows Beer's law. Sensitivity of the method is 5 - 50 μg/mL and recovery was 63 to 95%.

A second colorimetric method was reported by Rao and Husain (1987). Sensitivity of the method was 1μg CPA/mL.

There are two differences between the method of Rathinavelu and Shanmugasundaram (1984) and the one of Rao and Husain (1987). Sulphuric acid was used instead of hydrochloric acid as the Ehrlich reagent for spraying. The second method uses a shorter time with a better recovery rate at 92 to 97%.
5) **Filter paper method.**

Recently, a new screening method for directly detecting CPA from the fungi producer was proposed by Lund (1995). Filter paper was bathed with Ehrlich reagent in ethanol then directly applied to the spore of the fungi. The appearance of violet zones confirmed the presence of CPA. However, this method cannot differentiate the presence of indole metabolites of *Penicillium* between chaetoglobosin C, isoafumigaclavine, rugulovasin A-B or CPA. However, with additional morphological and physiological analysis, the method will allow the genera of *Penicillium* to be discerned.

6) **Spectrophotometric method.**

Developed by Chang-Yen and Bidasee (1990) and based on the reaction of Ehrlich reagent, the spectrophotometric method was used to detect CPA in feed and corn. The toxin was spectrophotometrically detected by using a wavelength of 580 nm with a limit of detection at 0.08 mg/Kg.

7) **Gas Chromatographic method.**

Goto *et al.*, (1990) reported on the use of gas chromatography with a fused silica capillary column to simultaneously identify 6 mycotoxins including CPA (Aflatoxin B₁, G₁, B₂, G₂, kojic acid and CPA). A mass spectrometer (MS) with gas chromatography (GC) and mass spectra’s data processing system was used for GC-MS. The limit of the detection was 0.5 ng and 1 ng for CPA and aflatoxins respectively. The minimum detectable level of CPA by mass chromatography was 0.5 ng.

8) **Liquid Chromatographic method.**

Based on the method for tenuazonic acid detection, Lansden (1984) initially proposed reverse phase high performance liquid chromatography to detect CPA in peanut samples. He used ligand-exchange chromatography on a C₈ or C₁₈ column loaded with 4-dodecyldiethylenetriamine with an aqueous mobile phase containing zinc acetate, ammonium acetate, 2-propanol, acetonitrile and 4-dodecyl-diethylenetriamine. Chloroform-methanol (80:20) was used for CPA extraction. CPA was detected under UV at 289 nm and limit of detection was 4 ng.
Norred et al., (1987) modified the method of Lansden (1984) to detect CPA in poultry meat with a mini column, which was only used in the phase of clean up. The overall recovery for 40 samples analysed was 56.3 - 84.5%.

Based on the method of high performance liquid chromatography (HPLC) with an alklyphenone retention index and UV-VIS spectra photo diode-array detector, Frisvad and Thrane (1987) developed a method capable of simultaneously detecting 182 mycotoxins including CPA. However, despite the same type of column being used, the retention times were not constant. Moreover, the UV detection wavelength of the method was limited to 245 nm. The detection of toxins with an end absorption was therefore not possible (Betina, 1989). Thereafter, HPLC methods provided CPA peaks, which were too broad (Betina, 1993).

Goto et al., (1987) proposed a more sensitive normal phase HPLC method. The mobile phase was the same as TLC system of solvents namely ethyl acetate - 2 propanol - 25% aqueous ammonia (55:20:5; v/v/v). The method used a silica gel (Devolsil 60-5, Nomura Chem., Seto, Japan) stainless steel column (4mm i.d. x 10 cm). Detection limit obtained was 0.2ng.

Normal phase HPLC was also used to purify CPA using a silica gel column (Peterson et al., 1989). Chloroform-methanol (99:1) or (99.5:0.5) were used as eluents. An amino bonded-phase column with a methanol-phosphate buffer (2:8) as the eluent was also proposed by the same authors. Peterson et al., (1989) reported that the use of such column with a weak anion-exchange mode would decrease "tailing" or irreversible binding of CPA to the column packing material.

Reverse phase liquid chromatography using a linear gradient was recently reported by Urano et al., (1992a; 1992b). CPA was partitioned into chloroform in a Sep-Pak silica cartridge. The dissolving solvent was methanol water (60:40 or 85:15). The toxin was measured with a linear gradient of 0 to 4mM ZnSO₄ in methanol-water (85-15) under UV 279 nm.

New methods for detecting CPA were recently reported (Matsudo and Sasaki, 1995). CPA detection in dairy products was also proposed by Simunek et al., (1992). However, the latter technique still relied on a colour reaction with Ehrlich's reagent and a colorimetric method.
SECTION (2): AFLATOXIN AND CYCLOPIAZONIC ACID IN MILK

Indirect contamination or carry-over of mycotoxins in milk is a consequence of the occurrence of the toxins in feedstuff consumed by dairy cattle (Van Egmond, 1989). Among mycotoxins, aflatoxin M₁ (AFM₁), which is the milk metabolite of aflatoxin B₁ (AFB₁) has the highest occurrence reported in milk (Brackett and Mart, 1982a; Blanc et al., 1983; El Nezami et al., 1995; Galvano et al., 1996). Compared to AFM₁, CPA, which can be co-produced by several genera of Aspergillus with AFB₁, has been reported on few occasions in literature.

I.3. OCCURRENCE IN FEEDS OF DAIRY CATTLE

The potential occurrence of mycotoxin in food and feed depends on the prevailing conditions contributing to the outgrowth of fungal producers and their production of toxin (Van Egmond, 1989). From the 1960’s when aflatoxins were originally discovered, worldwide reports on the occurrence of several mycotoxins in feed have been reported (Shreeve et al., 1979; Jelinek et al., 1989).

I.3.A. Occurrence of Aflatoxin in Feeds

Information on the excretion of a toxic compound into milk within a few hours of feeding lactating animals with feed contaminated by aflatoxin, was initially reported by Allcroft and Carnaghan (1962 and 1963). Despite measures being taken, the occurrence of mycotoxin in feed continues to be a constantly widespread problem that dairy farmers have to face (Screenivasamurthy et al., 1967; Stoloff, 1976; Scott, 1978; Dutton and Westlake, 1985; Jones et al., 1994).

However, aflatoxin occurrence in feedstuffs might depend on geographical situations. Nevertheless, aflatoxin contamination in grain is not confined only to under developed or poor countries considered prone to detrimental environmental and economic situations (Shotwell et al., 1977; Bryden et al., 1980). Most agricultural plant crops used as feedstuffs for dairy cattle are susceptible to contamination by aflatoxins including groundnut meal, cotton seed meal, maize meal, root crops and some forage crops (Afzal et al., 1979). The constant incidence of reports of aflatoxin in feed justifies the belief in the carry-over of aflatoxin into
milk (Fritz et al., 1977; Shreeve et al., 1979; Stuart Jones and Ewart, 1979; Jalon et al., 1994; El Nezami et al., 1995; Galvano et al., 1996).

I.3.B. Occurrence of Cyclopiazonic Acid in Feeds

The worldwide occurrence and current developments in mycotoxins have incited scientific communities to expand monitoring studies on mycotoxins other than aflatoxins, including cyclopiazonic acid (Jelinek et al., 1989; Trucksess, 1994; 1995; 1997). However, unlike aflatoxins, barely any information has been reported on CPA occurrence in feed.

It is well known that a retrospective study on Turkey “X” disease strongly supported the view of the involvement of CPA with aflatoxin in the aetiology in this disease (Cole, 1986a). CPA has been found as a natural contaminant of agricultural products used in feeds (Trucksess et al., 1987; Blaney et al., 1989; Urano et al., 1992b). Moreover, Aspergillus flavus co-producer of CPA and aflatoxin was constantly isolated from animal feeds and very recent reports confirm that the neurotoxin does occur in feed (Polster et al., 1990; Balachandran and Parthasarathy, 1996).

I.4. RESPONSES OF DAIRY CATTLE TO AFLATOXIN OR CYCLOPIAZONIC ACID

I.4.A. Rumen

The effects of CPA on the rumen and/or the role of detoxifying microflora in the rumen on CPA are still unknown. However, some of the previous reports discussed the insolubility of CPA in aqueous solution under pH 7. It was presumed that the neurotoxin would be an insoluble compound in acidic stomach conditions and absorption would be weak (Purchase, 1971). However, it was found that CPA absorption does occur and appears to be rapid depending on the animal species and the method of administration of the toxin.

Little is known on metabolism and the absorption mechanism of CPA in the alimentary tract. Some studies found that CPA absorption may encounter difficulties due to inflammation or necrosis of the enteric mucosal tissue caused by irritation effects of CPA in the epithelium (Dorner et al., 1983; Lomax et al., 1984). However, Elrington (1991) confirmed that in the near neutral pH in the rumen, CPA could be
more soluble and readily absorbed from the rumen and reticulum. The effect of rumen microflora/fauna on CPA still needs further study.

Compared to CPA, information on the effects of the rumen on aflatoxin or of the toxins on the rumen microflora was extensively reported (Fehr et al., 1969; Fehr and Delage, 1970; Mathur et al., 1976). The behaviour of aflatoxin in the alimentary tract of dairy cattle and degradation of the toxin by rumen in vivo and in vitro was known (Mathur et al., 1976; Engel and Hagemeister, 1978).

Since CPA and aflatoxin are co-produced by Aspergillus flavus and detected in feedstuffs, this suggests that consumption of the two mycotoxins by lactating animals is likely. It would be of great interest to evaluate the detoxifying ability of microflora in rumen in vivo or in vitro on CPA, including its behaviour in the alimentary tract.

I.4.B. Parameters that Reflect Health in Dairy Cattle.

The effects of CPA on lactating animals following oral doses were initially studied by Cole et al., (1988) then by Elrington (1991). Ewes fed for 2 days with 5 mg CPA/Kg body weight per day, had a feed intake fall of 25% or stopped eating completely. However, feed intakes subsequently increased and completely recovered 6 days later. The lactating animal lost weight then gradually regained it. However, the weight regained was not completely equal to that prior to CPA administration. The animals were confused, weak, hyperthermic, hypokinesic and some of them had diarrhea. Respiration rate increased but the heart rate was normal. Plasma were affected and plasma glucose, lactation and growth hormone were all decreased. In contrast, plasma urea increased. Other than the study done by Elrington (1991), no other report on the clinical effects of CPA on lactating animals is available.

In contrast to CPA, toxicity of aflatoxin on dairy cattle has been extensively reported (Van der Linde et al., 1965; Nabney and Burbage 1967; Keyl and Booth, 1968; Lynch et al., 1971; Applebaum and Marth, 1980a; Smith et al., 1994). In a review on toxicity of aflatoxin on dairy cattle, Applebaum et al., (1982b) described the effects of oral doses of the toxin on dairy calves and cows in Table 1.8 and 1.9.
Table 1.8. Effects of oral doses of aflatoxin on dairy calves

<table>
<thead>
<tr>
<th>Decrease in:</th>
<th>Increase in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed intake</td>
<td>Serum alkaline phosphates</td>
</tr>
<tr>
<td>Weight gain</td>
<td>Total bilirubin</td>
</tr>
<tr>
<td>Serum carotene</td>
<td>Urine volume</td>
</tr>
<tr>
<td>Serum vitamin A</td>
<td>Urine nitrogen</td>
</tr>
<tr>
<td>Serum inorganic phosphorus</td>
<td>Urinary creatinine</td>
</tr>
<tr>
<td>Nitrogen utilisation</td>
<td>Serum glutamic oxaloacetic transaminase</td>
</tr>
<tr>
<td>Faecal nitrogen</td>
<td></td>
</tr>
<tr>
<td>Hepatic glycogen</td>
<td></td>
</tr>
</tbody>
</table>


Table 1.9. Effects of aflatoxin on dairy cows

1. Unthriftiness, lethargy and anorexia
2. Decreased milk production.
3. Normal or below normal body temperature.
4. Dry, peeling skin on muzzle.
5. Prolapse of the rectum.
6. Liver damage.
7. Elevated blood levels of cholesterol, bilirubin, glutamic, oxaloacetic transaminase, lactic dehydrogenase and alkaline phosphatase.
8. Oedema in the abdominal cavity

*Mertens (1979) and Applebaum *et al.*, (1982b)*
1.4.C. Milk Production

Oral administration of CPA to lactating ewes induced a reduction in milk yield (Elrington, 1991). However, levels of milk yield recovered rapidly and reached the quantity of milk yielded prior to CPA treatment on the eighth day. Milk composition was also changed after CPA feeding to lactating animals. Milk fat and milk protein increased following CPA administration and decreased when oral dosing concluded.

1.5. AFLATOXIN AND CYCLOPIAZONIC ACID IN MILK AND MILK PRODUCTS.

Substantial quantities of CPA in the milk of orally dosed animals revealed the potential for carry-over of the toxin into milk used for human consumption (Elrington, 1991; Dorner et al., 1994). However, the presence and stability of CPA in milk and dairy products has not been reported as it has for aflatoxins AFB$_1$ and AFM$_1$.

1.5.A. Stability in Raw Milk

The stability and behaviour of CPA in raw milk are still not available in the literature. Up to date, information on the stability of CPA that in milk from the day it is yielded and collected from the farm until it is in a stage ready to be consumed has not been reported.

In contrast to CPA, the stability of aflatoxin following carry-over in raw milk has been extensively reported. The transmission of AFB$_1$ into the milk of dairy cattle was initially reported by Van der Linde et al., (1964). AFM$_1$, found in the milk was within 1 - 2% of AFB$_1$ ingested. AFM$_1$ secretion in milk corresponds to a linear regression with the amount of AFB$_1$ intake with feed. Other than being a potential threat to consumers’ health, the stability of AFM$_1$ in milk may render milk products to be discarded and economic loss in the dairy industry.

AFM$_1$ in naturally contaminated raw milk decreased by 40% and 80% following 4 days and 6 days of storage at 0°C respectively (McKinney et al., 1973). Stoloff et al., (1975) reported that AFM$_1$, artificially contaminated milk stored at 4°C was less affected compared to naturally contaminated milk. However, another study
found that AFM$_1$ in both artificially or naturally contaminated milks stored at 5°C decreased, but the naturally contaminated toxin was reduced at a faster rate (Kiermeier and Mashaley, 1977).

1.5.B. Inactivation by Treating Milk

Since the initial finding of CPA in milk (Elrington, 1991; Dorner et al., 1994) a report on treatment or the elimination of the mycotoxin from milk products has still not become available. Studies have not been conducted on how to prevent consumption of CPA contaminated feed by dairy cattle. Moreover, assessment of CPA inactivation using similar biological, physical or chemical methods as that of aflatoxin has still not been done. Thereafter, how to treat equipment following contaminated milk processing is still not known.

In contrast to CPA studies, the treating of milk to inactivate AFM$_1$ has been intensively reported. Decontamination of aflatoxin starts at the level of feed or feedstuffs (Hagler, et al., 1982; Cole, 1989; Oariyakul, 1991). Ammoniation has been one select method to treat aflatoxin in rations being fed to lactating cows (McKinney et al., 1973; Bailey et al., 1994). Sodium bisulfite, sodium hydroxide and aqueous ammonia were among chemical compounds used to eliminate aflatoxin from agricultural products or feed components (Moerck et al., 1980).

Physical treatments similar to dairy industrial processes using heat treatment including pasteurisation and sterilisation were reported by Purchase et al., (1972). Heat treatment of AFM$_1$ is still a topic of current research (Gelosa and Buzzetti, 1994). Ultraviolet energy is another physical method that can be used with or without chemical treatment to degrade AFM$_1$ in milk (Yousef and Marth, 1985; 1986; 1987; 1989). The elimination of AFM$_1$ using degratrafiltration and diafiltration of milk was recently reported (Higuera-Ciapara et al., 1995).

Chemical treatment to inactivate AFM$_1$, and AFB$_1$, has been extensively studied (Mukendi N'Gombo et al., 1992). The elimination of AFM$_1$ from milk is possible by using hydrogen peroxide alone or combined with riboflavin or lactoperoxidase (Applebaum and Marth, 1980b; 1980c; 1982a). Investigation on the use of food additives to degrade aflatoxins was recently reported (Tabata et al.,
1994). Until some methods of detoxification of mycotoxin are proved operative, treatment of mycotoxin is still a subject of further study (Piva, 1995; Rustom, 1997).

1.5.C. Stability During Handling and Processing of Milk

Raw milk commonly undergoes several treatments including collection and storage in a dairy farm, bulk milk handling and transportation, at 4°C, to the dairy plant. After it is received and held at 2 - 3°C at a dairy plant, milk is heat-processed, cooled then stored at <4°C prior to human consumption. Since processed milk may not be available in rural areas or in developing countries, milk can only be consumed safely after boiling.

Information on stability of CPA in raw milk following collection and storage at 4°C is still not available. The effect of transportation of milk from farm to dairy plant on CPA is not reported. It is not known if milk boiled or heat-processed at similar temperatures to those used in the dairy industry, will adequately eliminate CPA. Information on CPA stability under effects of cold storage of either milk culture or milk processing is not available in literature.

In general, milk can be processed into dairy products with or without the separation of milk components.

1.5.C.a. Effect of processing milk without component separation:

Processing milk into dairy products without component separation consists of heat treatment, cold storage and culture of dairy products (Van Egmond, 1989).

1) Heat treatment of milk

The heat treatment of milk constitutes pasteurisation, sterilisation or even boiling directly on a fire. Reports on the effect of heat treatment of milk on the stability of AFM, are contradicting due to several reasons including the mode of contamination and the efficiency of recovery of the extraction method used (Pons, 1973; Kiermeier and Mashaley, 1977). However, most reports revealed that no obvious change occurred in the amount of aflatoxin due to heat processing of milk (Van Egmond et al., 1977; Wiseman and Marth, 1983b; Wiseman et al., 1983b)

2) Cold storage and freezing of milk

Similar to heat treatment, the effect of cold storage and freezing of milk had variable results due to the different sensitivity of methods of extraction used (Van
Egmond, 1989). AFM$_1$ was reduced by 87% after 120 days of frozen storage at -18°C (McKinney et al., 1973). In contrast, AFM$_1$ in naturally contaminated milk at -18°C did not decrease after 68 days of frozen storage (Stoloff et al., 1975). Kiermeier and Mashaley (1977) also found that freezing AFM$_1$ in naturally contaminated milk at -18°C for 6 days, induced only a minor reduction of the toxin compared to that of artificially contaminated milk, which decreased by 32%. Frozen storage of AFM$_1$ in contaminated ice cream for eight months does not greatly affect the toxin (Wiseman and Marth, 1983d).

3) *Culture dairy product*

The combination of heat treatment followed by starter culture addition is a standard method for cultured dairy products that subsequently induces acid development. The fermentation process is ceased by storing the product at low temperatures. The effect of acidic pH on AFM$_1$ and AFB$_1$ was studied by using buffer solutions such as McIlvaine buffer (Doyle and Marth, 1978a; Wiseman and Marth, 1983b). The two aflatoxins were not affected by acidic pH. However, more recent studies reported that the aflatoxins were degraded by strong bases or acids in aqueous solution as well as in acidic milk (Considine and Considine, 1982; Rasic et al., 1991; Tabata et al., 1994).

Comparatively, no report on the effects of cultured milk on CPA are available. However, during a study on the effects of Japanese fermented foods on CPA, Goto and Manabe (1992) found that the toxin was also decreased by acidic pH but less by alkaline or neutral pH at 30°C in McIlvaine buffer.

Making yogurt is one method of processing milk without component separation. Acidity occurs due to fermentation. The effect of yogurt during processing and storage on CPA is currently not available in literature. In contrast to CPA, studies on AFM$_1$ found that the toxin was only slightly increased in yogurt made from naturally contaminated milk (Van Egmond et al., 1977; Wiseman and Marth, 1983a)

**1.5.C.b. Effect of processing milk on component separation**

Processing milk with component separation involves drying or concentrating milk, manufacture of ice cream, cream, butter and cheesemaking.
1) Manufacture of cheese

The effect of milk processing on CPA carry-over in contaminated milk or dairy products is not yet available in this literature. The only report on CPA in dairy products is due to the direct occurrence of the toxin in cheese, resulting from production by fungal growth used for fermentation or unintentional fungal growth (Van Egmond, 1989). Currently, no information on carry-over CPA into cheese is available.

For the production of mould ripened cheese, there are white surface mould cultures (*Penicillium camemberti*) and bluish to greenish moulds (*Penicillium roqueforti*) used to ripen the interior of cheese. CPA can also be produced by *Penicillium camemberti* (Still et al., 1978a; Le Bars, 1979; Engel, 1981; Schoch et al., 1984; Van Egmond, 1989). Reports on the finding of CPA in commercial cheese samples are shOWN in Table 1.10.

Based on the amount of CPA found in these cheeses, some reports claimed that the consumption of mould ripened cheeses would not generate health hazards for consumers (Van Egmond, 1989). However, no evidence has been presented on the circumstances where CPA carrying-over in milk used for making cheese was combined with the toxin resulting from the production by mould ripened cheese.

Allcroft and Carnaghan (1962; 1963) initially found that the rennet precipitated casein fraction from AMF, in naturally contaminated milk was similarly toxic to ducklings as the whole contaminated milk. The effect of making cheese on the toxin using milk contaminated with AMF, was contradicting and variable. AMF, was initially found only in whey (Purchase et al., 1972). Earlier study, however, revealed that the toxin was less concentrated than in the curd (Van Egmond et al., 1977).

The study on making different kinds of cheese by using contaminated milk revealed that AMF, was not degraded during cheesemaking and proved that carry-over of the toxin from milk into cheese did occur (McKinney, 1973; Stubblefield and Shannon, 1974; Van Egmond et al., 1977).
Table 1.10. Cyclopiazonic acid reported in commercial cheese samples  
(Camembert, Brie)  

<table>
<thead>
<tr>
<th>Number of examined cheeses</th>
<th>Number of positive samples</th>
<th>Concentration range of cyclopiazonic acid (mg/Kg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>9</td>
<td>5: 0.1 - 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Still  <em>et al.</em>, 1978a; 1978b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4: 0.7 - 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>11</td>
<td>3: 0.05 - 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Le Bars, 1979</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5: 0.1 - 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3: 0.4; 1; 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>0.06 - 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Frevel, 1981</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>0.08; 0.25; 0.37</td>
<td>Schoch <em>et al.</em>, 1983</td>
</tr>
</tbody>
</table>

<sup>a</sup> In the whole cheese,  
<sup>b</sup> Calculated for the cheese rind,  
<sup>c</sup> Van Egmond (1989).

The effect of ripening on AFM<sub>1</sub> was contradicting due to the inadequacy of the type of method used (Brackett and Marth, 1982b). The majority of reports maintained that AFM<sub>1</sub> was not greatly affected throughout long periods of ripening of most cheeses including Cheddar, Gouda or Mozzarella (Van Egmond *et al.*, 1977; Brackett and Marth, 1982b; 1982c). Most researchers reported that carry-over of AFM<sub>1</sub> did occur into cheese and the level of the toxin in cheese was 40-60% of the amount found in milk (Van Egmond, 1989). The association of the toxin with casein was proved by a higher concentration of AFM<sub>1</sub> in cheese than in milk used to make cheese.
2) **Manufacture of cream and butter**

The effect of the manufacture of cream and butter using CPA contaminated milk is not known.

The manufacture of cream and butter using AFM₁ contaminated milk revealed that the toxin remained more in skim milk and butter milk than in cream or butter (Grant and Carlson, 1971; Stubblefield and Shannon, 1974; Kiermeier and Mashaley, 1977).

3) **Drying or concentration of milk**

Removing water to dry or concentrate milk is known not to affect CPA in milk.

The concentration of naturally contaminated milk to half of its initial volume using reduced pressure at 40°C induced a reduction of AFM₁ by 64% (Purchase et al., 1972). Freeze-drying milk contaminated with the toxin decreased it by 12% compared to the original level (Kiermeier and Mashaley, 1977). Spray drying also reduced the amount of the toxin by 86% (Purchase et al., 1972)
SECTION (3): GENERAL CONCLUSION

I.6. CONCLUSIONS FROM THE LITERATURE

The review of the literature allows the following conclusions to be reached:

1. Cyclopiazonic acid is a secondary fungal metabolite co-produced with aflatoxin by several species of the fungal *Aspergillus*. It is highly possible the two mycotoxins occur simultaneously in food or feedstuffs. CPA is also produced by *Penicillium*. Fungal producers of CPA are widespread in nature and in commercial use. Limited reports on CPA occurrence in a number of agricultural products, animal products and some cultured dairy products lead to the conclusion that exposure of the toxin to both humans and animals through consumption of contaminated food and feed is likely.

2. CPA is an indole tetramic acid toxic to all animal species studied. CPA is unlikely to be hepato-carcinogenic but it has been shown to be either mutagenic or immunosuppressive (Purchase, 1971; Nuehring *et al.*, 1985; Pier, 1991). CPA has as principal toxic target organs the liver, kidneys and digestive system and acts primarily as an entero-nephrotoxin. The effects of CPA vary depending on the animal species. The LD$_{50}$ ranges from 2.3 to 69.6 mg/Kg. The toxin has pharmacological properties similar to chlorpromazine and reserpine. It can induce neurological symptoms in animals and acts similarly to anti-psychotic drugs. The occurrence of CPA in human food indicates a high potential for the toxin to cause neurotoxic effects in man.

3. CPA accumulates in skeletal muscle, carcasses and meat products. Significant amounts of CPA are found in the milk of orally dosed lactating animals. It can also occur in eggs. Multiple doses of CPA can increase the amount of carry-over in milk causing a cumulative effect.

4. Finding CPA in milk following oral dosing is a turning point as it discloses the potential for human exposure to the toxin through the consumption of contaminated milk or dairy products.
5. Current available information on CPA in dairy products is only due to the direct contamination of cheese through fungal growth that has been used for fermentation or because of unintentional fungal growth. However, no information on CPA carry-over into cheese from the milk is available. Knowledge of CPA during or following milk handling and milk processing is lacking.

6. Despite the fact that CPA and aflatoxin can be co-produced by Aspergillus flavus and have been shown to occur in the same commodities and to have additive toxic actions, information on CPA in milk and its exposure to humans is still not well reported in the literature compared to that for aflatoxins.

7. The effect of milk processing into dairy products on aflatoxin stability including with or without separation of the components of milk using heat or cold treatments, biological agents in culture, the removal of water, whey or cream has been evidently reported. However, the effect on CPA of the milk processing is not available in literature.

8. The treatment and decontamination of AFM₁ in milk using physical or chemical agents have economic significance. No information is available on the effects of such treatments on CPA in milk. It is not known if some chemicals used to treat AFB₁ have any effect on CPA.
SECTION (4): HYPOTHESIS AND AIM

1.7. HYPOTHESIS

1. Since the CPA-aflatoxin producing fungi are widespread, it is expected that the incidence of CPA in dairy products should also be widespread. The low incidence of CPA detection may be due to the lack of sensitive analysis methods for systematic surveys or due to the instability of CPA itself or the handling, treatment and processing of dairy products, that may affect the stability of CPA.

2. If this is the case, CPA may pose a potential health risk for dairy consumers specially children due to its neurotoxic and nephrotoxic properties.

1.8. AIM OF THE STUDY

The present study has the following objectives:

1. To find a sensitive method for CPA extraction from milk or dairy products and elaborate on a new method to analyse CPA by using a sensitive instrument, which is easy to manipulate and that has not been used before in CPA analysis such as Capillary Electrophoresis (CE).

2. To assess CPA behaviour in milk from farm to factory and its effect on milk analysis. Evaluate the effect of milk processing with or without milk component separation on CPA in contaminated milk. Assess CPA behaviour in milk under different pH levels, temperatures, UV energies and the duration of treatment.

3. To assess CPA stability during the processing of dairy products. Assess the rate of CPA carry-over from milk into dairy products and the partition behaviour of the toxin between fractions such as whey, curd or buttermilk.

4. Study the potential of reported methods to destabilise AFM to decontaminate CPA.
CHAPTER II

METHOD DEVELOPMENT:

ANALYSIS OF CYCLOPIAZONIC ACID IN MILK BY CAPILLARY ELECTROPHORESIS.

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II.2. CAPILLARY ELECTROPHORESIS.

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II.2.B. Theory.

II.2.B.a. Electrophoresis

II.2.B.b. Electroosmosis

II.2.B.c. Flow dynamics

II.2.B.d. Efficiency

II.2.B.e. Resolution

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II.2.D. Sample injection, separation, and detection

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II.4. RESULTS AND DISCUSSION

II.1. INTRODUCTION

The fungi producing CPA are described in Chapter I. They are widespread in nature, utilised commercially and, some of them, produce both CPA and aflatoxins. The toxicity of CPA both acute and chronic in both animals and humans was previously examined in Chapter I. The toxin is detectable in agricultural commodities and in animal feed. This indicates the high probability of its presence in animal products. Toxicity to lactating animals and laying birds confirms a potential health risk to animals and consumers (Cole et al., 1988; Elrington, 1991). CPA fed to a lactating animal induced milk carry-over within a day (Chapter I). The amount of CPA in milk increases double fold after a second dose. Withdrawal of the toxin from feeding to animals did not result in elimination of CPA from milk for several days (Dorner, et al., 1994). However, the incidence of CPA in milk and its exposure to humans is still not well reported in the literature compared to that for aflatoxins. The lack of reported CPA contamination in milk is possibly due to the lack of a rapid reproducible and low cost assays for the screening of this mycotoxin (Jelinek et al., 1989).

CPA detection in agricultural commodities and food has been performed by thin-layer chromatography (TLC) (Lansden, 1986), colorimetric (Rathinavelu and Shanmugasundaram, 1984; Rao and Husain, 1987) and spectrophotometry (Chang-Yen and 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 and Weiler, 1991) with a variability of 14% from day to day and 3.7% between intra-assays. Various normal and reverse phases of high performance liquid chromatography (RPLC) have been documented, however peaks obtained were too broad (Betina, 1993). Urano et al., (1992a) recently published a sensitive RPLC method applied to corn and peanuts. Simunek, et al., (1992) used a gel diffusion and the reported colorimetry method of Rao and Husain, (1987) to detect CPA in dairy products. A new method to detect CPA was recently reported (Matsudo and Sasaki, 1995). However, the reported methods are time consuming, laborious and complex. Few of them are sensitive but they are also expensive and material needed for the technique is not readily available.
in the market such as monoclonal antibodies for CPA. Few techniques require short
times and simultaneously detect CPA with other mycotoxins, allowing a screening of
multiple samples in a short period. Furthermore, few methods except TLC and gel
diffusion are used to detect CPA in milk and dairy products.

Recently, environmental pollutants including aflatoxins and six other
mycotoxins were successfully separated by Micellar electrokinetic capillary
chromatography (MEKC), one of modes of capillary Electrophoresis (CE) (Holland
and Sepaniak, 1993).

II.2. CAPILLARY ELECTROPHORESIS.

II.2.A. Overview

Despite the overall simplicity of the instrumentation, capillary
electrophoresis has properties similar to a combination of traditional polyacrylamide
gel electrophoresis (PAGE) and modern high performance liquid chromatography
(HPLC). CE is capable of separating several charged and water soluble molecules in
a single analysis. CE requires only a small amount of sample, reagents and uses
modern detector technology permitting electropherogram generation resembling a
chromatogram. In several applications, CE showed a greater efficiency compared to
gas and high performance liquid chromatography (Wätzig and Dette, 1993; Marina
and Torre, 1994).

CE is reported to be used in diverse fields including biological
macromolecules, amino acids, chiral drugs, vitamins, pesticides, inorganic ions,
organic acid, dyes, surfactants, peptides, proteins, carbohydrates, oligonucleotides,
DNA, cells, virus particles and food (Cancalon, 1995). From 1983, increasing
development of CE methods and applications has been shown by the rise of reports
in the literature. In our studies on method development of CE, we also found that one
of the CE mode was applicable in the separation of food antioxidants found in milk
or in wine (Prasongsidh and Skurray, 1998)*. The surfactant anionic (SDS) and

* Prasongsidh, B. C. and Skurray, G. R. (1998) Capillary electrophoresis analysis of
trans- and cis-resveratrol, quercetin, catechin and gallic acid in wine. Food
organic modifiers (7% acetonitrile) were added to the used buffer to help control the migration direction and separation. In a single analysis, the pure antioxidants such as gallic acid, catechin, quercetin, cis and trans-resveratrol were clearly separated in less than 11 minutes (Figure 2.1). Application of the CE method to detect antioxidants in wine samples proved to be successful (Figure 2.2). We used this CE method as a tool to study the presence of resveratrol, the compound known as an anticancer and cardio-protective agent in Australian wines (Skurray and Prasongsidh, 1997) **.

II.2.B. Theory. (Schwartz et al., 1992; Heiger, 1993)

II.2.B.a. Electrophoresis

The differential migration of solutes in an electric field induces separation by electrophoresis. The electric field strength depends on applied voltage and the length of the capillary. The electrophoretic mobility is given as follows:

\[
\mu_{ep} = \frac{v_{ep}}{E} \quad (1)
\]

where 
- \( v_{ep} \) = migration velocity (cm/s)
- \( \mu_{ep} \) = electrophoretic mobility (cm²/Vs)
- \( E \) = electric field strength (V/cm)

Figure 2.1. Electropherogram of the standards of resveratrol, catechin, gallic acid (250 µg/mL) and quercetin (100 µg/mL).

Conditions: Bare fused silica capillary-extended light path, 50µm i.d. x 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/50µA and power 4.5 W; cassette temperature, 40°C and run time 12 min. A peak of noise appeared at approximately 11 minutes was not observed in the other electropherograms of standards.
Figure 2.2. Electropherogram of the cis- and trans-Resveratrol, catechin and quercetin in a wine sample.
### 2.6b Electroosmosis

Electroosmosis is one of the key processes that allow CE to operate. Efficient separation of large and small molecules that occurs in narrow-bore capillaries (20 - 200μm id.) and is due to high voltages inducing electroosmotic (EOF) and electrophoretic flow of buffer solutions and ionic species respectively. EOF is the bulk flow of liquid in the capillary and a result of the surface charge on the wall. An electrical double layer occurs due to the attraction of positively charged ions from the buffer, by the wall of the capillary, which is negatively charged. Following a voltage application to the capillary, cations in the double layer move to the cathode bringing with them buffer solution. However, when untreated fused silica capillaries and neutral or alkaline buffer are used, most solutes migrate in the direction of the cathode regardless of the charge.

In general, the magnitude of the EOF depends on the surface charge on the capillary wall, pH and electrolyte concentration in the buffer used. Volume flow of the buffer is high at an alkaline pH. By controlling the EOF, all species including cations, anions or neutral can be analysed in a single analysis. The electroosmotic flow is given as follows:

\[
\nu_{eo} = \frac{\varepsilon \zeta}{4\pi\eta} E
\]

(2)

where

- \( \nu_{eo} \) = electroosmotic flow (nL/s)
- \( \varepsilon \) = dielectric constant (C²/J cm)
- \( \zeta \) = zeta potential (V)
- \( E \) = electric field strength (V/cm)
- \( \eta \) = viscosity of the buffer (N s/cm²)
II.2.B.2 Flow dynamics.

Compared to liquid chromatography where a pressure-driven system is used, CE has an electrical-driven system, which generates a uniform distribution of EOF inducing similar flow velocities in every part of the capillary.

From equation (1), the migration velocity is defined as follows:

\[ v_{ep} = \frac{\mu_{ep} E}{L} \quad (3) \]

where

- \( v_{ep} \) = migration velocity (cm/s)
- \( \mu_{ep} \) = electrophoretic mobility (cm²/Vs)
- \( E \) = electric field strength (V/cm)
- \( V \) = applied voltage (kV)
- \( L \) = total capillary length (cm)

Electrophoretic separation occurs in the capillary tubing at 500 Volts/cm or higher. The use of high electrical fields cuts analysis time and provides high resolution of peaks. Migration time or the period required a solute to reach the detection point depends on the length of capillary used, the voltage applied and the electric field. The migration time is defined as follows:

\[ t = \frac{L}{v_{ep}} = \frac{L^2}{\mu_{ep} V} \quad (4) \]
where \( t \) = migration time (s) 
\( v_{ep} \) = migration velocity (cm/s) 
\( \mu_{ep} \) = electrophoretic mobility (cm\(^2\)/Vs) 
\( L \) = total capillary length (cm) 
\( V \) = applied voltage (kV)

The application of high electric fields induces the solute to spend less time in the capillary and has less chance to diffuse. Molecular diffusion may occur while the solute migrates through the capillary and induces dispersion of the peaks. Efficiency of the system is related to the molecular diffusion. Standard deviation of the peak and diffusion coefficient of the solute are related as follows:

\[
\sigma = \frac{2DL^2}{\mu_{ep}V}
\]

where \( t \) = migration time (s) 
\( \sigma \) = standard deviation of the peak 
(in time, length or volume) 
\( \mu_{ep} \) = electrophoretic mobility (cm\(^2\)/Vs) 
\( L \) = total capillary length (cm) 
\( V \) = applied voltage (kV) 
\( D \) = diffusion coefficient of the solute (cm\(^2\)/s)
II.C.3.D. Efficiency:

The efficiency of CE is derived from several factors including migration velocity, migration time, peak dispersion due to molecular diffusion and the number of theoretical plates. The efficiency can be expressed in the number of theoretical plates as follows:

\[ N = \left( \frac{L}{\sigma} \right)^2 \]  \hspace{1cm} (6)

where

- \( N \) = number of theoretical plates
- \( L \) = total capillary length (cm)

Substituting equation (5) into equation (6), number of theoretical plates can be calculated by:

\[ \frac{\mu_{ep} V}{2D} \]  \hspace{1cm} (7)

where

- \( N \) = number of theoretical plates
- \( \mu_{ep} \) = electrophoretic mobility (cm\(^2\)/Vs)
- \( V \) = applied voltage (kV)
- \( D \) = diffusion coefficient of the solute (cm\(^2\)/s)
Based on equation (7), macromolecules having small diffusion molecules produce a high number of theoretical plates. It is understood that the use of high voltage induces great efficiency with a short separation time.

The theoretical plate number can also be calculated from an electropherogram using the following equation:

\[
N = 5.54 \left( \frac{t}{W^{1/2}} \right) \tag{8}
\]

where

- \( N \) = number of theoretical plates
- \( t \) = migration time (s)
- \( W \) = temporal peak width at height (s)

Other factors that also affect CE efficiency are temperature gradients generated by Joule heating, the injection plug length and the interaction of the solute with the walls of the capillary. Non-uniform temperature gradients, viscosity change in the capillary occur due to the heat cause by voltage and current applied. Therefore, employing high electric fields may induce great efficiency and resolution in the CE, but also generate Joule heating.

**II.2.B.e. Resolution.**

The resolution between two species in samples can be defined in several ways with respect to migration, dispersive process, and effect of EOF on resolution and efficiency. The resolution expressed with respect to efficiency is given as follows:
\[ R_s = \frac{1}{4} \frac{\Delta \mu_{ep}}{\mu_{ep}}N \]  
\[(9)\]

where 
- \( R_s \) = resolution between two species
- \( \Delta \mu_{ep} \) = difference in electrophoretic mobility
- \( \mu_{ep} \) = average in electrophoretic mobility
- \( N \) = number of theoretical plates

**II.2.C. Mode of CE.**

The basic modes of CE are capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF) and capillary isotachophoresis (CTTP). The basis of separation of each modes of CE is shown in Table 2.1.

Among CE modes so called “zonal” electrophoresis, CZE is the most simple to operate and possesses great flexibility. This explains why this mode is the most widely used. MEKC and CGE are also classified as zonal techniques. However, they have different separation mechanisms, which induce different selectivities. Separation mechanisms of CZE sometimes overlap that of MEKC when buffer additives are used. Correct selection of buffer, based on \( pK_a \) value of the substance to be analysed, is the factor that insures success in separation with CE.

**II.2.D. Sample injection, separation, and detection.**

The most common sample injections in CE are hydrodynamic and electrokinetic. Hydrodinamic injection is performed by application of pressure at the injection end of the capillary, vacuum at the exit end of the capillary or by siphoning action. In contrast, electrokinetic is conducted by replacing the injection-end reservoir with the sample vial and applying the voltage (Heiger, 1993). In CE, the
sample volume is not calculated in quantity but in pressure per time or voltage per time. Care has to be taken not to overload during sample injection in order to avoid acquiring a broad peak shape.

Table 2.1. Basis of separations of each modes of CE

<table>
<thead>
<tr>
<th>Mode</th>
<th>Basis of Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary zone electrophoresis</td>
<td>Free solution mobility, charge to mass ratio</td>
</tr>
<tr>
<td>Micellar electrokinetic chromatography</td>
<td>Hydrophobic / ionic interactions with micelle</td>
</tr>
<tr>
<td>Capillary gel electrophoresis</td>
<td>Size and charge</td>
</tr>
<tr>
<td>Capillary isoelectric focusing</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>Capillary isotachophoresis</td>
<td>Moving boundaries</td>
</tr>
</tbody>
</table>

(Heiger, 1993)

Volume of sample loaded by hydrodynamic injection is a function of the capillary dimension, the viscosity of the buffer, the applied pressure, the time used and can be calculated according to the Hagen-Poiseuille equation (Heiger, 1993) as follows:
\[
\text{Volume of sample loaded} = \frac{\Delta P d^4 \pi t}{128 \eta L} \quad (10)
\]

where \( \Delta P \) = pressure difference across the capillary
\( d \) = capillary diameter (\( \mu \)m)
\( t \) = time (s)
\( L \) = capillary length (cm)
\( \eta \) = viscosity of the buffer

Based on equation (10), pressure, time for injection and sample plug volume or length can be calculated.

Separation is performed in the capillary. Selection of the type of capillary depends on the nature of the substance to be separated (Marina and Torre, 1994). Chemically, electrically inert and UV-visible transparent materials are used for the capillary coating (Schwartz et al., 1992). Teflon capillaries with treated or untreated fused silica have been used in CE applications.

Several methods of detection have been used in CE. Similar to HPLC, UV-visible detection is the most commonly used due to its universal detection capability. For 10 nL injection volume, UV-visible absorption provides a limit of mass detection at \( 10^{-13} \) - \( 10^{-16} \) moles and a limit of concentration detection at \( 10^{-5} \) - \( 10^{-8} \). Other methods including fluorescence, laser induced fluorescence, amperometry, conductivity, mass spectrometry, radioactivity, thermal lens, refractive index, circular dichroism and Raman may provide higher limits of mass and concentration detection. However, they require special sample treatment, expensive, complicated instrumental modifications and are selective for certain types of samples.
The use of Diode-array detection (DAD) covers single and multiple wavelength detection, which presents a great benefit for new developing electrophoretic methods because there is no need to know beforehand the detector conditions and optimal wavelengths. A range of wavelengths from 190 to 600 nm can be selected. All solutes that can be absorbed between these wavelengths will be detected. DAD allows the monitoring of samples by using several wavelengths and can determine the absorbance maximum for all analytes. Currently, certain software can provide data in three-dimensional form or in isoabsorbance plots (time/wavelength/absorbance). Moreover, DAD allows the quantifying of non-separated peaks, validation of peak purity and confirmation of peak identity when there is doubt due to migration time or mobility measurement. DAD provides spectra at the peak apex and a spectral library that can give a statistical probability of the peak identity.

In recent years, the application of CE has been increasing in several areas due to CE offering a simple method development technique but also providing good separation. With a wide range of methods, CE has been used to separate and quantify substances in various fields including mycotoxins (Holland and Sepaniak, 1993). Since CPA is soluble in water and CE has several characteristics that allow a high separation of the mycotoxins in milk (Cole et al., 1992), practical application of CE for CPA detecting in milk was evaluated.

II.3. MATERIALS AND METHODS

II.3.A. Equipment, reagents and samples

II.3.A.a. Reverse phase liquid chromatography (RPLC)

The liquid chromatography system consists of two solvent delivery systems (Waters 501 and 510, Los Angeles, California, USA), injector Model U6K, Waters 484 tunable absorbance detector, Waters Baseline 810 Chromatography Workstation data handling system; LC column: Nova-pak, 150 x 3.9mm i.d., packed with C18, 5µm (Waters, Los Angeles, California, USA); Guard column: all-Guard GRP-2, Nova-Pak™ C18 (Waters, Waters, Los Angeles, California, USA); 16-port vacuum
II.3.A.b. Capillary electrophoresis (CE)

The capillary electrophoresis system HP10CE instrument was equipped with a replenishment system, vial tray, diode array detector (DAD), ChemStation data software (Hewlett-Packard, Waldbronn, Germany) and bare fused silica capillary (50μm i.d. x 64.5 cm, 60 cm effective length and alignment interface) with extended light path (150μm i.d. bubble) was used (Hewlett-Packard, Waldbronn, Germany).


Analar grade solvents were used. Tenuazonic acid (purity 99.9%), aflatoxins B₁, B₂, G₁, G₂ (purity 100%) were obtained from Sigma-Aldrich (Sydney, NSW, Australia). Milk samples were obtained from The Dairy Processing Plant at the University of Western Sydney, Hawkesbury, N.S.W., Australia. Artificial contamination of milk was done in similar fashion to the method of Wiseman and Marth (1983b), by adding CPA in methanol to 4°C milk agitated with a magnetic stirrer.

II.3.B. CPA Standards.


Pure solid CPA (Sigma-Aldrich, Sydney, NSW, Australia) was dissolved in methanol (liquid chromatography grade) at 1mg/mL and stored at 0°C in the dark.


Stock solution was diluted with methanol (liquid chromatography grade) with a CPA concentration of 40, 100, 500 ng/mL and 1, 5, 10 μg/mL.

II.3.C. Extraction and clean-up

Triple samples of 25 mL of milk were mixed with 25mL methanol-2% sodium bicarbonate mixture (7:3) (Urano et al., 1992a). The milk-methanol mixture was defatted with 100mL hexane (76°C boiling range) by shaking for 3 min. using a wrist action shaker (Flask shaker, Stuart Scientific, Melbourne, Victoria, Australia). Centrifugation (5,000 rpm for 5min.) assisted the hexane layer separation. The
sample layer was acidified to pH 3 with 6N HCl before being extracted twice for 30 min. with 100mL chloroform that was then dried with anhydrous sodium sulphate. The extract was evaporated at 40°C to dryness and redissolved in 5mL chloroform before being cleaned in a Sep-Pak plus silica gel cartridge by 10mL diethyl ether and 5mL chloroform-acetone mixture (1:1). CPA was eluted with 10mL chloroform-methanol mixture (75:25), which was subsequently evaporated to dryness. The residue was redissolved again with 1.5mL chloroform before being dried under nitrogen gas and kept in a dark screw-cap vial at 0°C until use.

II.3.D. Quantification

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

II.3.D.a. Capillary Chromatography Analysis

The temperature of the capillary’s cartridge was set at 40°C. In between analyses the capillary was washed with 0.1 M sodium hydroxide and bi-distilled water (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 watts of power, 20 kV of voltage and a current of 50 μA. As in similar reported studies on aflatoxins (Holland and 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 7s. The absorbance of CPA was recorded at 220nm wavelength. A standard curve was developed from 40 ppb to 100 ppm with a correlation coefficient at 0.99995.

II.3.D.b. Reverse Phase Liquid Chromatography Analysis

The analysis was conducted accordingly to the method of Urano et al., (1992a). The chromatograph 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 4mM zinc sulphate -methanol-water (85:15). The flow rate was set at 1mL/min. and the injection volume was 20µL. A standard curve was obtained from 500ppb to 100ppm with a correlation coefficient of 0.9999937.

II.3.D.c. Method of calculation of detectable CPA

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

\[ C_{cpa \, ng/mL} = R_{MEKC \, ng/µL} \times T_v \, µL \times (1/V \, mL) \times 2.5 \]  (11)

CPA analysed by RPLC was calculated with the equation:

\[ C_{cpa \, w \, ng/mL} = R_{RPLC \, ng/µL} \times T_v \, µL \times (1/V \, mL) \]  (12)

where

\[ C_{cpa \, w \, (ng/mL)} = \text{Concentration of CPA in test portion} \]
\[ T_v(µL) = \text{Total concentrated extracted elute (400 µL)} \]
\[ V(mL) = \text{Total volume of the test portion} \]
\[ R_{MEKC \, (ng/µL)} = \text{CPA in test solution injected and detected by MEKC} \]
\[ R_{RPLC \, (ng/µL)} = \text{CPA in test solution injected and detected by RPLC} \]
\[ 2.5 = \text{Coefficient of dilution of sample in MEKC} \]

Based on equation (11) and the Hagen-Poiseuille equation (10), the detectable limit of CPA by CE using MEKC mode was calculated.
II.3.E. **Presentation of Results and Statistic Analysis**

The results of the studies are in the form of means of triplicate samples along with the standard deviation of the mean (SD) and relative standard deviation (RSD). Variable measurements at the conclusion of the study were analysed using Student’s t-Test with two-tailed distribution. Significance is based on $p \leq 0.05$.

II.4. **RESULTS AND DISCUSSION.**

To minimise confusion, MEKC is hereafter referred as CE, unless otherwise specified.

The preliminary experiments involved optimising the 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 (Figure 2.3) found to detect and quantify CPA in milk are described in the method section. However, these conditions cannot ensure the 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 induced 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. (Figure 2.4). 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 high 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. Using the equation of Hagen-Poiseuille (10) and calculation of CPA in CE (11) in II.2.D and II.3.D.e respectively, the detectable limit of CPA by CE was $0.27 \times 10^{-7}$ pg/mL. The sensitivity of RPLC was restricted when the signal to noise ratio was <5:1.
Figure 2.3. Electropherograms of control milk (A); CPA (10μg/mL), aflatoxins B₁ and G₁ standards (5μg/mL) (B); and spiked milk extracts (CPA at 1μg/mL) (C).

Conditions: Bare fused silica capillary-extended light path, 50μm i.d. x 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/50μA and power 4.5 W; Cassette temperature, 40°C and run time 10 min.
Figure 2.4. Electropherograms of CPA (10μg/mL) and marker (A) and CPA (5μg/mL) with aflatoxins B₁ (3μg/mL) B₂ (1μg/mL) G₁ (1μg/mL) G₂ (1μg/mL), tenuazonic acid (5μg/mL) and marker sudan III (B).

Conditions: Bare fused silica capillary-extended light path, 50μm i.d. x 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/50μA and power 4.5 W; Cassette temperature, 30°C and run time 12 min.
In contrast, while still maintaining high theoretical plate numbers and resolution, CE was capable of isolating the CPA peak at lower quantity, although the sample injection volume (≈ 8.3 nL calculated with equation 10) was several times lower than that of RPLC (20 μL).

The ChemStation software (HP 3D CE, Hewlett Packard, Waldbronn, Germany) 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 the filtering out of spectral noise and quantification of CPA at low levels.

The extended light path capillary used in this study permitted the accumulation of a sample volume three times larger than that in the inner diameter of the capillary at the bubble cell where the light throughput was located. This allowed several fold increases in signal and thus improved the lower limit of detection as well as the linear detection range in CE. The variability in CE in an inter-assay basis between-days 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 every fourth injection. Conditioning of the capillary before each analysis and using a unique batch of mobile phase ensured a low variability between interassays. 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 concentrations than RPLC (Table 2.2)

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 and Torre, 1994).
Table 2.2. Recovery of CPA added to milk and quantified by Reverse Phase Liquid Chromatography (RPLC) and Micellar Electrokinetic Capillary Chromatography (MEKC)

<table>
<thead>
<tr>
<th>CPA spiked (ng/mL)</th>
<th>MEKC (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>RPLC (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg. (n=6)</td>
<td>Max.</td>
<td>Min.</td>
<td>SD</td>
<td>RSD</td>
<td>Avg. (n=6)</td>
<td>Max.</td>
<td>Min.</td>
<td>SD</td>
<td>RSD</td>
</tr>
<tr>
<td>20</td>
<td>81.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>89.9</td>
<td>66.7</td>
<td>8.3</td>
<td>6.7</td>
<td>nd&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>50</td>
<td>80.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90.0</td>
<td>74.4</td>
<td>6.6</td>
<td>5.3</td>
<td>70.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.0</td>
<td>62.3</td>
<td>5.8</td>
<td>4.1</td>
</tr>
<tr>
<td>100</td>
<td>79.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82.6</td>
<td>73.4</td>
<td>2.9</td>
<td>2.3</td>
<td>71.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83.2</td>
<td>67.7</td>
<td>5.7</td>
<td>4.1</td>
</tr>
<tr>
<td>200</td>
<td>80.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>89.7</td>
<td>76.8</td>
<td>4.4</td>
<td>3.5</td>
<td>80.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>85.6</td>
<td>69.8</td>
<td>7.2</td>
<td>5.8</td>
</tr>
<tr>
<td>500</td>
<td>77.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>84.3</td>
<td>69.9</td>
<td>4.7</td>
<td>3.7</td>
<td>76.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>96.0</td>
<td>70.4</td>
<td>8.9</td>
<td>6.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> not detectable

<sup>b</sup> p≤0.05 significant difference between CE and RPLC.

<sup>c</sup> no significant difference (p>0.05); SD- Standard deviation; RSD- Relative standard deviation
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. Sequential 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 a 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.

Figure 2.4 shows that CE method is capable of being used to detect and separate the other mycotoxins such as Tenuazonic acid, another indole acid, which occurs in tomatoes and tomato products (Stack et al., 1985). However, the method of extraction and cleaning of tenuazonic acid before injecting into CE needs to be further assessed.

These results indicate that CPA in liquid milk can adequately be extracted using the method as described in II.3.C and detected by CE techniques (II.3.D.a). However, it is important to know if CPA contaminated in other milk products could be adequately recovered by this method of analysis.

Duplicate samples of commercial dairy products from local supermarkets were spiked after grinding or using the method as described in II.3.A.c. with a solution of standard 1mg CPA/mL methanol (II.3.B.a.) to prepare contaminated samples equivalent to 1μg CPA/mL or 1μg CPA/g. Ten grams of powder products were mixed with water to make 30g prior to the spiking. Thirty grams of other dairy products were assayed. The following dairy products were tested and the mean of recovery rates were calculated as follows: Cheddar cheese (78%), butter (94%), ice cream (98%), evaporated milk (98%), concentrated milk (92%), yogurt (87%), full cream powdered (89%), skim milk powdered (93%), malted milk powdered (95%).

Capillary electrophoresis under the conditions used and described in this chapter proved to be sensitive enough to detect CPA at very low levels in milk and milk products. Using several facilities to confirm identity, quantify non-separated peaks and valid peak purity avoids false calculations or detections due to the change
of migration time and mobility measurement. With certain modifications according to the particular type of solute in each sample, the method can also be applied to detection and separation of other types of samples in other fields of analysis.

Since CE is adequately sensitive, the recovery of the analysis method is dependent on the method of extraction and optimum conditions of material and equipment. Especially, every step of CPA extraction from milk or dairy products needs to be carefully done to avoid the loss of the toxin. For instance, the speed applied to the shaker during every step of extraction needs to be optimised. Emulsification during sample defatting might also induce a possible loss of CPA if separation of different layers is not adequately done. At this stage, it is not known if acidic pH has an effect on the stability of CPA in aqueous solutions during the acidifying step prior to the extraction with chloroform. A prompt extraction appears to insure a good recovery. Moreover, every step of the cleaning of the extract following the evaporation to dryness by using Sep-Pak cartridge needs to be done carefully. The sensitivity of the method also depends on the quality of the defatting of the sample. Incomplete removal of the fat generates a partial blockage of cartridge, which causes poor elution by different mixtures of chloroform-methanol. This difficulty may be overcome by a quick filtration through 0.22 μm filter prior to cleaning in a Sep-Pak silica gel cartridge with diethyl ether. Incomplete removal of the fat following the cleaning in a Sep-Pak cartridge induces a carry over of fat following the second evaporation to dryness. Therefore, loss of CPA may also occur in the step where the residue is redissolved with methanol then with CE buffer solution. The extract of the sample should not be left over night and analysis by CE needs to be conducted on the same day.
CHAPTER III:

KINETIC STUDY OF CYCLOPIAZONIC ACID DURING THE HEAT-PROCESSING OF MILK. *

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III.1. INTRODUCTION

To render milk safe and to extend its shelflife, raw milk is commonly processed prior to human consumption. It undergoes several treatments including heating to eliminate microorganisms, inactivate enzymes and undergoes chemical or physical changes before further processing (Early, 1992). The thermal effect on the stability of CPA in milk may be similar to that of aflatoxins (Polzhofer, 1977; Gelosa and Buzzetti, 1994). Several model studies on heat processing of the aflatoxin M₁ have been documented (Purchase et al., 1972, Wiseman and Marth, 1983a).

Due to the significance of consumer health and the economics of dairy products (Early, 1992), the potential of thermally reducing and the level of CPA residues in milk following heat processing should be established. The overall objective of this study was to assess the stability of CPA to heat treatments similar to that used in the dairy industry, and to determine the thermo-resistance of the mycotoxin in milk to thermal destruction.

III.2. MATERIALS AND METHODS

III.2.A. Artificially contaminated milk

Whole unpasteurised milk was obtained from the same source as described in II.3.A.c. Considering the reports on CPA carry-over into milk of lactating animals, artificially contaminated milk was prepared at a concentration of 1µg CPA/mL (Elrington, 1991; Dorner et al., 1994). Cyclopiazonic acid (Sigma-Aldrich, Sydney, NSW, Australia) in methanol was used to artificially contaminated whole-unpasteurised milk as described in II.3.A.c.

III.2.B. Thermo-kinetics of CPA in milk heated to 60°, 80° and 100°C for one hour.

The heating of milk in this study was done similarly to the model of Stoloff, et al., (1975) to study aflatoxin M. Whole raw milk (500mL) containing 1µg CPA/mL was placed in a 1L Pyrex bottle. A magnetic stirrer was placed in the bottle prior it was hermetically closed with a screw cap, which was pierced by a thermocouple probe line connecting the milk to a squirrel data logger (model 1200, Grant Instruments, Cambridge, England). This data logger permitted the constant
monitoring and recording of the heat in the milk, which was initially warmed to the required temperatures by a hot plate stirrer (Cimarec 3, Barnstead/Termolyne, USA) before being transferred into a shaking water bath (Tecator 1024, Linbrook, Högana, Sweden) set at the same temperature. Pressure was released at regular intervals. Prior to any heat-treatment, control milk was extracted to determine if any natural contamination of CPA had occurred. Temperatures used to treat the contaminated milk samples were 60°C, 80°C and 100°C. Aliquots of the heated milk were taken at 15, 30, 45 and 60 minutes after reaching the desired temperature. The triplicate samples of 30mL of heated milk in bottle were then gradually cooled in water and then in ice water, before being extracted for CPA.

III.2.C. Thermo-resistance of CPA in milk after two hours of heating.

Using the same method and conditions, three trials of similar milk samples were heated for 2 hours. All temperatures in the milk were monitored and recorded. One group of three trials of heated milk samples was extracted for CPA analysis after cooling in ice water. Further decrease of CPA after high heating was assessed by leaving another set of treated milk at 4°C overnight before being extracted for CPA.

III.2.D. Stability of CPA in milk heated to 120°C.

Three samples of raw contaminated milk (500mL) at 1μg CPA /mL were canned (John Heine, Melbourne, Victoria, Australia) before being heated up to 120°C in a 1000L vertical retort (Ronald J. Murray & Son, Boiler Makers & Engineers, Town or Sydney, NSW, Australia). The thermo-evolution was monitored and recorded using a squirrel data logger. Pressure control in the retort permitted a rapid heating and cooling process.

Another set of similar milk samples contained in Pyrex bottles were heated to 120°C for 30 minutes in an autoclave (Atheron Equipment, Ballarat, Victoria, Australia). The milk samples were left for 30 minutes to cool in the autoclave before the CPA was extracted. Autoclave tape (3M, Ryde, NSW, Australia) and a thermometer indicated that the desired temperature and duration were reached.
III.2.E. CPA behaviour in milk under simulation of heat-treatments used by the dairy industry.

Similarly, contaminated raw milk (500mL) in 1L Pyrex bottles were heated according to the following method to study the thermo-kinetics of CPA 60°C for 30 minutes, 70°C, 90°C and 100°C for 15 seconds. The heat evolution was monitored and recorded. All samples were cooled down in ice water before extraction.

III.2.F. Analysis of Cyclopiazonic acid by capillary electrophoresis

Extraction of milk samples was conducted as previously reported in the section II.3.C. (Prasongsidh et al., 1998a). The samples were alkali with methanol-2% sodium bicarbonate mixture (7:3) before being defatted by hexane, acidified with hydrochloric acid, then extracted with chloroform. The samples were cleaned in a silica gel cartridge Sep-Pak® plus silica (Waters, los Angeles, California, USA) using diethyl ether, chloroform-acetone and chloroform-methanol. After evaporating the elution to dryness, the chloroform-methanol residue was eluted with liquid chromatography grade methanol then the mobile phase of the capillary electrophoresis prior to injection. The mobile phase of the CE and sample dilution were done as previously reported and as described in II.3.D.a. (Prasongsidh et al., 1998a; Prasongsidh and Skurray, 1998). They consisted of a mixture of sodium deoxycholate, disodium hydrogen phosphate, and disodium tetraborate at a pH 9.3. The capillary electrophoresis was equipped as described in section II.3.A.b. The temperature of the capillary cassette and the method of conditioning the capillary was the same as in section II.3.D.a. The polarity, applied voltage, current and power of the CE were set at the limits as described in II.3.D.a. The absorbance was recorded at 220 nm wavelength.

III.3. RESULTS AND DISCUSSION.


Data obtained from the sets of milk samples heated to 100°, 80° and 60°C for one hour are shown in Tables 3.1, 3.2, 3.3 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 any statistical significant difference (p>0.05) between 0.998 - 0.980 μg/mL. Every sample from three trials, taken each 15 minutes from when the milk reached 100°C had the amount of CPA significantly decreased (p≤0.05). In the first 15 minutes of heating at 100°C, more than 12% of the decrease was observed (Table 3.1). However, the decrease of the mycotoxin was less than 31% even after 1 hour of heating at this temperature.

Heating milk for a similar length of time to 80°C significantly (p≤0.05) reduced the amount of CPA by 14 - 18% (Table 3.2). In contrast, no significant decrease (p>0.05) in CPA in milk heated to 60°C was found in the first 15 minutes (Table 3.3). Significant decrease (p≤0.05) was observed after 30 minutes of heating in trials 2 and 3.

The original level of CPA was reduced by 3 - 9% after an hour of heating to 60°C. Even though all heat treatments failed to totally eliminate the mycotoxin from the milk in an hour, the decrease of CPA depends on time and temperature. Using the approach of the order of reaction study by using semilogarithm coordinates, Figure 3.1 was obtained and indicates that CPA in milk exposed to heat-treatment at 60°, 80° and 100°C induced kinetics of reaction following a pattern of a first order reaction.

**III.3.B. Effect of storage on CPA after long heat-treatment**

After 2 hours, the apparent decrease of CPA in milk heated to 100°C was pronounced (50%) (Table 3.4) Leaving overnight at 4°C induced a further loss of the mycotoxin to 54 - 62% of the original sample. Heating the milk at lower temperatures, 60° and 80°C for 2 hours resulted in a greater retention of CPA. Only 20 to 34% of CPA were denatured at 80°C but a further loss of 30 - 44% of the original level was observed after storage overnight at 4°C (Table 3.5). Two hours of heating to 60°C reduced the content of CPA to no more than 9 - 17% of the original value. A substantial further lost of 19 - 23% of CPA was observed after standing overnight at 4°C (Table 3.6).

Figure 3.2 reveals that the loss of CPA in milk after long time exposure to heat at 60°, 80° and 100°C was decrease and temperature dependent.
Table 3.1. Residue of CPA in contaminated milk at 1µg/mL heated to 100°C for 15 minutes to one hour.

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th></th>
<th></th>
<th></th>
<th>Trial 2</th>
<th></th>
<th></th>
<th></th>
<th>Trial 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA Residual µg/mL</td>
<td>SD</td>
<td>%</td>
<td>CPA Residual µg/mL</td>
<td>SD</td>
<td>%</td>
<td>CPA Residual µg/mL</td>
<td>SD</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>0.993</td>
<td>0.028</td>
<td>0.73</td>
<td>0.987</td>
<td>0.049</td>
<td>1.28</td>
<td>0.980</td>
<td>0.084</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td>15 min</td>
<td>0.879</td>
<td>0.042</td>
<td>12.08</td>
<td>0.879</td>
<td>0.018</td>
<td>12.10</td>
<td>0.877</td>
<td>0.112</td>
<td>12.34</td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>0.766</td>
<td>0.054</td>
<td>23.43</td>
<td>0.811</td>
<td>0.113</td>
<td>18.92</td>
<td>0.752</td>
<td>0.099</td>
<td>24.75</td>
<td></td>
</tr>
<tr>
<td>45 min</td>
<td>0.737</td>
<td>0.008</td>
<td>26.30</td>
<td>0.778</td>
<td>0.035</td>
<td>22.25</td>
<td>0.741</td>
<td>0.036</td>
<td>25.89</td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td>0.693</td>
<td>0.051</td>
<td>30.67</td>
<td>0.743</td>
<td>0.123</td>
<td>25.72</td>
<td>0.729</td>
<td>0.097</td>
<td>27.07</td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference with the original level p≤0.05
@ Triplicate samples;
'b' Milk heated at 100°C.
'c' Percentage degradation of CPA in milk.
'd' Relative standard deviation equal to 0.00; SD = Standard deviation.
Table 3.2. Residue of CPA in contaminated milk at 1μg/mL heated to 80°C for 15 minutes to one hour.

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th></th>
<th>Trial 2</th>
<th></th>
<th>Trial 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA Residual μg/mL&lt;sup&gt;®&lt;/sup&gt;</td>
<td>SD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>CPA Residual μg/mL&lt;sup&gt;®&lt;/sup&gt;</td>
<td>SD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>CPA Residual μg/mL&lt;sup&gt;®&lt;/sup&gt;</td>
<td>SD&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>&lt;sup&gt;80 °C&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>0.997</td>
<td>0.002</td>
<td>0.34</td>
<td>0.998</td>
<td>0.000</td>
<td>0.23</td>
</tr>
<tr>
<td>15 min</td>
<td>0.951&lt;sup&gt;·&lt;/sup&gt;</td>
<td>0.021</td>
<td>4.86</td>
<td>0.951&lt;sup&gt;·&lt;/sup&gt;</td>
<td>0.018</td>
<td>4.91</td>
</tr>
<tr>
<td>30 min</td>
<td>0.934&lt;sup&gt;·&lt;/sup&gt;</td>
<td>0.037</td>
<td>6.64</td>
<td>0.934&lt;sup&gt;·&lt;/sup&gt;</td>
<td>0.041</td>
<td>6.59</td>
</tr>
<tr>
<td>45 min</td>
<td>0.898&lt;sup&gt;·&lt;/sup&gt;</td>
<td>0.010</td>
<td>10.17</td>
<td>0.883&lt;sup&gt;·&lt;/sup&gt;</td>
<td>0.018</td>
<td>11.66</td>
</tr>
<tr>
<td>60 min</td>
<td>0.863&lt;sup&gt;·&lt;/sup&gt;</td>
<td>0.027</td>
<td>13.67</td>
<td>0.833&lt;sup&gt;·&lt;/sup&gt;</td>
<td>0.008</td>
<td>16.74</td>
</tr>
</tbody>
</table>

<sup>·</sup> Significant difference with the original level p≤0.05
<sup>®</sup> Triplicate samples;
<sup>80</sup>Milk heated at 80°C.
<sup>·</sup>Percentage degradation of CPA in milk
<sup>d</sup>Relative standard deviation equal to 0.00; SD = Standard deviation.
Table 3.3. Residue of CPA in contaminated milk at 1μg/mL heated to 60°C for 15 minutes to one hour.

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th></th>
<th></th>
<th>Trial 2</th>
<th></th>
<th></th>
<th>Trial 3</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA Residual μg/mL</td>
<td>SD</td>
<td>%</td>
<td>CPA Residual μg/mL</td>
<td>SD</td>
<td>%</td>
<td>CPA Residual μg/mL</td>
<td>SD</td>
<td>%</td>
</tr>
<tr>
<td>0 min</td>
<td>0.995</td>
<td>0.026</td>
<td>0.54</td>
<td>0.993</td>
<td>0.021</td>
<td>0.74</td>
<td>0.998</td>
<td>0.004</td>
<td>0.20</td>
</tr>
<tr>
<td>15 min</td>
<td>0.980</td>
<td>0.015</td>
<td>2.04</td>
<td>0.977</td>
<td>0.044</td>
<td>2.30</td>
<td>0.986</td>
<td>0.005</td>
<td>1.44</td>
</tr>
<tr>
<td>30 min</td>
<td>0.972</td>
<td>0.037</td>
<td>2.84</td>
<td>0.963 *</td>
<td>0.009</td>
<td>3.70</td>
<td>0.957 *</td>
<td>0.037</td>
<td>4.34</td>
</tr>
<tr>
<td>45 min</td>
<td>0.969</td>
<td>0.019</td>
<td>3.06</td>
<td>0.963 *</td>
<td>0.029</td>
<td>3.73</td>
<td>0.933 *</td>
<td>0.018</td>
<td>6.67</td>
</tr>
<tr>
<td>60 min</td>
<td>0.967</td>
<td>0.031</td>
<td>3.28</td>
<td>0.9555</td>
<td>0.080</td>
<td>4.52</td>
<td>0.913 *</td>
<td>0.092</td>
<td>8.70</td>
</tr>
</tbody>
</table>

* Significant difference with the original level p≤0.05
® Triplicate samples;
\(^b\) Milk heated at 60°C.
\(^c\) Percentage degradation of CPA in milk.
\(^d\) Relative standard deviation equal to 0.00; SD = Standard deviation
Figure 3.1. Kinetics of reaction of CPA in milk heated to 60°, 80° and 100°C follow a pattern of 1st order reaction.
Table 3.4. Residue of CPA in contaminated milk at 1 μg/mL heated to 100°C for 2 hours and left over night at 4°C.

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA Residual μg/mL</td>
<td>SD d</td>
<td>% c</td>
</tr>
<tr>
<td><em>100°C</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original level</td>
<td>0.993</td>
<td>0.028</td>
<td>0.73</td>
</tr>
<tr>
<td>2 h of heating</td>
<td>0.496 *</td>
<td>0.154</td>
<td>50.44</td>
</tr>
<tr>
<td>2 h of heating &amp; 4°C overnight</td>
<td>0.381 *</td>
<td>0.011</td>
<td>61.91</td>
</tr>
</tbody>
</table>

* Significant difference with the original level p ≤ 0.05

* Triplicate samples;

* Milk heated at 100°C.

* Percentage degradation of CPA in milk.

* Relative standard deviation equal to 0.00; SD = Standard deviation.
Table 3.5. Residue of CPA in contaminated milk at 1μg/mL heated to 80°C for 2 hours and left overnight at 4°C.

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th></th>
<th></th>
<th>Trial 2</th>
<th></th>
<th></th>
<th>Trial 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA Residual</td>
<td>SD</td>
<td>%</td>
<td>CPA Residual</td>
<td>SD</td>
<td>%</td>
<td>CPA Residual</td>
<td>SD</td>
</tr>
<tr>
<td><strong>80°C</strong></td>
<td>µg/mL®</td>
<td></td>
<td></td>
<td>µg/mL®</td>
<td></td>
<td></td>
<td>µg/mL®</td>
<td></td>
</tr>
<tr>
<td>Original level</td>
<td>0.977</td>
<td>0.002</td>
<td>0.34</td>
<td>0.998</td>
<td>0.00</td>
<td>0.23</td>
<td>0.980</td>
<td>0.021</td>
</tr>
<tr>
<td>2 h of heating</td>
<td>0.779*</td>
<td>0.021</td>
<td>22.06</td>
<td>0.836*</td>
<td>0.056</td>
<td>19.40</td>
<td>0.658*</td>
<td>0.042</td>
</tr>
<tr>
<td>2 h of heating &amp; 4°C overnight</td>
<td>0.560*</td>
<td>0.044</td>
<td>44.05</td>
<td>0.703*</td>
<td>0.026</td>
<td>29.71</td>
<td>0.570*</td>
<td>0.069</td>
</tr>
</tbody>
</table>

* Significant difference with the original level p≤0.05
® Triplicate samples.
*b Milk heated to 80°C.
%c Percentage degradation of CPA in milk.
\[d\] Relative standard deviation equal to 0.00; SD = Standard deviation.
Table 3.6. Residue of CPA in contaminated milk at 1µg/mL heated to 60°C for 2 hours and left overnight at 4°C.

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPA Residual µg/mL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>%&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>b 60°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original level</td>
<td>0.995</td>
<td>0.026</td>
<td>0.54</td>
</tr>
<tr>
<td>2 h of heating</td>
<td>0.907&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.031</td>
<td>9.29</td>
</tr>
<tr>
<td>2 h of heating &amp; 4 °C overnight</td>
<td>0.804&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.064</td>
<td>19.63</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significant difference with the original level p≤0.05
<sup>a</sup> Triplicate samples;
<sup>b</sup> Milk heated to 60°C.
<sup>c</sup> Percentage degradation of CPA in milk.
<sup>d</sup> Relative standard deviation equal to 0.00; SD = Standard deviation.
Figure 3.2. The loss of CPA in milk after long time exposure to heat to 60°C, 80°C and 100°C had a decrease and temperature dependence (Residues of CPA in contaminated milk at 1μg/mL heated to 60°C, 80°C and 100°C for 2 hours and left at 4°C over night.)
Storage even at a low temperature still led to a substantial further loss of the mycotoxin. However, this prolonging of heat-processing at high temperature, normally affects the milk quality and denatures certain milk components. This still failed to eliminate the toxin from the milk.

III.3.C. Resistivity of CPA in milk to heat at 120°C.

Heating milk to 120°C in a retort for 30 minutes achieved a reduction CPA to 36.5% of its original content (Table 3.7). No significant difference (p>0.05) was found in three trials of milk treatment. Keeping the milk in hermetically closed cans during the period of heating in the retort may explain this finding. In contrast, the milk placed in Pyrex bottles and autoclaved at 120°C had a significant difference (p<0.05) between the first, second and third trials. Non uniform heating and the slow process of heating and cooling in the autoclave might have affected the level of CPA in trial 1. However, after 30 minutes in the autoclave at 120°C, the amount of CPA residue was not significantly different from milk heated in the retort (Table 3.8).

Using the temperatures and duration times similar to the dairy industry failed to decrease CPA by more than 7% of its original content. Table 3.9 indicates that the same temperature and duration as milk pasteurisation (60°C for 30 min.) only decrease CPA by 3.5%. High temperature-short time systems such as 70°C, 90°C and 100°C for 15 seconds slightly reduced the level of the mycotoxin by 0.39%, 2.1% and 6.6% respectively.

The observation that heat-processing of milk at 60° to 120°C from 15 seconds to 2 hours did not destroy all the CPA, indicates the high possibility that processing conditions do not remove CPA from contaminated milk. Some of the levels of temperature used in this project were similar to that of conventional heat processing. However, a significant residue of CPA persisted even after one hour of over-processing the milk (Table 3.1). Half an hour at 120°C still left more than 60% of the original amount of the mycotoxin in the milk (Table 3.7 and 3.8). Using short time treatments (1 sec.) but with a higher temperature of 133°C such as UHT processing, would not lead to greater losses of CPA.
Table 3.7. Residue of CPA in canned milk contaminated by 1μg/mL after being heated to 120°C for 30 min in a retort.

<table>
<thead>
<tr>
<th></th>
<th>CPA Residual µg/mL @</th>
<th>SD</th>
<th>RSD</th>
<th>% degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA at original content</td>
<td>0.999</td>
<td>0.001</td>
<td>0.000</td>
<td>0.10 †</td>
</tr>
<tr>
<td>Trial 1</td>
<td>0.638 **</td>
<td>0.002</td>
<td>0.000</td>
<td>36.19 *</td>
</tr>
<tr>
<td>Trial 2</td>
<td>0.637 **</td>
<td>0.053</td>
<td>0.000</td>
<td>36.29 *</td>
</tr>
<tr>
<td>Trial 3</td>
<td>0.649 **</td>
<td>0.031</td>
<td>0.000</td>
<td>35.11 *</td>
</tr>
</tbody>
</table>

* Significant difference with the original level p≤0.05

** No Significant difference between trials p>0.05

† Amount of CPA (%) decreased from milk originally contaminated at 1μg/mL.

@ Triplicate samples, SD = Standard deviation; RSD = Relative standard deviation.
Table 3.8. Residue of CPA in milk contaminated by 1µg/mL autoclaved at 120°C for 30 min.

<table>
<thead>
<tr>
<th>CPA Residual µg/mL @</th>
<th>SD</th>
<th>RSD</th>
<th>% degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA at original content</td>
<td>0.980</td>
<td>0.023</td>
<td>0.00</td>
</tr>
<tr>
<td>Trial 1</td>
<td>0.661 *</td>
<td>0.008</td>
<td>0.00</td>
</tr>
<tr>
<td>Trial 2</td>
<td>0.624 **</td>
<td>0.015</td>
<td>0.00</td>
</tr>
<tr>
<td>Trial 3</td>
<td>0.638 **</td>
<td>0.018</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Significant difference with the original level p≤0.05
** No significant difference between trials p>0.05
* Significant difference between trials p≤0.05
▷ Amount of CPA (%) decreased from milk originally contaminated at 1µg/mL.
@ Triplicate samples
SD = Standard deviation; RSD = Relative standard deviation
Table 3.9. Residue of CPA in milk containing 1μg CPA/mL heated to 60°, 70°, 90° and 100°C for duration similar to that used in the dairy industry.

<table>
<thead>
<tr>
<th>Temperature and duration of treatment</th>
<th>Residue of CPA µg/mL @</th>
<th>SD</th>
<th>RSD</th>
<th>% degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA at original content</td>
<td>0.980</td>
<td>0.023</td>
<td>0.00</td>
<td>2.03 ‡</td>
</tr>
<tr>
<td>60°C for 30 min</td>
<td>0.965 *</td>
<td>0.014</td>
<td>0.00</td>
<td>3.49</td>
</tr>
<tr>
<td>70°C for 15 s</td>
<td>0.996 **</td>
<td>0.017</td>
<td>0.00</td>
<td>0.39</td>
</tr>
<tr>
<td>90°C for 15 s</td>
<td>0.979 **</td>
<td>0.028</td>
<td>0.00</td>
<td>2.14</td>
</tr>
<tr>
<td>100°C for 15 s</td>
<td>0.934 *</td>
<td>0.004</td>
<td>0.00</td>
<td>6.57</td>
</tr>
</tbody>
</table>

@ Triplicate samples.

* Significant difference with the original level p \leq 0.05

** No significant difference with the original level p > 0.05

‡ Amount of CPA (%) decreased from milk originally contaminated at 1μg/mL.

SD = Standard deviation RSD = Relative standard deviation
### CHAPTER IV:

STABILITY OF CYCLOPIAZONIC ACID DURING STORAGE AND PROCESSING OF MILK. *

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
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<td>IV.2.</td>
<td>MATERIALS AND METHODS</td>
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<td>Stability of CPA in frozen storage and in freeze-dried milk.</td>
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<td>Behaviour of CPA during the processing of concentrated and evaporated milks.</td>
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<td>RESULTS.</td>
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<tr>
<td>IV.3.A.</td>
<td>Stability of CPA in milk from farm to dairy plant.</td>
<td>99</td>
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<td>IV.3.B.</td>
<td>Plate counts of unpasteurised milk containing CPA.</td>
<td>101</td>
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<td>IV.3.C.</td>
<td>Effect of milk storage on CPA</td>
<td>102</td>
</tr>
<tr>
<td>IV.3.D.</td>
<td>Stability of CPA in processed milk with component separation.</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>IV.3.D. a. Effect of milk concentration on CPA</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>IV.3.D. c. Carry-over of CPA from liquid milk into milk powder</td>
<td>109</td>
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<tr>
<td>IV.4.</td>
<td>DISCUSSION AND CONCLUSION</td>
<td>109</td>
</tr>
</tbody>
</table>

IV.1. INTRODUCTION

Milk contamination by mycotoxins is a serious problem in food safety (Robinson et al., 1979; Lück and Wehner, 1979; Applebaum et al., 1982a; Maragos and Richard, 1994; Galvano et al., 1996) and the economy of the dairy industry. Most of the reported incidences of mycotoxins in milk (Jalon et al., 1994; Rajan et al., 1995) were due to aflatoxin M₁ (AFM₁). Information on the occurrence of CPA in milk and its exposure to humans is not well documented in the literature as for aflatoxin. Moreover, little is known on CPA stability during raw milk collection, storage and post milk processing.

Raw milk commonly undergoes several treatments including collection and storage on a dairy farm, bulk milk handling and transport to a dairy plant (≤4°C). After it is received and held at 2 - 3°C at a dairy plant, milk is tested, heat-processed, cooled then stored at <4°C prior to human consumption.

Before the era of pasteurisation (1930s), aetiology of several human diseases including typhoid, diphtheria and tuberculosis was from the bacteria contained in milk. Most important pathogens in raw milk are Salmonella, Campylobacter, Escherichia coli, Staphylococci, Listeria monocytogenes, Yersinta and others (Hughes, 1979; Bryan, 1983; Lovett et al., 1987).

The effect of CPA on certain bacteria has been reported and has become a tool for CPA detection in a disk diffusion assay (Madhyastha et al., 1994). However, the effect of CPA on viable bacteria in untreated milk or on Standard Plate Counts prior to pasteurisation of milk is not reported in the literature.

Chapter III shows evidence that simulated heat-treatments used by the dairy industry did not considerably degrade CPA (Prasongsidh et al., 1998b). The effect of processing and storage of milk on the stability of CPA may be similar to that of AFM₁ (Stoloff et al., 1975; Kiermeier and Mashaley, 1977). If this is true, CPA will likely be a potential risk to dairy consumers, especially children.

Due to public health and economic concerns, the stability of CPA following cold storage of milk and the effect of milk processing should be established. This is the objective of this study. Moreover, the effect of the presence of CPA at levels
similar to that reportedly found in milk of lactating animals on the results of Standard Plate Counts needs to be assessed.

IV.2. MATERIALS AND METHODS

IV.2.A. Preparation of artificially contaminated milk

Raw milk or pasteurised and homogenised whole milk were provided by the same source as described in III.2.A. Cyclopiazonic acid (Sigma-Aldrich, Sydney, NSW, Australia) in methanol was artificially contaminated in whole unpasteurised milk as described in II.3.A.c.

IV.2.B. Stability of CPA in storage of milk.

Three batches each of 1L of whole raw milk containing 1µg of CPA/mL were placed in each of 2L Pyrex bottles covered with aluminum foil, hermetically closed with a screw cap and kept at 4°C for 3 days to simulate collection and storage of raw milk on the dairy farm. On the fourth day, simulation of raw milk transport to the dairy plant was simulated by slowly shaking (10 shakes/min) the contaminated milk in a shaking water bath (Tecator 1024, Linbrook, Höganäs, Sweden) containing cold water (4°C) continuously circulated by a peristaltic pump (Vera, Manostat, California, USA) from an ice-water tank. The temperature of the cold water was monitored and continuously recorded by a Squirrel data logger 1200 (Grant Instruments, Cambridge, England). Triplicate samples from each batch of contaminated milk were taken daily, except for the sampling on day 4 being conducted after 4 h shaking and 4 h holding at 4°C.

The storage of processed milk at retail stores was simulated in a similar manner to the report by Stoloff et al., (1975). Three batches of 2L of homogenised, pasteurised contaminated milk (1µg CPA/mL) were refrigerated at 4°C for 21 days in polyethylene milk bottles. On day 1, 7, 14 and 21, triplicate milk samples of 30mL were extracted and analysed for CPA.


CPA stability during frozen storage was conducted similarly to the model of Stoloff et al., (1975) who studied aflatoxin M. Two litres of whole pasteurised and contaminated milk at 1µg CPA/mL was chilled and kept in several aliquots of 30mL at -18°C for 140 days.
Another 2L of contaminated milk (1 μg CPA/mL) was divided into aliquots of 30mL. These were kept in 50mL sterilised plastic sample flasks with screw caps, subsequently frozen to -18°C over night and placed in a freeze-drier TM FD2 (Dynavac High Vacuum, Melbourne, Australia). All freeze dried milk samples were then stored at -18°C for 140 days.

Triplicate samples from both frozen and freeze-dried milks were extracted weekly and analysed for CPA during the first 4 weeks. Thereafter, the analysis for CPA in both experimental groups was done monthly for 140 days.

**IV.2.D. Behaviour of CPA during the processing of concentrated and evaporated milks.**

The concentration of contaminated milk was done as reported by Caric’ (1994). Four litres of cold whole raw milk containing 1 μg CPA/mL were preheated using a hot plate stirrer (Cimarec 3, Barnstead/Termolyne, USA) and held at 100°C for 10 min. The contaminated milk was then concentrated in a pilot scale steam-jacketed kettle (Librit, Carlingford, Australia). The concentration of 4L of milk was discontinued when the volume of milk was reduced to 1.5L. The concentrated milk was immediately cooled in an ice bath to 10°C, then stored at 4°C. Prior to heating the milk, triplicate control samples were taken. Another sample was taken when the cooling of the concentration milk was completed. The concentrated milk was stored at 4°C for 8 weeks and triplicate analysis for CPA was done weekly.

Four litres of contaminated and preheated milk was evaporated in a rotavapor (Büchi, Zurich, Switzerland) under vacuum at 60°C. The evaporation was stopped when the volume of milk reached 1L. Triplicate aliquots of 30mL each samples were extracted for CPA before and after evaporation. Evaporated milk was stored at 4°C. Triplicate samples from this milk were then analysed weekly for CPA.

**IV.2.E. Occurrence of CPA in spray dried milk powder.**

Milk powder was processed using a method similar to the one described by Caric’ (1994). Four hundred mL of whole raw milk containing 1 μg CPA/mL were placed in a 1L closed screw cap Pyrex bottle, initially heated to 71°C on a hot plate stirrer (Cimarec 3, Barnstead/Termolyne, USA) for 15s before being transferred into a shaking water bath (Tecator 1024, Linbrook, Höganå, Sweden) set at 85°C. After
15 min at this temperature, contaminated milk was evaporated in a rotavapor at 60°C under vacuum until the volume of milk was reduced to 200mL. The evaporated milk was transferred to a 250mL Pyrex bottle with a screw cap, which was pierced with a polyester tube connecting the milk in the bottle to a laboratory spray dryer (Lab ST, Anhydro, Copenhagen, Denmark). The contaminated milk was continuously fed into the spray dryer by a peristaltic pump (Vera, Manostat, California, USA) and sucked into the spray drying chamber through an atomiser under vacuum. The temperature in the drying chamber was set at 80°C. Triplicate samples of 5 g of milk powder were randomly taken in 3 areas on the spray drying chamber such as the outlet, the wall and the bottom of the chamber, which was connected to a cyclone.

### IV.2.F. Effect of CPA on Plate Count.

Viable bacteria in milk without or with artificial CPA contamination were estimated by the Standard Plate Count (SPC), the Dairy Test method officially used by the New South Wales Dairy Corporation (Anon, 1995). This Pour Plate method used tryptone glucose yeast agar CM 325 and 0.1% peptone solution as the media and diluent respectively (Oxoid, Manchester, England). Aliquots of milk samples (25mL) were kept in test tubes with closures (Sigma-Aldrich, Sydney, NSW, Australia) at 4°C before being contaminated with 5 to 25 µL of 100µg CPA/mL or 1mg CPA/mL in order to have solutions with final concentrations of CPA at 1, 0.5, 0.2, 0.1, 0.05 and 0.02µg/mL. Testing of one batch was conducted within minutes of contamination with CPA. Another similar batch kept at 4°C was tested 24 h later.

The testing consisted of three serial dilutions of 1mL milk samples aseptically taken and mixed into 99mL of the diluent. Prepared agar medium was cooled to 48°C prior to pouring (15mL) into petri dishes and thoroughly mixed with the diluted sample. The mixture was allowed to stand until the agar solidified. Incubation of the mixture was conducted at 30°C for 3 days before colony counting was done under Darkfield colony counters M 3327. The results were based on the mean of triplicate samples of the count per mL multiplied by 1000 (3 dilutions). Six new petri dishes poured with agar media only, and agar with milk, were taken as controls for agar plates and for milk respectively. The effect of methanol alone on
the bacteria in milk was assessed by pouring another six petri dishes with agar mixed with milk sample containing 5 or 25µL of the solvent without CPA.

**IV.2.F. Analysis of CPA by capillary electrophoresis**

CPA was extracted from milk products and analysed using the method of Prasongsidh et al. (1998a) as described in **II.3.C.** All results were expressed as means of triplicate samples and Student’s t-Test was used to analyse the data.

**IV.3. RESULTS.**

**IV.3.A. Stability of CPA in milk from farm to dairy plant.**

Table 4.1 indicates that if CPA was carried over into raw milk obtained from lactating animals, it would be stable throughout the period of collection and storage at 4°C at the dairy farm and during transportation to the dairy plant. A significant decrease of CPA occurred on day 4 only when samples were taken after the milk was slowly shaken for 4 h and held at 4°C for another 4 h. Shaking contaminated milk in this study not only simulated milk transport in an insulated tanker but also milk agitation to test for the possible presence of off-flavours before the milk is unloaded. It is not known whether the decrease in CPA level (1.4%) was due to the length of storage or due to the shaking. It is also not known whether CPA may behave similarly to AFM, and be capable of binding to casein (Brackett and Marth, 1982a). A study on CPA’s association with casein or other milk proteins is needed before any further explanation can be attempted.

Since CPA seems to persist in raw milk and processed milk may not be available in rural areas, milk can only be consumed safely after boiling. However, boiling contaminated milk failed to completely eliminate CPA (Prasongsidh et al., 1998b). Moreover, a heat treatment similar to milk pasteurisation (60°C for 30 min) was also unable to eliminate CPA from processed milk. Thus, there is the great possibility that CPA remains undestroyed in heat processed milk.
<table>
<thead>
<tr>
<th>Period of storage</th>
<th>CPA Residual (μg/mL)</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.997</td>
<td>0.029</td>
<td>0.000</td>
<td>0.3</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.995</td>
<td>0.002</td>
<td>0.000</td>
<td>0.5</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.995</td>
<td>0.002</td>
<td>0.000</td>
<td>0.5</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.986</td>
<td>0.000</td>
<td>0.000</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*Under conditions of milk transport simulation from farm to dairy plant.

*Significant difference with other days (p<0.05).

** Sampling after 6 h of shaking at 10 shakes per min in 4°C water bath.

SD = Standard deviation; RSD = Relative standard deviation.
IV.3.B. Plate count of unpasteurised milk containing CPA.

Figure 4.1. Effect of CPA at 0.02, 0.05, 0.1, 0.2, 0.5 and 1 μg/mL and of methanol on the estimation of the count of viable bacteria in milk using a Plate Count with the Pour Plate method.

One batch was tested following contamination with CPA. Another batch was tested following 24 h of incubation at 4°C. The results were based on the mean of triplicate samples of the count per mL multiplied by the dilution factor. (Standard variation: 1 - 9.6)

Figure 4.1 shows that 5 μL of methanol alone has an effect (p≤0.05) on the viability of bacteria in unpasteurised milk (25 mL) either with or without 24 h incubation. A further decrease of colonies was observed when 25 μL of methanol was added to the milk. Compared to milk samples 0.02 μg CPA/mL and 0.2 μg CPA/mL contaminated with 5 μL of methanol containing 100 μg CPA/mL or 1 mg...
in colonies of bacteria was not only due to 5μL methanol, but also due to CPA at the concentration at 0.02μg/mL and 0.2μg/mL. Similar decrease was also observed in milk sample 0.05μg CPA/mL and 0.5 μg/mL contaminated with 12.5μL methanol containing 100μg/mL or 1mg/mL respectively. Milk samples added with CPA (0.1μg/mL or 1μg/mL) and 25μL methanol had fewer colonies than milk, which contained methanol only regardless 24 h incubation or not.

However, the number of colonies found in milk samples containing CPA at 1 and 0.5μg/mL tended to be higher than that of the sample containing 0.1 and 0.05μg/mL (p>0.05). This is not fully understood. It seems that CPA has some effect on the viability of bacteria in milk when comparing the number of colonies between the control unpasteurised milk and other milk samples containing CPA. However, it appeared that this decrease in colonies was not dependent on the amount of CPA used.

A similar trend towards a decrease in colonies was observed both in CPA contaminated samples with or without incubation for 24 h at 4°C. Since milk contains several organisms, a study on individual bacterium is needed. Moreover, the effect of natural contamination by CPA on bacteria in milk needs to be further assessed before any conclusion can be attempted.

IV.3.C. Effect of milk storage on CPA

Artificially contaminated (1μg CPA/mL) homogenised and pasteurised milk that had been stored at 4°C for 21 days to simulate storage in a retail shop showed a decrease in CPA after week 1 (Table 4.2). This became a significant decrease in CPA after 3 weeks (5.8%). The possibility for CPA to reach dairy consumers appeared high due to its stability throughout the simulated period of storage in retail stores.

The storage of milk products at very low temperatures favoured CPA stability despite long periods of storage (Tables 4.3 and 4.4). A significant decrease in levels of CPA was, however, observed after weeks 2 and 3 in frozen milk and freeze dried milk respectively. Evaporated milk, stored at less than 10°C, can be kept for up to a year without a significant quality change. CPA was also stable in liquid frozen milk and in powdered freeze dried milk stored at -18°C.
Table 4.2. Residue of CPA in homogenised and pasteurised milk stored at 4°C for 21 days.

<table>
<thead>
<tr>
<th>Period of storage</th>
<th>CPA Residual (µg/mL)</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.999</td>
<td>0.026</td>
<td>0.000</td>
<td>0.1</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.972&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.002</td>
<td>0.000</td>
<td>2.8</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.971&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.025</td>
<td>0.000</td>
<td>2.9</td>
</tr>
<tr>
<td>Day 21</td>
<td>0.942&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.004</td>
<td>0.000</td>
<td>5.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Under conditions of milk storage simulating commercial retail stores.

<sup>b</sup> No significant difference with day 1 (p>0.05).

<sup>c</sup> Significant difference with day 1 (p≤0.05).

<sup>d</sup> Triplicate samples.

SD = Standard deviation, RSD = Relative standard deviation.
Table 4.3. Residue of CPA in homogenised and pasteurised milk stored frozen at -18°C for 140 days.

<table>
<thead>
<tr>
<th>Period of storage</th>
<th>CPA Residual (µg/mL) ( {c} )</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.997</td>
<td>0.008</td>
<td>0.000</td>
<td>0.3</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.990( ^{a} )</td>
<td>0.046</td>
<td>0.000</td>
<td>1.0</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.959( ^{b} )</td>
<td>0.012</td>
<td>0.000</td>
<td>4.1</td>
</tr>
<tr>
<td>Day 21</td>
<td>0.950( ^{b} )</td>
<td>0.038</td>
<td>0.000</td>
<td>5.0</td>
</tr>
<tr>
<td>Day 28</td>
<td>0.932( ^{b} )</td>
<td>0.015</td>
<td>0.000</td>
<td>6.8</td>
</tr>
<tr>
<td>Day 56</td>
<td>0.922( ^{b} )</td>
<td>0.014</td>
<td>0.000</td>
<td>7.8</td>
</tr>
<tr>
<td>Day 84</td>
<td>0.906( ^{b} )</td>
<td>0.022</td>
<td>0.000</td>
<td>9.4</td>
</tr>
<tr>
<td>Day 112</td>
<td>0.904( ^{b} )</td>
<td>0.023</td>
<td>0.000</td>
<td>9.6</td>
</tr>
<tr>
<td>Day 140</td>
<td>0.892( ^{b} )</td>
<td>0.036</td>
<td>0.000</td>
<td>10.8</td>
</tr>
</tbody>
</table>

\( ^{a} \) No significant difference with day 1 (p>0.05).

\( ^{b} \) Significant difference with day 1 (p≤0.05).

\( ^{c} \) Triplicate samples; SD = Standard deviation; RSD = Relative standard deviation
Table 4.4. Residue of CPA in freeze dried homogenised and pasteurised milk stored at -18°C for 140 days.

<table>
<thead>
<tr>
<th>Period of storage</th>
<th>CPA Residual (μg/mL) (^c)</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.999</td>
<td>0.035</td>
<td>0.000</td>
<td>0.1</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.993 (^a)</td>
<td>0.113</td>
<td>0.001</td>
<td>0.7</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.958 (^a)</td>
<td>0.070</td>
<td>0.001</td>
<td>4.2</td>
</tr>
<tr>
<td>Day 21</td>
<td>0.917 (^b)</td>
<td>0.008</td>
<td>0.000</td>
<td>8.3</td>
</tr>
<tr>
<td>Day 28</td>
<td>0.917 (^b)</td>
<td>0.033</td>
<td>0.000</td>
<td>8.3</td>
</tr>
<tr>
<td>Day 56</td>
<td>0.912 (^b)</td>
<td>0.009</td>
<td>0.000</td>
<td>8.8</td>
</tr>
<tr>
<td>Day 84</td>
<td>0.913 (^b)</td>
<td>0.001</td>
<td>0.000</td>
<td>8.7</td>
</tr>
<tr>
<td>Day 112</td>
<td>0.894 (^b)</td>
<td>0.013</td>
<td>0.000</td>
<td>10.6</td>
</tr>
<tr>
<td>Day 140</td>
<td>0.890 (^b)</td>
<td>0.001</td>
<td>0.000</td>
<td>11.0</td>
</tr>
</tbody>
</table>

\(^a\) No significant difference with day 1 (p>0.05).

\(^b\) Significant difference with day 1 (p<0.05).

\(^c\) Triplicate samples; SD = Standard deviation; RSD = Relative standard deviation
When CPA residuals in both products were compared at day 21 of storage, the mycotoxin, decreased less in frozen milk (5%) than in freeze dried (8.3%). Tables 4.3 and Table 4.4 show a similar trend of CPA decrease in both frozen and freeze dried milk when stored for up to 140 days. CPA residuals in both products were not substantially different and the decrease of the mycotoxin was less than 12% on day 140.

IV.3.D. Stability of CPA in processed milk with component separation

Better keeping quality and extended shelf life of milk products are the main objectives of processing concentrated milk, evaporated milk or spray dried milk powders.

IV.3.D.a. Effect of milk concentration on CPA

Concentrating whole raw milk contaminated with 1µg CPA/mL in this study was achieved by boiling milk for 30 min in a steam-jacketed kettle following preheating at 100°C for 10 min. Based on the relative amount of milk, concentration from 4 to 1.5L, caused CPA to decrease in the final product by 39.7% (Table 4.5).

This decrease may be the result of CPA hydrolysis during preheating and boiling of the milk. However, it was previously reported that boiling of milk failed to eliminate CPA (Prasongsidh et al., 1998b). Moreover, the residual CPA left in concentrated milk was very stable throughout storage at 4°C for 8 weeks (Table 4.5)


Removal of water from milk containing 1µg CPA/mL by evaporation at a temperature of 60°C under vacuum appeared not to greatly affect the mycotoxin levels (0.7%). CPA decrease in the final evaporated product (Week 1) was accordingly estimated to be relative to the amount of milk evaporated from 4 to 1L (Table 4.6). However, a significant decrease in CPA in evaporated milk occurred from week 4 during storage at 4°C for 8 weeks. Heat treatment with a temperature lower than 60°C seemed to generate little hydrolysis of CPA during evaporation of the milk.
Table 4.5. Residue of CPA in 1.5L concentrated milk from 4L of whole raw milk containing 1µg CPA/mL and stored at 4°C for 8 weeks.

<table>
<thead>
<tr>
<th>Period of storage</th>
<th>CPA Residual (µg/mL)</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk prior to concentration</td>
<td>0.997</td>
<td>0.012</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>Week 1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.608&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.055</td>
<td>0.00</td>
<td>39.7</td>
</tr>
<tr>
<td>Week 2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.604&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.008</td>
<td>0.00</td>
<td>39.8</td>
</tr>
<tr>
<td>Week 3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.593&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.001</td>
<td>0.00</td>
<td>40.3</td>
</tr>
<tr>
<td>Week 4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.593&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.011</td>
<td>0.00</td>
<td>40.3</td>
</tr>
<tr>
<td>Week 5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.584&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.021</td>
<td>0.00</td>
<td>40.6</td>
</tr>
<tr>
<td>Week 6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.581&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.015</td>
<td>0.00</td>
<td>40.7</td>
</tr>
<tr>
<td>Week 7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.010</td>
<td>0.00</td>
<td>40.9</td>
</tr>
<tr>
<td>Week 8&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.574&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.083</td>
<td>0.00</td>
<td>41.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> No significant difference with week 1 (p>0.05). <sup>b</sup> Significant difference from milk prior concentrated (p≤0.05).
<sup>c</sup> Triplicate samples; SD = Standard deviation; RSD = Relative standard deviation.
<sup>d</sup> Decrease percentage of CPA in concentrated milk from week 1 to week 8.
<sup>e</sup> Milk concentrated and stored at 4°C.
Table 4.6. Residue of CPA in 1L evaporated milk from 4L of whole raw milk containing 1μg CPA/mL by using rotavapor under vacuum at 60°C.

The evaporated milk was then stored at 4°C for 8 weeks.

<table>
<thead>
<tr>
<th>Period of storage</th>
<th>CPA Residual (μg/mL)</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk prior to evaporation</td>
<td>0.998</td>
<td>0.012</td>
<td>0.000</td>
<td>0.2</td>
</tr>
<tr>
<td>Week 1 (^e)</td>
<td>3.973</td>
<td>0.024</td>
<td>0.001</td>
<td>0.7(^d)</td>
</tr>
<tr>
<td>Week 2 (^e)</td>
<td>3.929(^a)</td>
<td>0.037</td>
<td>0.001</td>
<td>1.8(^d)</td>
</tr>
<tr>
<td>Week 3 (^e)</td>
<td>3.931(^a)</td>
<td>0.006</td>
<td>0.000</td>
<td>1.7(^d)</td>
</tr>
<tr>
<td>Week 4 (^e)</td>
<td>3.873(^b)</td>
<td>0.015</td>
<td>0.001</td>
<td>3.2(^d)</td>
</tr>
<tr>
<td>Week 5 (^e)</td>
<td>3.846(^b)</td>
<td>0.021</td>
<td>0.001</td>
<td>3.8(^d)</td>
</tr>
<tr>
<td>Week 6 (^e)</td>
<td>3.834(^b)</td>
<td>0.032</td>
<td>0.001</td>
<td>4.1(^d)</td>
</tr>
<tr>
<td>Week 7 (^e)</td>
<td>3.803(^b)</td>
<td>0.035</td>
<td>0.001</td>
<td>4.9(^d)</td>
</tr>
<tr>
<td>Week 8 (^e)</td>
<td>3.764(^b)</td>
<td>0.064</td>
<td>0.002</td>
<td>5.9(^d)</td>
</tr>
</tbody>
</table>

\(^a\) No significant difference with week 1 (p>0.05).  \(^b\) Significant difference with week 1 (p≤0.05).

\(^c\) Triplicate samples; SD = Standard deviation; RSD = Relative standard deviation.

\(^d\) Decrease percentage of CPA based on milk evaporated and concentrated from 4 to 1L.

\(^e\) Milk evaporated and stored at 4°C.
A higher percentage of CPA residual in evaporated milk than in concentrated milk (Table 4.5 and 4.6) indicates that the processing of milk using low heat treatment to avoid loss of milk quality could also prevent CPA elimination from milk products.

IV.3. D. c. Carry-over of CPA from liquid milk into milk powder

Processing of milk powder by spray drying did not remove CPA. Its distribution was not uniform in milk powder following spray drying of milk at 80°C under pressure. Random sampling of milk powder confirmed a significant difference (p≤0.05) in CPA concentration. Triplicate 5 g samples were taken in several areas such as the door, the bottom and wall of the pilot scale spray dryer. A higher CPA residual was found in samples taken from the bottom of the drying chamber in the area next to the conduit to the cyclone (Table 4.7)

IV.4. DISCUSSION AND CONCLUSION

This study shows that CPA can be carried over into raw milk (Dorner et al., 1994) and the mycotoxin would be stable from milking throughout the retail period if milk is kept normally refrigerated at 4°C.

The occurrence of certain mycotoxins such as AFB₁ in milk may induce toxicity or carcinogenicity in consumers (Wogan and Newborne, 1967). AFB₁ is capable of increasing anaphylactic sensitivity to milk proteins (Prasongsidh et al., 1994). In contrast, certain mycotoxins such as AFM₁ do not boost anaphylactic reactions. CPA is an additive that induces certain diseases and is co-produced with AFB by the same group of fungi (Cole, 1986a; Cvetnic, 1994). CPA at low doses was found not to affect cutaneous hypersensitivity to Mycobacterium tuberculosis (Richard et al., 1986). However, since CPA was stable in simulated retail milk, it would be of great interest to evaluate the possible behaviour of CPA at the level found in milk and the anaphylactic reaction to milk protein. Moreover, further studies on the association of CPA with certain milk components such as casein and the effect on milk homogenisation during milk processing and CPA extraction and analysis are needed.
Table 4.7. Residue of CPA in milk powder obtained from spray drying of raw milk.

<table>
<thead>
<tr>
<th>Sampling areas (Spray dryer)</th>
<th>CPA Residual (µg/mL) (^b)</th>
<th>SD</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Door</td>
<td>0.658</td>
<td>0.023</td>
<td>0.000</td>
</tr>
<tr>
<td>Bottom of spray-drier tank</td>
<td>0.727 (^c)</td>
<td>0.020</td>
<td>0.000</td>
</tr>
<tr>
<td>Wall</td>
<td>0.692 (^d)</td>
<td>0.037</td>
<td>0.000</td>
</tr>
</tbody>
</table>

\(^a\) Two hundred mL contaminated milk continuously injected into pilot scale spray-drier and dried at 80°C under pressure.
\(^b\) Triplicate samples of 5 g milk powder.
\(^c\) Significant difference with samples taken from door of spray drier tank (p≤0.05).
\(^d\) No significant difference with samples taken from door and bottom of spray drier tank (p>0.05).

SD = Standard deviation; RSD = Relative standard deviation
Simulating industrial milk processing without component separation (Prasongsidh et al., 1998b) has been shown not to eliminate CPA. Processing milk with component separation in this study indicated that CPA is stable when a low heat treatment is used. However, high heat treatment such as boiling milk to remove water did not eliminate the mycotoxin. The potential of CPA to reach milk consumers appeared to be high since it persisted in all forms of processed milk products including liquid, frozen, freeze dried and spray dried milk.
CHAPTER V:
BEHAVIOUR OF CYCLOPIAZONIC ACID DURING
THE MANUFACTURE AND STORAGE OF YOGURT.

V.1. INTRODUCTION
V.2. MATERIALS AND METHODS
   V.2.A. Preparation of artificially contaminated milk
   V.2.B. Study of the effect of pH on pure cyclopiazonic acid at 4°C.
   V.2.C. Manufacture of yogurt and acidified milk.
      V.2.C.a. Yogurt
      V.2.C.b. Acidified milk
   V.2.D. Analysis of cyclopiazonic acid by capillary electrophoresis.
V.3. RESULTS AND DISCUSSION.
   V.3.A. Effect of pH on pure CPA.
   V.3.B. Effect of manufacture of yogurt on CPA.
   V.3.C. Effect of acidified milk on CPA.
V.4. CONCLUSION
V.1. INTRODUCTION

Cyclopiazonic acid appearance in animal products and its carry-over into the milk of lactating animals, as mentioned in Chapter I, emphasises the potential risk to dairy consumers. Although milk undergoes several treatments including heat processing prior to human consumption. Results presented in Chapter III demonstrate that CPA is quite stable during heat treatments at 60° and 90°C (Prasongsidh et al. 1998b). Exposure of CPA in milk to 120°C for 30 min still left more than 60% of its original levels.

Aflatoxins are known to be degraded by strong bases or acids in aqueous solutions and in acidic milk as described in Chapter I (Considine and Considine, 1982; Rasic et al., 1991; Tabata et al., 1994). CPA was also decreased by an acidic pH but less by alkaline or neutral pH at 30°C (Goto and Manabe, 1992). Making yogurt is one way of processing milk without component separation but using alteration of pH occurs. During their study on CPA in Japanese fermented foods, Goto and Manabe (1992) found that pure CPA in buffer at 30°C was degraded to less than 50% in a period of 30 days by the acidic pH. Although, cultured dairy foods are mostly stored at 4°C, there is no information on the stability of CPA at different pH levels at low temperature.

The present study investigated the behaviour of CPA in aqueous solutions at different pHs, stored at 4°C for a period of 30 days. Yogurt is a widely consumed acidic fermented milk product and, due to public health concerns (Wiseman & Marth, 1983a; Blanco et al., 1993), stability of CPA during the manufacture and refrigerated storage of yogurt needs to be evaluated. In comparison, the stability of CPA in chemically acidified milk was also studied.

V.2. MATERIALS AND METHODS

V.2.A. Preparation of artificially contaminated milk

Raw milk or pasteurised and homogenised whole milk were provided by the same source as described in III.2.A. Cyclopiazonic acid (Sigma-Aldrich, Sydney, NSW, Australia) in methanol was artificially contaminated in whole unpasteurised milk as described in II.3.A.c.
V.2.B. Study of the effect of pH on pure cyclopiazonic acid at 4°C.

McIlvaine buffers ranging from pH 2 to 8 were prepared using A.C.S. grade Na₂HPO₄, 12 H₂O, citric acid and KCl (Elving et al., 1956). The stock solutions of pH 10 and 12 were prepared using borax and NaOH or KCl and NaOH respectively. The pH was adjusted with 0.1M NaOH or 0.2 M NaOH. A known concentration of pure CPA in methanol was mixed with the aid of a magnetic stirrer into each buffer solution to obtain a final concentration of 1μg CPA/mL. All CPA contaminated buffers were sterilised by filtering through a 0.45μm filter and kept at 4°C in pre-sterilised opaque bottles. Samples of 30mL were taken aseptically at intervals of five days for 30 days. The effect of each pH on CPA was studied in triplicate.

V.2.C. Manufacture of yogurt and acidified milk.

V.2.C.a. Yogurt

The yogurt mix (16% solids not-fat content) in each batch consisted of 170g of skim milk powder (95% solid not-fat), 1.83L contaminated milk and 50g of yogurt stabiliser (NP 48C, Davis-Germantown, NSW, Australia). Yogurt was prepared in pre-sterilised 2.5L Pyrex bottles with screw caps. Four of the bottles were each filled with CPA contaminated milk. One control yogurt bottle instead contained normal pasteurised and homogenised whole milk. All milk bottles were pre-warmed to 40 - 45°C in a water bath, commercial non fat skimmed milk powder was added gradually to the warmed milk with high speed stirring. To this yogurt mix, the yogurt stabiliser was added while stirring. The mixtures were heated to 85°C and held at this temperature for 20 min then cooled in an iced water bath to 45°C. Commercial yogurt starter (DS 215, Gist-brocades, Australia) activated in milk at 45°C was added gradually at a ratio of 0.05 unit per 10L to the warmed milk. Estimation of bacteria was done by counting on coloured slides that had been applied with smears of 4 dilutions from 1 g of yogurt, at 30 min after inoculation of the starter and at the end of fermentation. Temperature and pH were measured at 1 h intervals and fermentation of yogurt at 45°C was concluded when pH 4.5 was reached. All yogurts including the control were refrigerated at 4°C for 21 days simulating a period of commercial shelf life (Blanco et al. 1993). Triplicate samples of 30mL from
individual batches of yogurt including control and experimental groups were taken aseptically at intervals of 4 days for CPA analysis.

V.2.C.h. Acidified milk

Using the model of Hassanin (1994), the contaminated milk at 1μg CPA/mL was acidified by adding to the milk a solution of 200g of acetic acid/L until pH 4 was reached. As a positive control, triplicate samples of 30mL acidified milk were extracted and analysed for CPA at the beginning. The acidified milk was refrigerated at 4°C for 20 days and triplicate samples of 30mL were taken for CPA analysis at 4 day intervals.

V.2.D. Analysis of cyclopiazonic acid by capillary electrophoresis.

CPA was extracted from products and contaminated McIlvaine buffer, then analysed using the method of Prasongsidh et al., (1998a) as described in II.3.C. However, milk and yogurt samples were alkalisised with MeOH-NaHCO₃, defatted with hexane, acidified with HCl, then extracted with CHCl₃. In contrast, the pH buffers were similarly extracted but the hexane step was omitted. The hexane or chloroform layers were centrifuged (5,000 rpm) to accelerate separation. The percentage recovery of CPA was 87% and 98% in yogurt or milk and buffers respectively.

Samples cleaning, CE conditioning and the mobile phase were similar to what described in II.3.C. All results were expressed as means of triplicate samples and Student’s t-Test was used to analyse the data.

V.3. RESULTS AND DISCUSSION.

V.3.A. Effect of pH on pure CPA.

The decomposition of pure CPA in acidic buffers at pH 2 and 4 was significant when compared to that at neutral pH. Higher acidic buffer (pH 2) induced quicker decomposition of pure CPA than other tested pHs (4, 6, 7, 8, 10,12). This is consistent with the report of Goto and Manabe (1992). Figure 5.1 shows that CPA levels also decreased at basic pH. The reductions of pure CPA in this study at 4°C on the 30th day were 30% and 36% at pH 4 and 2 respectively. These reductions were
less than that previously reported where the decrease of CPA was more than 50% at 30°C (Goto and Manabe 1992).

On day 5 (Figure 5.1), there was no significant decrease of mycotoxin (p>0.05) in the buffers at pH 6, 7, 8 and 10. In contrast, there was a significant decrease of CPA (p<0.05) observed at pH 4 and 2. However, the acidity of these two latter pHs did not completely degrade pure CPA in aqueous solution. Even on the 30\textsuperscript{th} day the remaining CPA content was more than 70 and 64% at pH of 4 and 2 respectively (Figure 5.1). In contrast, pure aflatoxin M\textsubscript{1} (AFM\textsubscript{1}) was found to be stable after 4 days in a buffer of pH 4. However, the length of this study on AFM\textsubscript{1} was confined to only 4 days and no information on pH 2 was provided (Wiseman and Marth 1983b). In a related study, slightly catalysed degradation of aflatoxins B\textsubscript{1} (AFB\textsubscript{1}) and G\textsubscript{1} (AFG\textsubscript{1}) at pH 4 was reported (Doyle and Marth 1978a).

\textbf{V.3.B. Effect of manufacture of yogurt on CPA.}

Similar changes in pH were observed in the control yogurt made from normal milk and in 4 batches of yogurt manufactured by using contaminated milk (Table 5.1). CPA was reduced after the manufacture of yogurt and the first day of storage (Figure 5.2). Certain authors reported that artificially and naturally contaminated AFM\textsubscript{1} was not affected by the manufacture of yogurt (Van Egmond \textit{et al.} 1977, Van Egmond 1983; Blanco \textit{et al.} 1993).

In contrast to AFM\textsubscript{1}, AFB\textsubscript{1} was transformed into its derivative aflatoxin B\textsubscript{2a} by acids during the manufacture of yogurt (Megalla and Hafez 1982). Making yogurt and acidified milk using AFB\textsubscript{1} added to milk produced similar results (Rasic \textit{et al.} 1991).

The decrease of pH along with the rise of acid resulting from the yogurt starter action affected the presence of CPA in milk (Figure 5.2). Similar to what occurred to the pure CPA in McIlvaine buffer, acidic pH in the yogurt samples caused degradation of CPA.

The development of certain bacteria may be inhibited by CPA (Madhyastha \textit{et al.}, 1994). However, the presence of CPA did not influence the development of bacteria during the manufacture of yogurt (Table 5.2). The fermentation time to reach pH 4.5 varied between 4.30 h to 5 hours.
Figure 5.1. Effect of pH (2 to 12) at 4°C on pure cyclopiazonic acid at 1μg/mL.
Table 5.1. Changes in pH in yogurt made from non-contaminated and artificially contaminated milks.

<table>
<thead>
<tr>
<th></th>
<th>Initial stage</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4h</th>
<th>4.30 - 5h</th>
<th>4 Days</th>
<th>8 Days</th>
<th>12 Days</th>
<th>16 Days</th>
<th>20 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control yogurt</td>
<td>6.60</td>
<td>6.43</td>
<td>6.31</td>
<td>5.45</td>
<td>4.71</td>
<td>4.50</td>
<td>4.48</td>
<td>4.43</td>
<td>4.38</td>
<td>4.32</td>
<td>4.20</td>
</tr>
<tr>
<td>Trial 2</td>
<td>6.50</td>
<td>6.41</td>
<td>6.30</td>
<td>5.40</td>
<td>4.60</td>
<td>4.50</td>
<td>4.49</td>
<td>4.44</td>
<td>4.40</td>
<td>4.39</td>
<td>4.22</td>
</tr>
<tr>
<td>Trial 3</td>
<td>6.50</td>
<td>6.39</td>
<td>6.27</td>
<td>5.42</td>
<td>4.75</td>
<td>4.50</td>
<td>4.49</td>
<td>4.45</td>
<td>4.45</td>
<td>4.4</td>
<td>4.38</td>
</tr>
<tr>
<td>Trial 4</td>
<td>6.50</td>
<td>6.40</td>
<td>6.28</td>
<td>5.41</td>
<td>4.72</td>
<td>4.50</td>
<td>4.45</td>
<td>4.41</td>
<td>4.38</td>
<td>4.32</td>
<td>4.28</td>
</tr>
</tbody>
</table>

\(a\) End of fermentation.

\(b\) Yogurt made from non contaminated milk

\(c\) Results of 4 trails of yogurt manufactured with artificially contaminated (1μg CPA/mL), homogenised and pasteurised whole milk.
Figure 5.2. Stability of CPA during the manufacture and storage of yogurt made from contaminated milk (1μg CPA/mL).

Storage of yogurt for 20 days induced a further decrease of CPA and a slight change of pH (Table 5.1). Proteolysis and lipolysis occur during the storage of yogurt (Early, 1992). However, the association of CPA with proteins or lipid in milk is not known. A study of CPA association with components of natural or artificially contaminated milk is needed before any further explanation is attempted.

Short duration (30 min to 15 sec) of heat treatments at 60° to 120°C similar to that employed in the dairy industry did not affect much of the CPA content in milk (Prasongsidh et al. 1998d). Heating contaminated milk to 80°C for an hour, however, induced a loss of CPA by up to 14-18% compared to its original level.
Table 5.2. Estimation of bacteria in CPA contaminated yogurt after 30 min and at the end of fermentation

<table>
<thead>
<tr>
<th>Dilution</th>
<th>30 min after the inoculation of starter culture</th>
<th>4:30 - 5 h @ after the inoculation of starter culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1 *</td>
<td>1:2 *</td>
</tr>
<tr>
<td>Control yogurt #</td>
<td>1.8 x 10^1</td>
<td>1.0 x 10^2</td>
</tr>
<tr>
<td>Trial 1 **</td>
<td>2.2 x 10^1</td>
<td>1.2 x 10^2</td>
</tr>
<tr>
<td>Trial 2 **</td>
<td>2.6 x 10^1</td>
<td>1.8 x 10^2</td>
</tr>
<tr>
<td>Trial 3 **</td>
<td>2.2 x 10^1</td>
<td>1.8 x 10^2</td>
</tr>
<tr>
<td>Trial 4 **</td>
<td>1.8 x 10^1</td>
<td>1.2 x 10^2</td>
</tr>
</tbody>
</table>

* Average of 5 fields/mm²

** manufactured with artificially contaminated (1μg CPA/mL), homogenised and pasteurised whole milk.

# Yogurt made from non contaminated milk

@ End of fermentation.
In the present study, pre-heating of milk to 85°C for 20 min was done in the making of yogurt, it was then rapidly cooled to 45°C prior to inoculation of the starter. This process may hydrolyse CPA, which may add to a further decrease in CPA due to development of acidity in the manufacture of yogurt.

V.3.C. Effect of acidified milk on CPA.

A decrease in CPA was observed in acidified milk (Figure 5.3), but to a lesser extent compared to that in yogurt (Figure 5.2).

![Graph showing CPA residue in acidified milk](image)

Figure 5.3. Effect of acidic pH on 1μg CPA/mL in acidified milk made from artificially contaminated whole milk.

[CPA in contaminated milk (1μg/mL) was considered as 100%]

Although the level of CPA was decreased due to the acidity and the heating effect, the manufacture of yogurt did not completely eliminate CPA. CPA, at 12%
compared to its original level, was still detectable at the end of the commercial life of yogurt (Figure 5.2). The potential of CPA reaching dairy consumers is still high if yogurt is made from a high level of CPA contaminated milk.

V.4. CONCLUSION

A neutral pH at 4°C affected CPA less than those of basic and acidic environments in the study over a period of 30 days. CPA was reduced by the 30th day to 70% at pH 4. Manufacture of yogurt reduces more than 70% CPA. However, more than 12% of the mycotoxin was still detectable in the yogurt at the end of storage. CPA was observed to decrease in a similar pattern in chemically acidified milk but the decrease was less than that in yogurt. Despite the extensive decrease of CPA by manufacture of yogurt or by acidic pH, CPA is still detectable in the final product. Moreover, even though CPA is degraded in yogurt, no information on the breakdown products that could still be toxic is available in the literature. Further work on the toxicity of CPA left in yogurt is suggested.
CHAPTER VI:

INFLUENCE OF MANUFACTURING AND STORING OF ICE CREAM ON CYCLOPIAZONIC ACID. *

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VI.1. INTRODUCTION

The presence and stability of CPA in milk during storage including frozen storage is not as well studied in the literature as for aflatoxins AFB₁ and AFM₁.

Despite an extensive study, stability of the AFM₁ in milk stored at low temperatures or after freezing, has been reported with contradicting results. Sometimes, AFM₁ was found completely eliminated or only to be partly reduced or even insignificantly decreased after a storage of 4 to 25 months (McKinney et al., 1973; McKinney and Cavanagh, 1977; Kiermeier and Mashaley, 1977). However, the study on AFM₁ during the manufacture and storage of contaminated ice cream revealed a stability of the toxin through an eight months period.

Compared to AFM₁, CPA in artificially contaminated milk is not greatly affected by storage for 4 to 21 days at 4°C as described in Chapter IV (Prasongsidh et al., 1998d). The stability of CPA is not greatly affected after long cold storage or after processing of liquid milk or milk powder. However, the stability of CPA during the manufacture using contaminated milk and subsequent storage of frozen dairy products such as ice cream has not been reported in the literature.

Since ice cream is a widely consumed frozen dessert and due to public health concerns, the stability of CPA during processing and storage of ice cream deserves to be evaluated.

The objective of the study in this chapter is to assess the stability of CPA to freezing during the preparation of ice cream and its storage over 3 months at -20°C, using a procedure similar to commercial methods and the model of Wiseman and Marth (1983d) to study aflatoxin M₁ in ice cream.

VI.2. MATERIALS AND METHODS

VI.2.A. Preparation of artificially contaminated milk

Raw whole milk was provided by the same source as described in III.2.A. Cyclopiazonic acid (Sigma-Aldrich, Sydney, NSW, Australia) in methanol was added to artificially contaminate whole unpasteurised milk at 1µg CPA/g using the method as described in II.3.A.c.
VI.2.B. Preparation of ice cream.

Based on information described by Arbuckle (1986), a batch of 30 Kg of plain ice cream was prepared using the basic formulation in Table 6.1. Non-fat dry milk powder, cream (milk fat 30%, Farmland, Victoria, Australia), Gemcol 645/c an integrated emulsifier-stabiliser (Germantown, NSW, Australia), CE 13669 vanilla flavour (Quest International) and cane sugar was obtained through the Dairy Processing Plant, University of Western Sydney, Hawkesbury, N.S.W. Contaminated milk was preheated to 40°C in a pasteuriser and ingredients were gradually mixed while stirring.

Table 6.1. Formulation of ice cream

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Kg per 30 kg of mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw whole milk</td>
<td>18.00</td>
</tr>
<tr>
<td>Cream (30% milk fat)</td>
<td>5.85</td>
</tr>
<tr>
<td>NFSMP (^a)</td>
<td>1.57</td>
</tr>
<tr>
<td>Emulsifier-Stabiliser (^b)</td>
<td>0.15</td>
</tr>
<tr>
<td>Sugar</td>
<td>3.60</td>
</tr>
<tr>
<td>Water</td>
<td>0.83</td>
</tr>
</tbody>
</table>

\(^a\) Non-fat skim milk powder.

\(^b\) Sheer (IC 9170) integrated emulsifier-stabiliser.
After continuing heating to 80°C then holding for 15 min, triplicate samples were taken prior to the mix being homogenised using a two stage homogeniser (APV Gaulin, Massachusetts, USA) at 2600 - 3000 psi on the first stage and 1000 psi on the second stage. Other triplicate samples were removed before returning the mixture to the pasteuriser (M30C, Mark SG Gluliano, Milanese, Italy). The mixture was pasteurised at 74°C for 10 min and heating was continued until 90°C. The mix was cooled to 10°C and vanilla was added at a rate of 0.08%. Another sampling was done in triplicate before the mix was aged overnight at 4°C. The following day, triplicate samples were taken from the mix before transferring to a batch ice cream freezer (GH 17A, Mark SG Gluliano, Milanese, Italy), packaging into 250mL containers and storing at -20°C for a period of 3 months.

VI.2.C. Analysis of CPA by capillary electrophoresis

Triplicate samples from artificially contaminated milk were taken as controls. Samples from mix making, homogenised mix, pasteurised mix, aged mix and ice cream from week 1 to 3 months were extracted for CPA and analysed using the method of Prasongsidh et al., (1998a) as described in II.3.C.

Sample cleaning, CE conditioning and the mobile phase were similar to that described in II.3.C. All results were expressed as means of triplicate samples and Student’s t-Test was used to analyse the data.

VI.3. RESULTS

VI.3.A. Stability of CPA during the processing of ice cream

Table 6.2 shows residual CPA in milk prior to any treatment and other samples taken during the manufacture of ice cream from mixing until ageing over night at 4°C. The levels of CPA were dramatically decreased by 42% in the mix following a preheating to 80°C for 15 min. CPA recovery was significantly (p≤0.05) less in the homogenised mix (50.8%). A CPA reduction occurred following a 10 min heat treatment at 74°C. After the ageing process at 4°C overnight, the reduction of CPA was significantly higher (53.7%).
Table 6.2. Residue of CPA in raw milk containing 1μg CPA/g and in the mix after adding ingredients.

<table>
<thead>
<tr>
<th>Samples (Unit operations)</th>
<th>CPA Residual (μg/g) ( ^a )</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw whole milk ( ^b )</td>
<td>0.993</td>
<td>0.066</td>
<td>0.001</td>
<td>0.7</td>
</tr>
<tr>
<td>Mix making &amp; pre heating ( ^c )</td>
<td>0.578 ( ^d )</td>
<td>0.002</td>
<td>0.000</td>
<td>42.2</td>
</tr>
<tr>
<td>Homogenising</td>
<td>0.492 ( ^d )</td>
<td>0.021</td>
<td>0.000</td>
<td>50.8</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>0.490</td>
<td>0.011</td>
<td>0.000</td>
<td>51.0</td>
</tr>
<tr>
<td>Holding and ageing</td>
<td>0.463 ( ^d )</td>
<td>0.010</td>
<td>0.000</td>
<td>53.7</td>
</tr>
</tbody>
</table>

\( ^a \) Triplicate samples; 
\( ^b \) Artificially contaminated raw whole milk at 1μg/g prior to any treatment and making the mix. 
\( ^c \) After all the ingredients (Table 6.1) were added and the mix was heated to 80°C. 
\( ^d \) Significant difference to that of preceding samples (p≤0.05). 
SD = Standard deviation; RSD = Relative standard deviation.
VI.3.B. Stability of CPA during the storage of ice cream

Storing the contaminated ice cream at -20°C did not affect the levels of mycotoxin at least during the first 3 weeks (Table 6.3). However, a significant decrease (p≤0.05) of CPA level was observed after 4 weeks of storage. Compared to its original level in milk prior to any processing, CPA in the ice cream stored for 4 weeks, was reduced by 59.3% (Table 6.3). However, this decrease of CPA in the ice cream mix was only 5% when compared to the content of the mycotoxin in ice cream that was ready for storage. CPA levels further decreased following 5 to 8 weeks of storage. CPA levels were clearly reduced in ice cream samples after 9 weeks of storage at -20°C.

VI.4. DISCUSSION

Homogenisation contributed to uniform dispersion of CPA in the mix. However, the reason for the significant decrease of CPA levels (50.8%) after the homogenisation of the mix compared to the content of the mycotoxin in the mix (42.2%) following the preheating at 80°C for 15 min, is not clear (Table 6.2). It appears that the recovery of CPA using the extraction method described earlier was significantly lower after homogenisation (p≤0.05).

The results in Table 6.2 also, reveal that CPA was not greatly affected during the manufacture of ice cream. The significant difference (p≤0.05) of CPA levels in the raw whole milk (control) and the mix following a short preheating was largely due to the effect of dilution of the mycotoxin by the addition of ingredients, which themselves, were nearly 50% of the total amount of the mix. The results of a small reduction of CPA are consistent with our previous findings (Prasongsidh et al., 1998c) on the mycotoxin stability to heat treatment using a similar method to that practiced in the dairy industry to pasteurise contaminated milk for a short period. In that previous study (Prasongsidh et al., 1998c), CPA was only reduced by less than 1% in the milk promptly taken after the heat treatment.

Table 6.2 reveals that further storage at 4°C overnight to age the ice cream contributed to an additional CPA decrease of nearly 2%. This finding was consistent with the previous study in Chapter III conducted upon on the kinetics of CPA to heat treatment.
Table 6.3. Residue of CPA in ice cream manufactured with CPA contaminated milk stored for 9 weeks at -20°C.

<table>
<thead>
<tr>
<th>Period of storage (Weeks)</th>
<th>CPA Residues (μg/g) c</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.455</td>
<td>0.029</td>
<td>0.000</td>
<td>54.5</td>
</tr>
<tr>
<td>2</td>
<td>0.455</td>
<td>0.004</td>
<td>0.000</td>
<td>54.5</td>
</tr>
<tr>
<td>3</td>
<td>0.447</td>
<td>0.009</td>
<td>0.000</td>
<td>55.3</td>
</tr>
<tr>
<td>4</td>
<td>0.407 a</td>
<td>0.005</td>
<td>0.000</td>
<td>59.3</td>
</tr>
<tr>
<td>5</td>
<td>0.395</td>
<td>0.015</td>
<td>0.000</td>
<td>60.5</td>
</tr>
<tr>
<td>6</td>
<td>0.375</td>
<td>0.048</td>
<td>0.000</td>
<td>62.5</td>
</tr>
<tr>
<td>7</td>
<td>0.362</td>
<td>0.053</td>
<td>0.000</td>
<td>63.8</td>
</tr>
<tr>
<td>8</td>
<td>0.321</td>
<td>0.006</td>
<td>0.000</td>
<td>67.9</td>
</tr>
<tr>
<td>9</td>
<td>0.271 a</td>
<td>0.012</td>
<td>0.000</td>
<td>72.9</td>
</tr>
</tbody>
</table>

a Significant difference to that of preceding samples (p≤0.05).

b % decrease compared to CPA levels in milk prior to the mix and manufacturing of ice cream.

c Triplicate samples each from different package of ice cream;

SD = Standard deviation; RSD = Relative standard deviation.
CPA in the heat treated contaminated milk further decreased despite overnight storage at 4°C (Prasongsidh et al., 1998b).

The decrease in CPA levels from mixing until ageing was nearly 12% in total (Table 6.2). Storage at -20°C appeared to restrict the loss of CPA after 9 weeks. However, comparing ice cream at week 1 and after 4 to 9 weeks of storage, a significant decrease of CPA levels (5%, 12% respectively) was observed.

VI.5. CONCLUSION

Manufacture of an ice cream with 1µg CPA/g contaminated milk and storage for 9 weeks at -20°C did not eliminate CPA residues and left more than 27% of CPA levels remaining compared to its initial amount in the milk.

Hot and cold treatment during the manufacture of ice cream did not greatly affect the levels of CPA in the mix. Only a small in reduction of CPA levels was observed from mixing until ageing. However, a slight effect on CPA levels occurred following pasteurisation. Storage of 9 weeks at -20°C adequately limits the loss of CPA.

The study in this chapter shows that if milk contains the carry over CPA, the manufacture and storage of the ice cream made with contaminated milk will not greatly affect the stability of this neurotoxic mycotoxin in the product and will not be able to prevent the CPA reaching consumers. Further studies using milk naturally contaminated with CPA may provide a different outcome due to the different association of milk components to CPA. However, this preliminary study on ice cream and CPA highlighted the possible risk from CPA in other dairy products manufactured with contaminated milk.
CHAPTER VII:

FATE OF CYCLOPIAZONIC ACID IN CHEDDAR CHEESE AND ITS PARTITION BEHAVIOUR IN CREAM AND BUTTER.*

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   VII.2.B Preparation of Cheddar cheese.
   VII.2.C Compositional analysis of milk, whey and cheese.
   VII.2.D Texture profile analysis (TPA) of cheese.
   VII.2.E Partition behaviour of CPA in a milk-cream system.
   VII.2.F Distribution of CPA in production of butter.
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      VII. 3.B.b. Texture of Cheddar cheese after ageing
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VII. 4. DISCUSSION


VII.1. INTRODUCTION

Except for a possible occurrence of the toxin by direct contamination in cheese, little has been known about behaviour, partition and stability of CPA in dairy products including milk, cream and butter.

The natural occurrence of CPA in cheese was reported to be mostly due to direct contamination resulting from mould ripened cheese (Le Bars, 1979; Le Bars et al., 1988; Schoch, 1981; Schoch et al., 1983; 1984; Pitt et al., 1986). In a study on international commercial cheese samples, Lund et al., (1995) found that nearly half of the fungi isolated were capable of producing CPA. However, little is known about cheese contamination resulting from the manufacture of cheese with CPA carried-over into milk.

If CPA is present in unpasteurised milk, the toxin will be carried over into pasteurised milk (Chapter III). Manufacture of cultured milk could not eliminate CPA from the products (Chapter V). Moreover, we found that manufacture of an ice cream with low level contaminated milk then storage for 9 weeks at -20°C did not eliminate CPA residues (Chapter VI) (Prasongsidh et al., 1998e). However, stability and occurrence of CPA in cream, butter, whey and cheese manufactured from contaminated milk are not reported.

The purpose of this study was to determine the fate of CPA in Cheddar cheese after manufacture and during a ripening period. This type of cheese is one of the varieties of cheese produced in greatest quantity in Australia. In addition, the distribution of CPA in milk-cream systems and the stability of the mycotoxin following the manufacture of butter were also investigated.

VII.2. MATERIALS AND METHODS

VII.2.A. Preparation of artificially contaminated milk

Pasteurised whole milk was provided by the same source as described in III.2.A. Cyclopiazonic acid (Sigma-Aldrich, Sydney, NSW, Australia) in methanol was artificially contaminated into whole unpasteurised milk at 1μg CPA/mL using the method as described in II.3.A.c.
VII.2.B Preparation of Cheddar cheese.

Sufficient quantity of CPA free milk was used to manufacture three batches of control cheese. Three other trial cheeses were similarly manufactured but with 10L of 1μg CPA/mL contaminated pasteurised whole milk. The manufacture of Cheddar cheese was performed similarly to the traditional method of Kosikowski (1977).

The cheese manufacture was conducted in a 10L stainless steel water-jacketed vat with a controlled paddle agitator (FT 20 Cheese Vat, Armfield, Abingdon, England). Temperature and pH probes allowed heat and acidity monitoring of the cheese milk. Warmed milk at 31°C was added with calcium chloride (50% w/v, Mauri Laboratories, Moorebank, Australia) and food grade colorant Annato (Home Cheese Making Supplies, Victoria, Australia) at 250mL/1000L and 30mL/1000L respectively.

After 15 min of agitation, starter culture inoculum (Lactococcus cremoris and Lactococcus lactis) at 2% prepared and activated 10 min before from a frozen concentrated culture (DS5 CRM, Direct Set®, Gist-brocaides, Moorebank, Australia) was added to 31°C milk. Rennet (Home Cheese Making Supplies, Victoria, Australia) was added (250mL/1000L) after 10 min of agitation.

The cutting curd was 30 - 45 min after the acidity reached 0.11-0.13%. Parallel to the pH meter, an acid test kit consisted of an acidimeter, 9N NaOH and phenolphthalein as indicator was used. The cutting curd was allowed to stand without agitation until the acidity read 0.09 - 0.11%. The slowly agitated curds were cooked with intermittent heating for 60 min and cooling conducted at the rate of 1°C for 10 min until the temperature reached 33°C then at the rate of 1°C for 5 min from the temperature 33 to 39°C. Whey was drained at 0.15-0.17% titratable acidity and agitation done until the acidity increased by a further 0.02%.

Cheddaring was continued until the titratable acidity was 0.70%, then the curds were milled and salted (25 g/Kg curd). Curds were pressed overnight at ambient temperature in cylindrical moulds then vacuum sealed in plastic film on the following day and matured in a cheese room at temperature 8-10°C for 10 months. Samples of 25mL were taken from milks prior to any processing and from whey following the complete drainage for CPA analysis. Twenty grams of cheese were
randomly taken. The effect of cheese making on CPA was studied with triplicate samples.

**VII.2.C Compositional analysis of milk, whey and cheese.**

Fat, protein and lactose content of milk samples were determined using a Milkoscan 104 (Newcastle, NSW, Australia) similar to the method described in the Dairy Test Manual, New South Wales Dairy Corporation, 1995 (Anon, 1995). The fat and protein of cheese and whey were determined using babcock method and semi-micro Kjeldahl method (N x 6.38) (AOAC, 1984) respectively. Milk acidity prior to or during cheese making was titrated according to the method of AOAC (1984).

Total solids in milk, whey and cheese moisture were determined by oven method (AOAC, 1984). Two microbiological tests (Coliform and total plate count) of milk were performed according to the Pour Plate Method (Australian standard) (Anon, 1995) using 0.1% Peptone Solution and Tryptone glucose yeast agar, CM325 (Oxoid, Manchester, England).

**VII.2.D Texture profile analysis (TPA) of cheese.**

Quality of cheese made in different trials was determined using texture evaluation after 9 months of maturation. Cheese cubes (1.3 cm) were randomly bored from the cheese blocks and hermetically kept at 4°C over night before the test using a computerised Texture profile analyser, TA-XT2, V. 5.15 (Stable Micro Systems, Blackdown Industries, Surrey, England). Coupled with software XT. RA version 3.7 (Stable Micro Systems, Blackdown Industries, Surrey, England), the instrument was set as follows: 200 pps sample rate, 0.5mm distance threshold, 20g force threshold, 5g contact force, sample area 1mm², speed 1mm/s, pre test and post test at 5mm/s, speed, 5g trigger type auto, distance 3mm and time 5s. The tested rheological properties of different trials of cheese were springiness, cohesiveness, chewiness, gumminess and hardness.
VII.2.E. **Partition behaviour of CPA in a milk-cream system.**

Equal volumes of cream and skim milk previously spiked with known amounts of CPA were used to determine the partition behaviour of the mycotoxin in a milk-cream system similar to the method of Grant and Carlson (1971).

Cream was prepared from a known quantity of fresh raw milk placed in aliquot in aseptic centrifuge tubes that were centrifuged at 2000g for 10 min. Skim milk and cream were separated by syphoning and saved in sterilised bottles, which were promptly kept at 4°C until used. Skim milk and cream (400g each) were warmed to 40°C then artificially contaminated with 1μg CPA/g similar to the method of Wiseman et al., (1983) while shaking and were left at 4°C overnight. Equal volumes (200mL) of skim milk and cream were mixed in 2L Pyrex bottles with screw cap that was shaken for 2 h in a shaking water bath (Tecator 1024, Linbrook, Höganäs, Sweden) containing cold water (4°C) continuously circulated by a peristaltic pump (Vera, Manostat, California, USA) from an ice-water tank to equilibrate the system. The mixture was kept at 4°C for 16 h then centrifuged at 2000g to separate cream and skim milk, which were promptly extracted for CPA. The partition behaviour of CPA in a skim milk-cream system was studied in triplicate.

VII.2.F. **Distribution of CPA in production of butter.**

Using the laboratory procedure of Grant and Carlson (1971) for processing butter, 4L of whole raw milk containing 1μg CPA/mL was pasteurised at 62°C for 30 min while agitating in a water bath shaker similar to the method of heat treatment of CPA in milk previously reported in Chapter III.

The milk was cooled in an ice water bath before being kept at 4°C overnight for creaming in a 5L Pyrex bottle. The skim milk was separated by siphoning the following day and kept in opaque plastic bottles at -18°C until analysis for CPA. The cream obtained was placed in an aliquot of 50mL in 100mL Pyrex bottles with screw cap. The bottles were horizontally mounted and shaken overnight on a shaking water bath (Tecator 1024, Linbrook, Höganäs, Sweden) set at 25°C.

The following day, the bottles were chilled in an ice water bath before the buttermilk was decanted and saved with the skim milk previously kept. Ice water
(100mL) was used to wash the obtained butterfat. Buttermilk and rinse ice water were taken for analysis of CPA after weighing of triplicate samples of butterfat.

VII.2.G. Analysis of CPA by capillary electrophoresis

Triplicate samples from artificially contaminated milk, cheese, whey, cream, skim milk, butter milk and rinse ice water were extracted for CPA and analysed using the method of Prasongsidh et al. (1998a) as described in II.3.C. Sample cleaning, CE conditioning and mobile phase were similar as described in II.3.C. All results were expressed as means of triplicate samples and Student’s t-Test was used to analyse the data.

VII. 3. RESULTS

VII. 3.A. Cheddar cheese.


Composition of raw whole milk was evaluated prior to contamination and pasteurisation. Acidity, fat content, protein content, lactose content, bacteria and ash content of the milk are given in Table 7.1. No significant difference (p>0.05) was observed between the composition of the control milk, or the milk used to manufacture the cheese trial 1 and 2. Milk from trial 4 had a lower fat content, protein content, and fewer bacteria. It appeared that the occurrence of CPA in milk did not influence the composition of cheese and whey.

Moisture contents of cheese, fat content, protein content and pH values of cheese and whey are shown in Table 7.2. Compared to the cheese made with milk free of CPA, the percentage of protein and ash in cheese made from contaminated milk were significantly lower (p≤0.05).

Table 7.3 shows the amount of milk in each trial and the CPA content used in the manufacturing of different cheese trials and in the obtained whey. The amount of Cheddar cheese yield was lower than 1kg. The concentration of CPA varied from 1.7 - 2.3μg/g of cheese and 0.6 - 0.7μg/mL of whey.
Table 7.1. Composition of cheese milk with or without contamination CPA.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Acidity (%)</th>
<th>Fat (%)</th>
<th>Ash (%)</th>
<th>Protein (%)</th>
<th>Lactose (%)</th>
<th>Coliform (cfu/mL)</th>
<th>Total count (cfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.14</td>
<td>4.40</td>
<td>0.42</td>
<td>3.17</td>
<td>4.35</td>
<td>&lt;1</td>
<td>2000</td>
</tr>
<tr>
<td>Control</td>
<td>0.11</td>
<td>4.14</td>
<td>0.43</td>
<td>3.16</td>
<td>4.34</td>
<td>&lt;1</td>
<td>2500</td>
</tr>
<tr>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.10</td>
<td>4.18</td>
<td>0.45</td>
<td>3.05</td>
<td>4.34</td>
<td>10</td>
<td>3000</td>
</tr>
<tr>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.11</td>
<td>3.83</td>
<td>0.34</td>
<td>3.03</td>
<td>4.31</td>
<td>&lt;1</td>
<td>500</td>
</tr>
<tr>
<td>3&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Average of triplicate determinations
<sup>b</sup> Milk without contamination with CPA
<sup>c</sup> Milk contaminated with 1 μg CPA/mL prior to processing of cheese.
Table 7.2. Composition of whey and Cheddar cheese

<table>
<thead>
<tr>
<th>Trial</th>
<th>pH</th>
<th>Fat (%)</th>
<th>Ash (%)</th>
<th>Protein (%)</th>
<th>Moisture (%)</th>
<th>Fat (%)</th>
<th>Ash (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (^b)</td>
<td>5.5</td>
<td>24.22</td>
<td>2.26</td>
<td>29.91</td>
<td>27.24</td>
<td>0.26</td>
<td>0.27</td>
<td>0.77</td>
</tr>
<tr>
<td>1 (^c)</td>
<td>5.1</td>
<td>26.02</td>
<td>2.15</td>
<td>27.81</td>
<td>40</td>
<td>0.19</td>
<td>0.20</td>
<td>0.81</td>
</tr>
<tr>
<td>2 (^c)</td>
<td>5.6</td>
<td>26.29</td>
<td>2.17</td>
<td>27.81</td>
<td>29.5</td>
<td>0.29</td>
<td>0.22</td>
<td>0.67</td>
</tr>
<tr>
<td>3 (^c)</td>
<td>5.4</td>
<td>24.42</td>
<td>2.12</td>
<td>25.01</td>
<td>26.55</td>
<td>0.26</td>
<td>0.18</td>
<td>0.74</td>
</tr>
</tbody>
</table>

\(^a\) Average of triplicate determinations

\(^b\) Whey and cheese made from milk without contamination of CPA

\(^c\) Whey and cheese made from milk contaminated with 1 µg CPA/mL.
Table 7.3. Concentration of CPA in cheddar cheese, whey and milk from which they were made

<table>
<thead>
<tr>
<th>Trial</th>
<th>Milk (L)</th>
<th>CPA (^b) (μg/mL)</th>
<th>Total CPA (^c) (mg)</th>
<th>Kg Cheese</th>
<th>Whey (L)</th>
<th>CPA in Cheese (μg/g)</th>
<th>Total CPA in Cheese (μg)</th>
<th>CPA in Whey (μg/mL)</th>
<th>Total CPA in Whey (μg)</th>
<th>% Total CPA recovered (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (^e)</td>
<td>10</td>
<td>0.0</td>
<td>0.0</td>
<td>0.88</td>
<td>8.70</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>1 (^f)</td>
<td>10</td>
<td>1.0</td>
<td>10</td>
<td>0.88</td>
<td>8.74</td>
<td>2.29</td>
<td>2012.01</td>
<td>0.67</td>
<td>5893.70</td>
<td>79.1</td>
</tr>
<tr>
<td>2 (^f)</td>
<td>10</td>
<td>1.0</td>
<td>10</td>
<td>0.92</td>
<td>8.48</td>
<td>1.70</td>
<td>1559.82</td>
<td>0.74</td>
<td>6308.12</td>
<td>78.7</td>
</tr>
<tr>
<td>3 (^f)</td>
<td>10</td>
<td>1.0</td>
<td>10</td>
<td>0.84</td>
<td>8.80</td>
<td>1.87</td>
<td>1568.56</td>
<td>0.71</td>
<td>6217.68</td>
<td>77.9</td>
</tr>
</tbody>
</table>

\(^a\) Average of triplicate determinations; \(^b\) CPA artificially contaminated in milk prior to cheesemaking.
\(^c\) Total CPA used in each trial; \(^d\) % of CPA added to milk recovered in cheese curd and liquid fractions;
\(^e\) Whey and cheese made from milk free of CPA; \(^f\) Whey and cheese made from milk contaminated with 1μg CPA/mL.
The CPA values indicated that the average increase in the final curd of the 3 trial cheeses was 1.95 fold over that in the milk originally contaminated. However, the total amount of CPA calculated according to the quantity of the cheese yielded and collected whey varied from 1568.6 to 2012µg and 6217.7 to 5893.7µg respectively.

Table 7.3 shows that the percentage of total CPA recovered in each trial of cheese manufacture from the initial amount contaminated in milk (10 mg/10L of milk) was from 77.9 to 79.1%. Using the method of Yousef and Marth (1989), the percentage of CPA carry-over from milk into cheese-curd and the enrichment factor of CPA in the making of cheese varied from 24.7 to 34.1% and 2.3 to 3.4 respectively (Table 7.4).

VII. 3.B. CPA in Cheddar cheese and its effect on textures.


Figure 7.1 shows the variation of CPA content in samples of cheeses (triplicate) as ageing proceeded. For instance, CPA levels detected in the sample taken in the first month of the ageing process started as high as 2.3 µg/g. The level of CPA became low at 1.7 µg/g initially then rose to 2.1µg/g. This phenomenon was again observed in the 4th and 5th month then the CPA levels decreased in the following month. The amount of CPA in the cheese of trial 1 was detected at 0.8µg/g. Similar variation in CPA values between months was also observed in the cheese of trial 2, which started at the first month lower (1.7 µg/g) than that of trial 1. The levels of CPA rose then decreased in 2nd, 4th and 7th month then declined to 0.7µg/g at the end of the ageing process. The CPA value in cheese trial 3, in contrast started low (1.9µg/g) then rose to 2.2µg/g in samples taken after one month of ageing. The level of CPA gradually decreased in the following months until it reached 1.2µg/g in the 6th month then increased to 1.5µg/g. This increase and decrease of CPA levels was observed in the following months. The trendline representing the average CPA levels of the 3 trials illustrates the decrease of CPA content of cheese as the process of ageing proceeded (Figure 7.1)
Table 7.4. Partition of CPA during cheddar cheese-making *.

<table>
<thead>
<tr>
<th>Trial $^d$</th>
<th>CPA carry-over $^b$ (%)</th>
<th>Enrichment factor $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34.1</td>
<td>3.4</td>
</tr>
<tr>
<td>2</td>
<td>24.7</td>
<td>2.3</td>
</tr>
<tr>
<td>3</td>
<td>25.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Average</td>
<td>28.0</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*a Based on the method of Yousef and Marth (1989)*

$^b$ % carry-over = (Amount of CPA in cheese-curd x 100) / Amount of CPA in cheese-milk

$^c$ Enrichment factor = Concentration of CPA in cheese-curd / Concentration of CPA in cheese-milk;

$^d$ Cheese made from milk contaminated with 1µg CPA/mL.
Figure 7.1. CPA content of Cheddar cheese (manufactured using 1μg CPA/mL contaminated milk) during 10 months of ageing.
VII. 3.B.b. Texture of Cheddar cheese after ageing

Due to the occurrence of CPA in the cheeses, sensory evaluation was impracticable. However, quality evaluation of the cheeses was done by using a Texture Profile Analysis (TPA). Under the method and conditions previously mentioned, the evaluation of springiness, cohesiveness, chewiness, gumminess and hardness of the cheese made from milk free of CPA and from contaminated milk was determined and averages of triplicate samples are given in Table 7.5.

VII. 3.C. Partition behaviour of CPA in a milk-cream system.

Table 7.6 illustrates the partition behaviour of CPA in an equal volume of milk cream system. Recovery of CPA in cream in 3 trials varied from 50 to 59%. Compared to skim milk, CPA was recovered in a greater amount than originally spiked. The amount of CPA found in skim milk varied from 101 to 108%. However, recovery of CPA in each skim milk sample varied substantially, which is indicated by the values of the standard deviation (SD). The percentages of CPA overall recovery in different trials of the treatment were 76%, 83% and 80%.

VII. 3.D. Distribution of CPA in the manufacture of butter.

Using a laboratory method to manufacture butter induced a low recovery of CPA in butter (4.8%). The average increase in the final butter was only 0.7% over that in the milk. In contrast, more than 92% of CPA artificially contaminated milk was found in butter milk. Only 1% of CPA was rinsed out of butter by using 100mL of rinse ice water (Table 7.7)

VII. 4. DISCUSSION

The composition of milk, cheese, whey, and texture profile analysis of cheeses indicated the quality of the obtained cheeses (Table 7.1 and 7.2). However, the results of the CPA levels in cheese suggest that the mycotoxin could definitely occur in the curd (Table 7.3).
Table 7.5. Texture profile analysis of cheeses from non contaminated and artificially contaminated milk. 

<table>
<thead>
<tr>
<th>Trial</th>
<th>Springiness(^d)</th>
<th>Cohesiveness(^d)</th>
<th>Chewiness (x 10(^{2}))(^d)</th>
<th>Gumminess (x 10(^{2}))(^d)</th>
<th>Hardness (x 10(^{2}))(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (^b)</td>
<td>0.78</td>
<td>0.58</td>
<td>2.9</td>
<td>3.5</td>
<td>5.5</td>
</tr>
<tr>
<td>1 (^c)</td>
<td>0.78</td>
<td>0.55</td>
<td>2.1</td>
<td>2.7</td>
<td>4.7</td>
</tr>
<tr>
<td>2 (^c)</td>
<td>0.79</td>
<td>0.56</td>
<td>2.7</td>
<td>3.4</td>
<td>5.8</td>
</tr>
<tr>
<td>3 (^c)</td>
<td>0.77</td>
<td>0.58</td>
<td>2.3</td>
<td>3.1</td>
<td>5.5</td>
</tr>
</tbody>
</table>

\(^a\) With 1µg CPA/mL pasteurised whole milk using Stable Micro Systems TA-XT2 and software XT. RA version 3.7. Conditions applied = Sample rate 200 PPS; Distance threshold = 0.5mm; force threshold = 20 g; contact force = 5g; sample area 1mm\(^2\); speed 1mm/s; Pre test and post test = speed 5mm/s; trigger type auto 5g, distance 3mm and time 5s

\(^b\) Cheese made from milk free of CPA;

\(^c\) Cheese made from milk contaminated with 1µg CPA/mL.

\(^d\) (Newtons/second)
Table 7.6. Distribution of CPA in an equal volume skim milk-cream system earlier spiked with 1μg CPA/g.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Product</th>
<th>CPA Residual (μg/g)</th>
<th>SD</th>
<th>RSD</th>
<th>Distribution of CPA residual (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cream</td>
<td>0.500</td>
<td>0.004</td>
<td>0.000</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>Skim milk</td>
<td>1.013</td>
<td>0.154</td>
<td>0.002</td>
<td>101.3</td>
</tr>
<tr>
<td>2</td>
<td>Cream</td>
<td>0.589</td>
<td>0.072</td>
<td>0.000</td>
<td>58.9</td>
</tr>
<tr>
<td></td>
<td>Skim milk</td>
<td>1.063</td>
<td>0.140</td>
<td>0.001</td>
<td>106.3</td>
</tr>
<tr>
<td>3</td>
<td>Cream</td>
<td>0.526</td>
<td>0.009</td>
<td>0.000</td>
<td>52.6</td>
</tr>
<tr>
<td></td>
<td>Skim milk</td>
<td>1.082</td>
<td>0.130</td>
<td>0.001</td>
<td>108.2</td>
</tr>
</tbody>
</table>

% overall recovery of CPA in trial 1 = 75.7% \(^a\); trial 2 = 82.6% \(^b\); trial 3 = 80.4% \(^c\)

\(^d\) % comparing to the amount of CPA spiked (1μg/g) in each product.

\(^e\) Triplicate samples; SD = Standard deviation; RSD = Relative standard deviation.
Table 7.7. Distribution of CPA during buttermaking.\(^a\)

<table>
<thead>
<tr>
<th>Products</th>
<th>Weight of products (^b) (g)</th>
<th>CPA Residual (^d) (µg/g)</th>
<th>SD</th>
<th>RSD</th>
<th>Distribution of CPA residual (%) (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buttermilk</td>
<td>3590</td>
<td>1.026</td>
<td>0.066</td>
<td>0.001</td>
<td>92.1</td>
</tr>
<tr>
<td>Rinse ice water</td>
<td>100</td>
<td>0.414</td>
<td>0.052</td>
<td>0.000</td>
<td>1.0</td>
</tr>
<tr>
<td>Butterfat</td>
<td>270</td>
<td>0.709</td>
<td>0.065</td>
<td>0.000</td>
<td>4.8</td>
</tr>
</tbody>
</table>

\(^a\) Manufactured with 4Kg of 1µg CPA/g artificially contaminated raw milk using laboratory procedure.

\(^b\) Weighted after a final removal of residual rinse ice water.

\(^c\) % of CPA residual according to the final weight of products.

\(^d\) Triplicate samples; SD = Standard deviation; RSD = Relative standard deviation.
CPA is classified as a lipophilic weak acid (McLaughlin and Dilger, 1980). However, the similar phenomenon that occurred in cheese was also observed following the manufacture of butter, where the toxin was found to be recovered at nearly 5% compared to the total amount of CPA contaminated. The percentage recovery in the testing of CPA extraction methods in butter and cheese samples with a spike of a known quantity of the mycotoxin was in the range of 67.2% - 86.5%. Distribution of CPA in skim milk-cream systems also revealed that occurrence of CPA in the cream portion of milk is also possible (Table 7.6). However, CPA carry-over in skim milk was higher than that of cream.

This study was undertaken as a preliminary exploration of the stability of cyclopiazonic acid following the manufacture of cheddar cheese, butter and the partition behaviour of the toxin in skim milk-cream systems by using milk containing the mycotoxin to simulate contamination through carry-over from milk. Extensive reporting was previously done on CPA directly contaminated cheese through fungal growth used for ripening or unintentional fungal growth (Still, 1978a; Le Bars, 1979; Schoch, 1981; Scott, 1981; Schoch et al., 1983 and 1984; Teuber and Engel, 1983; Tantaoui-Elaraki and Khabbazi, 1984). CPA was found in cheese rind rather than in interior parts, which suggested that CPA came from direct contamination and not from contamination from carry-over from milk (Le Bars, 1979). Since CPA is found carried-over into the milk of lactating animals (Dorner et al., 1994), the chance that cheese can be contaminated by carry-over from contaminated milk is high. The occurrence of CPA in cream or cheese curd in this study suggested that in association with the contamination of the mycotoxin through direct contamination in cheese, the contamination of CPA by using contaminated milk is possible.

The variation of the CPA value in 3 trials of cheese in the ageing period suggested that the distribution of CPA in cheese curd was not uniform. The sampling was randomly taken and the values shown in Figure 7.1 were the results of triplicate samples. However, the trendline obtained according to the average of the CPA values in each month of ageing of the 3 trials revealed that the CPA amount decreased during ageing. Nevertheless, CPA was residual after 10 months of ageing.
(Figure 7.1). Compared to the CPA amount previously contaminated in milk before any processing, the average quantity of CPA left in cheese after 10 months of processing was still 0.93μg/g, which is 93% of the concentration of CPA in milk. This phenomenon of the variation of the value of the mycotoxin was also observed during the ageing process of Cheddar cheese manufactured with milk containing AFM₁. This may be due to the changes within the cheese occurring during the ageing process that provoked a recovery of more or less mycotoxin (Brackett and Marth, 1982c). However, in the case of CPA, more study on the carry-over of the mycotoxin into different types of cheese is needed before any further explanation is attempted. Moreover, during ageing of Cheddar cheese, proteolysis and lipolysis occur (Brackett and Marth, 1982c). Previous study on the relation between the mycotoxin and milk components and evidence shows the influence of the proteolysis and lipolysis on the recovery of CPA during the extraction of cheese is still not available. Therefore, the behaviour of CPA with different components of milk such as proteins and casein need to be further studied.

The recovery of CPA in whey in Table 7.3 (0.6 - 0.7μg/mL) shows that the levels of the mycotoxin were much lower than that in milk. However, the total amount of CPA in whey was between 59 - 63% compared to the content of the mycotoxin initially in milk prior to any processing. Whey product utilisation is increasing in various industries. Whey product is also used in formulating animal feed and human food. Food product application for whey products includes infant foods, soups, confectionery, margarine, ice cream, salad dressing or prepared dry mixes (Morr, 1992). It is unknown if CPA could become a potential risk for the consumers of whey products. Since information on the stability of CPA in whey and whey products during or following processing is still not available, a study on CPA in whey products is needed before any further explanation is attempted. However, as has been previously mentioned, our earlier study revealed that CPA in milk products resisted the heat usually used in the dairy industry to process milk (Prasongsidh et al., 1998b). Moreover, processing of fermented milk including frozen, freeze dried and spray dried milk could not eliminate the mycotoxin from the products (Prasongsidh et al., 1998d). Therefore, it would be of great interest to further
evaluate the possible stability of CPA at its level found in whey and in the processing of whey products.
Chapter VIII:

EFFECTS OF ULTRAVIOLET ENERGY, HYDROGEN PEROXIDE AND RIBOFLAVIN ON CYCLOPIAZONIC ACID.

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* Submitted and revision for publication in “Food Research International”

VIII.1. INTRODUCTION

Increased incidence of mycotoxins in food or feed motivated studies on the inactivation or elimination of the toxins to significantly lower levels (Ellis et al., 1991; Bailey et al., 1994; Clavero et al., 1994; Saad et al., 1995). A number of methods have been evaluated including biological, chemical and physical treatments (Tabata et al., 1994; Rustom, 1997). Elimination of AFB$_1$ from feed or AFM$_1$ from milk by chemical inactivation using low concentrations of hydrogen peroxide (H$_2$O$_2$) in combination with riboflavin was generally found as a plausible approach (Screenivasamurthy et al., 1967; Applebaum and Marth, 1982a).

Despite the use of hydrogen peroxide in milk and milk products being prevented in some countries since 1962, detoxification or elimination of mycotoxins from food and feed using hydrogen peroxide with or without association to other types of physical or chemical treatment was extensively studied (Considine and Considine, 1982; Applebaum and Marth, 1982a, Yousef and Marth, 1986, Fouler et al., 1994). Treatment of aflatoxins by hydrogen peroxide appeared to be remarkable due to its feasibility of being conducted at low concentrations ($\leq 1\%$) in a sensitive food such as milk or at less than 6$\%$ in peanut meal (Screenivasamurthy et al., 1967; Applebaum and Marth, 1982a;).

Vitamin B$_2$ or riboflavin and hydrogen peroxide can engender singlet oxygen (Korycka-Dahl and Richardson, 1978). Applebaum and Marth (1982a) described riboflavin as a photosensitiser, which was electronically excited when it absorbed UV-visible light and generated singlet oxygen capable of reacting with high density electrons moieties. The structure of AFM$_1$ in milk appeared to be altered by the association of riboflavin and hydrogen peroxide.

Exposure of aflatoxins to 365 nm light generated photochemically driven reactions inducing a development of a less toxic photodegradation product (Andrellos et al., 1967). Many researchers considered application of UV-rays to enrich vitamin D in milk a physical method capable of reducing AFM$_1$ in aqueous solution and in milk (Yousef and Marth, 1985; 1987). In contrast, the success of practical applications of UV radiation as a treatment method for aflatoxin reduction was uncertain for other researchers (Considine and Considine, 1982). A combination
of physical and chemical methods in the treatment for aflatoxin reduction was also studied. Hydrogen peroxide and UV-visible radiation degrading AFM,

in milk was reported by Yousef and Marth (1985; 1986). AFB,

is sensitive to UV light at 222, 265, 362 nm and particularly 365 nm, which induces the aflatoxins to undergo a photochemically driven series of reactions when analysis is done on silica gel TLC plates (Rustom, 1997). Since CPA and AFB,

are co-produced by the same fungi and carried over in to milk like AFM,

, it would be of great interest to assess the effect of chemical and/or physical treatments of the aflatoxins on CPA (Cvetnic, 1994; Dorner et al., 1994).

The objective of the study in this chapter was to determine the effect of hydrogen peroxide, riboflavin and UV radiation on CPA in milk. Milk components could interfere in the outcome of CPA treatment, therefore, preliminary study was done on standard CPA dissolved in bi-distilled water (Milli-Q-Water). A comparison of the effect of various treatments in aqueous and in milk systems was conducted. Moreover, the effect of enzymes indigenous to milk and UV-visible radiation on CPA in milk was also evaluated using the model of Yousef and Marth (1986).

**VIII.2. MATERIALS AND METHODS**

**VIII.2.A. Preparation of aqueous solution and milk artificially contaminated with CPA.**

An aqueous solution consisted of bi-distilled water (Milli-Q-Water) or McIlvaine buffer. Whole milk was provided by the same source as described in **III.2.A.** Cyclopiazonic acid (Sigma-Aldrich, Sydney, NSW, Australia) in methanol was artificially contaminated into bi-distilled water (Milli-Q-Water) or pH buffer or whole milk at 4°C (2μg CPA/mL) constantly agitated with a magnetic stirrer in an opaque bottle as described in **II.3.A.c.**

**VIII.2.B. Treatment of CPA in aqueous solution.**

**VIII.2.B.a. UV-visible radiation on CPA in aqueous solution**

The source of UV-visible energy in this study was a Deuterium lamp (200 nm - 325 nm) 100 watt and a Tungsten halogen lamp (325 nm - 900 nm) of a UV-visible spectrophotometer (Ultrspec+ II, LKB, Biochrom 4050) set at ca. 222 and 365 nm (main wavelengths). An accessory base plate capable of accommodating a 100mm
diameter cylindrical cell was installed in the cell compartment. Adjustment of the position of the base plate was done to ensure that the light beam was located centrally in the cell holder’s window. The sample bottle containing 400mL of 2μg CPA/mL contaminated bi-distilled water (Milli-Q-Water) was shaken in an ice water bath shaker (Tecator 1024, Linbrook, Högana, Sweden). The solution was pumped at a flow of 200mL per min by a peristaltic pump (Vera, Manostat, California, USA) into the cylindrical cell then recirculated back again to the sample bottle. Constantly shaken and cooled sample (4°C) in the bottle was circulated to the cylindrical cell and exposed to the UV-visible light once every 2 min. This was maintained by adjusting the flow speed of the pump. The temperature of the sample was constantly monitored and recorded by Squirrel data logger 1200 (Grant Instruments, Cambridge, England). Ten mL was taken as a control then samplings were done in triplicate at 0, 5, 15, 30, 45 and 60 min following the exposure of the solution to the UV-visible light. All samples were kept in 100mL opaque bottles and promptly used for extraction of CPA.

VIII.2.B.b. Effect of UV-visible radiation and acidic pH on CPA.

McIlvaine buffer at pH 4 was prepared using A.C.S. grade Na₂HPO₄·12 H₂O, citric acid and KCl (Elving et al., 1956). Similar to the study on the effect of pH on CPA, known concentrations of pure CPA in methanol were mixed into 400mL buffer solution having a final concentration of 2μg CPA/mL with the aid of a magnetic stirrer (Prasongsidh et al., 1998c). The CPA contaminated buffer was sterilised by filtering through a 0.45μm filter and kept at 4°C in a 1L pre-sterilised opaque bottle in the water bath shaker containing ice water. The method and material used to irradiate the sample is described earlier. Triplicate samples of 10mL were taken as previously described after 1h. CPA was extracted promptly from all samples.

VIII.2.B.c. Treatment of UV-visible radiation and hydrogen peroxide or riboflavin.

Four batches of aqueous solutions containing 1% and 6% hydrogen peroxide and 0.5mM and 3.2mM riboflavin were placed each in 1L opaque bottles and contaminated with 2μg CPA/mL. The method and material of UV-visible radiation at 4°C as well as the sampling and extraction were conducted as previously described.

Three batches each of 400mL bi-distilled water (Milli-Q-Water) containing 1% hydrogen peroxide were placed in 1L opaque bottles hermetically closed with screw caps. A known concentration of pure CPA in methanol was mixed into the solution of hydrogen peroxide having a final concentration of 2μg CPA/mL and triplicate samples of 10mL were promptly taken. One of the bottles was kept at 4°C in a water bath shaker containing ice water. Two others were in other water bath shakers preset at 30°C and 60°C. Triplicate samples were taken from three bottles and promptly extracted for CPA as previously mentioned. A similar treatment was repeatedly done on 3 other batches of 400mL bi-distilled water (Milli-Q-Water) containing 6% hydrogen peroxide.


Three batches of 400mL bi-distilled water (Milli-Q-Water) containing 1mM riboflavin were artificially contaminated with 2μg CPA/mL and treated at 4, 30 and 60°C in similar conditions and methods previously described.

VIII.2.B.f. Effect of the combination of hydrogen peroxide and riboflavin on CPA.

A measured volume (400mL) of a 2μg CPA/mL contaminated aqueous solution containing 1% hydrogen peroxide and 0.5 riboflavin in a 1L bottle was shaken at 4°C in a water bath shaker containing ice water. Similar to the method of AFM, study, an equal volume of 2μg CPA/mL in aqueous solution containing 6% hydrogen peroxide associated with 3.2mM riboflavin was similarly shaken at 4°C (Yousef and Marth, 1987). Triplicate samples of 10mL were taken and promptly extracted as previously described.

VIII.2.C. Treatment of CPA in milk.


Three batches of 400mL contaminated raw whole milk at 2μg CPA/mL were placed in 1L opaque bottles sealed with screw caps. One bottle of milk was kept at
4°C in a water bath shaker containing ice water. The two other bottles of milk were placed in two water bath shakers preset at 30°C and 60°C. Radiation by UV-visible light including sampling and extraction were done as previously described for CPA in aqueous solution.

**VIII.2.C.b. Treatment of CPA in milk with hydrogen peroxide with or without riboflavin under different temperatures.**

Three batches of 400mL of 2μg CPA/mL contaminated raw whole milk containing 1% hydrogen peroxide were placed in 1L opaque bottles hermetically closed with screw caps. The three batches of samples were shaken in a water bath shaker set at 4, 30 and 60°C as previously described for the study on CPA in aqueous solution. The sampling and extraction were as mentioned previously. A similar study was also done on 2μg CPA/mL contaminated raw whole milk treated by 1% hydrogen peroxide in association with 1mM riboflavin.

**VIII.2.C.c. Effect of enzymes indigenous to milk combined with UV-visible radiation on milk contaminated with CPA.**

Four hundred mL of whole raw milk in a 1L bottle with screw cap was pre-heated on a hot plate stirrer (Cimarec 3, Barnstead/Termolyne, USA) until 90°C was reached before the bottle was transferred into a shaking water bath (Tecator 1024, Linbrook, Höganäs, Sweden) set at the same temperature and held for 10 min similar to the method of Yousef and Marth (1986). The heated milk was immediately cooled and stored at 4°C in an ice bath before being contaminated by 2μg CPA/mL with two other batches of 400mL non-heated whole raw milk. One of the latter batches was promptly extracted for CPA prior to any treatment and was taken as a control. The two batches left (heated and non heated milks) were similarly irradiated by UV-visible radiation at 4°C as previously described. All the obtained results were based on the mean of triplicate samples.
VIII.2.D. Analysis of CPA by capillary electrophoresis

CPA was extracted from aqueous solution or from milk and analysed using the modified method of Prasongsidh et al. (1998a) as described in II.3.C. Aqueous solution samples were alkalised with MeOH-NaHCO₃, acidified with HCl, then CPA extracted with CHCl₃. In contrast, milk samples were defatted prior to extraction then cleaned in a Silica gel cartridge Sep-Pak® (Waters, Los Angeles, California, USA) and eluted with chloroform-methanol. Samples cleaning, CE conditioning and mobile phase were similar as described in II.3.C. All results were expressed as means of triplicate samples and Student's t-Test was used to analyse the data.

VIII.3. RESULTS AND DISCUSSION.

VIII.3.A Effect of UV-visible radiation

To simulate the temperature at which milk is stored, preliminary study on the effect of UV-visible radiation on CPA in aqueous solution was conducted at 4°C. UV-visible radiation showed little effect on 2μg CPA/mL in the aqueous solution in the first 30 min of treatment (Figure 8.1). CPA levels in control aqueous solution and in samples treated for 30 min by UV-visible radiation were not significantly different (p>0.05). A slight decrease of CPA was observed after 45 min exposure (5.7%) (p>0.05) and 60 min (9.1%) (p≤0.05).

A similar trend was also observed when CPA in contaminated milk was exposed to UV-visible radiation at 4°C (Table 8.1) but with less intensity (6.5% after 45 min and 7.6% after 60 min). However, exposure of CPA in milk to UV-visible radiation at higher temperatures (30°C and 60°C) was followed by an intensified CPA decrease (p≤0.05) of 17.5% and 24.1% respectively (Table 8.1). In contrast to what was found in our previous study, heating contaminated raw milk to 60°C for 1h caused CPA levels to decrease less than 9% (Prasongsidh et al., 1998b). Association of UV-visible radiation and higher temperatures (60°C) seems to further decrease CPA levels in milk.

The decrease in CPA concentration by UV-visible radiation found in this study appeared to be less when compared to that of AFM₁ (Yousef and Marth, 1985; 1987).
Figure 8.1. Effect of UV-visible radiation with or without acidic pH (4) or hydrogen peroxide (1 and 6%) on 2μg CPA/mL in aqueous solution at 4°C in 1h. The results of the studies on the effect of UV-visible radiation on CPA in aqueous solution were in the form of means of triplicate samples.
Table 8.1. Effect of UV-visible radiation on 2μg CPA/mL in raw milk at different temperature (4°, 30° or 60°C) in 1h.

<table>
<thead>
<tr>
<th>Period of exposure</th>
<th>CPA Residual in milk at 4°C (μg/mL)</th>
<th>SD</th>
<th>RSD</th>
<th>CPA Residual in milk at 30°C (μg/mL)</th>
<th>SD</th>
<th>RSD</th>
<th>CPA Residual in milk at 60°C (μg/mL)</th>
<th>SD</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>1.817</td>
<td>0.030</td>
<td>0.001</td>
<td>1.811</td>
<td>0.050</td>
<td>0.001</td>
<td>1.811</td>
<td>0.040</td>
<td>0.001</td>
</tr>
<tr>
<td>5 min</td>
<td>1.783 b</td>
<td>0.012</td>
<td>0.000</td>
<td>1.783</td>
<td>0.041</td>
<td>0.001</td>
<td>1.760 b</td>
<td>0.081</td>
<td>0.001</td>
</tr>
<tr>
<td>15 min</td>
<td>1.761 b</td>
<td>0.024</td>
<td>0.000</td>
<td>1.692 b</td>
<td>0.058</td>
<td>0.001</td>
<td>1.691 b</td>
<td>0.001</td>
<td>0.000</td>
</tr>
<tr>
<td>30 min</td>
<td>1.725 b</td>
<td>0.009</td>
<td>0.000</td>
<td>1.652</td>
<td>0.231</td>
<td>0.004</td>
<td>1.619 b</td>
<td>0.013</td>
<td>0.000</td>
</tr>
<tr>
<td>45 min</td>
<td>1.700 b</td>
<td>0.016</td>
<td>0.000</td>
<td>1.610 b</td>
<td>0.034</td>
<td>0.001</td>
<td>1.573 b</td>
<td>0.015</td>
<td>0.000</td>
</tr>
<tr>
<td>60 min</td>
<td>1.680 b</td>
<td>0.041</td>
<td>0.001</td>
<td>1.495 b</td>
<td>0.033</td>
<td>0.000</td>
<td>1.375 b</td>
<td>0.078</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Period of exposure to UV-visible radiation and temperature (4°, 30° or 60°C);

b Significant difference from milk prior exposure to radiation and temperature (p≤0.05).

c Triplicate samples; SD = Standard deviation; RSD = Relative standard deviation.
The nature of the mycotoxins and the difference in UV-visible source, or the UV exposure process in this study may be the reason. Influence of the variety of CPA exposure processes to UV-visible radiation needs to be further evaluated.

Association of acidic pH (4) and exposure to UV-visible radiation at 4°C dramatically decreased (p≤ 0.05) CPA in aqueous solution for the first 15 min of exposure (Figure 8.1). Further CPA decrease (45%) occurred after 1h (p≤0.05).

Our previous study revealed that manufacture of yogurt with contaminated milk induced CPA reduction. However, in spite of CPA decreases due to the acidity and the heat of manufacture of yogurt, the mycotoxin was not completely eliminated (Prasongsich et al., 1998c). CPA reduction in this study seems to suggest that UV-visible radiation can further decrease CPA levels remaining in processed yogurt manufactured with CPA contaminated milk.

**VIII.3.B. Effect of hydrogen peroxide**

One % hydrogen peroxide reduced CPA at 4°C in aqueous solution after 5 min of the commencement of the treatment (Figure 8.2). Nearly 14% of CPA was removed after the first 15 min of treatment (p≤0.05). The amount of CPA decreased after 1h to 60% compared to its initial level. A dramatic CPA decrease (p≤0.05) in aqueous solution was obtained when 6% hydrogen peroxide was used at 4°C (Figure 8.2). The amount of CPA was lowered by 70% at the beginning and 86% at the end of the treatment (Figure 8.2).

The reduction of CPA by 1% or 6% hydrogen peroxide was enhanced (p≤0.05) by an exposure to higher temperatures (Figure 8.2) or to UV-visible radiation (Figure 8.1). More than 85% of CPA was reduced when the contaminated aqueous solution was heated to 60°C and treated with 1% hydrogen peroxide (Figure 8.2). In contrast, CPA was undetectable after the first 15 min and at 45 min following a treatment with 6% hydrogen peroxide at 60°C and 30°C respectively (Figure 8.2). However, a level of 6% hydrogen peroxide cannot be used in milk since it is impractical and illegal (Applebaum and Marth, 1982a).

Treatment with 1% hydrogen peroxide and exposure to 4°C, 30°C and 60°C in milk induced after the first 15 min, a similar decrease (p≤0.05) in CPA (Figure 8.3).
Figure 8.2. Effect of hydrogen peroxide (1 and 6%) at different temperatures (4°C, 30°C or 60°C) on 2μg CPA/mL in aqueous solution in 1h. The effect of hydrogen peroxide at different temperatures on CPA was studied in triplicate.
Figure 8.3. Effect of 1% Hydrogen peroxide with or without 1mM riboflavin on 2μg CPA/mL in raw milk at different temperatures (4°C, 30°C or 60°C) in 1h. The results of the studies on the effect of Hydrogen peroxide with or without riboflavin on CPA in milk were in the form of means of triplicate samples.
However, the reduction of CPA in milk was far less intense (p≤0.05) than in aqueous solution (Figures 8.4 and 8.2). After an hour of treatment with 1% hydrogen peroxide at 4°C, CPA contaminated levels in milk were lowered by 26% compared to 40% in the aqueous solution (Figure 8.3). CPA decrease in milk was boosted (41% and 51%) by exposure to an increased temperature of 30° and 60°C (Figure 8.3). However, these increases were less compared to those of CPA in aqueous solution (64% and 85% respectively) (Figure 8.2).

It was not understood why this phenomenon occurred. No evidence had been found in this study to suggest that CPA had any affinity with casein or the other proteins in milk in similar to AFM₁ that partially interfered with an inactivation or decrease of the mycotoxin in milk (Applebaum and Marth, 1982a and 1982b). Therefore, a study on CPA association with casein or other milk protein is needed before any further explanation is attempted.

VIII.3.C. Effect of riboflavin

The mechanism by which riboflavin alone or associated with hydrogen peroxide can alter CPA was not known. However, a decrease (p≤0.05) of CPA in aqueous solution by 1mM riboflavin at 4°C correlated with time (Figure 8.4). Moreover, a similar trend of CPA decrease was obtained when combined with UV-visible radiation even with only 0.5mM riboflavin in aqueous solution (Figure 8.4). It seemed that the degradation of CPA was boosted by UV-visible radiation. This appeared to be correlated with the amount of riboflavin. Increasing riboflavin to 3.2mM in the treatment associated with 6% hydrogen peroxide at 4°C induced complete elimination of CPA after 30 min of treatment (Figure 8.4).

Increasing temperature (30 and 60°C) in the treatment of CPA with 1mM riboflavin induced a further decrease (p≤0.05) of the mycotoxin (Figure 8.4). Combining a low concentration of riboflavin (0.5mM) and 1% hydrogen peroxide removed more (p≤0.05) CPA at 4°C (Figure 8.4) than the treatment with 1% hydrogen peroxide alone (Figure 8.2).
Figure 8.4. Effect of 1 mM riboflavin alone on 2 μg CPA/mL at different temperatures (4°C, 30°C or 60°C). This figure also shows the effect of combinations of 0.5 mM riboflavin with 1% hydrogen peroxide or 3.2 mM riboflavin with 6% hydrogen peroxide on 2 μg CPA/mL in aqueous solution 4°C. The effect of riboflavin with hydrogen peroxide on CPA was studied in triplicate.
In contrast, despite an association of 1% hydrogen peroxide to a higher concentration of riboflavin (1mM) in the treatment of CPA in milk at 4°C, a less significant decrease (p≤0.05) of CPA in contaminated milk (Figure 8.3) than that in aqueous solution (Figure 8.4) was observed after 15 min of treatment. It is not known if the reduction of the mycotoxin was interfered with its association with any of milk components.

A combination of riboflavin at 3.2mM with 6% hydrogen peroxide (Figure 8.4) was capable of completely eliminating CPA in aqueous solution following 30 min of treatment at 4°C. However, the use of such high concentrations of hydrogen peroxide and riboflavin in milk is illegal as previously mentioned (Applebaum and Marth, 1982a).

**VIII.3.D. Interference of indigenous enzymes**

The interference of indigenous enzymes in milk in the reduction of CPA by the UV-visible radiation was studied. No significant difference (p>0.05) between the amount of CPA in control milk and the two irradiated milks was observed (Table 8.2). Enzymes indigenous to milk seemed not to interfere with the reduction of CPA in milk by UV-visible radiation. However, a tendency for CPA to decrease was observed in both irradiated milks (Table 8.2).

**VIII.4. CONCLUSION**

It is not known if the opacity of the milk can prevent the effect of UV-visible radiation on CPA. However, there was more degradation of the toxin in aqueous solution than that in milk under the treatment by UV energy.

If contaminated milk was treated by UV-visible radiation with or without hydrogen peroxide and/or riboflavin to inactivate CPA, the carry over CPA in the milk would not be completely eliminated by the treatment. However, association of hydrogen peroxide, riboflavin and higher temperature (≤60°C) in the treatment could alter CPA. CPA residue would still be detectable in milk despite the treatment, because high concentrations of hydrogen peroxide (6%) and riboflavin (3.2mM) that are capable of completely eliminating CPA, is prohibited and impractical to use for milk treatment (Applebaum and Marth, 1982a). CPA is likely to be left over in milk despite the treatment and so may become a potential risk for dairy consumers.
Riboflavin is one of numerous vital nutrients naturally existing in milk. Since the occurrence of CPA in cheese curd through contamination by using contaminated milk is possible (Chapter VII) (Prasongsidh et al., 1998f), it is of great interest to further investigate the effect of B vitamin at normal levels in milk, especially on cheese naturally contaminated CPA. Moreover, the effect of B vitamin in cheese contaminated due to the use of fungal producers of CPA in the cheese making process is still required (Still et al., 1978a; Le Bars et al., 1988).

In contrast to other mycotoxins, inactivation of CPA in food to significantly lower levels has never been reported in literature (Chapter I; 1.5.B). Therefore, references to aflatoxin in this chapter are based on the method and the findings of this study.
Table 8.2. Effect of enzymes indigenous to milk on CPA exposed for 1h to UV-visible radiation at 4°C.

<table>
<thead>
<tr>
<th>Type of contaminated milk</th>
<th>CPA Residual (µg/mL)</th>
<th>SD</th>
<th>RSD</th>
<th>CPA residual in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control contaminated milk</td>
<td>1.742 d</td>
<td>0.020</td>
<td>0.000</td>
<td>87.1</td>
</tr>
<tr>
<td>Heated milk</td>
<td>1.727 d</td>
<td>0.058</td>
<td>0.001</td>
<td>86.4</td>
</tr>
<tr>
<td>Non heated milk</td>
<td>1.717 d</td>
<td>0.135</td>
<td>0.002</td>
<td>85.9</td>
</tr>
</tbody>
</table>

* Whole raw milk contaminated with 2µg CPA/mL was extracted without treatment.

* Heated whole raw milk at 90°C for 10 min to eliminate milk enzymes, cooled to 4°C, contaminated with 2µg CPA/mL and radiated by UV-visible for 1h.

* Non heated whole raw milk was contaminated with 2µg CPA/mL and radiated at 4°C by UV-visible for 1h.

* No significant difference between samples or control (p>0.05).

* Triplicate samples.

SD = Standard deviation, RSD = Relative standard deviation.
CHAPTER IX:

EFFECTS OF FOOD ADDITIVES AND AQUEOUS AMMONIA ON CYCLOPIAZONIC ACID.

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IX.1. INTRODUCTION

Despite abundant reports on fungi producers of CPA in food and feedstuffs and accumulation of the toxin in tissue or animal products as described in Chapter I (Norrred, 1990), inactivation or reduction of the mycotoxin into significantly lower levels has not been reported in literature.

CPA and aflatoxin have the possibility of being present in a single food commodity under the same circumstances. Compared to CPA, reduction of aflatoxin has been extensively reported as described in Chapter I. The potential of reported methods to destabilise aflatoxins in food to decontaminate CPA has not yet been reported in literature.

Special treatments reported to reduce mycotoxins in milk include biological, chemical and physical methods (Tabata et al., 1994; Rustom, 1997). Acid produced by bacteria or moulds can inactivate aflatoxins (Park and Liang, 1993). Reported treatments that physically remove mycotoxins were solvent extraction, diafiltration and adsorption by bentonite (Doyle et al., 1982; Hrong, et al., 1994; Higuera-Ciapara et al., 1995). Reduction of mycotoxins by using solar energy, heat, pH, moisture and UV-radiation with or without chemical treatment was also reported (Kiermeier and Mashaley, 1977; Mahjoub and Bullerman, 1988; Yousef and Marth, 1985; 1986; Nkama and Muller, 1988; Gelosa and Buzzetti, 1994). Among additives used to inactivate aflatoxins were hydrogen peroxide, sodium hypochlorite, sodium sulfite and ammonia, bisulfite (Screenivasamurthy et al., 1967, Doyle and Marth, 1978b; Applebaum and Marth, 1980c; Hagler et al., 1982; Mukendi N’Gombo et al., 1992; Cole, 1989; Bailey et al., 1994). Tabata et al., (1994) confirmed that acidic, neutral and alkaline food additives had potential to degrade or remove aflatoxins during food processing.

Food additives are not normally used in the dairy industry due to public health reasons. However, some food additives have been or are being used in the dairy industry such as in cheese production to prevent microbial spoilage (Smith and Harran, 1993). Since CPA and aflatoxins may be derived from the same source and can occur in similar food commodities or carry over into milk, it would be of great
interest to evaluate effects of food additives, reported to be used in the treatment of aflatoxins, to reduce CPA (Tabata et al., 1994).

Due to the significance of the consumer’s health and the economies of food and feed, the possibility of CPA reduction should be established. Since milk is a complex milieu, it is difficult to monitor CPA reaction products under a treatment with food additives. CPA in bi-distilled water (Milli-Q-Water) was used in this preliminary study.

The objective of the study in this chapter is preliminary to assess CPA behaviour from treatment with chemicals or food additives. A study on the effectiveness of food additives on CPA with different exposure times or temperature levels is needed. This chapter provides primary information before any in depth study on the treatment of CPA in food is attempted.

IX.2. MATERIALS AND METHODS

IX.2.A. Preparation of artificially contaminated CPA solutions.

Aqueous solutions consisted of appropriate concentrations of food additives or chemical substances in bi-distilled water (Milli-Q-Water). Pure solid cyclopiazonic acid (Sigma-Aldrich, Sydney, NSW, Australia) in methanol was stored at 0°C in the dark until used. Artificially contaminated aqueous CPA solutions at 2μg CPA/mL were prepared similar to the method of Wiseman and Marth (1983b) as described in II.3.A.c.

IX.2.B. Effect of treatment with 1% food additive α at 4°C for 16h period on CPA.

Fifty milliliters of bi-distilled water (Milli-Q-Water) in 100mL pyrex bottles with screw caps were kept in a water bath shaker (Tecator 1024, Linbrook, Höganä,

α Hydrochloric acid, sulfuric acid, sodium bicarbonate, sodium carbonate, sodium hydroxide, sodium sulfite, sodium hypochlorite, sodium metabisulfite, sodium dithionite, hydrogen peroxide, sodium chloride, ammonium peroxodisulfate, potassium bromate, potassium nitrate and sodium nitrite.
Sweden) containing cold water (4°C) continuously circulated by a peristaltic pump (Vera, Manostat, California, USA) from an ice-water tank. While shaking the samples in the water bath, pure CPA in methanol was added to all bottles to give a final concentration of 2μg CPA/mL. A known amount of the mixture from each bottle (in triplicate) was taken as control samples. A known quantity of concentrated food additive or ammonia was added to the CPA solution to have a final concentration of 1%. After closure with screw caps, all sample bottles were continually shaken for a 1h period and kept at 4°C over night then promptly used for extraction of CPA. The study was done in triplicate.

**IX.2.C. Effect of treatment time with 1% food additive at 4°C.**

Food additives that reduced CPA levels by more than 50% in the previously mentioned study (IX.2.B. Effect of treatment with 1% food additive at 4°C for 16h period on CPA.) were selected. Similar preparation of samples and methods of treatment were performed. Triplicate samples were taken at the beginning, as control of treatment with food additives. Sampling was done at 5, 15, 30, 45 and 60 min then at 2h, 4h and 8h periods after initiating the treatment. All samples were promptly extracted and analysed for CPA.

**IX.2.D. Influence of temperature on the effect of 1% food additives on CPA.**

The same selected food additives at 1% were similarly prepared and treated as mentioned previously. Instead of shaking the sample bottles in cool water (4°C), similar samples in screw cap bottles were shaken in a water bath set at a temperature of 30° and 60°C. Sampling was done after a 1h period of treatment. All samples treated at 30° and 60°C were cooled in ice water prior to CPA extraction and analysis.

**IX.2.E. Effect of concentration of food additives on CPA**

Preparation of samples was done as previously described (solution of 2μg CPA/mL). Known quantities of concentrated food additive were added to CPA

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\(^b\) See \(^a\) in the foot note of the page 169.
solutions to have final concentrations of 1, 0.5, 0.2 and 0.1%. Triplicate samples were promptly extracted after a 1h period of treatment then analysed for CPA.

**IX.2.F. Analysis of CPA by capillary electrophoresis**

CPA was extracted from contaminated samples and analysed using the modified method of Prasongsidh et al. (1998a) as described in **II.3.C** but omitting the defatting step. Sample cleaning, CE conditioning and mobile phase were similar as described in **II.3.C**. All results were expressed as means of triplicate samples and Student’s t-Test was used to analyse the data.

**IX.3. RESULTS AND DISCUSSION**

Similar to the model of Tabata et al., (1994), this study explored the possible effect of food additives on CPA in aqueous solutions. Trials for appropriate treatment conditions, capable of decreasing CPA were done. Since CPA can occur in feed and food, especially in dairy products usually kept at 4°C, the preliminary study was done at this temperature for a 16h period.

**IX.3.A. Effect of food additives\(^2\) on CPA at 4°C overnight.**

**IX.3.A.a. Effect of acidic food additives.**

Table 9.1 shows that CPA in aqueous solution is decreased by acidic food additives such as hydrochloric acid (HCl) and sulfuric acid (H\(_2\)SO\(_4\)). The decrease of the toxin was less than 30% of its original level (2μg CPA/mL) when subjected to such treatment. CPA in aqueous solution (Goto and Manabe, 1992) or in acidic milk (Prasongsidh et al., 1998c) was previously reported to be also decreased by an acidic pH.

Degradation by strong acids and bases was also observed in aflatoxins (Considine and Considine, 1982). Decrease of aflatoxin B\(_1\), which transformed into aflatoxin B\(_2\), in acidic aqueous solution (Tabata et al., 1994) and acidic milk (Rasic et al., 1991) was reported.

\(^2\) See \(^{a}\) in the foot note of the page 169.
Table 9.1. Effect of acidic food additives on 2μg CPA/mL in aqueous solution.

<table>
<thead>
<tr>
<th>Acidic food additives</th>
<th>Original pH</th>
<th>CPA Residual (μg/mL) (^a)</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1.811</td>
<td>0.028</td>
<td>0.001</td>
<td>9.4</td>
</tr>
<tr>
<td>hydrochloric acid</td>
<td>1.1</td>
<td>1.487</td>
<td>0.071</td>
<td>0.001</td>
<td>25.7</td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>1.2</td>
<td>1.413</td>
<td>0.225</td>
<td>0.003</td>
<td>29.4</td>
</tr>
</tbody>
</table>

\(^a\) CPA was treated with 1% of acidic food additive at 4°C for 16h.

\(^b\) Triplicate samples.

\(^b\) Percentage of CPA decreased comparing to amount contaminated.

SD = Standard deviation, RSD = Relative standard deviation.

CPA was also reduced by alkaline food additives (Table 9.2) including sodium bicarbonate (NaHCO₃), sodium carbonate (Na₂CO₃), sodium hydroxide (NaOH), sodium sulfite (Na₂SO₃) and sodium hypochlorite (NaOCl). CPA levels were reduced to nearly 39% with sodium bicarbonate or sodium carbonate. Insolubility was not observed following addition of CPA to sodium bicarbonate solution to suggest that CPA derivative was occurred (Holzapfel, 1968). This might due to a low level of CPA (2μg/mL). Nearly 50% of CPA was still recovered after treatment with 1% sodium hydroxide or sodium sulfite. In contrast, CPA was completely eliminated by 1% sodium hypochlorite. Previous reports revealed that CPA was reduced in aqueous solution by alkaline pH at 30°C (Goto and Manabe, 1992). A previous study conducted with a similar treatment for 24h, but at 4°C obtained a lesser reduction of CPA level (Prasongsidh et al., 1998c). Holzapfel (1968) reported that CPA was partially converted into an isomer when vigorously treated with aqueous alkali or mineral acid.


Table 9.3 shows that percentage of CPA decrease was more than 50% when treated with neutral food additives except sodium dithionite (Na₂S₂O₄). Potassium nitrate (KNO₃) and sodium nitrite (NaNO₂) reduce CPA levels by 58% and 64.5% respectively (Table 9.3).

Despite being unpopular due to formation of carcinogenic compounds, the use of nitrate and nitrite to prevent unwanted formation of gas in cheese is still practiced in some countries. Nitrate can reduce hydrogen formation due to coliform bacteria. In contrast, nitrite inhibits the growth of clostridia (Mogensen, 1993). Despite this finding, the effect of nitrate or nitrite in cheese on CPA still needs further study (Table 9.3).

Hydrogen peroxide and sodium hypochlorite reduced CPA levels in aqueous solutions by 62% and 100% respectively (Tables 9.2 and 9.3). Hydrogen peroxide and sodium hypochlorite are commonly used as oxidising agent to eliminate aflatoxins. Moreover, it has dramatic effects on aflatoxins B₁ and G₁ but less on B₂ and G₂ after a 16h period of treatment at 40°C (Tabata et al., 1994).
Table 9.2. Effect of alkaline food additives on 2µg CPA/mL in aqueous solution.

<table>
<thead>
<tr>
<th>Alkaline food additives</th>
<th>Original pH</th>
<th>CPA Residual (µg/mL) **</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.0</td>
<td>1.811</td>
<td>0.028</td>
<td>0.001</td>
<td>9.4</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>8.0</td>
<td>1.387</td>
<td>0.159</td>
<td>0.002</td>
<td>30.6</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>11.3</td>
<td>1.225</td>
<td>0.012</td>
<td>0.000</td>
<td>38.8</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>12.4</td>
<td>1.015</td>
<td>0.018</td>
<td>0.000</td>
<td>49.2</td>
</tr>
<tr>
<td>Sodium sulfite</td>
<td>9.9</td>
<td>1.062</td>
<td>0.038</td>
<td>0.000</td>
<td>46.9</td>
</tr>
<tr>
<td>Sodium hypochlorite</td>
<td>11.6</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* CPA was treated with 1% of alkaline food additives at 4°C for 16h

** Triplicate samples.

SD = Standard deviation, RSD = Relative standard deviation.
Table 9.3. Effect of neutral food additives on 2µg CPA/mL in aqueous solution.

<table>
<thead>
<tr>
<th>Neutral food additives</th>
<th>Original pH</th>
<th>CPA Residual (µg/mL) *a</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.0</td>
<td>1.811</td>
<td>0.028</td>
<td>0.001</td>
<td>9.4</td>
</tr>
<tr>
<td>sodium metabisulfite</td>
<td>2.9</td>
<td>0.416</td>
<td>0.002</td>
<td>0.000</td>
<td>79.2</td>
</tr>
<tr>
<td>sodium dithionite</td>
<td>5.5</td>
<td>1.019</td>
<td>0.175</td>
<td>0.002</td>
<td>49.1</td>
</tr>
<tr>
<td>hydrogen peroxide</td>
<td>4.4</td>
<td>0.758</td>
<td>0.050</td>
<td>0.000</td>
<td>62.1</td>
</tr>
<tr>
<td>sodium chlorite</td>
<td>7.2</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>100.0</td>
</tr>
<tr>
<td>ammonium peroxodisulfate</td>
<td>4.7</td>
<td>0.296</td>
<td>0.012</td>
<td>0.000</td>
<td>85.2</td>
</tr>
<tr>
<td>potassium bromate</td>
<td>6.0</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>100.0</td>
</tr>
<tr>
<td>potassium nitrate</td>
<td>5.7</td>
<td>0.841</td>
<td>0.005</td>
<td>0.000</td>
<td>58.0</td>
</tr>
<tr>
<td>sodium nitrite</td>
<td>6.1</td>
<td>0.709</td>
<td>0.225</td>
<td>0.002</td>
<td>64.5</td>
</tr>
</tbody>
</table>

* CPA was treated with 1% of neutral food additives at 4°C for 16h

*a TriPLICATE samples.

SD = Standard deviation, RSD = Relative standard deviation.
Since CPA and aflatoxin have the possibility of being present in a single food commodity (Lee and Hagler, 1991) under the same circumstances (Bryden, 1987), the use hydrogen peroxide and sodium hypochlorite to eliminate aflatoxins could also affect CPA.

Table 9.3 shows that sodium metabisulfite (Na₂S₂O₅) and ammonium peroxodisulfate [(NH₄)₂S₂O₈] decreased CPA by 79% and 85% respectively. CPA was also completely eliminated by sodium chlorite (NaClO₂) and by potassium bromate (KBrO₃).

**IX.3.A.d. Effect of organic acids used in dairy products and aqueous ammonia on CPA.**

One percent sorbic acid completely eliminated 2μg CPA/mL. In contrast, 1% benzoic acid decreased CPA by only 80% (Table 9.4). Sorbic acid and benzoic acid have been used to control fungal spoilage in cheese, or as a preservative in juice despite being illegal in numerous countries (Mogensen, 1993). One percent aqueous ammonia at 4°C for 16h induced CPA reduction by 72% (Table 9.4). The effectiveness of ammonia in inactivating aflatoxins in feedstuff has been reported (Moerck et al., 1980; Bailey et al., 1994). However, efficacy of the treatment still needed a high temperature environment (Mukendi N'Gombo et al., 1992). Co-occurrence of the aflatoxin and CPA was well known, but no report on the effect of ammoniation of CPA was available. Holzapfel et al., (1970) reported that after treatment of CPA with 25% aqueous ammonia, there was a formation of a less toxic derivative cyclopiazonic acid imine. Moreover, aqueous ammonia as low as 0.1N could still convert CPA into the imine (Holzapfel et al., 1970). Unfortunately, no CPA derivative was detected using conditions and method of analysis of this study.

**IX.3.B. Effect of concentration food additives and exposure temperatures on CPA.**

Of all food additives tested⁸, 10 were capable of reducing CPA by 60 - 100% after a 16h period treatment at 4°C (Tables 9.1, 9.2, 9.3 and 9.4).

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⁸ See a in the foot note of the page 169.
Table 9.4. Effect of organic acid used in dairy products and aqueous ammonia on 2μg CPA/mL in aqueous solution.

<table>
<thead>
<tr>
<th>Food additives</th>
<th>Original pH</th>
<th>CPA Residual (μg/mL) *a</th>
<th>SD</th>
<th>RSD</th>
<th>% decrease of CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.0</td>
<td>1.811</td>
<td>0.028</td>
<td>0.001</td>
<td>9.4</td>
</tr>
<tr>
<td>sorbic acid</td>
<td>2.9</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>100.0</td>
</tr>
<tr>
<td>benzoic acid</td>
<td>2.8</td>
<td>0.406</td>
<td>0.001</td>
<td>0.000</td>
<td>79.7</td>
</tr>
<tr>
<td>ammonia</td>
<td>10.8</td>
<td>0.567</td>
<td>0.042</td>
<td>0.000</td>
<td>71.6</td>
</tr>
</tbody>
</table>

* CPA was treated with 1% of organic acid and ammonia at 4°C for 16h

*a Triplicate samples.

SD = Standard deviation, RSD = Relative standard deviation.
Among them 6 were the neutral food additives sodium metabisulfite, hydrogen peroxide, ammonium peroxodisulfate, potassium bromate, sodium nitrate, and sodium chlorite. The others were sodium hypochlorite; sorbic acid, benzoic acid and ammonia.

**IX.3.B.a. Effect of time of treatment on CPA by some food additives at 4°C.**

Among 4 food additives capable of completely eliminating 2μg CPA/mL after 16h of treatment (Tables 9.2, 9.3 and 9.4), sodium hypochlorite (Figure 9.1), potassium bromate, sodium chlorite (Figure 9.2) still had the same effect of CPA elimination after 1h of treatment. These reductions in CPA occurred within 5 minutes of initiation the treatment (Figure 9.1 and 9.2). In contrast, sorbic acid completely eliminated CPA after 16h of treatment and was capable of reducing CPA by 74%, 81%, 84% and 86% after 1h, 2h, 4h and 8h period of treatment (Figure 9.3). Figure 9.1 shows that in the first 15 min of treatment by sorbic acid, CPA was reduced by 65 to 70%. Among the remaining food additives and ammonia, hydrogen peroxide was the least effective in reducing CPA. CPA levels decreased by 30%, 40%, 45% and 56% after a treatment of 30 min, 1h, 2h and 8h periods respectively (Figure 9.2 and 9.4). In contrast, the most effective was ammonium peroxodisulfate, which was capable of decreasing CPA by 57% in the first 15 min, 71% after 1h and 75% after 2h period (Figure 9.2 and 9.4). A similar decrease in the level of CPA was observed after 8h to 16h periods of treatment by sodium metabisulfite or benzoic acid (Figure 9.3). However, benzoic acid seemed to be more effective in a treatment of 1h to 4h (Figure 9.3). Despite the fact that sodium nitrite decreased CPA in the first 15 min by 48% (Figure 9.2) the toxin was not reduced by more than 60% after an 8h period of treatment (Figure 9.4). CPA was decreased by 60 to 62% in a 15 min to 1h period by ammonia treatment. Further reduction of CPA (67%) was observed after 8h of treatment (Figure 9.2 and 9.4)

**IX.3.B.b. Effect of temperature on the treatment of CPA.**

It appeared that selected food additives reduce more CPA in aqueous solutions at higher temperatures (Figure 9.5 and 9.6).
Figure 9.1. Effect of treatment 2μg CPA/mL in aqueous solution for 60 min at 4°C with 1% sodium hypochlorite, sodium metabisulfite, sorbic acid, benzoic acid and ammonia.
Figure 9.2. Effect of treatment 2µg CPA/mL in aqueous solution for 60 min at 4°C with hydrogen peroxide, ammonium peroxodisulfate, potassium bromate, sodium nitrate and sodium chlorite.
Figure 9.3. Effect of treatment 2μg CPAML in aqueous solution from 1h to 16h at 4°C with 1% sodium hypochlorite, sodium metabisulfite, sorbic acid, benzoic acid, and ammonia.
Figure 9.4. Effect of treatment 2μg CPA/mL in aqueous solution from 1h to 16h at 4°C with hydrogen peroxide, ammonium peroxodisulfate, potassium bromate, sodium nitrate and sodium chlorite.
Figure 9.5. Effect of temperature (4°, 30° or 60°C) on one hour of treatment 2µg CPA/mL in aqueous solution with 1% sodium metabisulfite, hydrogen peroxide, ammonium peroxodisulfate, potassium bromate, sodium nitrate and sodium chlorite.
Figure 9.6. Effect of temperature (4°C, 30°C or 60°C) on one hour of treatment 2μg CPA/mL in aqueous solution with sorbic acid, benzoic acid, ammonia and sodium hypochlorite.
Sodium chlorite, potassium bromate (Figure 9.5) and sodium hypochlorite (Figure 9.6) completely eliminated CPA at 4°C. Sodium metabisulfite, ammonium peroxodisulfate, sodium nitrite, sorbic acid, benzoic acid and ammonia completely eliminated CPA at 30°C. In contrast, hydrogen peroxide only reduced CPA by 64% and 85% at 30° and 60°C respectively (Figure 9.5).

**IX.3. B.c. Effect of concentrations of some food additives on CPA.**

Figure 9.8 shows that 0.5% sodium hypochlorite completely eliminated 2μg CPA/mL in aqueous solution after a 1h period of treatment at 4°C. Treatment by 0.1% and 0.25% sodium hypochlorite reduced CPA by 74% and 89% respectively. Among 6 selected neutral food additives, sodium chlorite and potassium bromate were the most effective at low concentrations. Reductions of CPA levels were 60% and 81% when treated with 0.1% of sodium chlorite and potassium bromate respectively (Figure 9.7) while both, at 0.25%, decreased CPA by 84%. In contrast, 0.1% ammonium peroxodisulfate, sodium nitrite, hydrogen peroxide and sodium metabisulfite reduced CPA by 45, 26, 18 and 14% respectively. Figure 9.8 shows that among organic acids, benzoic acid appeared to be the most effective, which decreased CPA by 49, 36 and 29% at the concentration of 0.5, 0.25 and 0.1% respectively. CPA was reduced by 38, 24 and 20% when treated by 0.5, 0.25 and 0.1% sorbic acid. Benzoic acid and ammonia showed a similar trend for CPA reduction. At 0.5, 0.25 and 0.1%, ammonia reduced CPA levels by 45, 39 and 27%.

**IX.4. CONCLUSION**

In conclusion, acidic food additives were less effective in the treatment of CPA in aqueous solution at 4°C when compared to alkaline, neutral food additives and organic acids. CPA in aqueous solution was completely eliminated by certain food additives at high temperatures.
Figure 9.7. Effect of sodium metabisulfite, hydrogen peroxide, ammonium peroxodisulfate, potassium bromate, sodium nitrate, sodium chlorite in different concentrations (0.1%, 0.25%, 0.5% and 1%) on 2µg CPA/mL in aqueous solution at 4°C.
Figure 9.8. Effect of sorbic acid, benzoic acid, ammonia, sodium hypochlorite in different concentrations (0.1%, 0.25%, 0.5% and 1%) on 2μg CPA/mL in aqueous solution at 4°C.
Since CPA and aflatoxin have the potential of being present in a single food commodity under the same circumstances, the use of food additives to eliminate aflatoxins could also affect CPA. Presence of nitrate or nitrite in cheese may have some effect on CPA in the product. However, a study on the effect of nitrate or nitrite on CPA during the manufacturing and ripening of cheese is needed before any further explanation is attempted.

Many food additives and ammonia are prohibited in food. Most chemical treatment methods of mycotoxins were performed under extreme conditions such as temperature, concentration and pressure, which was unsafe and unfavourable for food (Rustom, 1997). However, these methods may be used in feed to prevent the possible carry over into milk. Sorbic and benzoic acids are naturally occurring and are also legal additives to be used in dairy products especially in cheese in certain countries (Mogensen, 1993). The presence of these organic acids may affect the stability of CPA in cheese. It would be of great interest to further study the effect of sorbic acid at the levels used in cheese on CPA during the ripening period. In this study, CPA levels were affected by various food additives especially at high temperatures. However, the reaction mechanism and the kinetic reaction of each food additive on CPA need to be further studied. Certain results of CPA treatment by food additives did not correspond to reaction kinetics. Since the mean of the recovery rates of the procedure of extraction and analysis used was high, especially CPA extraction conducted from a water mixture sample (Chapter II), it could not be speculated that the reason was due to a confound between chemical loss and recovery losses. Similar observations were also found in the study of aflatoxins (Tabata et al., 1994), the loss of CPA might not be due to an experimental artefact. It is not known if CPA in naturally contaminated milk under treatment by food additives will be similarly degraded. It is of great interest to further investigate the effect each food additive has on CPA in more depth.
CHAPTER X:
GENERAL DISCUSSION

The objectives of this thesis were to assess and study the stability of CPA in milk and in dairy products processed from contaminated milk as well as to explore the possibility of eliminating the toxin from such products. However, before these studies could be commenced, a sensitive method for CPA analysis from milk and milk products needed to be developed.

The reported methods to determine CPA in agricultural commodities are laborious, complex and provide limited sensitivity and selectivity. Only TLC and gel diffusion are used to detect CPA in milk and dairy products. The report on separation, in a single analysis, of aflatoxins along with six other mycotoxins by CE induces this study on its application in the detection of CPA in milk.

The potential for applying the CE method in a single analysis to detect CPA and other compounds found in milk and milk products has shown CPA at levels similar to that found in naturally contaminated milk (Chapter II). CE methods could also be used in several areas including the detection of other mycotoxins such as tenuazonic acid in tomato juice.

CPA was not eliminated by heat treatment during milk processing, storage and manufacturing of dairy products (Chapter III). CPA in milk exposed to heat-treatment induced kinetics of reaction following a pattern of a first order reaction (Figure 3.1). Long time exposure of CPA in milk to heat-treatment induces denaturation of the toxin with a temperature dependent (Figure 3.2). However, this heat-treatment failed to eliminate the toxin from the milk. Short time heat-treatment even with a higher temperature such as 133°C would not lead to greater losses of CPA.

CPA was stable throughout the period of collection at the dairy farm and during transportation to the dairy plant (Chapter IV). CPA was stable throughout the period of storage in retail stores. Frozen milk and freeze dried milk favoured CPA
stability. Concentration of milk caused a decrease of CPA in the final product, but the toxin residual left was stable throughout storage at 4°C for 8 weeks. Evaporation of milk under vacuum appeared not to greatly affect CPA levels. Processing of milk powder by spray drying at 80°C under pressure did not remove CPA (Table 4.7). However, distribution of CPA in the product was not uniform.

CPA was reduced after the manufacture of yogurt and the first day of storage (Chapter V). Storage of yogurt for 20 days induced a further decrease of CPA. A decrease in CPA was observed in acidified milk but to a lesser extent compared to that in yogurt (Table 5.1). CPA was not greatly affected during the manufacture of ice cream (Chapter V). Storing the ice cream for 9 weeks at -20°C did not affected CPA residues.

The occurrence of CPA in Cheddar cheese curd, butter or cream following manufacture with contaminated milk is firmly established (Chapter VII). However, CPA distribution in cheese curd was not uniform. Despite the fact that CPA amounts decrease with time, the toxin was residual after 10 months of ageing (Figure 7.1).

CPA remains in milk following treatment by UV-visible radiation with or without hydrogen peroxide and/or riboflavin (Figure 8.1). However, chemical treatment capable of completely eliminating CPA, is prohibited to use as a milk treatment.

Following this study, CPA would be found in milk and milk products for human consumption as the toxin is carried-over into milk. Stability of CPA in milk and milk products confirmed the potential of the toxin to reach consumers of dairy products. CPA may be a possible potential health risks for dairy consumers especially children due to its neurotoxic and nephrotoxic properties.

The objectives of the thesis may have been fulfilled. However, the present study still unveils many areas that need further study.

It would seem appropriate that the toxin be added to feed then fed to lactating animals in order to obtain naturally contaminated milk for experiments. However, due to complex ethical problems in the use of animals in such research and the considerable volume of milk required that would be obtained only by dosing several lactating animals, the present studies were confined to using artificially contaminated
milk. Since CPA artificially contaminated in milk was in a pure form, the results obtained maybe different to that of CPA in naturally contaminated milk. The degradation products of CPA may also be toxic and this should be further investigated. Moreover, the association of CPA to milk components, especially in naturally contaminated milk, needs to be further assessed before any attempt at speculation on the increase or decrease of the toxin following milk processing is possible. The possibility exists that toxin may be liberated by processing after adhering or connecting to certain components of milk. Treatment of CPA by food additives needs to be studied in more depth before any attempt at speculation on the application of the method.

Despite the fact that CPA artificially contaminated milk was used in these studies, the high residue of CPA in milk and milk products still shows that public attitudes toward CPA in milk need to be changed in order to ensure health safety and the good quality of dairy products. In the areas having a high incidence of aflatoxin, the dairy industry needs to be survey milk for CPA.

There is no correlation between production of CPA and aflatoxin by the fungal co-producers of the mycotoxins (Luk et al., 1977; Gallagher et al., 1978; Blaney et al., 1989). This may explain the reason why the incidence and information of CPA contamination are still not well reported in the literature compared to that for aflatoxins. However, the results of these present studies proved that carry-over and stability of CPA in milk and milk products, was highly possible. Establishment of legislation and permitted levels of CPA in milk products is needed.

Actual legislation and control only defined maximum permitted levels of aflatoxins and some other mycotoxins have been gazetted in some countries. No legislation and permitted levels of CPA in food products have been implemented in any country. Moreover, a further study and survey of CPA in feed is needed to fully understand and assess the extent of CPA contamination. Furthermore, the scope of study on the effect of dairy product processing on CPA needs to be extended, for instance, to different types of cheese or cultured milk. The use of whey obtained from the manufacture of cheese by using contaminated milk should be assessed since whey product utilisation is increasing in various industries including formulating.
animal feed and human food, infant foods, soups, confectionery, margarine, ice cream, salad dressing or prepared dry mixes (Morr, 1992). Currently, it is not known if CPA could become a potential risk for the consumers of whey products. Finally, with the accumulation of more data on the occurrence and toxicity of CPA, legislation will then be in a position to undertake detailed risk assessment of this often neglected mycotoxin.
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